INTERNATIONAL STANDARD

ISO 21470

First edition
2020-11

Infant formula and adult nutritionals — Simultaneous determination of total vitamins B_1, B_2, B_3 and B_6 — Enzymatic digestion and LC-MS/MS

Formules infantiles et produits nutritionnels pour adultes — Détermination simultanée de la teneur en vitamines B_1, B_2, B_3 et B_6 *— Digestion enzymatique et CL-SM/SM*

Reference number
ISO 21470:2020(E)

COPYRIGHT PROTECTED DOCUMENT

ISO copyright office
CP 401 • Ch. de Blandonnet 8
CH-1214 Vernier, Geneva
Phone: +41 22 749 01 11
Email: copyright@iso.org
Website: www.iso.org

Published in Switzerland

Contents

Page

Foreword ... **iv**

1 Scope ... **1**

2 Normative references ... **1**

3 Terms and definitions ... **1**

4 Principle ... **1**

5 Reagents and materials ... **2**

6 Standard and solution preparation ... **3**
6.1 Mobile phases and prepared solutions ... 3
6.2 Stable isotope labelled compounds, individual, internal standard stock solutions ... 4
6.3 Stock standard solutions of native compounds ... 5
6.4 Working standard solution preparation ... 6
6.5 Summary of standard and solution preparation ... 7

7 Apparatus ... **7**

8 Procedure ... **9**
8.1 Sample preparation ... 9
8.1.1 Powdered products ... 9
8.1.2 Reconstituted powders and liquid products ... 9
8.2 Enzymatic digestion ... 9
8.3 UHPLC-MS/MS analysis ... 9
8.3.1 UHPLC conditions ... 9
8.3.2 MS tune conditions ... 10
8.3.3 Mass transitions ... 10
8.3.4 LC-MS/MS equilibration ... 11
8.4 Quality control ... 11
8.4.1 General ... 11
8.4.2 Calibration curve ... 11

9 Calculations ... **11**

10 Precision data ... **13**
10.1 General ... 13
10.2 Repeatability ... 13
10.3 Reproducibility ... 13

11 Test report ... **15**

Annex A (informative) **Precision data** ... **16**

Annex B (informative) **Comparison between this document and EN 14122** ... **25**

Annex C (informative) **Comparison between this document and EN 14152** ... **27**

Annex D (informative) **Comparison between this document and EN 14164** ... **29**

Bibliography ... **31**

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.

The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

For an explanation of the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT) see www.iso.org/iso/foreword.html.

This document was prepared by Technical Committee 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 document is equivalent to the AOAC Official Method 2015.14: *Simultaneous Determination of Total Vitamins B_1, B_2, B_3, and B_6 in Infant Formula and Related Nutritionals by Enzymatic Digestion and LC-MS/MS.*

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

Infant formula and adult nutritionals — Simultaneous determination of total vitamins B_1, B_2, B_3 and B_6 — Enzymatic digestion and LC-MS/MS

1 Scope

This document specifies a method for the simultaneous quantitative determination of four water-soluble vitamins in infant formula and related nutritional products, including relevant forms of vitamins B_1, B_2, B_3 and B_6 by enzymatic digestion and UHPLC-MS/MS. This document is not intended to be used on products where vitamins have not been added.

2 Normative references

There are no normative references in this document.

3 Terms and definitions

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

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at http://www.electropedia.org/

3.1
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

3.2
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]

4 Principle

Samples are prepared by enzymatic digestion with papain and α-amylase to hydrolyse protein and complex carbohydrate and acid phosphatase to free phosphorylated vitamin forms. Stable-isotope labelled internal standards are incorporated into the sample preparation to correct for variability in both the sample preparation and instrument response. A series of six mixed working standard solutions spanning two orders of magnitude in vitamin concentration are used to generate calibration curves based on the peak response ratio of the analyte to its stable-isotope labelled internal standard.

Prepared samples and working standard solutions are injected onto ultra-high pressure liquid chromatograph (UPLC) interfaced to a triple-quadrupole mass spectrometer (MS/MS) for analysis. The MS/MS is configured to monitor precursor-fragment ion pairs for each analyte and internal standard. This reaction forms the basis for method selectivity. Analytes are quantified by least squares regression using the response ratio of the analyte to its internal standard.

5 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.

5.1 Niacinamide (nicotinamide) (MW = 122,12), primary reference standard, e.g. USP Reference Standard, catalogue #1462006[1)]. Follow the manufacturer's storage and handling directions.

5.2 Niacin (nicotinic acid) (MW = 123,11), primary reference standard, e.g. USP Reference Standard, catalogue # 1461003[1)]. Follow the manufacturer's storage and handling directions.

5.3 Pyridoxine hydrochloride (MW = 205,64), primary reference standard, e.g. USP Reference Standard, catalogue # 1587001[1)]. Follow the manufacturer's storage and handling directions.

5.4 Riboflavin (MW = 376,36), primary reference standard, e.g. USP Reference Standard, catalogue # 1603006[1)]. Follow the manufacturer's storage and handling directions.

5.5 Thiamine hydrochloride (MW = 337,27), primary reference standard, e.g. USP Reference Standard, catalogue #1656002[1)]. Follow the manufacturer's storage and handling directions. Measure the moisture content of the powder prior to use or use the supplier certificate of analysis (COA) moisture value.

5.6 Pyridoxamine dihydrochloride, Fluka Analytical Standard, catalogue #P9380[1)].

5.7 Pyridoxal hydrochloride, Sigma, catalogue #P9130[1)].

5.8 $^{2}H_{4}$-Niacinamide, CDN Isotopes, catalogue #D-3457[1)].

5.9 $^{2}H_{4}$-Nicotinic acid, CDN Isotopes, catalogue #D-4368[1)].

5.10 $^{13}C_{4}$-Pyridoxine: pyridoxine:HCl (4,5-bis(hydroxymethyl)-$^{13}C_{4}$), Cambridge Isotope Laboratory, catalogue #CLM-7563[1)].

5.11 $^{2}H_{3}$-Pyridoxal, IsoSciences, catalogue #7098[1)].

5.12 $^{2}H_{3}$-Pyridoxamine, IsoSciences, catalogue #7099[1)].

5.13 $^{13}C_{4}$-Thiamine chloride, IsoSciences, catalogue #9209[1)].

5.14 $^{13}C_{4}$,$^{15}N_{2}$-Riboflavin, IsoSciences, catalogue #7072[1)].

5.15 Acid phosphatase, type II from potato, 0,5 U/mg to 3,0 U/mg, Sigma, catalogue #P3752[1)].

5.16 Papain from *Carica papaya*, ≥ 3 U/mg, Sigma, catalogue #76220[1)].

5.17 α–amylase from *aspergillus oryzae*, 150 U/mg, Sigma, catalogue #A9857[1)].

5.18 Hydrochloric acid concentrated (substance concentration c = 12 mol/l), ACS grade, or equivalent.

5.19 Ammonium formate, for mass spectrometry (purity ≥ 99,0 %), Fluka 70221 or equivalent[1)].

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.

5.20 Glacial acetic acid, Sigma ACS reagent grade, or equivalent[1)].

5.21 Formic acid, Sigma ACS reagent grade, or equivalent[1)].

5.22 Laboratory water, 18,0 MΩ, < 10 µg/kg TOC, or equivalent.

5.23 Methanol, Fisher LC-MS/MS Optima grade or EMD Omni-Solve LC-MS grade[1)].

5.24 Ethylenediaminetetracetic acid, disodium salt dihydrate (EDTA), ACS grade (99 % to 101 %), or equivalent.

5.25 Potassium phosphate dibasic, ACS grade (purity > 98 %), or equivalent.

5.26 meta-Phosphoric acid, ACS grade (33,5 % to 36,5 %), or equivalent.

5.27 Buffer solutions for pH meter calibration, pH = 4,0, 7,0 and 10,0.

5.28 Phosphoric acid, 85 g/100 g, ACS grade, or equivalent.

5.29 Potassium hydroxide, 40 g/100 g, ACS grade, or equivalent.

6 Standard and solution preparation

6.1 Mobile phases and prepared solutions

6.1.1 Mobile phase A, substance concentration c = 0,020 mol/l ammonium formate in water.

Using a graduated cylinder, transfer 500 ml laboratory water to a mobile phase reservoir. Add 0,631 g of ammonium formate (5.19) and mix well. Expiration is three days.

6.1.2 Mobile phase B, methanol.

6.1.3 HCl solution, c = 0,12 mol/l.

Add approximately 300 ml of water to a 500 ml graduated cylinder. Add 5,0 ml ± 0,1 ml of concentrated HCl solution (5.18) and swirl to mix. Bring to 500 ml with laboratory water and mix well.

6.1.4 Acetic acid solution, 1,0 ml/100 ml.

Add approximately 30 ml of water to a 500 ml graduated cylinder. Add 5,0 ml ± 0,1 ml of glacial acetic acid (5.20) and swirl to mix. Bring to 500 ml with laboratory water and mix well.

6.1.5 Weak needle wash, 10 ml/100 ml methanol in water, expiration three months. Alternatively, use week needle wash as recommended by the supplier.

6.1.6 Strong needle wash, methanol or as recommended by the supplier.

6.1.7 Ammonium formate solution, c = 0,050 mol/l.

Using a graduated cylinder, transfer 1 400 ml of laboratory water to an appropriate reservoir. Add 4,41 g of ammonium formate (5.19) and mix well. One 400 ml is adequate for 6 working standards and 32 samples. Scale as needed. Expiration is three days.

6.1.8 Mixed enzyme solution.

Using a graduated cylinder, transfer 200 ml of ammonium formate buffer (6.1.7) to an appropriate reservoir. Add 200 mg ± 10 mg of acid phosphatase (5.15), 80 mg ± 5 mg of α-amylase (5.17) and 400 mg ± 10 mg of papain (5.16). Mix for 10 min with a magnetic stir plate and stir bar. Check pH and adjust to 4,25 ± 0,25 with formic acid (5.21, approximately 100 µl). 200 ml is adequate for 6 working standards and 32 samples. Scale as needed. Prepare fresh daily.

6.2 Stable isotope labelled compounds, individual, internal standard stock solutions

6.2.1 Internal standard stock solutions have an expiration of six months. However, the following guidelines can be used to troubleshoot internal standards and, when documented as part of routine system suitability checks, extend the expiration dates indefinitely.

Based on US FDA bioanalytical method validation guidelines, which state that the lowest-level calibration shall be five times the analyte response of the blank,[9] the channel of the non-labelled analyte of interest shall be monitored to ensure the stable isotope-labelled internal standard does not contribute more than 20 % of the area count of the lowest-level calibration standard. No response should be generated in any other channels being monitored in the method, as this is a sign of contamination, in which case fresh solution should be prepared or fresh lot of material should be ordered.

The area count of the internal standard should be at least three times the area count of the analyte in the lowest-level calibration standard and the lowest level matrix-based QC sample.

6.2.2 **$^{2}H_{4}$-Niacinamide stock solution**, mass concentration $\rho \approx 560$ µg/ml.

Weigh 14,0 mg ± 0,1 mg into a tared weighing vessel. Quantitatively transfer to a 25 ml volumetric flask with laboratory water and fill to the mark with laboratory water. Mix well and transfer to a 50 ml amber bottle and store refrigerated (2 °C to 8 °C). For expiration, see 6.2.1.

6.2.3 **$^{2}H_{4}$-Nicotinic acid stock solution**, $\rho \approx 500$ µg/ml.

Weigh 12,5 mg ± 0,1 mg into a tared weighing vessel. Quantitatively transfer to a 25 ml volumetric flask with laboratory water and fill to the mark with laboratory water. Mix well and transfer to a 50 ml amber bottle and store refrigerated (2 °C to 8 °C). For expiration, see 6.2.1.

6.2.4 **$^{13}C_{4}$-Pyridoxine stock solution**, $\rho \approx 70$ µg/ml.

Weigh 7,0 mg ± 0,1 mg into a tared weighing vessel. Quantitatively transfer to a 100 ml volumetric flask with laboratory water and fill to the mark with laboratory water. Mix well and transfer to a 100 ml amber bottle and store refrigerated (2 °C to 8 °C). For expiration, see 6.2.1.

6.2.5 **$^{2}H_{3}$-Pyridoxal stock solution**, $\rho \approx 40$ µg/ml.

Weigh 4,0 mg ± 0,1 mg into a tared weighing vessel. Quantitatively transfer to a 100 ml volumetric flask with laboratory water and fill to the mark with laboratory water. Mix well and transfer to a 100 ml amber bottle and store refrigerated (2 °C to 8 °C). For expiration, see 6.2.1.

6.2.6 **$^{2}H_{3}$-Pyridoxamine stock solution**, $\rho \approx 40$ µg/ml.

Weigh 4,0 mg ± 0,1 mg into a tared weighing vessel. Quantitatively transfer to a 100 ml volumetric flask with laboratory water and fill to the mark with laboratory water. Mix well and transfer to a 100 ml amber bottle and store refrigerated (2 °C to 8 °C). For expiration, see 6.2.1.

6.2.7 $^{13}C_4$-Thiamine chloride stock solution, $\rho \approx 100$ µg/ml.

Weigh 5,0 mg ± 0,1 mg of $^{13}C_4$-thiamine into a tared weighing vessel. Quantitatively transfer to a 50 ml volumetric flask with HCl solution (6.1.2) and fill to the mark with HCl solution (6.1.2). Mix well and transfer to a 100 ml amber bottle and store refrigerated (2 °C to 8 °C). For expiration, see 6.2.1.

6.2.8 $^{13}C_4$,$^{15}N_2$-Riboflavin stock solution, $\rho \approx 73$ µg/ml.

Weigh 7,3 mg ± 0,1 mg of $^{13}C_4$,$^{15}N_2$-riboflavin into a tared weighing vessel. Quantitatively transfer to a 100 ml volumetric flask with acetic acid solution (6.1.3) and fill to the mark with acetic acid solution (6.1.3). Mix well and transfer to a 100 ml amber bottle and store refrigerated (2 °C to 8 °C). For expiration, see 6.2.1.

6.2.9 Internal standard stock mixture (ISSM).

Combine 2 500 µl of ammonium formate solution (6.1.7) with 250 µl of $^{2}H_4$-niacinamide stock solution (6.2.2), 250 µl of $^{2}H_4$-nicotinic acid stock solution (6.2.3), 250 µl of $^{13}C_4$-pyridoxine stock solution (6.2.4), 200 µl of $^{2}H_3$-pyridoxal stock solution (6.2.5), 50 µl of $^{2}H_3$-pyridoxamine stock solution (6.2.6), 250 µl of $^{13}C_4$-thiamine stock solution (6.2.7) and 250 µl of $^{13}C_4$,$^{15}N_2$-riboflavin stock solution (6.2.8). Volume provides sufficient ISSM for 6 working standards and 32 samples. Scale as needed. Prepare fresh daily.

6.2.10 Phosphate buffer solution, pH = 5,0 (0,010 mol/l potassium phosphate dibasic, 1 g/100 g EDTA, 2 g/100 g metaphosphoric acid.

Weigh 20,0 g ± 0,2 g of EDTA into a tared weighing vessel and quantitatively transfer to a 2 000 ml beaker containing approximately 1 800 ml laboratory water and add a magnetic stir bar.

Weigh 34,8 g ± 0,1 g of potassium phosphate dibasic into a tared weighing vessel and quantitatively transfer to the 2 000 ml beaker already containing approximately 1 800 ml laboratory water and EDTA. Mix by stirring on a magnetic stir plate until both the EDTA and potassium phosphate dibasic is completely dissolved.

Weigh 40,0 g ± 0,2 g of metaphosphoric acid into a tared weighing vessel and quantitatively transfer to the 2 000 ml beaker containing approximately 1 800 ml laboratory water, EDTA, and potassium phosphate dibasic. Mix by stirring on a magnetic stir plate until the metaphosphoric acid is completely dissolved.

Adjust the pH of the solution to pH = 5,00 ± 0,02 using 40 g/100 g potassium hydroxide or 85 g/100 g phosphoric acid. Quantitatively transfer the solution to a 2 000 ml volumetric flask and dilute to volume with laboratory water. Expiration: 48 hours.

6.3 Stock standard solutions of native compounds

6.3.1 Vitamin standard stock mixture (VSSM).

Accurately weigh the indicated amounts for the following standards using separate weighing funnels or other appropriate weighing vessels and quantitatively transfer to a 100 ml volumetric flask using phosphate buffer (pH = 5).

a) Niacinamide (5.1): 70,5 mg ± 0,5 mg.

b) Thiamine hydrochloride (5.5): 10,5 mg ± 0,2 mg.

   Determine the moisture of the thiamine hydrochloride reference standard (5.5) as directed on the container immediately prior to weighing or use moisture content from the supplier COA. The per cent moisture determined for the reference standard is used to calculate the concentration of thiamine in the VSSM.

c) Riboflavin (5.4): 7,0 mg ± 0,2 mg.

d) Pyridoxine hydrochloride (5.3): 10,8 mg ± 0,2 mg.

Fill to volume with phosphate buffer (pH = 5) solution. Heat and slowly stir until the standards have completely dissolved (riboflavin dissolves more slowly) and the solution is clear. Do not heat the solution for more than 40 min and do not exceed 90 °C. Store refrigerated (2 °C to 8 °C). Expiration: three months.

6.3.2 Nicotinic acid stock solution, ρ = 550 mg/ml.

Accurately weigh 13,7 mg ± 0,1 mg niacin primary reference standard (5.2). Quantitatively transfer the nicotinic acid to a 25 ml volumetric flask. Add laboratory water to a total volume of about 20 ml and swirl until completely dissolved. Bring to volume with laboratory water. Mix well. Expiration: three months.

6.3.3 Pyridoxal stock solution, ρ = 140 mg/ml.

Accurately weigh 17,0 mg ± 0,5 mg pyridoxal dihydrochloride standard (5.7). Quantitatively transfer to a 100 ml volumetric flask. Add laboratory water to a total volume of about 70 ml and swirl until completely dissolved. Bring to volume with laboratory water. Mix well. Expiration: three months.

6.3.4 Pyridoxamine stock solution, ρ = 160 mg/ml.

Accurately weigh 23,0 mg ± 0,5 mg pyridoxamine hydrochloride standard (5.6). Quantitatively transfer to a 100 ml volumetric flask. Add laboratory water to a total volume of about 70 ml and swirl until completely dissolved. Bring to volume with laboratory water. Mix well. Expiration: three months.

6.3.5 Mixed working standard (MWS).

Combine 500 µl VSSM (6.3.1), 25 µl pyridoxamine stock (6.3.4), 25 µl pyridoxal stock (6.3.3), and 65 µl nicotinic acid stock solutions (6.3.2) in a 10 ml volumetric flask containing approximately 5 ml of ammonium formate solution (6.1.7). Bring to volume with ammonium formate solution (6.1.7) and mix well. Prepare fresh daily.

6.4 Working standard solution preparation

6.4.1 Working solution (WS) 1.

Add 20 µl of MWS (6.3.5) and 980 µl of ammonium formate (6.1.7) to a 50 ml centrifuge tube. Add 100 µl of ISSM (6.2.9), and vortex to mix. Prepare fresh daily.

6.4.2 Working solution (WS) 2.

Add 50 µl of MWS (6.3.5) and 950 µl of ammonium formate (6.1.7) to a 50 ml centrifuge tube. Add 100 µl of ISSM (6.2.9), and vortex to mix. Prepare fresh daily.

6.4.3 Working solution (WS) 3.

Add 100 µl of MWS (6.3.5) and 900 µl of ammonium formate (6.1.7) to a 50 ml centrifuge tube. Add 100 µl of ISSM (6.2.9), and vortex to mix. Prepare fresh daily.

6.4.4 Working solution (WS) 4.

Add 200 µl of MWS (6.3.5) and 800 µl of ammonium formate (6.1.7) to a 50 ml centrifuge tube. Add 100 µl of ISSM (6.2.9), and vortex to mix. Prepare fresh daily.

6.4.5 Working solution (WS) 5.

Add 500 µl of MWS (6.3.5) and 500 µl of ammonium formate (6.1.7) to a 50 ml centrifuge tube. Add 100 µl of ISSM (6.2.9), and vortex to mix. Prepare fresh daily.

6.4.6 Working solution (WS) 6.

Add 1 000 µl of MWS (6.3.5). Add 100 µl of ISSM (6.2.9), and vortex to mix. Prepare fresh daily.

6.5 Summary of standard and solution preparation

See Table 1.

Table 1 — Summary of standard and solution preparation

Compound	Mass	Purity	Moisture correction	Volume stock solution	Aliquot stock	Volume of MWS	Aliquot of MWS (6.3.5)	Aliquot of ISSM (6.2.9)	Final volume
	mg			ml	µl	ml	µl	µl	ml
Niacinamide (5.1)	70,5 ± 0,5	0,999[a]	1,000	100	500	10	see 6.4	100	30
Thiamine HCl (5.5)	10,5 ± 0,2	0997[a]	0961[b]	100	500	10	see 6.4	100	30
Riboflavin (5.4)	7,0 ± 0,2	0,986[a]	1,000	100	500	10	see 6.4	100	30
Pyridoxine (5.3)	10,8 ± 0,2	0,999[a]	1,000	100	500	10	see 6.4	100	30
Pyridoxal (5.7)	17,0 ± 0,5	0,990[a]	1,000	100	25	10	see 6.4	100	30
Pyridoxamine (5.6)	23,0 ± 0,5	0,980[a]	1,000	100	25	10	see 6.4	100	30
Niacin (nicotinic acid) (5.2)	13,7 ± 0,1	0,998[a]	1,000	25	65	10	see 6.4	100	30

[a] Purity of the standard as defined by the manufacturer.

[b] Moisture correction (1 – moisture content, from measurement or from the COA provided by the manufacturer).

7 Apparatus

7.1 Waters® Acquity BEH C18 column[2] or equivalent, 2,1 mm x 100 mm, 1,7 µm.

7.2 UHPLC system, Waters Acquity Classic[2], or equivalent.

7.3 Tandem quadrupole mass spectrometer with ESI probe, Waters Xevo TQ-S[2], or equivalent.

7.4 Analytical balances.

A balance capable of accurately weighing 5,00 mg (for standards), a six-place balance, an analytical five-place balance for samples and a top-loading two-place balance capable of weighing to several hundred grams.

7.5 Water purifier, Millipore Milli-Q Water Purification System[2], or equivalent.

2) 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.

7.6 Water bath shaker, capable of maintaining 37 °C, Lab-Line Orbit[2)], or equivalent.

7.7 Bottle-top dispenser, capable of dispensing volumes of approximately 24 ml.

7.8 pH meter, capable of measuring pH = 4,0 to pH = 5,0.

7.9 Vortex mixer.

7.10 Multi-position magnetic stir plate.

7.11 Room light shields, A.L.P. Protect-A-Lamp[2)], UV cutoff at 460 nm, or equivalent. Alternatively, the use of amber (brown) glassware and vials can be used.

7.12 Graduated cylinders, various sizes, including 10 ml, 100 ml, 500 ml and 1 000 ml.

7.13 Beakers, various sizes, including 100 ml, 200 ml, 400 ml, 600 ml, 1 000 ml and 2 000 ml.

7.14 Volumetric flasks, various sizes, including 10 ml, 25 ml, 50 ml, 100 ml, 250 ml and 2 000 ml.

7.15 Mobile phase bottles, glass, various sizes, including 250 ml, 500 ml, 1 000 ml and 2 000 ml.

7.16 Disposable plastic Pasteur pipettes.

7.17 Amber bottles, volume capacity of 50 ml and 100 ml (for stock standard storage).

7.18 Weighing vessels, various, including disposable weighing boats and glass weighing funnels.

7.19 Positive displacement pipettes, 10 µl, 100 µl, 250 µl and 1 000 µl, Gilson Microman: Part #F148501, #F148504, #F148505 and #F148506[2)].

7.20 Positive displacement pipette tips, 10 µl, 100 µl, 250 µl and 1 000 µl, Gilson Capillary Piston: Part #F148312, #F148314, #F148014 and #F148560[2)].

7.21 Plastic syringes, 3 ml.

7.22 Syringe filters, polytetrafluorethylene (PTFE) 0,45 µm syringe filters, Acrodisc[2)] 25 mm, or equivalent.

7.23 Plastic centrifuge tubes, 50 ml, self-standing, Superior Scientific, Ltd.[2)], or equivalent.

7.24 Autosampler vials, Waters autosampler vials; 9 mm amber with screw top 12 mm x 32 mm pre-split PTFE-silicon septa; Waters Part # 186000847C, or equivalent[2)].

7.25 PTFE coated magnetic stir bars.

8 Procedure

8.1 Sample preparation

8.1.1 Powdered products

Using a tared beaker or low-density polyethylene (LDPE) cup, accurately weigh 10,0 g ± 0,3 g of sample. Record the mass to at least four significant figures. This is the powder mass. Add room temperature laboratory water to bring the total reconstituted sample mass (to include the product mass) to 100 g ± 2 g. Record the mass to at least four significant figures. This is the reconstitution mass. Carefully add a stir bar so as not to splash the liquid from the beaker/cup and place it onto a stir plate. Set the stir plate to stir the sample as fast as possible without causing the sample to splatter or froth. Powder samples should stir for at least 10 min but not more than 30 min.

8.1.2 Reconstituted powders and liquid products

Using a tared, 50 ml centrifuge tube, accurately weigh the appropriate sample amount (1,000 g ± 0,100 g for infant formula, 0,500 g ± 0,050 g for paediatric formulas and the NIST SRM, and 0,250 g ± 0,050 g for adult nutritionals). Record the mass to 0,000 1 g. This is the sample mass. Add 100 µl of the internal standard stock mixture (6.2.9) via positive-displacement pipette. Vortex to mix.

8.2 Enzymatic digestion

Add 5 ml of mixed enzyme solution (6.1.8) to all prepared samples and working standards. Cap and vortex immediately. Incubate at 37 °C overnight with agitation in water bath shaker. Remove from water bath and add ammonium formate solution (6.1.7) to bring volume to approximately 30 ml and vortex to mix. Filter approximately 2 ml aliquot of the sample extract into an appropriate size vial using a 0,45 µm PTFE syringe filter. Transfer 60 µl of filtrate to an autosampler vial with 940 µl of ammonium formate solution (6.1.7). Cap and vortex. The sample is ready for analysis. Samples have been determined to be stable for at least 48 h at room temperature.

8.3 UHPLC-MS/MS analysis

8.3.1 UHPLC conditions

Place freshly prepared mobile phases, weak needle wash and strong needle wash onto the UHPLC system. Purge old solvents from the solvent lines and needle washes. Injection volume is 10 µl and column temperature is 40 °C. Mobile phase flow rate is 0,350 ml/min. Hold at 99 % mobile phase A and 1,0 % mobile phase B for 0,50 min, then ramp to 8,0 % B over 2,00 min, ramp to 90 % B over the next 2,50 min, and hold at 90 % B for 1,00 min. Return to 99 % mobile phase A and 1,0 % mobile phase B over 0,10 min and hold for 1,9 min for re-equilibration. Total gradient program is 8,00 min long. See Table 2 for a summary.

Table 2 — Summary of gradient programme

Time min	Mobile phase A %	Mobile phase B %	Flow rate ml/min
0,00	99	1	0,350
0,5	99	1	0,350
2,5	92	8	0,350
5,0	10	90	0,350
6,0	10	90	0,350
6,1	99	1	0,350
8,0	99	1	0,350

8.3.2 MS tune conditions

Clean the sample cone and MS source with 5 g/100 g aqueous formic acid prior to analysis. Tune conditions can vary between instrument models and an appropriate balance shall be struck to achieve an adequate signal for each compound. Determine the appropriate conditions experimentally for each instrument model. On a Waters TQ-S[3)], ionization is performed by ESI+ at 2,5 kV. Additional tune conditions include: source offset of 50 V, ion block temperature of 150 °C, desolvation gas temperature of 500 °C, desolvation gas flow of 800 l/h, cone gas flow of 150 l/h, nebulizer gas pressure of 700 kPa (7,00 bar), and collision gas flow of 0,15 ml/min with argon. Both quadrupoles are set to unit mass resolution.

8.3.3 Mass transitions

Mass transitions for each vitamin and its corresponding internal standard are given in Table 3. Retention time windows are also given in the table. Like the tune parameters, these parameters may need adjusted based upon instrument model.

Table 3 — Conditions for MS transitions on a Waters TQ-S[a] and retention time windows

Compound	Function no.	Start min	End min	Molecular ion	Fragment ion	Cone voltage	Collision energy V	Dwell time s
Niacinamide[b]	1	2,71	3,20	122,9	80,1	20,0	16,0	0,025
Niacinamide	1	2,71	3,20	122,9	96,0	20,0	16,0	0,025
$^{2}H_4$-Niacinamide[b]	1	2,71	3,20	127,0	84,0	20,0	16,0	0,025
$^{2}H_4$-Niacinamide	1	2,71	3,20	127,0	100,0	20,0	16,0	0,025
Nicotinic acid[b]	2	0,50	1,70	124,0	80,0	20,0	16,0	0,025
Nicotinic acid	2	0,50	1,70	124,0	106,0	20,0	16,0	0,025
$^{2}H_4$-Nicotinic acid[b]	2	0,50	1,70	128,0	84,1	20,0	16,0	0,025
$^{2}H_4$-Nicotinic acid	2	0,50	1,70	128,0	109,0	20,0	16,0	0,025
Pyridoxal	3	1,76	2,70	168,0	94,0	20,0	22,0	0,025
Pyridoxal[b]	3	1,76	2,70	168,0	150,0	20,0	12,0	0,025
$^{2}H_3$-Pyridoxal	3	1,76	2,70	171,0	97,0	20,0	22,0	0,025
$^{2}H_3$-Pyridoxal[b]	3	1,76	2,70	171,0	153,0	20,0	12,0	0,025
Pyridoxamine	4	0,50	1,70	169,0	134,0	20,0	20,0	0,025
Pyridoxamine[b]	4	0,50	1,70	169,0	152,0	20,0	12,0	0,025
$^{2}H_3$-Pyridoxamine	4	0,50	1,70	172,0	136,0	20,0	20,0	0,025
$^{2}H_3$-Pyridoxamine[b]	4	0,50	1,70	172,0	155,0	20,0	12,0	0,025
Pyridoxine[b]	5	2,41	3,00	170,0	134,0	20,0	18,0	0,025
Pyridoxine	5	2,41	3,00	170,0	152,0	20,0	12,0	0,025
$^{13}C_4$-Pyridoxine[b]	5	2,41	3,00	174,0	138,0	20,0	18,0	0,025
$^{13}C_4$-Pyridoxine	5	2,41	3,00	174,0	156,0	20,0	12,0	0,025
Thiamine	6	3,01	3,60	265,1	81,0	20,0	30,0	0,025
Thiamine[b]	6	3,01	3,60	265,1	122,0	20,0	12,0	0,025
$^{13}C_4$-Thiamine	6	3,01	3,60	269,0	81,0	20,0	30,0	0,025

[a] While the mass transitions are expected to remain the same across instrument platforms, the other parameters may need to be adjusted to maximize sensitivity.

[b] Indicates primary transition used in quantitation.

3) 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.

Table 3 *(continued)*

Compound	Function no.	Start min	End min	Molecular ion	Fragment ion	Cone voltage	Collision energy V	Dwell time s
$^{13}C_4$-Thiamine[b]	6	3,01	3,60	269,0	122,0	20,0	12,0	0,025
Riboflavin	7	4,21	5,00	377,0	172,0	20,0	35,0	0,025
Riboflavin[b]	7	4,21	5,00	377,0	243,0	20,0	20,0	0,025
$^{13}C_4$,$^{15}N_2$-Riboflavin	7	4,21	5,00	383,0	175,0	20,0	35,0	0,025
$^{13}C_4$,$^{15}N_2$-Riboflavin[b]	7	4,21	5,00	383,0	249,0	20,0	20,0	0,025

[a] While the mass transitions are expected to remain the same across instrument platforms, the other parameters may need to be adjusted to maximize sensitivity.

[b] Indicates primary transition used in quantitation.

8.3.4 LC-MS/MS equilibration

The instrument should be held at initial conditions (with mobile phase flow on and MS at temperature) for 30 min to 60 min prior to injection. Alternatively, 6 to 10 blank injections at the start of a sequence can be used for the same purpose.

8.4 Quality control

8.4.1 General

Bracket each calibration curve with blanks of ammonium formate solution (6.1.7) to enable a check for laboratory background and instrumental carryover. Background should be no more than 5 % of the signal for the lowest working standard.

8.4.2 Calibration curve

Calibration curves are set up to bracket the sample injections. Calibration residuals (relative error from known concentration) are expected to be ≤ 20 % for pyridoxal and ≤ 8 % for the other vitamins. A standard injection outside of this range can be excluded with evidence of a standard preparation error in a single calibration level leading to a high or low response for all vitamins or evidence of a one-off instrumental error, such as a missed injection.

9 Calculations

Calculate the mass concentration of vitamin stock solutions using Formula (1):

$$\rho_{stk} = \frac{m_s \times M \times S \times P \times 1\,000}{V} \quad (1)$$

where

ρ_{stk} is the vitamin standard stock solution mass concentration, in µg/ml;

m_s is the mass of the standard, in mg;

M is the moisture content correction factor for the standard, if applicable;

S is the stoichiometric correction factor to convert the standard's form, in Clause 5, to the form that is reported (i.e. to report as thiamine ion [MW = 265,36] S = 265,36/337,27 = 0,7868);

P is the purity of the standard as defined by the manufacturer;

1 000 is the units conversion factor, from mg to µg;

V is the dissolution volume, in ml.

Calculate the vitamin mass concentrations in the mixed working standard (MWS, see 6.3.5) using Formula (2):

$$\rho_{MWS} = \rho_{stk} \times \frac{V_{SS}}{10} \quad (2)$$

where

ρ_{MWS} is the vitamin mass concentration in the MWS, in ng/ml;

ρ_{stk} is the vitamin mass concentration in the stock standard solution [see Formula (1)] in µg/ml;

V_{SS} is the volume of stock solution added to MWS, in µl;

10 in ml.

Calculate the vitamin mass concentration in the working standard (WS, see 6.4.1 to 6.4.6) using Formula (3):

$$\rho_{WSi} = \frac{V_{MWS} \times \rho_{MWS}}{500} \quad (3)$$

where

ρ_{WSi} is the vitamin mass concentration in the working standard, in ng/ml;

V_{MWS} is the volume of the mixed working standard fortified in working standard, in µl;

ρ_{MWS} is the mass concentration of vitamin in the mixed working standard [MWS, see Formula (2)] in ng/ml;

500 is the standard preparation dilution factor.

Calculate the vitamin mass fraction in the product, w_{pr}, in µg/kg, using Formula (4):

$$w_{pr} = \frac{\rho_{as} \times m_r \times 500}{m_s \times m_p} \quad (4)$$

where

ρ_{as} is the vitamin mass concentration in the analytical sample as calculated from the calibration curve, in ng/ml;

m_r is the total reconstitution mass, in g. For direct mass (liquid) samples, $m_r = 1$;

500 is the dilution factor;

m_s is the analytical sample mass, in g;

m_p is the powder mass (for reconstituted samples), in g. For liquid samples, $m_p = 1$.

For vitamin B_3 and vitamin B_6, the reported concentration of the individual forms is summed to report total. For example, concentration of niacinamide and nicotinic acid are summed to report "Total

vitamin B_3" and concentration of pyridoxal, pyridoxamine, and pyridoxine are summed to report "Total vitamin B_6".

10 Precision data

10.1 General

Details of the interlaboratory test of the precision of the method are summarized in Annex A. The values derived from the interlaboratory test may not be applicable to analyte concentration ranges and/or matrices other than those given in Annex A.

10.2 Repeatability

The absolute difference between two single test results found on identical test material by one operator using the same apparatus within the shortest feasible time interval will exceed the repeatability limit r in not more than 5 % of the cases. The values of r are given in Table 4.

10.3 Reproducibility

The absolute difference between two single test results found on identical test material reported by two laboratories will exceed the reproducibility limit R in not more than 5 % of the cases. The values of R are given in Table 4. The results of NIST SRM 1849a are in µg/100 g of powder. All other powders are expressed as per 100 g of reconstituted powder (25 g powder plus 200 g water). Vitamin B_1 is reported as thiamine ion, vitamin B_2 as riboflavin, vitamin B_3 as niacin and vitamin B_6 as pyridoxine.

Table 4 — Precision data

Sample	$\bar{x}$ µg/100 g	r µg/100 g	R µg/100 g
Precision data for vitamin B_1			
IF powder milk protein-based	122	6,87	23,6
IF powder soy-based	120	7,42	13,7
IF powder partially hydrolysed milk-based	82,5	4,80	28,3
IF powder partially hydrolysed soy-based	83,9	3,00	8,46
AN RTF high fat	185	8,19	98,7
AN RTF high protein	169	13,99	64,1
Child formula powder milk-based	48,8	3,14	9,97
IF powder stage 1 milk-based	112	6,32	20,8
IF RTF milk-based	34,0	3,44	13,1
IF RTF milk-based placebo	2,43	1,06	4,01
NIST SRM 1849a (results in µg/100 g powder)	1 307	126,4	176,6
Child formula powder milk-based	341	14,4	83,9
AN powder low fat	208	20,7	30,6
Infant elemental powder	176	12,0	14,7
IF powder FOS/GOS-based	65	3,03	11,0
Key IF: infant formula AN: adult nutritional			

Table 4 *(continued)*

Sample	$\bar{x}$ µg/100 g	r µg/100 g	R µg/100 g
Precision data for vitamin B_2			
IF powder milk protein-based	204	23,0	41,9
IF powder soy-based	147	8,30	30,8
IF powder partially hydrolysed milk-based	175	11,8	23,7
IF powder partially hydrolysed soy-based	107	6,47	18,1
AN RTF high fat	485	81,5	128
AN RTF high protein	375	36,7	55,3
Child formula powder milk-based	140	16,84	27,5
IF powder stage 1 milk-based	183	14,24	28,7
IF RTF milk-based	94,0	23,1	3,7
IF RTF milk-based placebo	43	3,13	22,7
NIST SRM 1849a (results in µg/100 g powder)	2 143	328	397
Child formula powder milk-based	378	16,3	51,7
AN powder low fat	217	35,1	39,7
Infant elemental powder	102	9,94	12,4
IF powder FOS/GOS-based	139	11,5	30,3
Precision data for vitamin B_3			
IF powder milk protein-based	953	61,7	132
IF powder soy-based	980	56,6	261
IF powder partially hydrolysed milk-based	742	66,9	149,4
IF powder partially hydrolysed soy-based	836	65,6	211
AN RTF high fat	4 152	209	631
AN RTF high protein	3 073	345	410
Child formula powder milk-based	514	41,9	96,9
IF powder stage 1 milk-based	695	65,3	184
IF RTF milk-based	929	37,4	126,3
IF RTF milk-based placebo	11	0,00	29,5
NIST SRM 1849a (results in µg/100 g powder)	10 544	1 263	1 581
Child formula powder milk-based	1 197	79,26	283
AN powder low fat	1 649	215,9	359
Infant elemental powder	1 473	63,2	152
IF powder FOS/GOS-based	477	18,0	82,5
Key IF: infant formula AN: adult nutritional			

Table 4 *(continued)*

Sample	$\bar{x}$ µg/100 g	r µg/100 g	R µg/100 g
Precision data for vitamin B_6			
IF powder milk protein-based	72,3	4,54	13,2
IF powder soy-based	67,7	3,49	9,21
IF powder partially hydrolysed milk-based	58,3	2,79	6,49
IF powder partially hydrolysed soy-based	59,5	2,47	6,34
AN RTF high fat	479	24,1	58,6
AN RTF high protein	373	27,6	41,0
Child formula powder milk-based	45,1	4,68	21,3
IF powder stage 1 milk-based	72,8	3,26	8,63
IF RTF milk-based	53,7	2,95	5,28
IF RTF milk-based placebo	1,83	0,660	3,01
NIST SRM 1849a (results in µg/100 g powder)	1 404	147	161
Child formula powder milk-based	339	18,8	32,1
AN powder low fat	212	16,2	32,2
Infant elemental powder	54,3	3,30	12,4
IF powder FOS/GOS-based	39,5	1,93	8,23
Key IF: infant formula AN: adult nutritional			

11 Test report

The test report shall contain the following data:

a) all information necessary for the identification of the sample (type of sample, origin and designation of the sample);

b) a reference to this document, i.e. ISO 21470;

c) the date and type of sampling procedure (if known);

d) the date of receipt;

e) the date of test;

f) the test results and the units in which they have been expressed;

g) any operations not specified in the method or regarded as optional, which might have affected the results.

Annex A
(informative)

Precision data

The data given in Tables A.1 to A.4 were obtained in an interlaboratory study and published in 2019[1], in accordance with ISO 5725-2[2] and the 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 References [4], [5], [6] and [7].

The results of NIST SRM 1849a are in µg/100 g of powder. All other powders are expressed as per 100 g of reconstituted powder (25 g powder plus 200 g water). Vitamin B_1 is reported as thiamine ion, vitamin B_2 as riboflavin, vitamin B_3 as niacin and vitamin B_6 as pyridoxine.

Table A.1 — Precision data for vitamin B_1

Sample	1[a]	2[b]	3[c]	4[d]	5[e]	6[f]	7[g]	8[h]	9[i]	10[j]	11[k]	12[l]	13[m]	14[n]	15[o]
Year of interlaboratory test	2016 to 2018	2016 to 2018	2016 to 2018	2016 to 2018	2016 to 2018	2016 to 2018	2016 to 2018	2016 to 2018	2016 to 2018	2016 to 2018	2016 to 2018	2016 to 2018	2016 to 2018	2016 to 2018	2016 to 2018
Number of laboratories	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
Number of non-compliant laboratories	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Number of laboratories retained after eliminating outliers	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
Number of outliers (laboratories)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Number of accepted results	18	18	18	18	18	18	18	18	18	18	18	18	18	18	18
Mean value, $\bar{x}$, µg/100 g	122	120	82,5	83,9	185	169	48,8	112	34,0	2,43	1 307	341	208	176	65
Repeatability standard deviation, s_r, µg/100 g	2,45	2,65	1,71	1,07	2,93	5,00	1,12	2,26	1,23	0,38	45,1	5,14	7,38	4,27	1,08
Reproducibility standard deviation, s_R, µg/100 g	8,44	4,89	10,1	3,02	35,3	22,9	3,56	7,44	4,68	1,43	63,1	30,0	10,9	5,25	3,93
Coefficient of variation of repeatability, $C_{V,r}$, %	2,00	2,20	2,08	1,28	1,58	2,95	2,30	2,02	3,61	15,5	3,45	1,51	3,55	2,42	1,66

[a] Infant formula powder milk protein-based, [b] Infant formula powder soy-based, [c] Infant formula powder partially hydrolysed milk-based, [d] Infant formula powder partially hydrolysed soy-based, [e] Adult nutritional RTF high fat, [f] Adult nutritional RTF high protein, [g] Child formula powder milk-based, [h] Infant formula powder stage 1 milk-based, [i] Infant formula RTF milk-based, [j] Infant formula RTF milk-based placebo, [k] NIST SRM 1849a (results in µg/100 g powder), [l] Child formula powder milk-based, [m] Adult nutritional powder low fat, [n] Infant elemental powder, [o] Infant formula powder FOS/GOS-based.

Table A.1 *(continued)*

Sample	1[a]	2[b]	3[c]	4[d]	5[e]	6[f]	7[g]	8[h]	9[i]	10[j]	11[k]	12[l]	13[m]	14[n]	15[o]
Coefficient of variation of reproducibility, $C_{V,R}$, %	6,90	4,07	12,26	3,60	19,01	13,51	7,29	6,66	13,76	58,9	4,82	8,80	5,26	2,98	6,04
Repeatability limit, r [$r = 2{,}8 \times s_r$], µg/100 g	6,87	7,42	4,80	3,00	8,19	13,99	3,14	6,32	3,44	1,06	126,4	14,4	20,7	12,0	3,03
Reproducibility limit, R [$R = 2{,}8 \times s_R$], µg/100 g	23,6	13,7	28,3	8,46	98,7	64,1	9,97	20,8	13,1	4,01	176,6	83,9	30,6	14,7	11,0
HorRat value, according to Reference [8]	0,445	0,261	0,744	0,219	1,304	0,914	0,409	0,423	0,731	2,10	0,444	0,661	0,367	0,203	0,354

[a] Infant formula powder milk protein-based, [b] Infant formula powder soy-based, [c] Infant formula powder partially hydrolysed milk-based, [d] Infant formula powder partially hydrolysed soy-based, [e] Adult nutritional RTF high fat, [f] Adult nutritional RTF high protein, [g] Child formula powder milk-based, [h] Infant formula powder stage 1 milk-based, [i] Infant formula RTF milk-based, [j] Infant formula RTF milk-based placebo, [k] NIST SRM 1849a (results in µg/100 g powder), [l] Child formula powder milk-based, [m] Adult nutritional powder low fat, [n] Infant elemental powder, [o] Infant formula powder FOS/GOS-based.

Table A.2 — Precision data for vitamin B_2

Sample	1[a]	2[b]	3[c]	4[d]	5[e]	6[f]	7[g]	8[h]	9[i]	10[j]	11[k]	12[l]	13[m]	14[n]	15[o]
Year of interlaboratory test	2016 to 2018	2016 to 2018	2016 to 2018	2016 to 2018	2016 to 2018	2016 to 2018	2016 to 2018	2016 to 2018	2016 to 2018	2016 to 2018	2016 to 2018	2016 to 2018	2016 to 2018	2016 to 2018	2016 to 2018
Number of laboratories	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
Number of non-compliant laboratories	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Number of laboratories retained after eliminating outliers	9	8	8	8	9	8	8	8	9	8	9	9	9	8	9
Number of outliers (laboratories)	0	1	1	1	0	1	1	1	0	1	0	0	0	1	0
Number of accepted results	18	16	16	16	18	16	16	16	18	16	18	18	18	16	18
Mean value, $\bar{x}$, µg/100 g	204	147	175	107	485	375	140	183	94,0	43	2143	378	217	102	139
Repeatability standard deviation, s_r, µg/100 g	8,22	2,97	4,21	2,31	29,1	13,1	6,02	5,08	8,23	1,12	117	5,8	12,6	3,55	4,09
Reproducibility standard deviation, s_R, µg/100 g	15,0	11,0	8,46	6,48	45,8	19,7	9,82	10,3	13,1	8,09	142	18,5	14,2	4,41	10,8
Coefficient of variation of repeatability, $C_{V,r}$, %	4,03	2,02	2,41	2,15	6,01	3,49	4,30	2,77	8,76	2,63	5,46	1,54	5,79	3,48	2,95

[a] Infant formula powder milk protein-based, [b] Infant formula powder soy-based, [c] Infant formula powder partially hydrolysed milk-based, [d] Infant formula powder partially hydrolysed soy-based, [e] Adult nutritional RTF high fat, [f] Adult nutritional RTF high protein, [g] Child formula powder milk-based, [h] Infant formula powder stage 1 milk-based, [i] Infant formula RTF milk-based, [j] Infant formula RTF milk-based placebo, [k] NIST SRM 1849a (results in µg/100 g powder), [l] Child formula powder milk-based, [m] Adult nutritional powder low fat, [n] Infant elemental powder, [o] Infant formula powder FOS/GOS-based.

Table A.2 *(continued)*

Sample	1[a]	2[b]	3[c]	4[d]	5[e]	6[f]	7[g]	8[h]	9[i]	10[j]	11[k]	12[l]	13[m]	14[n]	15[o]
Coefficient of variation of reproducibility, $C_{V,R}$, %	7,33	7,50	4,83	6,03	9,45	5,26	7,02	5,60	13,93	19,0	6,62	4,89	6,55	4,33	7,82
Repeatability limit, r [$r = 2{,}8 \times s_r$], µg/100 g	23,0	8,30	11,8	6,47	81,5	36,7	16,84	14,24	23,1	3,13	328	16,3	35,1	9,94	11,5
Reproducibility limit, R [$R = 2{,}8 \times s_R$], µg/100 g	41,9	30,8	23,7	18,1	128	55,3	27,5	28,7	36,7	22,7	397	51,7	39,7	12,4	30,3
HorRat value, according to Reference [8]	0,510	0,496	0,328	0,381	0,749	0,401	0,461	0,383	0,863	1,05	0,656	0,373	0,460	0,271	0,514

[a] Infant formula powder milk protein-based, [b] Infant formula powder soy-based, [c] Infant formula powder partially hydrolysed milk-based, [d] Infant formula powder partially hydrolysed soy-based, [e] Adult nutritional RTF high fat, [f] Adult nutritional RTF high protein, [g] Child formula powder milk-based, [h] Infant formula powder stage 1 milk-based, [i] Infant formula RTF milk-based, [j] Infant formula RTF milk-based placebo, [k] NIST SRM 1849a (results in µg/100 g powder), [l] Child formula powder milk-based, [m] Adult nutritional powder low fat, [n] Infant elemental powder, [o] Infant formula powder FOS/GOS-based.

Table A.3 — Precision data for vitamin B_3

Sample	1[a]	2[b]	3[c]	4[d]	5[e]	6[f]	7[g]	8[h]	9[i]	10[j]	11[k]	12[l]	13[m]	14[n]	15[o]
Year of interlaboratory test	2016 to 2018	2016 to 2018	2016 to 2018	2016 to 2018	2016 to 2018	2016 to 2018	2016 to 2018	2016 to 2018	2016 to 2018	2016 to 2018	2016 to 2018	2016 to 2018	2016 to 2018	2016 to 2018	2016 to 2018
Number of laboratories	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
Number of non-compliant laboratories	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Number of laboratories retained after eliminating outliers	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
Number of outliers (laboratories)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Number of accepted results	18	18	18	18	18	18	18	18	18	18	18	18	18	18	18
Mean value, $\bar{x}$, µg/100 g	953	980	742	836	4 152	3 073	514	695	929	11	10 544	1 197	1 649	1 473	477
Repeatability standard deviation, s_r, µg/100 g	22,1	20,2	23,9	23,4	74,6	123,1	15,0	23,3	13,4	0,0	450,9	28,3	77,1	22,6	6,4
Reproducibility standard deviation, s_R, µg/100 g	47,0	93,4	53,3	75,2	225	146	34,6	65,7	45,1	10,5	565	101	128,3	54,3	29,4
Coefficient of variation of repeatability, $C_{V,r}$, %	2,31	2,06	3,22	2,80	1,80	4,00	2,91	3,36	1,44	0	4,28	2,36	4,68	1,53	1,35

[a] Infant formula powder milk protein-based, [b] Infant formula powder soy-based, [c] Infant formula powder partially hydrolysed milk-based, [d] Infant formula powder partially hydrolysed soy-based, [e] Adult nutritional RTF high fat, [f] Adult nutritional RTF high protein, [g] Child formula powder milk-based, [h] Infant formula powder stage 1 milk-based, [i] Infant formula RTF milk-based, [j] Infant formula RTF milk-based placebo, [k] NIST SRM 1849a (results in µg/100 g powder), [l] Child formula powder milk-based, [m] Adult nutritional powder low fat, [n] Infant elemental powder, [o] Infant formula powder FOS/GOS-based.

Table A.3 *(continued)*

Sample	1[a]	2[b]	3[c]	4[d]	5[e]	6[f]	7[g]	8[h]	9[i]	10[j]	11[k]	12[l]	13[m]	14[n]	15[o]
Coefficient of variation of reproducibility, $C_{V,R}$, %	4,93	9,53	7,19	9,00	5,43	4,76	6,74	9,46	4,86	94,9	5,35	8,44	7,78	3,69	6,17
Repeatability limit, r [$r = 2{,}8 \times s_r$], µg/100 g	61,7	56,6	66,9	65,6	209	345	41,9	65,3	37,4	0,00	1263	79,26	215,9	63,2	18,0
Reproducibility limit, R [$R = 2{,}8 \times s_R$], µg/100 g	132	261	149,4	211	631	410	96,9	184	126,3	29,5	1581	283	359	152	82,5
HorRat value, according to Reference [8]	0,430	0,840	0,610	0,770	0,590	0,500	0,540	0,790	0,420	4,261	0,670	0,770	0,740	0,350	0,490

[a] Infant formula powder milk protein-based, [b] Infant formula powder soy-based, [c] Infant formula powder partially hydrolysed milk-based, [d] Infant formula powder partially hydrolysed soy-based, [e] Adult nutritional RTF high fat, [f] Adult nutritional RTF high protein, [g] Child formula powder milk-based, [h] Infant formula powder stage 1 milk-based, [i] Infant formula RTF milk-based, [j] Infant formula RTF milk-based placebo, [k] NIST SRM 1849a (results in µg/100 g powder), [l] Child formula powder milk-based, [m] Adult nutritional powder low fat, [n] Infant elemental powder, [o] Infant formula powder FOS/GOS-based.

Table A.4 — Precision data for vitamin B_6

Sample	1[a]	2[b]	3[c]	4[d]	5[e]	6[f]	7[g]	8[h]	9[i]	10[j]	11[k]	12[l]	13[m]	14[n]	15[o]
Year of interlaboratory test	2016 to 2018	2016 to 2018	2016 to 2018	2016 to 2018	2016 to 2018	2016 to 2018	2016 to 2018	2016 to 2018	2016 to 2018	2016 to 2018	2016 to 2018	2016 to 2018	2016 to 2018	2016 to 2018	2016 to 2018
Number of laboratories	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
Number of non-compliant laboratories	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
Number of laboratories retained after eliminating outliers	9	9	9	9	9	9	9	9	9	9	9	9	9	9	9
Number of outliers (laboratories)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Number of accepted results	18	18	18	18	18	18	18	18	18	18	18	18	18	18	18
Mean value, $\bar{x}$, µg/kg	72,3	67,7	58,3	59,5	479	373	45,1	72,8	53,7	1,83	1 404	339	212	54,3	39,5
Repeatability standard deviation, s_r, µg/100 g	1,62	1,25	1,00	0,88	8,59	9,86	1,67	1,16	1,05	0,24	52,7	6,73	5,77	1,18	0,69
Reproducibility standard deviation, s_R, µg/100 g	4,70	3,29	2,32	2,27	20,9	14,6	7,60	3,08	1,89	1,07	57,6	11,5	11,5	4,45	2,94
Coefficient of variation of repeatability, $C_{V,r}$, %	2,24	1,84	1,71	1,48	1,79	2,65	3,70	1,60	1,96	12,9	3,75	1,99	2,72	2,17	1,74

[a] Infant formula powder milk protein-based, [b] Infant formula powder soy-based, [c] Infant formula powder partially hydrolysed milk-based, [d] Infant formula powder partially hydrolysed soy-based, [e] Adult nutritional RTF high fat, [f] Adult nutritional RTF high protein, [g] Child formula powder milk-based, [h] Infant formula powder stage 1 milk-based, [i] Infant formula RTF milk-based, [j] Infant formula RTF milk-based placebo, [k] NIST SRM 1849a (results in µg/100 g powder), [l] Child formula powder milk-based, [m] Adult nutritional powder low fat, [n] Infant elemental powder, [o] Infant formula powder FOS/GOS-based.

Table A.4 *(continued)*

Sample	1[a]	2[b]	3[c]	4[d]	5[e]	6[f]	7[g]	8[h]	9[i]	10[j]	11[k]	12[l]	13[m]	14[n]	15[o]
Coefficient of variation of reproducibility, $C_{V,R}$, %	6,50	4,86	3,98	3,81	4,37	3,93	16,9	4,23	3,51	58,6	4,10	3,39	5,42	8,19	7,44
Repeatability limit, r [$r = 2{,}8 \times s_r$], µg/100 g	4,54	3,49	2,79	2,47	24,1	27,6	4,68	3,26	2,95	0,660	147	18,8	16,2	3,30	1,93
Reproducibility limit, R [$R = 2{,}8 \times s_R$], µg/100 g	13,2	9,21	6,49	6,34	58,6	41,0	21,3	8,63	5,28	3,01	161	32,1	32,2	12,4	8,23
HorRat value, according to Reference [8]	0,387	0,287	0,229	0,220	0,346	0,299	0,935	0,252	0,200	2,01	0,382	0,254	0,380	0,467	0,404

[a] Infant formula powder milk protein-based, [b] Infant formula powder soy-based, [c] Infant formula powder partially hydrolysed milk-based, [d] Infant formula powder partially hydrolysed soy-based, [e] Adult nutritional RTF high fat, [f] Adult nutritional RTF high protein, [g] Child formula powder milk-based, [h] Infant formula powder stage 1 milk-based, [i] Infant formula RTF milk-based, [j] Infant formula RTF milk-based placebo, [k] NIST SRM 1849a (results in µg/100 g powder), [l] Child formula powder milk-based, [m] Adult nutritional powder low fat, [n] Infant elemental powder, [o] Infant formula powder FOS/GOS-based.