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Infant formula and adult nutritionals — Determination of total folate content by trienzyme extraction and ultra high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS)

Préparations pour nourrissons et produits nutritionnels pour adultes — Détermination de la teneur en folates totaux par extraction trienzymatique et chromatographie liquide à ultra performance (CLUHP) couplée à une spectrométrie de masse en tandem (SM/SM)

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Foreword

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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 document 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).

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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 2011.06 Total Folate in Infant Formula and Adult Nutritionals by Trienzyme Extraction and LC-MS/MS Quantitation.

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 — Determination of total folate content by trienzyme extraction and ultra high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS)

1 Scope

This document specifies a method for the analysis of total folate in infant formula and adult nutritionals using trienzyme extraction and ultra high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS).

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 terminology databases for use in standardization at the following addresses:

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

— IEC Electropedia: available at https://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 month of life up to the introduction of appropriate complementary feeding

[SOURCE: Codex Standard 72-1981]

4 Principle

Folates in a sample are extracted in a buffer (pH = 6,0) containing internal standards by treatments with protease, amylase and rat plasma conjugase (trienzyme digestion). The extract is purified and concentrated using a weak anion exchange (WAX) solid phase extraction (SPE). Polyglutamate forms of folates in the sample are deconjugated to monoglutamates during the extraction and are analysed by UHPLC-MS/MS. Folic acid, 5-methyl-tetrahydrofolate (5-CH_3-THF) and, 5-formyl-tetrahydrofolate (5-CHO-THF) are quantified, and total folate is estimated and expressed as folic acid. Isotopically labelled folic acid (^{13}C-folic acid), 5-CH_3-THF (^{13}C-5-CH_3-THF) and 5-CHO-THF (^{13}C-5-CHO-THF) are used as the internal standards (IS).

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.

Most of the chemicals used are LC-MS grade unless specified. Product numbers and suppliers, when listed, reflect those used in validation. Equivalent chemicals can be used.

All chemicals used in preparation of standards were stored at a minimum at −20 °C or as directed by the manufacturer. Because folate compounds are light sensitive, all samples and standards shall be prepared, handled and stored in the dark or under yellow-shielded lighting or UV-filtered lighting. If standards and samples shall be transported through or into an area without UV-filtered lighting, they shall be tightly wrapped in foil.

Store chemicals per manufacturer guidelines or set conventions.

5.1 List of reagents

5.1.1 Folic acid (standard) (FA), Schircks Laboratories, Cat. No. 16.203[1)].

5.1.2 (6R,S)-5-Methyl-5,6,7,8-Tetrahydrofolic acid (5-CH_3-THF), calcium salt, Schircks Laboratories, Cat. No. 16.235[1)].

5.1.3 (6S)-5-Formyl-5,6,7,8-Tetrahydrofolic acid (5-CHO-THF), calcium salt, Schircks Laboratories, Cat. No. 16.221[1)].

5.1.4 Pteroyltri-γ-L-glutamic acid (Folic acid triglutamate) (Pte-Glu3), Schircks Laboratories, Cat. No. 16.253[1]

An alternate source for this reagent is Toronto Research Chemicals, Toronto, Canada, Cat No P840220[1)].

5.1.5 $^{13}C_5$-Folic Acid (internal standard) ($^{13}C_5$-FA), IsoSciences, PA, Cat# 14139[1)].

5.1.6 $^{13}C_5$-labelled (6S)-5-methyltetrahydrofolic acid ($^{13}C_5$-CH_3-THF), calcium salt (internal standard), IsoSciences, PA, Cat# 14168Ca[1)].

5.1.7 $^{13}C_5$-labelled (6S)-5-formyltetrahydrofolic acid ($^{13}C_5$-CHO-THF), calcium salt, (internal standard), Merck Cie[1)].

An alternate source is asi chemicals, 1837 University Circle, Sci. Bldg, Rm. 308, Cheyney, PA 19319, USA. Web: www.asichemicals.com; email: info@asichemicals .com, Phone: 609-440-0020[1)]. Available in 1 mg size. A kit of all the three internal standards each in 1 mg size is also available from this source.

5.1.8 α-Amylase from *Aspergillus oryzae*, powder, ≥ 150 units/mg protein, Sigma-Aldrich, Cat. No. A9857[1)].

5.1.9 Protease from *Bacillus licheniformis*, Subtilisin A, lyophilized powder, Sigma-Aldrich Cat. No. P3910[1)].

1) Listed chemicals were used in the validation study. They 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.

5.1.10 Conjugase (Male Sprague Dawley Rat Plasma) with lithium and heparin (not filtered), BIOIVT Cat. No. RAT00PLLHMNN[1)].

An alternate source for the rat plasma reagent is Odin Bioscience[1)], 1621 Central Ave., Cheyenne, WY 82001, info@odinbioscience.com or Lampire Biological Laboratories, Pippersville, 18947. PA, USA[1)].

5.1.11 Ammonium hydroxide solution, (certified ACS Plus) 28 % to 30 % (mass fraction), (aqueous ammonia), Fisher, Cat. No. A669-500[1)].

5.1.12 Sodium phosphate, dibasic anhydrous (granular or powder/certified ACS, ≥ 99 %), Fisher Cat. No. S-374-500[1)].

5.1.13 Methanol, Optima LC/MS grade, 99,9 % minimum, Fisher Cat. No. A456-4[1)].

5.1.14 Glacial acetic acid, (certified ACS), Fisher, Cat. No. A38S-212[1)].

5.1.15 Sodium hydroxide, (pellets/ACS Certified), ≥ 97 %, Fisher Cat. No. S318-1[1)].

5.1.16 Ammonium acetate, (crystalline/certified ACS), ≥ 97 %, Fisher Cat. No. A637-500[1)].

5.1.17 2-Mercaptoethanol, (electrophoresis grade), ≥ 98 %, Fisher Cat. No. BP176-100; Sigma M6250[1)].

5.1.18 Ascorbic acid, (white crystalline powder), ≥ 99 %, Fisher Cat. No. BP351-500[1)].

5.1.19 Tris-(2-carboxyethyl)phosphine, hydrochloride (TCEP-HCl), Fisher, Cat. No. AC 363830100[1)].

5.1.20 Charcoal, (Darco G-60 activated carbon), Fisher Cat. No. D127-500[1)].

5.1.21 Formic acid, (reagent grade), ≥ 95 %, water ≤ 2,5 %, acetic acid < 1 %, Sigma F0507[1)].

5.1.22 Water, (high purity, suitable for HPLC mobile phase), resistivity up to 18 MΩ·cm[1)].

5.2 Preparation of solvent for standard solutions

5.2.1 Stock solvent, substance concentration c = 15,6 × 10^{-3} mol/l ammonium acetate buffer with 25 % ascorbic acid and 1 % mercaptoethanol, pH = 5,5.

Accurately weigh 0,6 g ± 0,01 g of ammonium acetate and transfer into a 500 ml beaker. In a fume hood, slowly add approximately 300 ml of HPLC water and 2,4 ml of glacial acetic acid. Add 125 g of ascorbic acid and 5 ml of 2-mercaptoethanol. Stir to dissolve completely. Adjust pH to = 5,5 using concentrated ammonium hydroxide (28 % to 30 %). Transfer the solution into a 500 ml volumetric flask and bring to volume (500 ml) with HPLC water. Mix thoroughly.

5.2.2 Intermediate solvent, c = 1,6 × 10^{-3} mol/l ammonium acetate buffer with 1 % ascorbic acid, pH = 5,5.

Accurately weigh 0,12 g ± 0,001 g ammonium acetate and transfer into a 1 l beaker. In a fume hood, slowly add approximately 700 ml of HPLC water and 480 µl of glacial acetic acid. Add 10 g of ascorbic acid and stir until completely dissolved. Adjust pH to pH = 5,5, if necessary, with concentrated ammonium hydroxide. Transfer the solution to a 1 l volumetric flask and bring to volume with HPLC water. Mix thoroughly.

5.2.3 Solvent for SPE elution and folates standards, methanol with 10 % formic acid and 1 % ascorbic acid.

Take 1 g of ascorbic acid and 10 ml of concentrated formic acid into a 100 ml volumetric flask. Add approximately 50 ml methanol. Sonicate in a water bath (at room temperature) for complete dissolution (usually takes about 2 min). Bring to 100 ml volume with methanol. Mix thoroughly.

Use this solution to elute folates from SPE sorbent after clean up, to make analytical standards and as a blank in the LC-MS/MS analysis. Prepare fresh on the day of use.

5.3 Preparation of folate standard solutions

5.3.1 Folate stock standards, mass concentration ρ = 500 µg/ml.

Accurately weigh approximately 25 mg of each of folic acid, 5-CH_3-THF, 5-CHO-THF and Pte-Glu3 into separate 50 ml low-actinic volumetric flasks. Add approximately 35 ml of the stock solvent to each flask and sonicate in a water bath for about 1 min for complete dissolution of the folate chemical. Add the least amount of ammonium hydroxide solution (28 % to 30 %) to aid in dissolution of folic acid; it can take approximately 30 drops to 48 drops (1,5 ml to 2,4 ml). Make up to 50 ml with folate stock solvent in each of the flask and mix the contents.

Transfer solutions to glass vials of capable of maintaining integrity at the targeted low temperature. The use of plastic vials has no demonstrable issues in storage. Store at –20 °C or lower temperatures (i.e. –70 °C for better stability). Folate stock standard solutions (folic acid, 5-CHO-THF and Pte-Glu3) stored at –70 °C are stable for six months, and 5-CH_3-THF is stable for three months. Solutions stored at –20 °C are stable for 30 days.

Calculate the correct concentration of each of the folate compound's respective stock solution after adjusting for the moisture content in the folate chemical and their respective purity (HPLC) based on the certificate provided by the supplier. Folic acid may have moisture content as high as 7,9 % and 5-formyl-THF as high as 14,9 %. Typical purity (HPLC based) of the folate compounds is usually 98 % to 99 %. 5-CH_3-THF and 5-CHO-THF chemicals used are often calcium salts.

Calculate the concentration of the stock standard solutions, ρ_{ss}, in micrograms per millilitre, as salt free form based on the differences in their respective molecular weights, using Formula (1):

$$\rho_{ss} = \frac{(m_s \times P) \times (10^6)}{V} \times \frac{M_{rsff}}{M_{rf}} \quad (1)$$

where

m_s is the mass of the standard, in grams, e.g. m_s = 0,025 g;

P is the purity of analyte and moisture contents, e.g. P = 90 % or P = 0,9 g/g;

V is the final volume, in millilitre, e.g. V = 50 ml;

M_{rsff} is the molecular weight of free base or salt free folate;

M_{rf} is the molecular weight of folate salt used.

For example, with the values above and a molecular weight of free base or salt free 5-CHO-THF of 473,4 and a molecular weight of 5-CHO-THF calcium salt of 511,5, the calculation using Formula (1) for ρ_{ss} (salt free) is:

$$\rho_{ss} = \frac{(0{,}025 \times 0{,}9) \times (10^6)}{50} \times \frac{473{,}4}{511{,}5} = 416{,}5$$

5.3.2 Folate intermediate standards, ρ = 20 µg/ml.

Add about 5 ml of the intermediate solvent to a 10 ml low-actinic volumetric flask. Accurately add 0,4 ml of each of the stock standard solutions (5.3.1) of FA, 5-CH_3-THF and 5-CHO-THF to the same flask. Make up volume to 10 ml with the folate intermediate solvent and mix contents.

Calculate the concentration of each folate vitamer in the intermediate standard solution, ρ_{is}, in micrograms per millilitre, using Formula (2):

$$\rho_{is} = \frac{(\rho_{ss} \times V_1)}{V_2} \quad (2)$$

where

V_1 is 0,4 ml;

V_2 is 10 ml.

The folate intermediate standard solution can be stored at −20 °C for 30 days and can be stable up to three months at −70 °C.

5.4 Preparation of folate internal standard stock solutions

5.4.1 $^{13}C_5$-Folic acid internal standard stock solution, ρ = 1 mg/ml.

The chemical is often supplied in a 1 mg amount. Dissolve the entire 1 mg amount of labelled folic acid in 1 ml of the stock solvent. Folic acid is difficult to dissolve. The addition of 10 µl of ammonium hydroxide solution (28 % to 30 %) aids in dissolution. A higher amount of the chemical, if available, can be used to make a stock solution of a final concentration of around 1 mg/ml. Sonication and vortex for 1 min to 2 min can help to complete dissolution.

5.4.2 $^{13}C_5$-(6S)-5-Methyl-5,6,7,8-Tetrahydrofolate internal standard stock solution, ρ = 1 mg/ml.

The labelled methyl-THF may be supplied in a 1 mg amount. Dissolve the entire 1 mg amount of labelled methyl-THF in 1 ml of the stock solvent. A higher amount of the chemical, if available, can be used to make a stock solution of a final concentration of around 1 mg/ml. Dissolve the chemical completely with the aid of vortex and brief (30 s) sonication.

5.4.3 $^{13}C_5$-(6S)-5-formyl-5,6,7,8-Tetrahydrofolate internal standard stock solution, ρ = 1 mg/ml.

Weigh about 10 mg of labelled formyl-THF in a 10 ml volumetric flask. Add about 7 ml of the stock solvent. Dissolve the chemical completely with the aid of vortex and brief (30 s) sonication. Make up volume to 10 ml with the stock solvent and mix the solution.

Folate internal standard stock solutions in 5.4.1, 5.4.2 and 5.4.3 if stored at −70 °C, folic acid and 5-CHO-THF can be stable for six months and 5-CH_3-THF for three months. Solutions can be stable for shorter time periods if stored at −20 °C.

Concentration of each of internal standard stock solution should be calculated after adjustment for their respective purity (based on the manufacturer's certificate).

5.4.4 Internal standard intermediate solution cocktail, ρ = 20 µg/ml of each folate.

Take about 5 ml of the intermediate solvent into a 10 ml low-actinic volumetric flask. Transfer accurately 0,2 ml of each of the internal standard stock solutions, i.e. $^{13}C_5$-FA (5.4.1), $^{13}C_5$-CH_3-THF (5.4.2) and $^{13}C_5$-CHO-THF (5.4.3) into the same 10 ml volumetric flask. Make up 10 ml with folate intermediate solvent. Thoroughly mix the solution.

Calculate the actual concentrations of each internal standard ρ_{iis}, in micrograms per millilitre, using Formula (3):

$$\rho_{iis} = \frac{(\rho_{ss} \times V_1)}{V_2} \quad (3)$$

where

ρ_{iis} is concentration of the internal standard intermediate solution after adjusting for purity;

V_1 is 0,2 ml;

V_2 is 10 ml.

The folate standard intermediate solution can be stored at −20 °C for 30 days and can be stable up to three months at −70 °C.

5.4.5 Internal standard solution for calibration standards 1, ρ = 2 µg/ml of each internal standard folate.

Take 20 ml of solvent for SPE elution (5.2.3) in a 50 ml centrifuge tube. Add 1 ml of ammonium hydroxide (28 % to 30 %) and mix (neutralized solvent for SPE elution). Prepare fresh before use.

Accurately pipette 100 µl of the folate internal standard intermediate solution (ρ is approximately 20 µg/ml) (5.4.4) into a microcentrifuge tube. Accurately add 900 µl of the freshly prepared neutralized solvent for SPE elution.

Mix thoroughly by a brief vortex for about 30 s. Prepare solution fresh before use. Store at 4 °C if necessary but not for more than 6 h. Calculate the concentration of each folate vitamer, ρ_{is1} in micrograms per millilitre using Formula (4):

$$\rho_{is1} = \rho_{is} \frac{V_1}{V_2} \quad (4)$$

where

V_1 is 0,1 ml;

V_2 is 1 ml.

5.4.6 Internal standard solution for calibration standards 2, ρ = 40,0 ng/ml each folate.

Take 12 ml of solvent for SPE elution in a scintillation vial or a 50 ml plastic centrifuge tube. Add 600 µl of ammonium hydroxide (28 % to 30 %) (neutralized solvent for SPE elution). Mix. Prepare fresh before use.

To prepare an internal standard solution for calibration standards 2, pipette about 5 ml of neutralized solvent for SPE elution into a 10 ml low actinic volumetric flask. Pipette 20,0 µl (ρ is approximately 20 µg/ml of each folate) of the internal standard intermediate solution into the same 10 ml volumetric flask. Make up volume to 10 ml with the neutralized solvent for SPE elution. Mix thoroughly. Prepare solution fresh before use. If the next step has to be delayed, the solution can be stored at the lower temperature up to 6 h. Alternatively, the solution can be prepared in advance and stored up to 6 h at 4 °C.

The concentration, in µg/ml, of each folate vitamer in the 40,0 ng/ml internal standard solution equals the concentration, in µg/ml, of the 20 µg/ml internal standard solution times 0,020 0 ml, divided by 10 ml.

5.5 Preparation of calibration standard solutions

Prepare fresh before the analysis. Store at 4 °C if necessary but not for more than 6 h.

Take 12 ml of solvent for SPE elution in a scintillation vial or a 50 ml plastic centrifuge tube. Add 600 µl of ammonium hydroxide (neutralized solvent for SPE elution). Mix. Prepare fresh before use. This solution is also used as an instrument blank in analysis.

The calibration standard solutions are prepared as detailed in Table 1. Set up microfuge tubes labelled calibration standard A through G separately. Standards A and B are prepared using folate intermediate standard solutions whereas calibrants C through G are prepared by dilutions of higher concentration calibrants.

Add intermediate standard solution as specified in Table 1 in the corresponding calibration tube A and B. Add 20 µl of the 2 µg/ml internal standard solution (5.4.5) to tubes A and B. Add neutralized elution solvent to tubes A and B as specified in Table 1. Close the tubes and mix thoroughly by brief vortex for about 20 s.

Prepare calibration standards C through G by dilutions of the Table 1 specified calibration standards. Pipette the specified calibration standard in specified volume. Make up volume to 1 ml with the 40,0 ng/ml internal standard solution (5.4.6) as specified in Table 1. Close the tubes and mix thoroughly by brief vortex for about 20 s.

Calculate exact concentration of each folate compound including internal standard in each calibration standard by following the dilution of the intermediate standard solution or the respective calibrant in the solution and the used internal standard solution.

Table 1 — Preparation of folate calibration standard solutions

Calibration standard	Volume of intermediate/ calibration STD solution µl	Volume internal standard solution 1 (5.4.5) µl	Volume internal standard solution 2 (5.4.6) µl	Volume of neutralized elution solvent µl	Final volume ml	Concentration of each folate compound in the standard solution ng/ml
A	25 [a]	20	0	955	1,0	500
B	10 [a]	20	0	970	1,0	200
C	100 [b]	0	900[f]	0	1,0	50
D	100 [c]	0	900[f]	0	1,0	20
E	100 [d]	0	900[f]	0	1,0	5
F	100 [e]	0	900[f]	0	1,0	2
G	50 [e]	0	950[f]	0	1,0	1

NOTE The internal standard concentration in every calibration standard solution (A through G) is 40 ng/ml of each folate.

[a] Amount of intermediate standard solution.

[b] Amount of calibration standard solution A.

[c] Amount of calibration standard solution B.

[d] Amount of calibration standard solution C.

[e] Amount of calibration standard solution D.

[f] Amount of internal standard solution for calibration standard 2.

5.6 Preparation of substrate solution to test plasma conjugase activity

5.6.1 Substrate solution to test plasma conjugase activity, ρ = 20 µg/ml of Pte-Glu3.

Add about 5 ml of intermediate solvent into a 10 ml low-actinic volumetric flask. Pipette 0,4 ml, of Pte-Glu3 stock solution (500 µg/ml). Make up volume to 10 ml with intermediate solvent. Mix thoroughly. Store at −20 °C for one month; can be stable for longer time periods at −70 °C.

5.7 Reagent for folate analysis

5.7.1 Protease solution, ρ = 2 mg/ml.

Prepare fresh before use. Store at 4 °C to 8 °C if necessary but not for more than 4 h. Dissolve 0,05 g protease in 25 ml of water in a 100 ml beaker or conical shaped flask by proper mixing. Each sample requires 1 ml.

5.7.2 α-Amylase solution, ρ = 20 mg/ml; about 300 units/ml.

Prepare fresh before use. Store at 4 °C to 8 °C if necessary but not for more than 4 h. Dissolve 0,5 g in about 20 ml of water in a 25 ml volumetric flask or a centrifuge tube. Make up volume to 25 ml. Mix gently for complete mixing. Make sure no foams develop.

The amylase solution is transferred to a 50 ml centrifuge tube if not already in one. Treat amylase solution with charcoal. Add 20 mg charcoal per ml of solution (0,5 g in 25 ml). Gently mix on a vortex mixer for about 45 s. Let stand for 5 min at 4 °C to 8 °C. Filter through a 0,45 µm PVDF syringe filter. Each sample requires 1 ml.

5.7.3 Male rat plasma conjugase.

The plasma is kept frozen at −20 °C or lower temperature until use. Check viability of plasma stored over three months. Avoid repeated freezing thawing of plasma to avoid losses in its conjugase activity. Plasma not subjected to repeated freezing and thawing will usually retain most of its conjugase activity even after 6 months of storage at -20 °C. Thaw plasma as required. Treat plasma with 20 mg charcoal per ml. Take 5 ml of plasma in a tube. Add 100 mg of charcoal. Gently mix with vortex mixer for about 30 s. Let stand for 5 min at 4 °C to 8 °C. Filter through a PVDF syringe filter. About 4,2 ml of plasma from 5 ml is usually obtained after charcoal treatment. Prepare fresh. Store at 4 °C to 8 °C if necessary but not for more than 4 h. Each sample requires 0,3 ml of the charcoal-treated plasma. Efficiency of the conjugase shall be determined prior to use but only once for every new lot of plasma.

To check the efficiency of rat plasma conjugase:

— Add 30 µl of conjugase test solution to a 50 ml centrifuge tube with 10 ml extraction buffer (containing only $^{13}C_5$ folic acid internal standard, 4 ng/ml) and 50 µl TCEP solution. Mix with a vortex mixer (reaction tube). For the conjugase test blank, add 30 µl of the intermediate solvent to another 50 ml centrifuge tube with 10 ml extraction buffer and 50 µl TCEP solution. Mix with a vortex mixer. Add 300 µl of charcoal-treated conjugase to both tubes, fill headspace with nitrogen, cover, and incubate for 16 h in a shaking water bath at 37 °C and 60 r/min shaking speed. Follow steps 7 through 15 of the procedure for analysis of folates. The amount of folic acid liberated by the conjugase action in the reaction tube and that is present in the test blank are determined by following the analysis method starting from 7.3 (extract purification) followed by the instrumental analysis (7.4) and the relevant calculations as explained in Clause 8.

— Using the result obtained and concentration of PteGlu3 in test solution prepared, determine conversion of PteGlu3 to folic acid. Conjugase is considered acceptable for use if conversion of the PteGlu3 to folic acid is ≥ 90 %. The protocol to calculate conversion of PteGlu3 to folic acid is located in Clause 8.

5.7.4 TCEP solution, c = 1 mol/l.

Dissolve 1 g of TCEP-HCl in 3,5 ml of water in a test tube or a vial.

5.7.5 Extraction buffer, $c = 10 \times 10^{-3}$ mol/l phosphate buffer, 1 % ascorbic acid, pH = 6,0.

Weigh 0,355 g of sodium phosphate, dibasic, anhydrous Na_2HPO_4 in a 250 ml beaker. Add 2,5 g of ascorbic acid and approximately 200 ml of HPLC water. Stir until completely dissolved. Adjust pH with phosphoric acid and/or sodium hydroxide solution (c = 10 mol/l NaOH) to pH = 6,0. Transfer into a 250 ml volumetric flask and dilute to volume with HPLC water. Mix. This can be stored at 4 °C for 5 days.

5.7.6 Extraction buffer with internal standard, $c = 10 \times 10^{-3}$ mol/l phosphate buffer, 1 % ascorbic acid, pH = 6,0, internal standard = 4 ng/ml of each folate.

Add exactly 0,05 ml of the 20 µg/ml internal standard intermediate solution to 250 ml of the buffer. Mix thoroughly. Internal standard concentration is 4 ng/ml of each folate. Prepare fresh on day of use. Store at 4 °C if necessary but not for more than 6 h after addition of internal standards. Each sample requires 10 ml of the extraction buffer.

5.7.7 Extraction buffer with only folic acid internal standard to check efficiency of rat plasma conjugase, $c = 10 \times 10^{-3}$ mol/l phosphate buffer, 1 % ascorbic acid, pH = 6,0, folic acid internal standard = 4 ng/ml.

This requires first a preparation of the folic acid (only) internal standard intermediate solution cocktail, $\rho = 20$ µg/ml folic acid. Take about 5 ml of the intermediate solvent into a 10 ml low-actinic volumetric flask. Transfer accurately 0,2 ml of the folic acid internal standard stock solutions, i.e. $^{13}C_5$-FA (5.4.1) into a 10 ml volumetric flask. Make up to 10 ml with folate intermediate solvent. Thoroughly mix the solution. Calculate the actual concentrations of folic acid internal standard ρ_{iisf}, in micrograms per millilitre, using Formula (3) with the following change to limit calculation to folic acid (replace ρ_{iis}, ρ_{iisf}). Where ρ_{iisf} is concentration of the folic acid internal standard intermediate solution after adjusting for purity.

Add exactly 0,05 ml of the 20 µg/ml folic acid internal standard intermediate solution prepared as described above to 250 ml of the extraction buffer (5.7.5). Mix thoroughly. Folic acid internal standard concentration = 4 ng/ml. Prepare fresh on day of use. Store at 4 °C if necessary but not for more than 6 h after addition of folic intermediate internal standard. Each assay to check efficiency of rat plasma conjugase requires 10 ml of the extraction buffer containing folic acid internal standard.

5.7.8 UHPLC mobile phase A.

Thoroughly mix 500 ml of water (UHPLC/MS) and 5 ml of glacial acetic acid.

5.7.9 UHPLC mobile phase B.

Methanol (LC/MS) as is.

6 Apparatus

6.1 Precision balance, reading 0,1 mg.

6.2 UHPLC system, Agilent 1290 Infinity Binary UHPLC System[2)] or Dionex UltiMate 3000 LC[2)] (used in validation) or equivalent.

6.3 LC-MS/MS system, Sciex 6500 or 5500 Triple QUAD[2)] (used in validation) or equivalent.

6.4 pH meter.

6.5 Refrigerator, capable of cooling to 4 °C.

6.6 Freezer, capable of cooling to −20 °C and −70 °C; explosion proof.

6.7 Water bath, with shaker.

6.8 Fume hood.

2) These 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.

6.9 Centrifuge, capable of up to 1 400 g and cooling capability (4 °C to 5 °C) equivalent to Sorvall Legend X1R[2] and rotor F15-8x50c with radius (R_{max} = 10,4 cm)[2].

6.10 Microcentrifuge, capable of up to 9 500 *g*.

6.11 Adjustable mechanical/electronic pipettes, volumes of 100 µl to 1 000 µl.

6.12 Vortex mixer.

6.13 SPE vacuum manifold, 24 well.

6.14 SPE columns, mixed mode weak anion exchange (WAX SPE), 33 µm polymeric, 60 mg/3 ml, Phenomenex, 8B-S038-UBJ[2].

6.15 UHPLC column, Waters ACQUITY UPLC HSS T3 Column[2] (100 Å, 1,8 µm, 2,1 mm × 50 mm), or equivalent.

6.16 Glass test tubes, 16 mm × 125 mm.

6.17 Centrifuge tubes, 50 ml polypropylene.

6.18 Syringe filters, polyvinyl difluoride (PVDF), 0,45 µm.

6.19 Microcentrifuge tubes, polypropylene, volume 1,5 ml to 2,0 ml.

6.20 Volumetric flasks, assorted sizes.

6.21 Glass Pasteur pipettes.

6.22 Volumetric pipettes, Class A, assorted sizes.

6.23 Repeating pipettes, 5 ml and 25 ml.

6.24 Glass beakers, assorted sizes.

6.25 Amber glass HPLC vials with septa and caps.

6.26 Low-actinic glass bottles, 250 ml to 500 ml.

7 Procedure

7.1 Sample preparation

7.1.1 Sample processing to make them homogenous

All samples should be as uniform and representative of the product as possible. This should be accomplished by a thorough mixing or stirring the sample before sampling. The dry samples are reconstituted to liquid samples and reconstituted liquid samples are used for analysis.

7.1.2 Reconstitution of powder sample into liquid

Weigh 25,0 g ± 0,1 g of dry sample into a low-actinic glass 250 ml beaker or bottle. If low-actinic bottles or beakers are not available, cover the outside of the bottle or beaker with aluminium foil to protect the vitamins from light. Note the masses. Add 200,0 g ± 0,1 g of warm water (40 °C ± 5 °C). Mix well until complete dissolution/suspension, making sure there are no lumps. Record final mass for each sample. Calculate mass (g) of dry sample in the corresponding reconstituted liquid sample in every case. Dry samples are analysed as reconstituted liquid samples, and ready to feed (RTF) samples are analysed as is. Reconstituted samples, as well as RTF samples, are thoroughly mixed immediately before weighing the aliquot for the analysis.

7.2 Extraction

Weigh 0,1 g to 2,5 g of sample, depending on anticipated total folate (in range of 20 ng to 500 ng), into a 50 ml centrifuge tube and record the mass to 0,000 1 g. Prepare a method blank containing reagents only. This is treated like a sample throughout the entire following method.

Add 10 ml extraction buffer (containing internal standards) and 50 µl of TCEP solution to each tube and mix with a vortex mixer. Add 1 ml protease solution to each tube, fill the headspace with nitrogen, cover and mix with a vortex mixer. Incubate for 3 h in a shaking water bath at 37 °C at 60 r/min shaking speed.

Inactivate the protease by placing the tubes in a boiling water bath (100 °C) for 5 min. Shake tubes every 1,5 min, and then remove from bath and cool to room temperature.

Add 1 ml of α-amylase solution (charcoal-treated) and 300 µl of rat plasma conjugase (charcoal-treated) to each tube. Fill the headspace with nitrogen, cover and incubate for 16 h in a shaking water bath at 37 °C at 60 r/min shaking speed.

Inactivate enzymes after 16 h incubation by placing the tubes in a boiling water bath (100 °C) for 5 min. Shake tubes every 1,5 min, and then remove from bath and cool to room temperature.

Centrifuge the tubes at 4 °C to 5 °C for 10 min at 1 400 *g*.

Filter supernatant from each tube through a 0,45 µm PVDF syringe filter into separate sample labelled glass tubes (16 mm × 125 mm).

7.3 Extract purification

Set up a 24-well SPE manifold. Set up one SPE tube for every sample and one for the method blank. Condition each SPE tube as follows, without vacuum:

— first, condition with 2 ml methanol;

— before the SPE sorbent is dry, repeat the first step with 2 ml of water.

Load samples into corresponding activated SPE tubes over sorbent, without vacuum. First, load all of the filtered supernatant (about 12 ml) for every sample into the corresponding SPE tube (no vacuum). Then, wash each SPE tube with 3 ml water with no vacuum. Push out any residual water by gentle push with a pipette bulb.

Elute the analytes from the SPE sorbent. Empty the waste reservoir and place a clean microcentrifuge tube to collect the eluent corresponding to every SPE tube. Add 1 ml of the freshly prepared solvent for SPE elution to each SPE tube. Allow gravity to elute the effluent into the tubes and use a pipette bulb to push out any remaining solvent. Add 50 µl ammonium hydroxide (28 % to 30 %) to each tube and immediately mix with a vortex mixer for 20 s. Centrifuge the tubes in a microcentrifuge at 9 500 *g* for 5 min.

Transfer supernatant from each tube into a corresponding HPLC vial for LC-MS/MS analysis.

Prepare LC and mass spectrometer for analysis and load vials in autosampler. The sequence of the analysis includes instrument blank (neutralized SPE elution solvent), calibration standards (G to A), instrument blank, method blank and all of the samples.

7.4 Instrumental analysis

7.4.1 Analysis of instrument blank, calibration standards, method blank and samples

Instrument blank and calibration standards G to A, as detailed in Table 1, are analysed. This is followed by analysis of an instrument blank, method blank and the sample extracts.

7.4.2 UHPLC conditions and MS parameters for different systems

UHPLC conditions with Dionex UltiMate 3000 LC/Agilent 1290 LC[2)].

— UHPLC Column: Waters ACQUITY UPLC HSS T3[2)] (100 Å), 1,8 µm, 2,1 mm × 50 mm.

— Mobile phases: A = 1 % acetic acid, B = 100 % methanol.

— Solvent composition: Gradient, see Table 2.

— UHPLC column temperature: 40 °C.

— Autosampler temperature: 5 °C ± 1 °C.

— Injection volume: 2 µl.

Table 2 — LC gradient

Time min	Mobile phase B %	Flow ml/min
0,00	0	0,5
0,50	0	0,5
5,00	40	0,5
5,05	100	0,5
6,50	100	0,5
6,55	0	0,5
9,00	0	0,5

MS source conditions for some of the Sciex triple quads[2)] are provided in Table 3.

Table 3 — MS source conditions Sciex triple quads[2)]

Source condition parameters	Sciex triple quad		
	5500	6500 Qtrap	6500 Plus
Curtain gas (nitrogen), kPa (psi)	172 (25)	207 (30)	207 (30)
Collision-activated dissociation	medium	medium	8
IonSpray voltage, V	5 500	5 500	5 500
Temperature, °C	700	400	400
Ion source gas 1 (zero air), kPa (psi)	414 (60)	414 (60)	414 (60)
Ion source gas 2 (zero air), kPa (psi)	345 (50)	345 (50)	345 (50)

Multiple reaction monitoring (MRM) transitions for different folates are provided in Table 4.

Table 4 — MRM transitions for different folates and MS parameters for their detection

Folate	Q1[a]	Q2[b]	DP[c]	EP[d]	CXP[e]	CE[f]
FA	442,2	295,2	75	6	6	20
	442,2	176,2	75	6	6	45
5-CH_3-THF	460,2	313,2	75	6	6	28
	460,2	194,2	75	6	6	46
5-CHO-THF	474,2	327,2	75	6	6	25
	474,2	299,2	75	6	6	40
$^{13}C_5$-FA	447,2	295,2	75	6	6	20
$^{13}C_5$-5-CH_3-THF	465,2	313,2	75	6	6	28
$^{13}C_5$-5-CHO-THF	479,2	327,2	75	6	6	25

[a] Q1 = Precursor ion (m/z).

[b] Q2 = Product ion (m/z).

[c] DP = declustering potential (V).

[d] EP = entrance potential (V).

[e] CXP = collision cell exit potential (V).

[f] CE = collision cell potential (V).

An example of a folate analysis elution pattern of different folate vitamers is: 5-CH_3-THF at 2,82 min, 5-CHO-THF at 3,60 min and folic acid at 3,91 min.

8 Calculations

8.1 Calculation of ratios of peak areas of the folate compounds to the respective internal standard peak areas

Calculate the ratios of peak areas of the folate vitamer against the respective internal standard area for all calibration standards, method blank and all of the sample extracts.

8.2 Calibration curve

Draw a calibration curve by plotting the concentration of each calibration standard (ng/ml) of a specific folate compound against the corresponding ratio of its peak area to the relevant internal standard peak area. Prepare the calibration curves for all the targeted folate compounds. Do not force the calibration curves through zero (weighting 1/x.) This calculation is usually done by the software used for the processing of MS-acquired data.

8.3 Calculation of concentration of folate compounds in the method blank

Calculate the ratios of peak areas of the detected folate compounds to the respective internal standards in the method blank. Calculate the concentration of each of the detected folate compound as ng/ml based on the respective calibration curve. This is usually done by the software used for the processing of the MS data.

8.4 Calculation of concentration of folate compounds in the sample extracts

Calculate the ratios of peak areas of the detected folate vitamers to the respective internal standard areas for every sample. Calculate the concentration of each of the folate compound as ng/ml based on the calibration curve of the targeted folate compound. This calculation is usually performed by the software used for processing of MS data.

The concentration of each of the folate compounds as ng/ml in a sample extract is adjusted by subtracting the amount of respective folate compound detected in the method blank ran in the batch.

Calculate the corrected folate compound mass concentration in the sample extracts, ρ_{cfc}, in ng per ml using Formula (5):

$$\rho_{cfc} = \rho_{sfc} - \rho_{sfcmb} \quad (5)$$

where

ρ_{cfc} is the corrected folate compound mass concentration in sample extracts, in ng/ml;

ρ_{sfc} is the mass concentration of the specific folate compound, in ng/ml;

ρ_{sfcmb} is the mass concentration of the respective specific folate compound in the method blank, in ng/ml.

8.5 Calculation of folate compounds and total folate in reconstituted or RTF samples (µg/100 g)

Multiply estimated folate vitamer concentration (ng/ml) in the extract by fold dilutions (if any) and the final volumes of the relevant step (volume after SPE elution, 1,05 ml). Adjust the total amount of folate vitamer in the sample for the aliquot of the sample used for the extraction. The folate vitamer is then calculated as µg/100 g of the reconstituted or RTF sample.

Calculate the mass fraction of folate vitamer in the reconstituted or RTF sample, w_{fvrec}, in µg per 100 g using Formula (6):

$$w_{fvrec} = \rho_{ve} \times \frac{V_f}{m_{sa}} \times 0,1 \quad (6)$$

where

w_{fvrec} is the mass fraction of folate vitamer in the reconstituted or RTF sample, in µg/100 g;

ρ_{ve} is the mass concentration of vitamin in the extract, in ng/ml;

V_f is the final volume, V_f = 1,05 ml;

m_{sa} is the mass of the sample aliquot, in g.

The amount of CH_3-THF and CHO-THF (µg/100 g) in the samples is calculated as folic acid by multiplying (µg/100 g) concentration of the folate compound by the corresponding conversion factors provided in Table 5. Total folate in the reconstituted (RCS) or RTF sample is calculated by the sum of all folate compound as folic acid (µg/100 g).

Table 5 — Conversion of amount of 5-CH_3-THF and 5-CHO-THF to folic acid equivalent

Folate vitamers	Formula weight	Multiplication factor to convert to FA	Example value µg/100 g	Value as FA µg/100 g
FA	441,4	1	100,0	100,0
5-CH_3-THF	459,5	0,960 6	100,0	96,1
5-CHO-THF	473,4	0,932 4	100,0	93,2
Total folates as FA			sum as FA	289,3

Total folate as folic acid µg/100 g in RCS or RTF samples = folic acid (µg/100 g) + (5-CH_3 – THF (µg/100 g) × 0,960 6) + (5-CHO-THF (µg/100 g) × 0,932 4).

8.6 Calculation of total folate in powder samples (µg/100 g) as-is basis

Calculate the mass fraction of total folate, w_{tfai}, in µg per 100 g in dry sample as-is basis from total folate (µg/100 g) in reconstituted beverage samples using Formula (7):

$$w_{tfai} = w_{tfrec} \times \frac{m_{tot}}{m_{ali}} \quad (7)$$

where

w_{tfai} is the mass fraction of total folate in an as-is basis in dry samples analysed as RCS, in µg/100 g;

w_{tfrec} is the mass fraction of total folate in the reconstituted sample, in µg/100 g;

m_{tot} is the total mass of the reconstituted beverage sample, in g;

m_{ali} is the mass of the aliquot of the powder sample used for reconstitution, in g.

8.7 Calculation of folic acid nanograms released in the conjugase test

The ratios of peak areas of folic acid and folic acid internal standard are calculated in the assay solution. Folic acid (ng/ml) in solution is calculated based on the calibration curve. Folic acid (ng/ml) is also calculated in conjugase test blank.

Blank corrected folic acid, in ng/ml, in the assayed mixture equals folic acid, in ng/ml, in the assayed mixture minus the folic acid, in ng/ml, in the conjugase test blank.

Adjusted folic acid released in whole assayed mixture equals the blank corrected folic acid, in ng/ml, times the final volume of assay mixture after purification (SPE elute volume is 1,05 ml).

8.8 Calculation of per cent conversion of Pte-Glu3 to folic acid in the conjugase assay

Folic acid (ng) released in the conjugase assay is converted to folic acid (nanomoles) by the following equation. The folic acid in nanomoles in the assay mixture is the folic acid, in ng, in the assay divided by 441,4, the molecular weight of folic acid. Pte-Glu3 in the conjugase assay is 0,03 ml times 20 µg/ml (in conjugase activity substrate solution) is 600 ng.

Pte-Glu3, in nanomoles, in the conjugase assay is 0,03 ml times 20 µg/ml in the conjugase activity substrate solution divided by 699,6, the molecular weight of Pte-Glu3.

Each molecule of Pte-Glu3 on hydrolysis yields one molecule of folic acid.

The percentage conversion of Pte-Glu3 to folic acid is the amount of folic acid in nanomoles released in the assay divided by Pte-Glu3 in nanomoles in the assay times 100.

9 Precision

9.1 General

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

9.2 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. A summary of the repeatability data obtained in the study of the method is given in Table 6.