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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 haute  
performance (CLUHP) couplée à une spectrométrie de masse en  
tandem (SM/SM)*

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## Foreword

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

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](http://www.iso.org/directives)).

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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](http://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 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](http://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<sub>3</sub>-THF) and, 5-formyl-tetrahydrofolate (5-CHO-THF) are quantified, and total folate is estimated and expressed as folic acid. Isotopically labelled folic acid (<sup>13</sup>C-folic acid), 5-CH<sub>3</sub>-THF (<sup>13</sup>C-5-CH<sub>3</sub>-THF) and 5-CHO-THF (<sup>13</sup>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  $-20\text{ }^{\circ}\text{C}$  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<sup>1)</sup>.

**5.1.2 (6R,S)-5-Methyl-5,6,7,8-Tetrahydrofolic acid (5-CH<sub>3</sub>-THF)**, calcium salt, Schircks Laboratories, Cat. No. 16.235<sup>1)</sup>.

**5.1.3 (6S)-5-Formyl-5,6,7,8-Tetrahydrofolic acid (5-CHO-THF)**, calcium salt, Schircks Laboratories, Cat. No. 16.221<sup>1)</sup>.

**5.1.4 Pteroyltri-γ-L-glutamic acid (Folic acid triglutamate) (Pte-Glu3)**, Schircks Laboratories, Cat. No. 16.253<sup>1)</sup>

An alternate source for this reagent is Toronto Research Chemicals, Toronto, Canada, Cat No P840220<sup>1)</sup>.

**5.1.5 <sup>13</sup>C<sub>5</sub>-Folic Acid (internal standard) (<sup>13</sup>C<sub>5</sub>-FA)**, IsoSciences, PA, Cat# 14139<sup>1)</sup>.

**5.1.6 <sup>13</sup>C<sub>5</sub>-labelled (6S)-5-methyltetrahydrofolic acid (<sup>13</sup>C<sub>5</sub>-CH<sub>3</sub>-THF)**, calcium salt (internal standard), IsoSciences, PA, Cat# 14168Ca<sup>1)</sup>.

**5.1.7 <sup>13</sup>C<sub>5</sub>-labelled (6S)-5-formyltetrahydrofolic acid (<sup>13</sup>C<sub>5</sub>-CHO-THF)**, calcium salt, (internal standard), Merck Cie<sup>1)</sup>.

**5.1.8 α-Amylase from *Aspergillus oryzae***, powder,  $\geq 150$  units/mg protein, Sigma-Aldrich, Cat. No. A9857<sup>1)</sup>.

**5.1.9 Protease from *Bacillus licheniformis*, Subtilisin A**, lyophilized powder, Sigma-Aldrich Cat. No. P3910<sup>1)</sup>.

**5.1.10 Conjugase (Male Sprague Dawley Rat Plasma) with lithium and heparin (not filtered)**, BIOIVT Cat. No. RAT00PLLHMNN<sup>1)</sup>.

An alternate source for the rat plasma reagent is Odine Bioscience, 1621 Central Ave., Cheyenne, WY 82001, USAinf@odinbioscience.com<sup>1)</sup> or Lampire Biological Laboratories, Pipersville, 18947. PA, USA<sup>1)</sup>.

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.

After careful evaluation of the activity on the substrate, pancreatic conjugase can be also used. A source for this reagent is  $\gamma$ -glutamyl hydrolase, turkey pancreas, order# 2800, ASA Enzymes, Niedersachsen, 38302, Germany<sup>1)</sup>.

**5.1.11 Ammonium hydroxide solution**, (certified ACS Plus) 28 % to 30 % (mass fraction), (aqueous ammonia), Fisher, Cat. No. A669-500<sup>1)</sup>.

**5.1.12 Sodium phosphate**, dibasic anhydrous (granular or powder/certified ACS,  $\geq 99$  %), Fisher Cat. No. S-374-500<sup>1)</sup>.

**5.1.13 Methanol**, Optima LC/MS grade, 99,9 % minimum, Fisher Cat. No. A456-4<sup>1)</sup>.

**5.1.14 Glacial acetic acid**, (certified ACS), Fisher, Cat. No. A38S-212<sup>1)</sup>.

**5.1.15 Sodium hydroxide**, (pellets/ACS Certified),  $\geq 97$  %, Fisher Cat. No. S318-1<sup>1)</sup>.

**5.1.16 Ammonium acetate**, (crystalline/certified ACS),  $\geq 97$  %, Fisher Cat. No. A637-500<sup>1)</sup>.

**5.1.17 2-Mercaptoethanol**, (electrophoresis grade),  $\geq 98$  %, Fisher Cat. No. BP176-100; Sigma M6250<sup>1)</sup>.

**5.1.18 Ascorbic acid**, (white crystalline powder),  $\geq 99$  %, Fisher Cat. No. BP351-500<sup>1)</sup>.

**5.1.19 Tris-(2-carboxyethyl)phosphine, hydrochloride (TCEP-HCl)**, Fisher, Cat. No. AC 363830100<sup>1)</sup>.

**5.1.20 Charcoal**, (Darco G-60 activated carbon), Fisher Cat. No. D127-500<sup>1)</sup>.

**5.1.21 Formic acid**, (reagent grade),  $\geq 95$  %, water  $\leq 2,5$  %, acetic acid  $< 1$  %, Sigma F0507<sup>1)</sup>.

**5.1.22 Water**, (high purity, suitable for HPLC mobile phase), resistivity up to 18 M $\Omega$ -cm<sup>1)</sup>.

## 5.2 Preparation of solvent for standard solutions

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

Accurately weigh 0,6 g  $\pm$  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**,  $c = 1,6 \times 10^{-3}$  mol/l ammonium acetate buffer with 1 % ascorbic acid, pH = 5,5.

Accurately weigh 0,12 g  $\pm$  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  $\mu$ l 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  $\rho = 500 \mu\text{g/ml}$ .

Accurately weigh approximately 25 mg of each of folic acid, 5-CH<sub>3</sub>-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<sub>3</sub>-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<sub>3</sub>-THF and 5-CHO-THF chemicals used are often calcium salts.

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

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

where

$m_s$  is the mass of the standard, in grams, e.g.  $m_s = 0,025 \text{ g}$ ;

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

$V$  is the final volume, in millilitre, e.g.  $V = 50 \text{ ml}$ ;

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

$M_{\text{rf}}$  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  $\rho_{\text{ss}}$  (salt free) is:

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



### 5.3.2 Folate intermediate standards, $\rho = 20 \mu\text{g/ml}$ .

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<sub>3</sub>-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,  $\rho_{is}$ , 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  $-20 \text{ }^\circ\text{C}$  for 30 days and can be stable up to three months at  $-70 \text{ }^\circ\text{C}$ .

## 5.4 Preparation of folate internal standard stock solutions

### 5.4.1 <sup>13</sup>C<sub>5</sub>-Folic acid internal standard stock solution, $\rho = 1 \text{ mg/ml}$ .

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  $\mu\text{l}$  of ammonium hydroxide solution (28 % to 30 %) aids in dissolution. A higher amount of the chemicals, 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 <sup>13</sup>C<sub>5</sub>-(6S)-5-Methyl-5,6,7,8-Tetrahydrofolate internal standard stock solution, $\rho = 1 \text{ mg/ml}$ .

<https://standards.iteh.ai/catalog/standards/iso/b0650c23-91b4-48f4-bd6f-70607d07f24c/iso-prf-20631>

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 chemicals, 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 <sup>13</sup>C<sub>5</sub>-(6S)-5-formyl-5,6,7,8-Tetrahydrofolate internal standard stock solution, $\rho = 1 \text{ mg/ml}$ .

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  $-70 \text{ }^\circ\text{C}$ , folic acid and 5-CHO-THF can be stable for six months and 5-CH<sub>3</sub>-THF for three months. Solutions can be stable for shorter time periods if stored at  $-20 \text{ }^\circ\text{C}$ .

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, $\rho = 20 \mu\text{g/ml}$ 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. <sup>13</sup>C<sub>5</sub>-FA ([5.4.1](#)), <sup>13</sup>C<sub>5</sub>-CH<sub>3</sub>-THF ([5.4.2](#)) and <sup>13</sup>C<sub>5</sub>-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  $\rho_{iis}$ , in micrograms per millilitre, using [Formula \(3\)](#):

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

where

- $\rho_{iis}$  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  $-20\text{ }^\circ\text{C}$  for 30 days and can be stable up to three months at  $-70\text{ }^\circ\text{C}$ .

**5.4.5 Internal standard solution for calibration standards 1,  $\rho = 2\text{ }\mu\text{g/ml}$  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  $\mu\text{l}$  of the folate internal standard intermediate solution ( $\rho$  is approximately 20  $\mu\text{g/ml}$ ) ([5.4.4](#)) into a microcentrifuge tube. Accurately add 900  $\mu\text{l}$  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  $4\text{ }^\circ\text{C}$  if necessary but not for more than 6 h. Calculate the concentration of each folate vitamer,  $\rho_{is1}$  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,  $\rho = 42,2\text{ ng/ml}$  to  $\rho = 44,4\text{ ng/ml}$  each folate.**

Take 12 ml of solvent for SPE elution in a scintillation vial or a 50 ml plastic centrifuge tube. Add 600  $\mu\text{l}$  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 2a, pipette about 5 ml of neutralized solvent for SPE elution into a 10 ml low actinic volumetric flask. Pipette 22,2  $\mu\text{l}$  ( $\rho$  is approximately 20  $\mu\text{g/ml}$  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  $4\text{ }^\circ\text{C}$ .

The concentration, in  $\mu\text{g/ml}$ , of each folate vitamer in the 44,4 ng/ml internal standard solution equals the concentration, in  $\mu\text{g/ml}$ , of the 20  $\mu\text{g/ml}$  internal standard solution times 0,022 2 ml, divided by 10 ml.

To prepare an internal standard solution for calibration standards 2b, prepare the internal standard solution as described above for the internal standard solution for calibration standards 2a, except