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Secretariat: AFNOR

# 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)



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## Contents





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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 documentsdocument should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directiveswww.iso.org/directives).

Attention is drawnISO draws attention to the possibility that some of the elementsimplementation of this document may beinvolve the subjectuse of (a) patent(s). ISO takes no position concerning the evidence, validity or applicability of any claimed patent rights. in respect thereof. As of the date of publication of this document, ISO had not received notice of (a) patent(s) which may be required to implement this document. However, implementers are cautioned that this may not represent the latest information, which may be obtained from the patent database available at www.iso.org/patents. 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'sISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT), see www.iso.org/iso/foreword.htmlwww.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.htmlwww.iso.org/members.html.

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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)

## 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~~https://www.iso.org/obp

— IEC Electropedia: available at ~~https://www.electropedia.org/~~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]

## ~~5~~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






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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).

## ~~6~~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.

### ~~6.1~~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)].

[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.





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**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)].

**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 Odine Bioscience, 1621 Central Ave., Cheyenne, WY 82001, ~~USAinf@odinbioscience.com[1)]~~USAinf@odinbioscience.com[1)] or Lampire Biological Laboratories, Pippersville, 18947. PA, USA[1)].

After careful evaluation of the activity on the substrate, pancreatic conjugase can be also used. A source for this reagent is γ-glutamyl hydrolase, turkey pancreas, order# 2800, ASA Enzymes, Niedersachsen, 38302, Germany[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)].

### ~~6.2~~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 ± 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

 

 



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