

**FDA USE ONLY**

DEPARTMENT OF HEALTH AND HUMAN SERVICES  
Food and Drug Administration

**GENERALLY RECOGNIZED AS SAFE  
(GRAS) NOTICE** (Subpart E of Part 170)

GRN NUMBER	DATE OF RECEIPT
ESTIMATED DAILY INTAKE	INTENDED USE FOR INTERNET
NAME FOR INTERNET	
KEYWORDS	

Transmit completed form and attachments electronically via the Electronic Submission Gateway (see *Instructions*); OR Transmit completed form and attachments in paper format or on physical media to: Office of Food Additive Safety (HFS-200), Center for Food Safety and Applied Nutrition, Food and Drug Administration, 5001 Campus Drive, College Park, MD 20740-3835.

**SECTION A – INTRODUCTORY INFORMATION ABOUT THE SUBMISSION**

1. Type of Submission (Check one)  
 New       Amendment to GRN No. \_\_\_\_\_       Supplement to GRN No. \_\_\_\_\_

2.  All electronic files included in this submission have been checked and found to be virus free. (Check box to verify)

3. Most recent presubmission meeting (if any) with FDA on the subject substance (yyyy/mm/dd): 12/17/2018

4. For Amendments or Supplements: Is your (Check one)  
 amendment or supplement submitted in  Yes If yes, enter the date of  
 response to a communication from FDA?  No communication (yyyy/mm/dd): \_\_\_\_\_

**SECTION B – INFORMATION ABOUT THE NOTIFIER**

<b>1a. Notifier</b>	Name of Contact Person William Turney	Position or Title Head of Regulatory Affairs NA	
	Organization (if applicable) DSM Nutritional Products		
	Mailing Address (number and street) 45 Waterview Blvd.		
City Parsippany	State or Province New Jersey	Zip Code/Postal Code 07054	Country United States of America
Telephone Number 9732578325	Fax Number 9732578414	E-Mail Address william-george.turney@dsm.com	
<b>1b. Agent or Attorney (if applicable)</b>	Name of Contact Person	Position or Title	
	Organization (if applicable)		
	Mailing Address (number and street)		
City	State or Province	Zip Code/Postal Code	Country
Telephone Number	Fax Number	E-Mail Address	

**SECTION C – GENERAL ADMINISTRATIVE INFORMATION**

1. Name of notified substance, using an appropriately descriptive term

Calcium L-Methylfolate

2. Submission Format: (Check appropriate box(es))

Electronic Submission Gateway                       Electronic files on physical media

Paper

If applicable give number and type of physical media

1 CD

3. For paper submissions only:

Number of volumes \_\_\_\_\_

Total number of pages \_\_\_\_\_

4. Does this submission incorporate any information in CFSAN's files? (Check one)

Yes (Proceed to Item 5)     No (Proceed to Item 6)

5. The submission incorporates information from a previous submission to FDA as indicated below (Check all that apply)

a) GRAS Notice No. GRN \_\_\_\_\_

b) GRAS Affirmation Petition No. GRP \_\_\_\_\_

c) Food Additive Petition No. FAP \_\_\_\_\_

d) Food Master File No. FMF \_\_\_\_\_

e) Other or Additional (describe or enter information as above) \_\_\_\_\_

6. Statutory basis for conclusions of GRAS status (Check one)

Scientific procedures (21 CFR 170.30(a) and (b))     Experience based on common use in food (21 CFR 170.30(a) and (c))

7. Does the submission (including information that you are incorporating) contain information that you view as trade secret or as confidential commercial or financial information? (see 21 CFR 170.225(c)(8))

Yes (Proceed to Item 8)

No (Proceed to Section D)

8. Have you designated information in your submission that you view as trade secret or as confidential commercial or financial information (Check all that apply)

Yes, information is designated at the place where it occurs in the submission

No

9. Have you attached a redacted copy of some or all of the submission? (Check one)

Yes, a redacted copy of the complete submission

Yes, a redacted copy of part(s) of the submission

No

**SECTION D – INTENDED USE**

1. Describe the intended conditions of use of the notified substance, including the foods in which the substance will be used, the levels of use in such foods, and the purposes for which the substance will be used, including, when appropriate, a description of a subpopulation expected to consume the notified substance.

Calcium L-methylfolate is intended to be used in non-exempt and exempt infant formulas as a partial or complete replacement for folic acid as a source of the vitamin, folate. Use levels will be equivalent on a molar basis to current folic acid use levels, to provide the same amount of folate. Because the intended use is as a replacement for folic acid, dietary exposure to calcium L-methylfolate from the intended use will not increase folate consumption by the intended population

2. Does the intended use of the notified substance include any use in product(s) subject to regulation by the Food Safety and Inspection Service (FSIS) of the U.S. Department of Agriculture?

(Check one)

Yes     No

3. If your submission contains trade secrets, do you authorize FDA to provide this information to the Food Safety and Inspection Service of the U.S. Department of Agriculture?

(Check one)

Yes     No, you ask us to exclude trade secrets from the information FDA will send to FSIS.



**SECTION E – PARTS 2 -7 OF YOUR GRAS NOTICE**

*(check list to help ensure your submission is complete – PART 1 is addressed in other sections of this form)*

- PART 2 of a GRAS notice: Identity, method of manufacture, specifications, and physical or technical effect (170.230).
- PART 3 of a GRAS notice: Dietary exposure (170.235).
- PART 4 of a GRAS notice: Self-limiting levels of use (170.240).
- PART 5 of a GRAS notice: Experience based on common use in foods before 1958 (170.245).
- PART 6 of a GRAS notice: Narrative (170.250).
- PART 7 of a GRAS notice: List of supporting data and information in your GRAS notice (170.255)

**Other Information**

Did you include any other information that you want FDA to consider in evaluating your GRAS notice?

Yes  No

Did you include this other information in the list of attachments?

Yes  No

**SECTION F – SIGNATURE AND CERTIFICATION STATEMENTS**

1. The undersigned is informing FDA that DSM Nutritional Products  
*(name of notifier)*

has concluded that the intended use(s) of Calcium L-Methylfolate  
*(name of notified substance)*

described on this form, as discussed in the attached notice, is (are) not subject to the premarket approval requirements of the Federal Food, Drug, and Cosmetic Act based on your conclusion that the substance is generally recognized as safe under the conditions of its intended use in accordance with § 170.30.

2. DSM Nutritional Products  
*(name of notifier)* agrees to make the data and information that are the basis for the conclusion of GRAS status available to FDA if FDA asks to see them; agrees to allow FDA to review and copy these data and information during customary business hours at the following location if FDA asks to do so; agrees to send these data and information to FDA if FDA asks to do so.

45 Waterview Blvd., Parsippany, NJ 07054  
*(address of notifier or other location)*

The notifying party certifies that this GRAS notice is a complete, representative, and balanced submission that includes unfavorable, as well as favorable information, pertinent to the evaluation of the safety and GRAS status of the use of the substance. The notifying party certifies that the information provided herein is accurate and complete to the best of his/her knowledge. Any knowing and willful misinterpretation is subject to criminal penalty pursuant to 18 U.S.C. 1001.

3. Signature of Responsible Official,  
Agent, or Attorney  


Printed Name and Title  
William Turney - Head of Regulatory Affairs NA

Date (mm/dd/yyyy)  
12/06/2019

**SECTION G – LIST OF ATTACHMENTS**

List your attached files or documents containing your submission, forms, amendments or supplements, and other pertinent information. Clearly identify the attachment with appropriate descriptive file names (or titles for paper documents), preferably as suggested in the guidance associated with this form. Number your attachments consecutively. When submitting paper documents, enter the inclusive page numbers of each portion of the document below.

Attachment Number	Attachment Name	Folder Location (select from menu) (Page Number(s) for paper Copy Only)
	GRAS Notice for the use of Calcium L-Methylfolate in infant formula	Submission

**Add Continuation Page**

**OMB Statement:** Public reporting burden for this collection of information is estimated to average 170 hours per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to: Department of Health and Human Services, Food and Drug Administration, Office of Chief Information Officer, [PRASStaff@fda.hhs.gov](mailto:PRASStaff@fda.hhs.gov). (Please do NOT return the form to this address). An agency may not conduct or sponsor, and a person is not required to respond to, a collection of information unless it displays a currently valid OMB control number.

## GRAS NOTICE FOR THE USE OF CALCIUM L-METHYLFOLATE IN INFANT FORMULA

Prepared for:

Office of Food Additive Safety (HFS-200)  
Center for Food Safety and Applied Nutrition  
Food and Drug Administration  
5100 Campus Drive  
College Park, MD 20740-3835

Submitted by:

DSM Nutritional Products  
45 Waterview Blvd  
Parsippany, NJ 07054

December 6<sup>th</sup>, 2019

# GRAS Notice for the use of Calcium L-Methylfolate in infant formula

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# 1 SIGNED STATEMENTS AND CERTIFICATION

## 1.1 Claim of GRAS notice

In accordance with 21 CFR §170 Subpart E, DSM Nutritional Products (hereafter referred to as DSM or the Notifier) hereby informs the United States (U.S.) Food and Drug Administration (FDA) of their view that the use of calcium L-methylfolate in infant formula as described in this GRAS notice is not subject to the premarket approval requirements of the Federal Food, Drug and Cosmetic Act (FD&C Act) based on DSM's conclusion through scientific procedures that this use is Generally Recognized As Safe (GRAS).

## 1.2 Name and address of the Notifier

William Turney  
Head of Regulatory Affairs North America  
DSM Nutritional Products  
45 Waterview Blvd  
Parsippany, NJ 07054  
Tel: (973) 257 8325  
E-mail: William-george.turney@dsm.com

## 1.3 Name of the notified substance

The subject of this GRAS notification is calcium L-methylfolate.

## 1.4 Intended conditions of use

Calcium L-methylfolate is intended to be used in non-exempt and exempt infant formulas as a partial or complete replacement for folic acid as a source of the vitamin, folate. Use levels will be equivalent on a molar basis to current folic acid use levels, to provide the same amount of folate. Because the intended use is as a replacement for folic acid, dietary exposure to calcium L-methylfolate from the intended use will not increase folate consumption by the intended population.

## 1.5 Basis conclusion of GRAS status

Pursuant to 21 CFR §170.30(a) and (b), the use of calcium L-methylfolate in infant formula has been determined to be GRAS based upon scientific procedures. A comprehensive search of the scientific literature served as the basis for preparation of a monograph. There exists sufficient qualitative and quantitative scientific evidence, including human and animal data to determine that the use of calcium L-methylfolate in infant formula is safe. A panel of experts qualified by scientific training and experience evaluated the available information on the safety of use of calcium L-methylfolate in infant formula and reached a consensus opinion that this use is GRAS by scientific procedures.

## 1.6 Not subject to pre-market approval

DSM concludes that calcium L-methylfolate under the intended conditions of use in infant formula is GRAS and therefore does not fall under the definition of "food additive" and thus is not subject to the premarket approval requirements outlined in section 201(s) of the FD&C Act.

## 1.7 Availability of information

The Notifier will retain copies of all of the data and information that form the basis for the GRAS determination. Upon request, DSM will either provide the availability for the review and copying of the data and information during customary business hours at the address specified in Part 1.2 or will provide complete copies in an electronic or paper format.



## 1.8 Exemption from disclosure under the Freedom of Information Act

None of the information in this GRAS notice is considered exempt from disclosure under the Freedom of Information Act, 5 U.S.C. 552, as trade secrets and/or commercial or financial information that is privileged or confidential.

## 1.9 Certification

To the best of our knowledge this GRAS notice is a complete, representative, and balanced submission that includes both unfavorable information, as well as favorable information, known to us and pertinent to the evaluation of the safety and GRAS status of the use of calcium L-methylfolate.

## 1.10 Name and position of signatory

William Turney  
Head of Regulatory Affairs North America  
DSM Nutritional Products

Signature:  Digitally signed by William Turney  
DN: cn=William Turney, c=US, o=DSM  
Nutritional Products LLC, ou=Head of  
Regulatory Affairs North America,  
email=william-george.turney@dsm.com  
Date: 2019.12.06 13:41:46 -0500

Date: 12/6/2019

## 2 IDENTITY, METHOD OF MANUFACTURE, SPECIFICATIONS, AND PHYSICAL OR TECHNICAL EFFECT

### 2.1 Scientific data and information that identifies the notified substance

#### 2.1.1 Chemical name

The systematic name of calcium L-methylfolate is: N-{4-[[[(6S)-2-amino-1,4,5,6,7,8-hexahydro-5-methyl-4-oxo-6-pteridiny]methyl]amino]benzoyl}-L-glutamic acid, calcium salt.

#### 2.1.2 Synonyms, trade names and abbreviations

Synonyms, trade names and [abbreviations] include:

- Calcium L-5-methyltetrahydrofolate;
- L-methylfolate, calcium<sup>1</sup>;
- L-5-methyltetrahydrofolic acid, calcium salt [L-5-MTHF-Ca];
- (6S)-5-methyltetrahydrofolic acid, calcium salt [(6S)-5-MTHF-Ca];
- L-5-methyltetrahydrofolic acid [L-5-MTHF], without the cation being specified;
- (6S)-5-methyl-5,6,7,8-tetrahydropteroyl-L-glutamic acid, calcium salt; and
- Metafolin<sup>®2</sup>

#### 2.1.3 CAS registry numbers

The CAS registry numbers are:

- 129025-21-4 (Calcium salt with an unspecified ratio of L-5-MTHF/Ca<sup>2+</sup>) and
- 151533-22-1 (Calcium salt with specified 1:1 ratio of L-5-MTHF/Ca<sup>2+</sup>)

#### 2.1.4 Molecular and structural formula

The empirical formula is C<sub>20</sub>H<sub>23</sub>CaN<sub>7</sub>O<sub>6</sub> and the structural formula is:

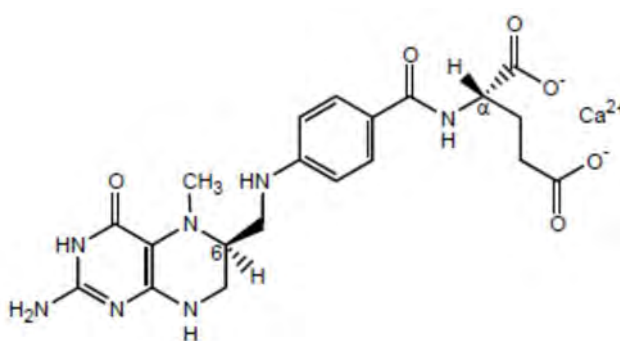


Figure 1: Structural formula of calcium L-methylfolate

Calcium L-methylfolate (L-5-MTHF-Ca) has two chiral carbon atoms: the C-atom in position 6 of the pteroyl moiety and the  $\alpha$ -C atom in the L-glutamic acid moiety. Consequently, there exists the possibility for four stereoisomers: (6S, $\alpha$ S), (6S, $\alpha$ R), (6R, $\alpha$ S), (6R, $\alpha$ R). The naturally occurring isomers of tetrahydrofolic acid and its 5-substituted derivatives are the (6S, $\alpha$ S) diastereomers, whereas the

<sup>1</sup> This term is used in more than 100 scientific articles in the title and/or abstract according to a literature search in Medline, Biosis, Chemical Abstracts, Toxline and Pascal.

<sup>2</sup> Metafolin<sup>®</sup> is a registered trademark for the calcium L-methylfolate manufactured by Merck KGaA. DSM Nutritional Products is the distributor of Metafolin<sup>®</sup>. The information provided in this notification is for Metafolin<sup>®</sup>.

natural isomers of 10-substituted and 5,10-bridged compounds are the (6R,αS) diastereomers. The configuration of the C-atom in position 6 of the natural 10-substituted and the 5,10-bridged compounds is not inverse compared to naturally occurring tetrahydrofolic acid, but according to the Cahn-Ingold-Prelog (CIP) nomenclature rules the configuration is (6R).

To avoid confusion about the stereochemistry, all natural diastereomers of reduced folates are defined as L-diastereomers and all others as D-diastereomers. A chiral HPLC-method can separate the diastereomers.

During synthesis of Metafolin® (the L-5-MTHF-Ca described in this dossier) the chiral centre at the C-atom in position 6 of the tetrahydropteroyl moiety is formed by reduction of the starting material, folic acid (See Part 2.2). The α-C atom in the L-glutamic acid moiety of L-5-MTHF-Ca stems from the starting material, folic acid, and its configuration (αS or L) remains unchanged during synthesis. Thus, both chiral centers in L-5-MTHF-Ca have the natural L-configuration.

The molecular weight of L-5-MTHF-Ca is 497.5 Daltons and the molecular weight of L-5-MTHF is 459.5 Daltons.

### 2.1.5 Physical and chemical properties

A brief summary of some physical and chemical properties of Metafolin®, the L-5-MTHF-Ca described in this GRAS notification, is provided in Table 1.

**Table 1: Physical and chemical properties of Metafolin®**

Parameter	Value
Appearance	White to yellow or beige crystalline powder
Particle size	Micrometer range
Solubility	Sparingly soluble in water, very slightly soluble or insoluble in most organic solvents and soluble in alkaline solutions
Melting point	Degradation occurs at ca. 300°C
Specific rotation $[\alpha]_D^{25}$	ca. +42° (0.5% w/v L-5-MTHF-Ca in 1% w/v aqueous sodium ascorbate, corrected for the solvent contribution)
pH of aqueous solution	7.7 (For a 0.5% w/v aqueous solution of L-5-MTHF-Ca)
Hygroscopicity	Slightly hygroscopic

## 2.2 Method of manufacture

Metafolin® is synthesized from folic acid in a 3-step synthesis conducted under Good Manufacturing Practice (cGMP) conditions (Figure 2):

- Step 1:** Catalytic hydrogenation or sodium borohydride reduction (2 alternatives)
- Step 2:** Condensation of the resulting tetrahydrofolic acid benzenesulfonate intermediate with formaldehyde, reduction of the formed 5,10-methylenetetrahydrofolic acid to L-5-methyltetrahydrofolic acid with NaBH<sub>4</sub>
- Step 3:** Crystallization as the calcium salt of L-5-methyltetrahydrofolic acid

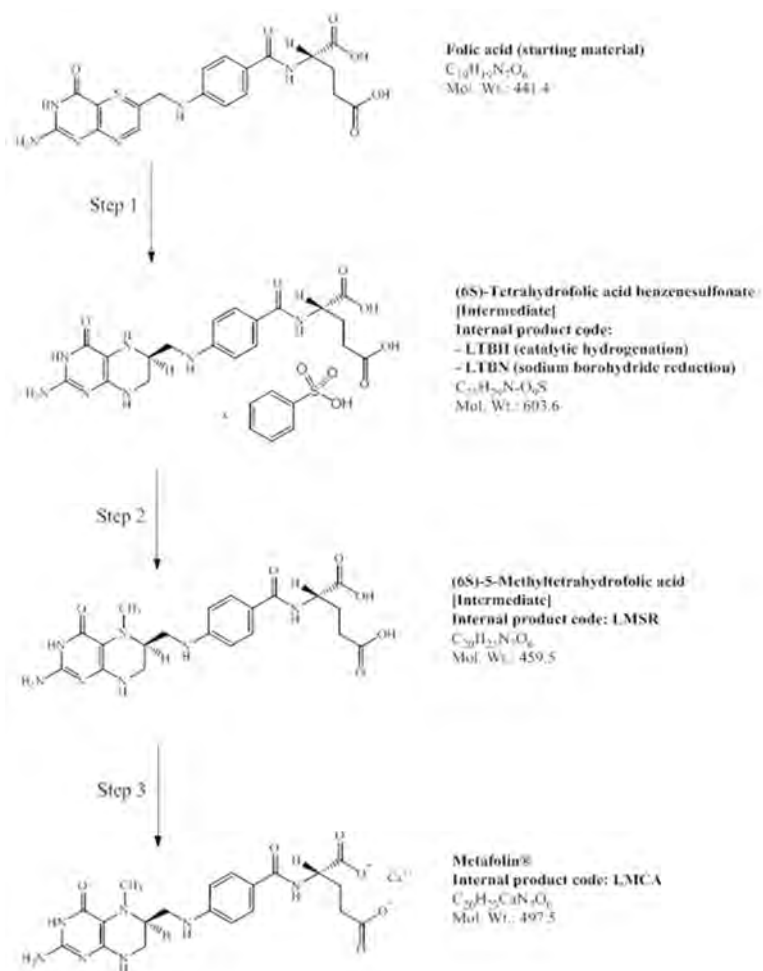


Figure 2: Flow diagram showing the steps to manufacture Metafolin®

The final product is prepared using either an impact mill or a jet mill depending on production capacity. The resulting products, called respectively “ground” or “micronized”, comply with the provided specifications and have similar particle size distributions.

Residues of starting materials, by-products, intermediates and reagents potentially present in the final material are listed and limited by the product specifications. The synthesis is performed in aqueous solution or in water/ethanol and the water soluble process chemicals are removed in the final steps of the manufacturing process, including the final washing steps in water/ethanol. Non water-soluble process materials, e.g. activated charcoal, are removed by filtration.

## 2.3 Specifications

### 2.3.1 Specifications for L-5-MTHF-Ca

Specifications for L-5-MTHF-Ca manufactured by the process outlined in Part 2.2 are provided in Table 2. Specifications meet or exceed the U.S. Pharmacopeia (USP) specifications for L-5-MTHF-Ca to be used in dietary supplements (Appendix A). Analytical results for 3 lots of Metafolin® confirming compliance with USP specifications for L-5-MTHF-Ca can be found in Table 3. The methods of analysis used to determine compliance with specifications are described in the USP specifications (Appendix A).



Table 2: Specifications for L-5-MTHF-Ca for use in infant formula

Test Parameter	Specification
Appearance	White to yellow or beige powder
Identity (IR spectrum)	Conforms to reference
Identity proof of calcium	Positive
Water content	6.0 to 17.0%
Residual solvents	
Assay ethanol	≤ 0.5%
Assay Isopropanol	≤ 0.5%
Chloride content	≤ 0.5%
Calcium content on dried basis	7.0 - 8.5%
Elemental impurities	
Boron	≤ 10 ppm <sup>1</sup>
Platinum	≤10 ppm
Arsenic	≤ 1.5 ppm
Cadmium	≤ 0.5 ppm
Lead	≤ 1.0 ppm
Mercury	≤ 1.5 ppm
Assay & related compounds (HPLC)	
Identity retention time HPLC	Conforms to reference
Assay L-5-MTHF-Ca on dried basis	95.0 to 102.0%
Assay 4-Aminobenzoylglutamic acid (ABGA)	≤ 0.5%
Assay 4 $\alpha$ -hydroxy-5-methyltetrahydrofolic acid (HoMeTHFA)	≤ 1.0%
Assay Mefox	≤ 1.0%
Assay Tetrahydrofolic acid (THFA)	≤ 0.5%
Assay 7,8-Dihydrofolic acid (DHFA)	≤ 0.5%
Assay Folic acid (FA)	≤ 0.5%
Assay 5,10-Methylenetetrahydrofolic acid (CH <sub>2</sub> THFA)	≤ 0.5%
Assay 5-Methyltetrahydroptericoic acid (MeTHPA)	≤ 0.5%
Assay Dimethyltetrahydrofolic acid (DiMeTHFA)	≤ 0.15%
Sum of all related compounds	≤ 2.5%
(6R)-Mefolate	≤1.0% area
Microbiological purity	
Total aerobic microbial count	≤100 CFU/g
Total combined yeasts/molds count	≤100 CFU/g

<sup>1</sup> USP specifications limit Boron to NMT 50  $\mu$ g/g

Table 3: Analytical results for three lots of Metafolin®

Test Parameter	LMCG045801	LMCG046801	LMCM048001
Appearance	Light yellowish powder	Light yellowish powder	Light yellowish powder
Identity (IR spectrum)	Conforms to reference	Conforms to reference	Conforms to reference
Identity proof of calcium	Positive	Positive	Positive
Water content	13.0%	12.1%	12.3%
Residual solvents			
Assay ethanol	< LOQ (0.04)%	< LOQ (0.04)%	< LOQ (0.04)%
Assay Isopropanol	< LOQ (0.003)%	< LOQ (0.003)%	< LOQ (0.003)%
Chloride content	0.21%	< LOQ (0.10)%	< LOQ (0.10)%
Calcium content on dried basis	8.0%	8.0%	8.0%
Elemental impurities			
Boron	< LOQ (5)ppm	< LOQ (5)ppm	< LOQ (5)ppm
Platinum	< LOQ (5)ppm	< LOQ (5)ppm	< LOQ (5)ppm
Arsenic	< LOQ (1.5)ppm	< LOQ (1.5)ppm	< LOQ (1.5)ppm
Cadmium	< LOQ (0.5)ppm	< LOQ (0.5)ppm	< LOQ (0.5)ppm
Lead	< LOQ (1.0)ppm	< LOQ (1.0)ppm	< LOQ (1.0)ppm
Mercury	< LOQ (1.5)ppm	< LOQ (1.5)ppm	< LOQ (1.5)ppm
Assay & related compounds (HPLC)			
Identity retention time HPLC	Conforms to reference	Conforms to reference	Conforms to reference
L-5-MTHF-Ca on dried basis	101.0%	100.6%	100.5%
Mefolate, acid as is	81.7%	81.7%	81.4%
ABGA	0.09%	0.09%	0.05%
HoMeTHFA	0.35%	0.28%	0.29%
Mefox	0.16%	0.19%	0.16%
THFA	<LOQ (0.01)%	<LOQ (0.01)%	<LOQ (0.01)%
DHFA	0.04%	0.03%	0.04%
FA	0.02%	0.02%	0.01%
CH <sub>2</sub> THFA	0.03%	0.02%	0.02%
MeTHPA	0.03%	0.03%	0.03%
DiMeTHFA	0.06%	0.07%	0.07%
Sum of all related compounds	1.08%	1.10%	0.91%
(6R)-Mefolate	0.6% area	0.5% area	0.5% area
Microbiological purity			
Total aerobic microbial count	<LOQ (10) CFU/g	<LOQ (10) CFU/g	<LOQ (10) CFU/g
Total combined yeasts/molds count	<LOQ (10) CFU/g	<LOQ (10) CFU/g	<LOQ (10) CFU/g

In addition to the USP specifications, food grade material specifications for L-5-MTHF-Ca have also been established by the Joint Food and Agricultural Organization of the United Nations (FAO)/World Health Organization (WHO) Expert Committee on Food Additives (JECFA) at their 65<sup>th</sup> meeting in 2005 for use in dietary supplements, foods for special dietary uses and other foods (Appendix C). L-5-MTHF-Ca to be used in infant formula also complies with the JECFA specifications.

### 2.3.2 Detailed description of specifications

#### 2.3.2.1 Purity

The purity of L-5-MTHF-Ca is not less than 95.0% and not more than 102.0% of calcium 5-methyltetrahydrofolate, the sum of the L- and D-diastereomers, calculated on the anhydrous and solvent-free basis, of which not more than 1.0% corresponds to calcium D-5-methyltetrahydrofolate (Appendix A).

#### 2.3.2.2 Elemental Impurities

Limits for the elemental impurities arsenic, cadmium, lead and mercury comply with limits set in the USP L-5-MTHF-Ca monograph (Appendix A).

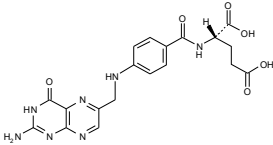
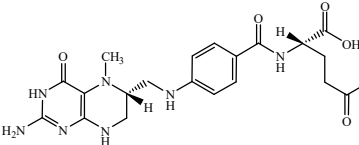
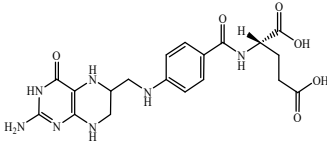
Sodium borohydride is used in the synthesis step 1 (sodium borohydride reduction) and in step 2 (reductive methylation). The USP specifies boron levels NMT 50 µg/g, however, manufacturer specifications of Metafolin<sup>®</sup> list boron as ≤10 mg/kg. Certificates of analysis for three batches of L-5-MTHF-Ca confirm that concentrations are below the LOQ of 5 mg/kg for the ICP-MS method (Appendix B).

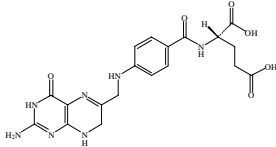
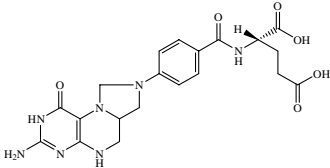
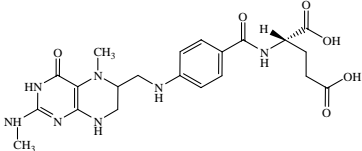
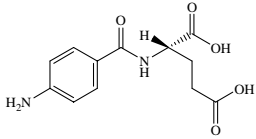
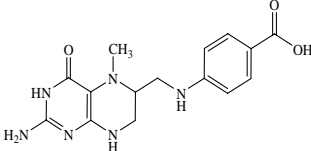
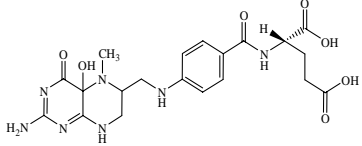
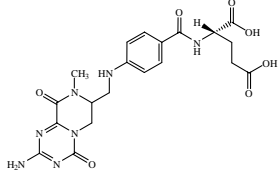
Platinum (Pt) is used as a catalyst in production step 1 (catalytic hydrogenation). Limits for platinum are set at ≤10 mg/kg in accordance with limits set in the USP L-5-MTHF-Ca monograph (Appendix A).

#### 2.3.2.3 Organic Impurities

Batches of L-5-MTHF-Ca may contain residues of folic acid (the starting material) and other organic by-products or degradation products. Potential organic impurities in L-5-MTHF-Ca and the possible source of the impurity are presented in Table 4.

**Table 4: Potential organic impurities in L-5-MTHF-Ca and their sources**

Organic impurity and possible source	Molecular structure
Folic acid (FA) is the starting material used for the synthesis of L-5-MTHF-Ca	
(6R)-5-Methyltetrahydrofolic acid (6R-Methylfolate (6R,αS isomer), D-5-Methylfolate, D-5-Methyltetrahydrofolate) is the unnatural diastereomer of 5-methyltetrahydrofolic acid. During the 1 <sup>st</sup> synthesis step most of the undesirable diastereomer is separated by crystallization (removal via mother liquor). During each further crystallization step (synthesis step 2 and 3) the isomer is further depleted. There is no increase observed during storage	
Tetrahydrofolic acid (THFA) is isolated as benzenesulfonate in the 1 <sup>st</sup> synthesis step. This intermediate is then converted to 5-methyltetrahydrofolic acid in the 2 <sup>nd</sup> synthesis step. THFA can be present in small quantities due to incomplete conversion or reduction of FA	

Organic impurity and possible source	Molecular structure
7,8-Dihydrofolic acid (DHFA) is formed by degradation of THFA	
5,10-Methylenetetrahydrofolic acid (CH <sub>2</sub> THFA) is a non isolated intermediate formed during synthesis step 2 (reaction of tetrahydrofolic acid with formaldehyde). This intermediate is then reduced to 5-methyltetrahydrofolic acid. This impurity can be present in small quantities due to incomplete conversion	
Dimethyltetrahydrofolic acid (DiMeTHFA) can be formed as a result of "over methylation" in the 2 <sup>nd</sup> synthesis step	
4-Aminobenzoylglutamic acid (ABGA) is a degradation product formed under oxidative, alkaline and acid conditions. During storage ABGA increases depending on storage conditions	
5-Methyltetrahydropteroic acid (MeTHPA) is formed under acid conditions by cleavage of the glutamic acid moiety. There is no increase observed during storage of L-5-MTHF-Ca	
4α-hydroxy-5-methyltetrahydrofolic acid (HoMeTHFA) is a degradation product formed in water, in alkaline medium and in the presence of oxidants. No significant increase has been observed during storage	
Pyrazino-S-triazine derivative (Mefox) is formed in water, in alkaline medium and in the presence of oxidants. Mefox increases under the recommended storage condition of 5°C	

#### 2.3.2.4 Microbial impurities

Microbial specifications are not listed in the USP specifications for L-5-MTHF-Ca. The manufacturer tests every batch of Metafolin® for total aerobic microbial counts (TAMC) and total yeast/mold counts (TYMC) and specifies ≤100 CFU/g. Certificates of analysis of three batches of L-5-MTHF-Ca confirm that microbial counts are below manufacturer specified limits (Table 3; Appendix B).

Methods of analysis for the two microbial tests are:

**Determination of TAMC:** membrane filter technique. A sample is dissolved in a sterile 0.1% peptone solution. Two aliquots each containing 1 g are filtered through sterile membrane filters. The filters are disposed after washing onto the surface of Soybean-Casein nutrient digest agar and incubated for three to five days at 30-35°C.

**Determination of TYMC:** membrane filter technique. A sample is dissolved in a sterile 0.1% peptone solution. Two aliquots containing 1 g are filtered through sterile membrane filters. The filters are disposed after washing onto the surface of Sabouraud dextrose agar and incubated for five to seven days at 20-25°C.



#### 2.3.2.4.1 Residual solvents

Residues of ethanol could potentially be present in L-5-MTHF-Ca because a mixture of ethanol and purified water is used to wash the isolated product in the final synthesis step (See Part 2.2). Residues of ethanol in L-5-MTHF-Ca are specified by the USP and the manufacturer to be  $\leq 0.5\%$  in agreement with USP specifications. This is in agreement with ICH<sup>3</sup> Guidelines for residual solvents<sup>4</sup> which list ethanol as a class 3 solvent that should be limited by cGMP or other quality-based requirements. Solvents in this class may be regarded as less toxic and of lower risk to human health and it is considered that amounts of these residual solvents of 50 mg/day corresponding to 0.5% would be acceptable without justification.

The ethanol used in the final synthesis step is denatured with ca. 5% isopropanol and residues of isopropanol could also potentially be present in L-5-MTHF-Ca. Like ethanol, isopropanol is classified as a class 3 solvent; therefore the manufacturer analyzes batches of L-5-MTHF-Ca to confirm that residues of isopropanol are also below the USP specified limit of  $\leq 0.5\%$ .

Certificates of analysis for three batches of L-5-MTHF-Ca confirm that concentrations of the two solvents are below 0.5% (Appendix B).

#### 2.3.3 Description of physical state

L-5-MTHF-Ca is a white-to-yellow or beige crystalline powder containing 6.0 to 17.0% adhering and/or structurally bound water in a non-stoichiometric ratio<sup>5</sup>.

#### 2.3.4 Solubility

L-5-MTHF-Ca is sparingly soluble in water, very slightly soluble or insoluble in most organic solvents and soluble in alkaline solutions (Appendix A).

#### 2.3.5 Stability

The manufacturer of L-5-MTHF-Ca specifies a shelf-life of 24 months from the date of production when stored in the unopened original container at a temperature of 2-8°C (Appendix D).

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<sup>3</sup> The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human use (ICH).

<sup>4</sup> [https://www.ich.org/fileadmin/Public\\_Web\\_Site/ICH\\_Products/Guidelines/Quality/Q3C/Q3C\\_R6\\_Step\\_4.pdf](https://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q3C/Q3C_R6_Step_4.pdf).

<sup>5</sup> Dynamic Vapor Sorption experiments were performed to evaluate the bonding form of the water.

## 3 DIETARY EXPOSURE

### 3.1 Proposed use and levels

L-5-MTHF-Ca is intended to replace folic acid as a source of the vitamin folate in conventional infant formula for full term infants<sup>6</sup>. L-5-MTHF-Ca is also intended to replace folic acid as a source of the vitamin folate in exempt infant formula insofar as exempt infant formulas may only deviate from the infant formula nutrient specifications listed in 21 CFR 107.100 under specific limited circumstances in which deviation is deemed necessary and will protect the public health.

Infant formula is a food which purports to be or is represented for special dietary use solely as a food for infants by reason of its simulation of human milk or its suitability as a complete or partial substitute for human milk<sup>7</sup>. The composition of infant formula should serve to meet the particular nutritional requirements and to promote normal growth and development of the infants for whom it is intended. Breast feeding is the ideal form of infant feeding, and data on the composition of human milk of healthy well-nourished women provides guidance for the composition of infant formula. Infant formula is a food with a standard of identity which specifically provides for the addition of folic acid. According to the nutrient requirements for infant formula (Section 412 of FD&C Act and 21 CFR 107.100), the minimum amount of folic acid in infant formula is 4.0 µg folic acid (folacin) per 100 kcal; no maximum amount is specified in the FD&C Act.

While the FDA has not set maximum upper levels for inclusion of folic acid in infant formula, the Codex Alimentarius provides a Guidance Upper Level (GUL) (Codex Alimentarius, 1981). GULs are set for nutrients without sufficient information for a science-based risk assessment and are derived on the basis of meeting the nutritional requirements of infants and an established history of apparent safe use. The purpose of GULs is to provide guidance to manufacturers; they should not be interpreted as goal values, and nutrient contents in infant formulas should usually not exceed the GULs. The Codex Alimentarius set the minimum amount of folic acid in infant formula to 10 µg/100 kcal and the GUL to 50 µg/100 kcal.

Dietary Reference Intakes for folate have been developed by the Food and Nutrition Board (FNB) at the Institute of Medicine (IOM) of the National Academies (IOM, 1998). The Adequate Intake (AI)<sup>8</sup> of folate per day, based on the mean intake of folate in healthy breastfed infants in the U.S., is 65 µg dietary folate equivalents (DFE)<sup>9</sup> for infants up to 6 months of age and 80 µg DFE from 7 to 12 months. Expressed in terms of folic acid, this would be 39 and 48 µg per day, respectively for each age group. Tolerable Upper Intake Levels (ULs)<sup>10</sup> for synthetic forms of folate in dietary supplements and fortified foods were established by the FNB to limit metabolic interactions between folate and vitamin B<sub>12</sub>, but no limits were set for infants from birth to 12 months of age as the FNB considered that breast milk, formula and food should be the only sources of folate for infants.

The amount of L-5-MTHF-Ca needed to supply the AI values of folate for infants set by the IOM, and the concentration needed to replace the amount of folate provided by folic acid when used at the

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<sup>6</sup> Infants are persons not more than 12 months old (21 CFR 105.3(e)).

<sup>7</sup> Federal Food, Drug and Cosmetic Act [as amended through P.L. 115-176, Enacted May 30, 2018] Chapter II - Definitions. Section 201.

<sup>8</sup> Adequate Intake (AI): a recommended daily intake value based on observed or experimentally determined approximations of nutrient intake by a group (or groups) of healthy people that are assumed to be adequate—used when an RDA cannot be determined.

<sup>9</sup> DFEs reflect the higher bioavailability of folic acid than that of food folate. At least 85% of folic acid is estimated to be bioavailable when taken with food, whereas only about 50% of folate naturally present in food is bioavailable. 1 µg DFE = 1 µg food folate = 0.6 µg folic acid from fortified food or as a supplement consumed with food = 0.5 µg of a folic acid supplement taken on an empty stomach.

<sup>10</sup> Tolerable Upper Intake Level (UL): the highest level of daily nutrient intake that is likely to pose no risk of adverse health effects to almost all individuals in the general population. As intake increases above the UL, the risk of adverse effects increases.

legal minimum of 4.0 µg folic acid per 100 kcal, or to not exceed the Codex GUI levels that can be calculated based upon an understanding of the chemistry of L-5-MTHF-Ca and an assessment of the relative bioavailability of the two folate sources.

In aqueous media, e.g. in prepared infant formula, L-5-MTHF-Ca dissociates readily and completely to Ca and L-5-MTHF ions. Following consumption, L-5-MTHF is absorbed and enters the circulation and its fate becomes indistinguishable from that of other absorbed and metabolized natural folates or L-5-MTHF formed from synthetic folic acid.

The bioavailability of L-5-MTHF-Ca compared to folic acid is extensively reviewed in Part 6 of this dossier. Based upon publicly available literature, it can be concluded that L-5-MTHF-Ca is bioavailable to an extent similar or slightly higher than folic acid. This conclusion is supported by a recently published infant growth and development study that found no major differences in growth and tolerance among infants who consumed an infant formula with either L-5-MTHF-Ca or folic acid at equimolar doses (Troesch et al., 2019).

Because folic acid and L-5-MTHF-Ca have equimolar equivalence, the use level of L-5-MTHF-Ca to meet the minimum, adequate and upper intake levels can be calculated based upon the molecular weights of the folate sources (Table 5).

Table 5: Use levels of folate sources to meet minimum, Guidance Upper Level and Adequate Intake levels for infants

Folate source	Molecular weight	Minimum (FD&C Act)	Minimum (Codex)	GUL (Codex)	AI 0-6 months (IOM)	AI 7-12 months (IOM)
	g	(µg/100 kcal)			µg/day	
Folic acid	441.4	4.0	10	50	39	48
L-5-MTHF-Ca	497.5	4.5	11	56	44	54
L-5-MTHF	459.5	4.1	10	52	41	50

GUL = Guidance Upper Level; AI = Adequate Intake; AI values are calculated from dietary folate equivalents (DFE) with 1 DFE = 0.6 µg folic acid.

Folic acid use levels of several commercially-available infant formulas currently on the market are as follows:

- [Enfamil](#): 16 µg per 100 kcal (5 fl oz, 147.9 ml)
- [Gerber](#): 15 µg per 100 kcal (5 fl oz, 147.9 ml)
- [Similac](#): 16 µg per 100 kcal (5.3 fl oz, 156.7 ml)
- [Perrigo](#) (store brands: Target, Walgreens, Walmart, etc): 16 µg per 100 kcal (5 fl oz, 147.9 ml)

The commercially-available formulas contain folic acid above the required minimum, but well below the Codex GUL. The amount of L-5-MTHF-Ca needed to replace folic acid in a typical commercially available infant formula would be 17 to 18 µg/100 kcal.

## 3.2 Estimated daily intakes

### 3.2.1 L-5-MTHF-Ca

Grimes et al. (2015) analyzed NHANES data from 2005 to 2012 to determine dietary intakes of energy and nutrients by US infants and toddlers. The mean calorie intake by infants aged 0-5.9 months was determined to be 612.5 ± 6.4 kcal/day while 6-to-11.9 month-olds consume about 40% more energy, 847.3 ± 13.3 kcal/day. Infant formula is the largest source of total energy intake,

comprising 65.4% of daily energy intake in 0-5.9 month-old infants and 47.1% in 6-to-11.9 month-olds.

The contribution of infant formula to the mean total energy intake can be calculated for the two age groups from these data. As Table 6 shows, daily energy intake from infant formula for both age groups is almost identical (~400 kcal/day). The higher mean energy intake of older infants is due to increased consumption of baby food and introduced new foods such as milk, fruits, grain products and beverages, rather than an increase in infant formula intake.

Considering the energy intake from infant formula and typical folic acid use levels in commercial infant formulas (16 µg/100kcal), the amount of folic acid and the equivalent amount of L-5-MTHF-Ca ingested per day from consumption of a typical infant formula can be calculated (Table 6).

Table 6: Folic acid and L-5-MTHF-Ca intake from infant formula calculated using data from Grimes et al. (2015)

Age (months)	Mean energy intake (kcal/day)	Energy intake from infant formula		Calculated intake (µg/day)	
		(%)	(kcal/day)	folic acid <sup>1</sup>	L-5-MTHF-Ca <sup>2</sup>
0 - 5.9	612.5 ± 6.4	65.4	401	64	72
6 - 11.9	847.3 ± 13.3	47.1	399	64	72

<sup>1</sup>Calculated assuming folic acid use level of 16 µg/100 kcal typical of commercial brands.

<sup>2</sup>Calculated assuming L-5-MTHF-Ca use level of 18 µg/100 kcal to replace folic acid in typical commercial brands.

Replacement of folic acid in typical infant formulas by L-5-MTHF-Ca to provide an equivalent amount of folate would result in an estimated daily intake of 72 µg of L-5-MTHF-Ca by infants aged 0-to-12 months.

Infant intake of L-5-MTHF-Ca can also be calculated on a per kg body weight basis for each month of age using mean formula intake values for infants aged 0-to-11 months as described by Neal-Kluever et al. (2014). Considering that L-5-MTHF-Ca would be added at a concentration of 18 µg/100 kcal to replace folic acid in typical commercial brands, that the energy content of typical formulae on the market is 100 kcal per 150 mL of formula, and that the density of infant formula is 1.03 g/mL in the ready-to-drink form, infant formula would contain 0.12 µg/g L-5-MTHF-Ca.

Table 7: Mean and 90<sup>th</sup> percentile formula and L-5-MTHF-Ca intake per kg body weight from Neal-Kluever et al. (2014)

Age (months)	Mean formula intake (g/kg bw/d)	Mean L-5-MTHF-Ca intake (µg/kg bw/d)	90 <sup>th</sup> percentile formula intake (g/kg bw/d)	90 <sup>th</sup> percentile L-5-MTHF-Ca intake (µg/kg bw/d)
0	170.4	20.4	250.5	30.1
1	150.0	18.0	240.6	28.9
2	140.0	16.8	199.3	23.9
3	123.1	14.8	183.0	22.0
4	113.1	13.6	177.9	21.3
5	98.5	11.8	157.9	18.9
6	103.9	12.5	158.4	19.0
7	89.2	10.7	144.7	17.4
8	79.9	9.6	122.0	14.6
9	76.8	9.2	114.8	13.8



Age (months)	Mean formula intake (g/kg bw/d)	Mean L-5-MTHF-Ca intake ( $\mu\text{g}/\text{kg bw/d}$ )	90 <sup>th</sup> percentile formula intake (g/kg bw/d)	90 <sup>th</sup> percentile L-5-MTHF-Ca intake ( $\mu\text{g}/\text{kg bw/d}$ )
10	66.2	7.9	99.5	11.9
11	59.6	7.2	101.7	12.2

At the intended use level to replace folic acid in infant formula, mean daily intake of L-5-MTHF-Ca would range from 7.2 to 20.4  $\mu\text{g}/\text{kg bw/d}$ , whereas high level intake would range from 11.9 to 30.1  $\mu\text{g}/\text{kg bw/d}$  for infants between the ages of 0-and-11 months.

The NOAEL of L-5-MTHF-Ca in the 90-d toxicity study in rats was established at 400 mg/kg bw/d (See Part 6.4.2.3), which is 19,608 times above the highest mean intake (400 mg/kg bw/d; 20.4  $\mu\text{g}/\text{kg bw/d}$ ) and 13,289 times the highest 90<sup>th</sup> percentile intake of L-5-MTHF-Ca in the highest consuming infant age group (400 mg/kg bw/d; 30.1  $\mu\text{g}/\text{kg bw/d}$ ). In addition, the NOAEL in the prenatal developmental toxicity study in rats was established at 1000 mg/kg bw/d (See Part 6.4.2.4), providing an even higher margin of safety (MoS) for infants in both the mean and high intake groups:

Highest mean intake group MoS = 1000 mg/kg bw/d : 20.4  $\mu\text{g}/\text{kg bw/d}$  = 49,020

Highest 90<sup>th</sup> percentile intake MoS = 1000 mg/kg bw/d : 30.1  $\mu\text{g}/\text{kg bw/d}$  = 33,223

Guidance Upper levels of folic acid in infant formula set by the Codex Alimentarius would also apply to limit the amount of L-5-MTHF-Ca used in infant formulas to ensure that infant formulas are comparable in composition to breast milk.

Finally, use of L-5-MTHF-Ca at the intended use level does not result in any adverse effects in infants as demonstrated in the tolerance and safety study in infants (See Part 6.3).

### 3.2.2 Folate

Infant formula has been estimated to contribute 79.2% of the  $78.6 \pm 1.7$   $\mu\text{g}/\text{day}$  folate consumed by 0-5.9 month-olds and 44.5% of the  $136.2 \pm 4.0$   $\mu\text{g}/\text{day}$  folate consumed by 6-11.9 month-olds (Grimes et al., 2015). Therefore, replacement of folic acid with L-5-MTHF-Ca, as DSM proposes, would not alter daily folate intake.

### 3.2.3 Calcium

In solution, L-5-MTHF-Ca readily dissociates into L-5-MTHF-Ca and calcium ion ( $\text{Ca}^{2+}$ ). Ca comprises about 8% of L-5-MTHF-Ca, therefore ingestion of 72  $\mu\text{g}/\text{day}$  of L-5-MTHF-Ca would include intake of approximately 6  $\mu\text{g}/\text{day}$  of Ca. Considering the mean Ca intake of infants reported by (Grimes et al., 2015):

- 0-5.9 month-olds:  $469.7 \pm 9.6$  mg/day
- 6-11.9 month olds:  $649.0 \pm 12.4$  mg/day

Inclusion of L-5-MTHF-Ca in infant formula would increase Ca intake by infants by 6  $\mu\text{g}/\text{day}$ , which is an increase of <0.00001% of their normal daily Ca intake.

Infant formula is regulated to contain a minimum of 60 mg/100 kcal Ca with typical commercial formulas containing 67 to 82 mg/100 kcal. The additional Ca from inclusion of L-5-MTHF-Ca at 18  $\mu\text{g}/100$  kcal would be 1.4  $\mu\text{g Ca}/100$  kcal, i.e. 0.00002% of the typical Ca concentration in infant formulas.

It can therefore be concluded that, considering normal daily intake of Ca, the additional intake of Ca from replacement of folic acid with L-5-MTHF-Ca is insignificant.

### 3.2.4 Impurities

Boron is an essential trace element that is found in high concentrations (10-45 mg/kg) in foods such as nuts, dried fruits, legumes and avocados. Sodium borohydride which is used in the synthesis of L-5-MTHF-Ca, is specified to be  $\leq 10$  mg/kg in the finished product. At the maximum specified concentration of boron in infant formula of 10 mg/kg and an estimated daily intake of 72  $\mu\text{g}$  of L-5-MTHF-Ca (see Part 3.2.1), infants aged 0-to-12 months could potentially be exposed to 0.72 ng Boron/day. Considering that breast milk has been reported to contain 27 to 42  $\mu\text{g/L}$  boron (Hunt et al., 2004; Hunt et al., 2005) and boron intake by infants is estimated to be 0.55 mg/day (Hunt & Meacham, 2001), any additional intake of the essential trace element from use of L-5-MTHF-Ca is insignificant.

Platinum (Pt) is used as a catalyst in the production of L-5-MTHF-Ca and is specified to be  $\leq 10$  mg/kg in the finished product. According to ICH Q3D Guidance<sup>11</sup>, the oral permitted daily exposure (PDE) of platinum is 108  $\mu\text{g/day}$  for a 50-kg adult, which can be calculated on a kg basis, to be 13  $\mu\text{g/day}$  for a 6-kg infant and 19  $\mu\text{g/day}$  for a 9-kg infant. At the maximum specified concentration of Pt in formula of 10 mg/kg and an estimated daily intake of 72  $\mu\text{g}$  of L-5-MTHF-Ca (see Part 3.2.1), infants aged 0-to-12 months could potentially be exposed to 0.72 ng Pt per day. Compared to the ICH Q3D PDE for Pt recalculated for infant body weight, the margin of safety for 0-6-month old infants (6 kg) and 6-12 month-old infants (9 kg) is more than 18000. Levels of Pt in breast milk were not found in the published literature, however, dietary Pt intake of German children aged 14-to-83 months were reported to range from  $<0.81$ -to-32 ng/kg bw/week (Wittsiepe et al., 2003). Calculating from the study by Wittsiepe et al., individuals of 6-kg and 9-kg at the upper end of the range consume 27-and-41 ng Pt/day. Inclusion of L-5-MTHF-Ca in infant formula could expose infants to a maximum of 0.72 ng Pt per day, an intake that is at the lower end of the normal range that young children are exposed to from their normal diet.

In the infant feeding study described in Part 6.3 (Troesch et al., 2019), 0-4 month-old infants received L-5-MTHF-Ca at levels of 11.3  $\mu\text{g}$  L-5-MTHF-Ca/100ml. If Pt levels in L-5-MTHF-Ca used in the trial were at the maximum specified limit, the formula given to infants in the feeding trial would have contained 0.00113 ng/g Pt. Reported Pt concentrations in food items such as eggs and offal - 5.8 ng/g, meat - 3.2 ng/g, grain products - 3.2 ng/g, fish - 1.8 ng/g, fruit and vegetables - 0.82 ng/g and dairy products - 0.27 ng/g (WHO, 2000) are more than 200 times higher. In comparison to normal dietary ingredients, the amount of Pt provided by L-5-MTHF-Ca is very low and the infants in the feeding trial thrived under the study conditions.

All potential elemental and organic impurities in L-5-MTHF-Ca are limited by the conservative specifications. No other substances are expected to be formed in or on food under the intended use of L-5-MTHF-Ca in infant formula.

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<sup>11</sup> [https://www.ich.org/fileadmin/Public\\_Web\\_Site/ICH\\_Products/Guidelines/Quality/Q3D/Q3D\\_Step\\_4.pdf](https://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q3D/Q3D_Step_4.pdf)

#### 4 SELF-LIMITING LEVELS OF USE

There are no known inherent self-limiting levels of use associated with calcium L-methylfolate.

## 5 EXPERIENCE BASED ON COMMON USE IN FOOD BEFORE 1958

The statutory basis for the GRAS determination is through scientific procedures in accordance with the Code of Federal Regulations 21 CFR §170.30(a) and (b). Therefore, the elements of this Part do not apply.



## 6 NARRATIVE

The conclusion that L-5-MTHF-Ca is GRAS under the conditions of its intended use as a source of the nutrient folate in infant formula is based upon scientific procedures using generally available data and information. A comprehensive and detailed search of the published scientific literature was conducted to identify information related to the safety of L-5-MTHF-Ca<sup>12</sup>. In this Part, the generally available literature is summarized, demonstrating both the general availability of the information and the evidence for safety.

### 6.1 Authorizations and safety evaluations by authoritative bodies

The safety of use of L-5-MTHF-Ca as a source of the nutrient folate has been assessed by several authoritative bodies. The conclusions of the safety assessments and the authorized uses of L-5-MTHF-Ca are described below, along with relevant safety data.

#### 6.1.1 United States

##### 6.1.1.1 Dietary supplements

New Dietary Ingredient Notification ("75-Day Notice"). In accordance with the requirements of Section 413(b) of the FD&C Act and Section 8 of the Dietary Supplement Health and Education Act, Merck KGaA filed a 75-day premarket notification with the FDA (Docket Number 95S-0316, filing date March 13, 2001) for the calcium salt of L-5-methyltetrahydrofolate (L-5-methyl-THF) as a new dietary ingredient for use in dietary supplements<sup>13</sup>. With no objections from the FDA, Merck KGA was able to lawfully use L-5-MTHF-Ca in dietary supplements in the United States after the 75-day period.

An earlier New Dietary Ingredient Notification for 5-MTHF (calcium-salt) was submitted to the FDA by General Nutrition Corporation (GNC) in cooperation with BASF<sup>14</sup>. In contrast to the product which is the subject of the present dossier, GNC/BASF's New Dietary Ingredient was a mixture containing 50% each of the (6R) and (6S) diastereoisomers<sup>15</sup>.

##### 6.1.1.2 United States Pharmacopeia (USP)

A monograph for calcium L-5-methyltetrahydrofolate has been included in the USP Dietary Supplements Compendium (Appendix A).

#### 6.1.2 European Union

In 2004, the European Food Safety Authority (EFSA) published the opinion of its Scientific Panel which concluded that the use of L-5-MTHF-Ca as a source of folate in foods for particular nutritional uses, food supplements and foods for the general population, with a tolerable upper level of 1 mg/adult person/day is not of concern from a safety point of view (EFSA, 2004).

On the basis of the EFSA opinion, L-5-MTHF-Ca is included in the list of vitamins authorized for use in food supplements (Directive 2002/46/EC as amended<sup>16</sup>) and in the list of vitamins for use in food for special medical purposes and in total diet replacement for weight control (Regulation (EU) 609/2013 as amended<sup>17</sup>).

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<sup>12</sup> Terms used in the search were the name and synonyms of L-5-MTHF-Ca in addition to terms for absorption, distribution, metabolism, excretion, safety, toxicity and infant.

<sup>13</sup> <https://www.regulations.gov/document?D=FDA-2001-S-0575-0029>.

<sup>14</sup> <https://www.regulations.gov/document?D=FDA-1998-S-1294-0023>.

<sup>15</sup> BASF's racemic product has been sold for many years in Italy for oral and parenteral application of high doses (15 mg/day) as a medicinal product (Trademark: "Prefolic").

<sup>16</sup> <https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:02002L0046-20170726&qid=1535537044673&from=EN>.

<sup>17</sup> <https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:02013R0609-20170711&qid=1535537251102&from=EN>.

To extend the use of L-5-MTHF-Ca to fortified foods, in 2007, Merck & Cie (formerly Merck Eprova AG), Switzerland submitted a novel food application to Ireland as the first assessing Member State. A favorable opinion of the Irish competent authority (FSAI) was issued and forwarded to all other Member States for comment. No 'reasoned objection' was received within the comment period. The Applicant received authorization for L-5-MTHF-Ca (Metabolin<sup>®</sup>) as a novel food on January 4<sup>th</sup> 2008<sup>18</sup>.

Following this approval, Merck & Cie requested the inclusion of L-5-MTHF-Ca in the EU positive list of vitamins that may be added to foods. As a result, Regulation (EC) 1925/2006 (with amendments) lists "Calcium-L-methylfolate"<sup>19</sup>.

### 6.1.3 JECFA

At its 65<sup>th</sup> meeting in 2005, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) evaluated the safety of calcium L-5-methyltetrahydrofolate as an alternative to folic acid in food fortification and supplementation. The Committee had no concern about the safety of the proposed use of L-5-MTHF-Ca as an alternative to folic acid in food supplements, foods for special dietary uses and other foods<sup>20 21</sup>.

### 6.1.4 Australia

In 2005, an application was submitted on behalf of Merck & Cie (formerly Merck Eprova AG) to Food Standards Australia New Zealand (FSANZ) requesting the approval of L-5-methyltetrahydrofolate, calcium salt (L-MTHF) as a permitted form of the vitamin folate for use in specified foods where voluntary folate fortification is currently permitted in the FSANZ Code. The application submitted was based on and included the information provided in the JECFA and EFSA dossiers.

FSANZ performed a full scientific evaluation of L-MTHF to assess its safety for human consumption and suitability for fortification of certain foods and concluded in 2008 that the use of L-MTHF for voluntary fortification purposes would raise no public health and safety concerns. L-methyltetrahydrofolate, calcium is listed as a permitted form of folic acid in Schedule 15 of the Australia New Zealand Food Standards Code<sup>22</sup>.

Based on the evaluation of 5-L-MTHF by the Australian Therapeutic Goods Administration (TGA), levomefolate calcium is listed as a permitted ingredient for use in Complimentary Medicines, which is equivalent to food supplements<sup>23</sup>.

### 6.1.5 Canada

L-5-Methyltetrahydrofolate, calcium salt (L-5-MTHF-Ca) is listed as a chemical substance in the Natural Health Products Ingredients Database of Health Canada<sup>24</sup>.

## 6.2 Metabolic fate of L-5-MTHF-Ca

L-5-MTHF-Ca is the calcium salt of L-5-methyltetrahydrofolate (L-5-MTHF). In aqueous solution or following ingestion, L-5-MTHF-Ca dissociates readily and completely into Ca<sup>2+</sup> and L-5-MTHF ions (EFSA, 2004). The publicly available information on the metabolic fate of L-5-MTHF and calcium are summarized separately in this part of the GRAS dossier.

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<sup>18</sup> [https://www.fsai.ie/uploadedFiles/Science\\_and\\_Health/Novel\\_Foods/Applications/2007%20%20L%20%20MTHF%20Ca.pdf](https://www.fsai.ie/uploadedFiles/Science_and_Health/Novel_Foods/Applications/2007%20%20L%20%20MTHF%20Ca.pdf).

<sup>19</sup> <https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:02006R1925-20170726&qid=1535537508786&from=EN>.

<sup>20</sup> [https://apps.who.int/iris/bitstream/handle/10665/43407/9241660562\\_eng.pdf;jsessionid=9E8B7C9CA98835C711492AF384706A18?sequence=1](https://apps.who.int/iris/bitstream/handle/10665/43407/9241660562_eng.pdf;jsessionid=9E8B7C9CA98835C711492AF384706A18?sequence=1).

<sup>21</sup> [https://apps.who.int/iris/bitstream/handle/10665/43408/WHO\\_TRS\\_934\\_eng.pdf?sequence=1](https://apps.who.int/iris/bitstream/handle/10665/43408/WHO_TRS_934_eng.pdf?sequence=1).

<sup>22</sup> <https://www.legislation.gov.au/Details/F2017C00328>.

<sup>23</sup> <https://www.legislation.gov.au/Details/F2017L00926>.

<sup>24</sup> <http://webprod.hc-sc.gc.ca/nhpid-bdipsn/ingredsReq.do?srchRchTxt=folate&srchRchRole=-1&mthd=Search&lang=eng>.

## 6.2.1 Absorption, distribution, metabolism and excretion of L-5-MTHF

### 6.2.1.1 Absorption of L-5-MTHF

Following consumption of L-5-MTHF-Ca and dissociation into ions, L-5-MTHF is absorbed primarily across the apical brush-border membrane of the duodenum and jejunum by the proton-coupled folate transporter (PCFT) (Visentin et al., 2014). The PCFT, a highly pH-dependent carrier-mediated transporter, is specific for the monoglutamate form of folate substrates. Absorption of L-5-MTHF requires monoglutamation, whereas polyglutamate folate forms, e.g. folates from natural dietary sources, require hydrolysis to monoglutamate forms prior to transport (Visentin et al., 2014). Once inside the intestinal cells, the various folate monoglutamates are reduced and methylated into L-5-MTHF (if they were not already in that form) and exported into the blood stream (Scott, 2001; EFSA, 2014).

The capacity of this system to convert various folate forms into L-5-MTHF is saturable and excess natural folates or synthetic L-5-MTHF not absorbed in the duodenum or jejunum may be passively absorbed during passage through the ileum or large intestine (EFSA, 2014; Visentin et al., 2014). Under normal circumstances, L-5-MTHF is the only form of folate that crosses into the human circulation; however, when excess folic acid is ingested as a supplement (>300 µg) and the capacity for reduction and methylation in the duodenum and jejunum is exceeded, unmetabolized folic acid may cross the membrane and enter the plasma via a diffusion-like process (Scott, 2001; Sweeney et al., 2007; EFSA, 2014). The unaltered folic acid is rapidly excreted by the kidneys, but during the time it is in the plasma, the unaltered folic acid comes into contact with and diffuses into cells, where it is converted to dihydrofolate or tetrahydrofolate and made into a polyglutamate. This system is not subject to the usual control of cellular uptake to which circulating L-5-MTHF is subjected (Scott, 2001).

The intestinal folate transport system has similar functional affinity for the monoglutamyl forms of synthetic L-5-MTHF and folic acid (Selhub et al., 1984; Gregory et al., 1992), whereas absorption of naturally present food folates is less efficient. Food folates are a mixture of reduced mono- and polyglutamates, typically in a methylated form and protein bound with an absorption efficiency of around 50%, whereas the absorption efficiency of folic acid from either fortified foods or from a supplement ingested with food is assumed by the Institute of Medicine (IOM) to be 85% (IOM, 1998). The IOM defined Dietary Folate Equivalents (DFE) to account for the variable absorption efficiency of synthetic and natural folates, where 1 µg DFE = 1 µg food folate = 0.6 µg folic acid from fortified food or as a supplement consumed with food = 0.5 µg of a folic acid supplement taken on an empty stomach.

Absorption of synthetic folates may also be influenced by dietary components. Using an in vitro gastrointestinal model consisting of glass compartments, Verwei et al. (2003) showed that milk is a suitable carrier for folate, as both folic acid and L-5-MTHF are easily released from the milk matrix and made available for absorption. The milk folate-binding protein (FBP) has a slightly lower binding affinity to L-5-MTHF compared to folic acid in the pH range of 5 to 7.4 resulting in possibly less stable FBP-L-5-MTHF complexes and higher release of L-5-MTHF during gastric passage, whereas folic acid remains partly bound to FBP during passage. Fortification of milk with L-5-MTHF leads to a slightly higher folate bioaccessibility (ca. 70%) than fortification with folic acid (ca. 60%) (Verwei et al., 2003).

### 6.2.1.2 Transport, distribution and storage of L-5-MTHF

Once absorbed across the intestinal lining, L-5-MTHF monoglutamate is transferred via portal circulation to the liver, the major storage tissue for folate, where it is either metabolized to polyglutamate derivatives (storage form of folate) and retained, or released back into the circulation for distribution to other tissues (Shane, 2010). A variable proportion of plasma folate in the circulation is L-5-MTHF monoglutamate bound to albumin, a low-affinity FBP, which accounts for about 50% of bound folate, increasing in folate deficiency (Shane, 2010; EFSA, 2014). L-5-MTHF

monoglutamate is taken up by cells via passive or facilitated diffusion, after which it either passes back out again or is used by the vitamin B<sub>12</sub>-dependent enzyme, methionine synthase (Scott, 2001). Methionine synthase takes the methyl group from L-5-MTHF and supplies it to the cell's methylation cycle and the remaining tetrahydrofolate monoglutamate is converted into a polyglutamate and retained in the cell. Methionine synthase can use both the monoglutamate and the polyglutamate forms of L-5-MTHF. Under normal conditions, the concentration of the polyglutamate in cells is high and the monoglutamate is low and diffuses out of the cells. Only when cells start to become folate-deficient and the ratio of polyglutamate to monoglutamate lowers, do the cells start to use more of the L-5-MTHF monoglutamate (Scott, 2001).

While plasma folate levels increase with increasing dietary intake, tissue concentrations saturate at high intakes as a result of the decreased ability for polyglutamation (Clifford et al., 1990) and storage of folates in excess of amounts required for normal metabolism is limited (Lowe et al., 1993). Some L-5-MTHF polyglutamates in tissues are bound to FBPs, with expression of FBP varying widely among tissues. For example, the presence of FBP in mammary gland tissue concentrates folate in breast milk at an average concentration of 80 µg/L, i.e., 5 to 10 times higher than in maternal plasma (Tamura et al., 1980).

Folate accumulates in red blood cells during erythropoiesis and appears to be retained mainly as L-5-MTHF polyglutamates through the life span of the cell (Shane, 2010). When cells divide, the daughter cells will initially have half of the original concentration of folate that was present in the parent cells, resulting in uptake of L-5-MTHF monoglutamate from the circulating plasma (Scott, 2001). To satisfy the high requirements for folate during fetal development, folate accumulates in the placenta due to the abundance of folate receptors, reduced folate carrier and proton-coupled folate transporter. The concentration of folate in fetal blood is maintained at two-to-four times that of maternal blood, even in women with low folate intakes (Thorand et al., 1996).

To measure long-term folate status, folate concentrations in red blood cells are often used because levels are higher than in plasma. Fasting plasma folate levels can also be a good indicator of status, but the folate pool is smaller, has fast turnover rates, and may also be influenced by recent dietary intake (Shane, 2010). Folate polyglutamates in tissues represent a large folate pool with slow turnover.

#### 6.2.1.3 Metabolism and excretion of L-5-MTHF

Within the cell, folate is present in the cytosolic, mitochondrial or nuclear compartments. Folate in the mitochondria is involved in generating formate which is utilized in the cytoplasm for remethylation of homocysteine to methionine and synthesis of nucleotides. Folate in the nuclear compartment is for DNA synthesis. Unbound folate may undergo catabolism, generating p-aminobenzylglutamates which are acetylated in the liver before excretion (Shane, 2010).

Most of the folate that is filtered through the kidney glomerulus is reabsorbed in the proximal tubule and most of the folate in urine is in the form of folate cleavage products (Shane, 2010). The excretion rate of endogenous folate in feces is about the same as that excreted via urine, and the majority of folate in feces is synthesized by intestinal microorganisms (Krumdieck et al., 1978).

Folate is also excreted in breast milk. The majority of folate in breast milk is in the form of L-5-MTHF and breastfeeding infants are exposed to L-5-MTHF from birth (Büttner et al., 2014).

#### 6.2.1.4 Summary of absorption, distribution, metabolism and excretion of L-5-MTHF

The following conclusions about the ADME of L-5-MTHF-Ca can be made based upon the publicly available scientific information:

- In aqueous media or once ingested and exposed to the aqueous environment of the digestive tract, L-5-MTHF-Ca dissociates readily and completely into Ca and L-5-MTHF ions;

- L-5-MTHF is subsequently monoglutamated and absorbed mainly in the small intestine by carrier-mediated transport; and
- Once absorbed, the fate of synthetic L-5-MTHF becomes indistinguishable from that of all other absorbed and metabolized natural folates, or L-5-MTHF formed from synthetic folic acid.

## 6.2.2 Absorption, distribution, metabolism and excretion of calcium

### 6.2.2.1 Absorption of calcium

Soluble calcium, mainly as ionized  $\text{Ca}^{2+}$ , is absorbed across the intestinal mucosa by two general mechanisms: active transport through a saturable mechanism via calcium-binding proteins that is controlled by the active form of vitamin D; and passive diffusion through tight junctions and intercellular spaces mainly in the distal regions of the intestine (Bronner, 2003; IOM, 2011). Absorption of calcium is affected by the physiological state of the individual (growth, pregnancy, lactation, and aging), presence of dietary components and total calcium intake (Bronner, 1987). Infants have fractional calcium absorption of about 30-to-60%, decreasing to 25-to-40% in older children, decreasing further to about 25% of calcium intake in adult men and non-pregnant women (Abrams et al., 1997; Abrams et al., 2001; Hunt & Johnson, 2007; Lynch et al., 2007; Abrams, 2010). Calcium absorption in newborns is largely passive, but with increasing age passive diffusion decreases and vitamin D-mediated active uptake becomes more important (Kocian et al., 1973; Kobayashi et al., 1975; EFSA, 2015). The presence of lactose in breast milk or cow-milk-based infant formula increases the fractional and total calcium absorbed by infants (Abrams et al., 2002; IOM, 2011).

It has been hypothesized that some components in infant formulas, such as the fat blend and the protein and carbohydrate sources, may lead to lower calcium absorption from formula than from breast milk. Whether or not this is the case, the higher concentration of calcium in infant formula than in breast milk ensures that the total absorption of calcium from infant formula exceeds that expected for breastfed infants (Abrams et al., 2002).

### 6.2.2.2 Transport, distribution and storage of calcium

Calcium is maintained in the serum of humans within a narrow physiological range between 2.1 and 2.6 mmol/L through the action of an endocrine system that involves vitamin D and parathyroid hormone (IOM, 2011; EFSA, 2015). Calcium is the most abundant cation in the human body, with 99 % existing in the mineral phase of bone as hydroxyapatite crystals which are responsible for bone strength and rigidity (Bae & Kratzsch, 2018). Calcium deposition into bone is an on-going process during periods of growth and deposits provide a reservoir for other essential calcium-dependent functions in the body (EFSA, 2015).

### 6.2.2.3 Metabolism and excretion of calcium

Within the body, bone is constantly undergoing remodeling and almost the entire adult skeleton is remodeled over a 10-year cycle (IOM, 2011). Rates of bone turnover are determined by age, weight bearing activities, diet and genetic factors (EFSA, 2015).

The net intake and output of calcium for the body are controlled by the activities of the kidneys and gastrointestinal tract. Absorbed calcium is excreted in urine, feces, skin and sweat (EFSA, 2015). Approximately 98 % of calcium filtered by the kidney is reabsorbed and urinary calcium increases only slightly with increasing dietary calcium intake because of lower calcium absorption (EFSA, 2015). Unabsorbed dietary calcium is lost in the feces.

During the first three months of lactation, calcium is excreted in breast milk at a homostatically regulated concentration ranging from 5.0 to 7.5 mmol/L. These levels are unrelated to maternal calcium intake (EFSA, 2015). During lactation, calcium is liberated from bones and there is up-regulation of intestinal calcium absorption so that more calcium can be liberated into the breast milk (Bae & Kratzsch, 2018).

#### 6.2.2.4 Summary of absorption, distribution, metabolism and excretion of calcium

Calcium is an integral component of the skeleton; approximately 99 % of total body calcium is found in bones and teeth as calcium hydroxyapatite, the remaining 1% acts as essential intracellular messenger in cells and tissues. The following can be summarized about calcium ADME:

- Intestinal calcium absorption occurs through an active, saturable, transcellular process and a non-saturable, passive process;
- Active transport is controlled by vitamin D and passive transport is paracellular;
- Calcium absorption varies considerably throughout the lifespan, being higher during periods of rapid growth and lower in old age; and
- The main routes of obligatory (endogenous) calcium loss are urine, feces, skin and sweat (dermal losses).

### 6.3 Bioequivalence and bioavailability

Bioavailability is the proportion of an ingested nutrient that is absorbed and becomes available for metabolism or storage. The bioavailability of L-5-MTHF-Ca compared to folic acid has been investigated in rats and humans. Dietary studies in humans have been performed in healthy adults of both sexes and at different life stages and also in patients suffering from coronary artery disease (Table 8).

Adult male rats receiving a single intragastric dose of labeled L-5-MTHF and monitored for 8 days exhibited similar excretion kinetics to those receiving folic acid, with both urine and feces as important excretory routes (Bhandari & Gregory, 1992). Male rats divided into three groups and given a single capsule of folic acid, (6S)5-MTHF calcium salt or Quatrefolic at a dose of 70 µg/Kg L-5-MTHF-Ca and monitored for 8 hours had numerically, but not significantly, higher maximum L-5-MTHF concentrations ( $C_{max}$ ) in plasma compared to animals given folic acid (Miraglia et al., 2016). The 8-hour area under the curve (AUC) for L-5-MTHF-Ca-treated rats was more than eight times higher than for the folic acid treatment, indicating higher overall absorption of L-5-MTHF; however, the speed of absorption (time to reach  $C_{max}$ ) was the same for both folate forms.

A longer study (4-weeks) in male rats receiving growing-up milk (milk for children between 1-and-3 years of age) as their sole food source found that, compared to folic acid, inclusion of L-5-MTHF increased folate concentration in erythrocytes and liver but not in plasma (Pérez-Conesa et al., 2009).

In healthy adult men, the short-term absorption kinetics of a single dose of 500 µg L-5-MTHF-Ca (given as a capsule) was found to be equivalent to that of folic acid (Pentieva et al., 2004).  $C_{max}$ , time to reach  $C_{max}$  and AUCs were similar for both folate sources. A single high oral dose (5g) of racemic 6[R,S] 5-MTHF-Ca administered to patients with coronary artery disease resulted in seven times higher plasma levels of L-5-MTHF within 1-3h of administration than patients given the same dose of folic acid indicating higher bioavailability of orally administered L-5-MTHF-Ca compared to folic acid (Willems et al., 2004).

Longer term dietary supplementation of healthy adults with 100 or 200 µg/d folic acid or equimolar amounts of L-5-MTHF-Ca for 16-or-24 weeks resulted in similar increases in plasma and erythrocyte folate levels (Venn et al., 2002; Venn et al., 2003; Wright et al., 2010). Supplements containing higher levels (400 µg/d folic acid or up to 416 µg/d L-5-MTHF-Ca) taken by healthy women of childbearing age for 24 weeks also resulted in similar or slightly higher increases in plasma and erythrocyte folate levels in women taking the L-5-MTHF-Ca supplements compared to those taking folic acid (Lamers et al., 2004; Lamers et al., 2006). In lactating women, 416 µg/d L-5-MTHF-Ca was at least as effective as 400 µg/d folic acid in preserving maternal plasma and erythrocyte folate concentrations in the first 16 weeks of lactation (Houghton et al., 2006). For middle-aged women, plasma folate levels did not differ between those taking folic acid or L-5-MTHF-Ca supplements for 5 weeks (de Meer et al., 2005). Interestingly, intestinal absorption of folate sources (folic acid and L-



5-MTHF-Ca) may be age-dependent with middle-aged women having lower absorption rates compared to young adult women.

Similar to naturally occurring folates, the bioavailability of L-5-MTHF-Ca or folic acid used to fortify foodstuffs may be affected by the food matrix (EFSA, 2004). Naturally occurring folates in food such as broccoli, spinach and legumes are a mixture of mono- and polyglutamates that may not be completely released from the food matrix and may have some losses during digestion, leading to incomplete bioavailability (EFSA, 2014). In laboratory studies, FBP in cow milk was shown to protect L-5-MTHF (and other folates) against degradation (Jones & Nixon, 2002) and inclusion of cow milk in the diet of young women improved the bioavailability of folates naturally present in food, possibly due to the presence of FBP in the milk (Picciano et al., 2004). Both folic acid and L-5-MTHF are easily released from a milk matrix and made available for absorption (Verwei et al., 2003). The slightly lower binding affinity of milk FBP to L-5-MTHF compared to folic acid results in higher release of L-5-MTHF during gastric passage leading to slightly higher folate bioaccessibility from L-5-MTHF-Ca than folic acid.

To specifically investigate the suitability and safety of L-5-MTHF (supplied as L-5-MTHF-Ca) as a substitute for folic acid as the folate source in infant formula, Troesch et al., 2019 performed a feeding study in healthy term infants. The growth and tolerance among infants receiving formula containing folic acid (10 µg/100ml) was compared to that of infants receiving formula containing the equimolar dose of L-5-MTHF-Ca. The study was performed as a randomized, double-blind, parallel, controlled trial with an additional group of breastfed infants as a reference group. Infants of parents who independently chose not to breastfeed and decided to start full formula-feeding within the first 28 days of life were randomly assigned to one of the two formula groups. Infants were examined and anthropometric data were collected at a baseline visit (age 1-27 days) and at four additional visits (Visits 1-4 at mean ages of 28, 56, 84 and 112 days). A total of 360 healthy term infants from singleton pregnancies were enrolled. Of the recruited infants, 120 were breastfed, 120 were allocated to the control formula (folic acid), and 120 were allocated to the intervention formula group (L-5-MTHF-Ca). A total of 315 infants completed the first visit and 298 completed the fourth visit. The number of drop-outs did not differ between groups. Both formulae were well-accepted and no differences in acceptance and tolerability or consistency, color and odor of stool were reported. There were no adverse effects, or blood chemistry and hematology results that gave reason for safety concerns and all results were within the expected range and not different between the intervention and control groups. Most markers for folate status did not differ between the intervention and control groups; however, at visit 4, plasma level of unmetabolized folic acid was significantly higher in the control compared to the intervention group, with comparable concentrations of unmetabolized folic acid in the intervention and breastfed groups. Red cell folate levels were significantly higher in infants consuming the formula containing L-5-MTHF-Ca compared to control subjects (adjusted means of 907 nmol/L versus 839 nmol/L). The primary outcome, weight gain during the intervention period was within the predefined interval of ±3.5g/day, thus demonstrating equivalence. While there was not enough evidence to support equivalence for length growth, the gain in head circumference demonstrated equivalence of the two folate sources. It could be concluded that an infant formula with L-5-MTHF-Ca did not show significant differences in growth and tolerance compared to infants fed the same formula with folic acid at equimolar doses.

The studies performed in rats and humans indicate that the bioavailability of L-5-MTHF-Ca, whether consumed as a supplement or as a folate source in milk or infant formula, is equivalent to or slightly higher than folic acid.

**Table 8: Bioequivalence studies in humans with L-5-MTHF-Ca and folic acid**

Reference	Study type	Population group (number) (age)	Duration	Treatment group and Dose (n)	Plasma L-5-MTHF (nmol/L)	RBC MTHF (nmol/L)	L-5-MTHF	Main findings
<i>Venn et al., 2002</i>	Double-blind, randomized	Women (104) (18-49y)	24 weeks	113 µg/d L-5-MTHF-Ca 100 µg/d FA Placebo	27.2 34.8 25.2	1015 1131 870		L-5-MTHF-Ca and FA increased blood folate indices to a similar extent
<i>Venn et al., 2003</i>	Double-blind, randomized	Healthy adults (117F, 38M) (mean age 45y)	24 weeks	113 µg/d L-5-MTHF-Ca 100 µg/d FA Placebo	25.6 34.5 20.5	984 1137 848		Increases in RBC and plasma folate did not differ between the FA and L-5-MTHF-Ca groups
<i>Lamers et al., 2004</i>	Double-blind, randomized	Healthy women (135) (18-35y)	24 weeks	416 µg/d L-5-MTHF-Ca 208 µg/d L-5-MTHF-Ca 400 µg/d FA Placebo	35.5 27.9 34.7 13.7	Not measured		L-5-MTHF-Ca and FA equally affected plasma folate
<i>Pentieva et al., 2004</i>	Double-blind, crossover	Men (13) (18-45y)	Single dose followed by 10h sampling	500 µg L-5-MTHF-Ca 500 µg folic acid Placebo	47.7 47.1 44.8	Not measured		Rmax was 1.5h for FA, 2h for L-5-MTHF. AUC calculated up to 7h post administration was 145.8 after FA and 141.6 after L-5-MTHF (p=0.9). L-5-MTHF-Ca and FA display equivalent short term bioavailability
<i>Willems et al., 2004</i>	Two-way, two-period, blinded, randomized, crossover	Patients with coronary artery disease (19M, 5F) (44-64y)	Single dose followed by 12h sampling. 1 wk wash-out before crossover	5mg 6[R,S] 5-MTHF-Ca 5mg folic acid	129 ng/ml 14.1 ng/ml	Not measured		The bioavailability of 5-MTHF is higher compared to FA. Peak concentration was 7 times higher.
<i>de Meer et al., 2005</i>	Double-blind, randomized	Healthy adults (5M, 7F) (<30y) Healthy adults (5M, 7F) (≥50y)	5 weeks 5 weeks	416 µg/d L-5-MTHF-Ca 400 µg/d folic acid 416 µg/d L-5-MTHF-Ca 400 µg/d folic acid	23.4 29.2 25.6 20.5	Not measured		L-5-MTHF-Ca and FA increased plasma folate to a similar extent. FA increased folate turnover in young adults but not older adults

Reference	Study type	Population group (number) (age)	Duration	Treatment group and Dose (n)	Plasma L-5-MTHF (nmol/L)	RBC MTHF (nmol/L)	L-5-MTHF	Main findings
Lamers et al., 2006	Double-blind, randomized	Healthy women (144) (19-33y)	24 weeks	416 µg/d L-5-MTHF-Ca 208 µg/d L-5-MTHF-Ca 400 µg/d FA Placebo	~58 ~52 ~42 ~20	~1400 ~1100 ~1250 ~700		L-5-MTHF-Ca was more effective than FA at increasing folate status
Houghton et al., 2006	Double-blind, randomized	Lactating mothers (64) (mean age 32y)	16 weeks	416 µg/d L-5-MTHF-Ca 400 µg/d folic acid Placebo	91.1 93.5 43.5	2178 1967 1390		Short term bioavailability of L-5-MTHF-Ca and FA are equivalent
Wright et al., 2010		Healthy adults (64M, 99F) (18-65y)	16 weeks	Food folate 208 µg/d L-5-MTHF-Ca 200 µg/d folic acid Placebo	30.4 36.8 43.1 25.5	1009 1136 1154 927		L-5-MTHF-Ca and FA increased RBC and plasma folate to a similar extent
Troesch et al., 2019	Double-blind, randomized	Healthy term infants (298) (0-4 m)	4 months	11.3 µg/100ml L-5-MTHF-ca 10 µg/100ml FA Breast milk	55.3 52.7 33.0	907 839 484		L-5-MTHF-Ca increased RBC folate levels compared to FA

## 6.4 Safety

### 6.4.1 Human studies

The safety of L-5-MTHF-Ca as a source of folate for use in infant formula was investigated in the recent study by Troesch et al. (2019) as described in Part 6.3. Infants were given formula containing 10 µg/100ml folic acid (15.2 ug/100 Kcal) or the equimolar dose of 10.4 µg/100 ml L-5-MTHF which was added as 11.3 µg/100 ml of L-5-MTHF-Ca. Infants received the formula for 4 months. Both formulae were well accepted without differences in tolerance or occurrence of adverse effects and weight gain and gain in head circumference were equivalent between groups. The study confirmed that L-5-MTHF-Ca is suitable for use in infant formula.

Besides the tolerability study in infants (Troesch et al., 2019) and the studies demonstrating the bioequivalence of the folate sources (Part 6.3), additional studies in patients suffering from various illnesses provide evidence to support high tolerance for L-5-MTHF-Ca and L-5-MTHF.

Adult patients suffering from major depressive disorder (MDD) taking daily doses of 7.5 or 15 mg L-5-MTHF or L-5-MTHF-Ca for up to 12 months reported significant improvements in some mental health outcomes, L-5-MTHF was well-tolerated, and the incidence of treatment-related adverse events was comparable to the placebo (Papakostas et al., 2012; Shelton et al., 2013; Zajecka et al., 2016). L-5-MTHF taken at a daily dose of 5.23 mg has also been explored in an initial 12-week pilot study in MDD patients planning pregnancy or during pregnancy and no major concerns related to L-5-MTHF were raised (Freeman et al., 2019). No adverse effects were mentioned in studies in which hemodialysis patients were treated for up to 12 weeks with 15-or-17 mg/d L-5-MTHF and homocysteine-lowering efficacy was measured (Perna et al., 1997; Bostom et al., 2000).

### 6.4.2 Pre-clinical studies

A series of pre-clinical studies with the L-5-MTHF-Ca that is the subject of this Notification were sponsored by the manufacturer, Merck & Cie, and were described in detail in the publication, Niederberger et al. (2019). The studies were performed under Good Laboratory Practice and according to the respective Organization for Economic Co-operation and Development (OECD) Guidance. Taken together, these studies provide additional support for the safety of L-5-MTHF-Ca. An overview of the genotoxicity, subchronic oral toxicity and developmental toxicity studies performed with L-5-MTHF-Ca is presented in Table 9. Details of each of the studies are provided in the following Parts.

Table 9: Summary of pre-clinical studies with L-5-MTHF-Ca\*

Study type	Test system/Species	Concentrations /Dosages	Results
<b>In vitro studies</b>			
Ames test, plate incorporation	S. typhimurium TA98, TA100, TA102, TA1535, TA1537. E. coli WP2 uvr A pKM101	5.00, 15.8, 50.0, 158, 500, 1580, 5000 µg/plate	Non-mutagenic
Mammalian gene mutation test	L5178Y TK(+/-) mouse lymphoma cells	50.0, 158, 500, 1580, 2810, 5000 µg/ml	Non-mutagenic
<b>In vivo studies</b>			
Mammalian erythrocyte micronucleus test	Male Wistar rats (HsdCpb:WU)	2000 mg/kg bw by oral gavage	Non-genotoxic

Study type	Test system/Species	Concentrations /Dosages	Results
Unscheduled DNA Synthesis test	Male Han Wistar rats (CrI:WI (Glx/BRL/Han) BR)	800 or 2000 mg/kg bw by oral gavage	Non-genotoxic
Acute toxicity	Hsd:Cpb:WU strain	2000 mg	LD50 higher than 2000 mg/kg bw
13-Week sub-chronic oral toxicity test with 4-week recovery period	Male and female HanIbm:Wistar (SPF) rats	25, 100 or 400 mg/kg bw/d by oral gavage	No treatment-related adverse effects. NOAEL at the highest dose tested, 400 mg/kg bw/d
Prenatal developmental toxicity test	Female Wistar rats (HsdCpb:WU)	100, 300 or 1000 mg/kg bw/d by oral gavage	No treatment-related adverse effects. NOAEL at the highest dose tested, 1000 mg/kg bw/d

\*Niederberger et al. (2019)

#### 6.4.2.1 Genotoxicity

The mutagenic potential of L-5-MTHF-Ca was investigated in an Ames test using *S. typhimurium* strains TA98, TA100, TA102, TA1535 and TA1537 and *E. coli* WP2 uvr A pKM101 exposed to concentrations of 5-5000 µg/plate with and without metabolic activation. Toxicity to the bacteria was not observed and the number of revertants did not increase in any bacterial strain. L-5-MTHF-Ca was thus found to be not mutagenic under the conditions of this test (Niederberger et al., 2019).

Using the same Ames test protocol, the mutagenic potential of D,L-5-MTHF-Ca (the racemic mixture of D-5-MTHF-Ca and L-5-MTHF-Ca), D-5-MTHF-Ca (the biologically inactive enantiomer of L-5-MTHF-Ca), L-Mefox-Ca (the s-triazine derivative of L-5-MTHF) and L-METHPA-Ca (the hydrolysis product of L-MTHF-Ca) were investigated (EFSA, 2004). None of the compounds were toxic to the bacteria and no increases in the number of revertants were seen in any bacterial strain with any of the tested compounds.

The mutagenic potential of L-5-MTHF-Ca was also investigated in an in vitro mouse lymphoma thymidine kinase gene mutation assay at concentrations of 5-5000 µg/ml using a microwell method (Niederberger et al., 2019). In the presence of S9 mix with an exposure time of 3 hours, no cytotoxicity occurred up to the highest concentration tested and the frequency of mutations at the TK locus was not increased. In the absence of S9 mix with an exposure time of 3-or-24 hours, cytotoxicity was observed at the three highest concentrations (1580, 2810 and 5000 µg/ml). In experiments without S9 mix, mutation frequency increased by less than 2-fold after a 3-hour exposure and 3.4-fold after 24-hour exposure. Test materials showing a weak effect in one series and no effect in another were assessed as negative and it was concluded that L-5-MTHF-Ca did not induce mutagenic effects at the TK locus in mouse lymphoma cells (Niederberger et al., 2019).

L-5-MTHF-Ca was tested for its potential to induce repairable DNA damage in a standard Unscheduled DNA Synthesis (UDS) test in male rats receiving the material at 800 and 2000 mg/kg bw via oral gavage (Niederberger et al., 2019). Animals were killed 2-4 or 12-14 hours after the administration, livers were removed, primary cultures of hepatocytes were prepared, incubated and then processed for autoradiography. The net number of nuclear grains was counted. The results demonstrated that oral administration of L-5-MTHF-Ca at 500 and 2000 mg/kg bw did not increase the unscheduled DNA synthesis in the liver of rats and exhibited no genotoxic activity under the conditions of this test.

A standard micronucleus test was performed in which L-5-MTHF-Ca was administered by gavage to male rats at a dose of 2000 mg/kg bw. Animals were killed after 24-or-48 hours. Bone marrow

smears were prepared and the erythrocytes examined for the presence of micronuclei. No increase in micronucleated polychromatic erythrocytes was observed, demonstrating that L-5-MTHF-Ca was not mutagenic under the conditions of the test (Niederberger et al., 2019).

#### 6.4.2.2 Acute toxicity

The acute toxicity of L-5-MTHF-Ca was examined in rats at a dose of 2000 mg/kg bw by gavage (EFSA, 2004). After the 15-day observation period, animals were killed and subjected to gross necropsy. All rats gained weight normally and survived until the end of the study. No gross changes in organs were observed. It was concluded that L-5-MTHF-Ca had an LD<sub>50</sub> greater than 2000 mg/kg bw.

Using the same protocol, the acute toxicity of D,L-5-MTHF-Ca, D-5-MTHF-Ca, L-Mefox-Ca and L-METHPA-Ca were investigated (EFSA, 2004). All rats on these treatments gained weight normally and survived until the end of the study. No gross changes in organs were observed at necropsy. It was concluded that each of these substances have an LD<sub>50</sub> greater than 2000 mg/kg bw.

#### 6.4.2.3 Subchronic toxicity

In a 13-week toxicity study rats received L-5-MTHF-Ca at doses of 0, 25, 100 and 400 mg/kg bw/day via gavage (Niederberger et al., 2019). After 13 weeks of treatment ten animals per sex per group were sacrificed. Extra five animals per sex per group in the control and high-dose groups were retained for an additional 4-week treatment-free recovery period. All animals survived until scheduled necropsy except for one female from the high-dose group which died after a gavage error which was confirmed by necropsy. All animals gained weight and showed no adverse effects due to treatment. Body weights and food consumption did not differ between treated-groups and controls. The functional observational battery and locomotor activity tests did not reveal any changes in response to the treatment. There were no hematological changes. Plasma analyses revealed statistically significantly lower levels of aspartate amino-transferase, lactate dehydrogenase and creatine kinase in males, but not females, from the high-dose group. The observed changes were no longer present after the recovery phase. Organ weights did not change in response to the treatment. The histopathological examinations did not reveal any abnormalities that could be attributed to treatment. The slight and reversible decreases in three plasma enzymes in the males from the high-dose group were the only changes noted and were not considered adverse. Therefore, the no-observed-adverse-effect-level (NOAEL) was the highest dose tested in the study, 400 mg/kg bw/day in male and female rats.

#### 6.4.2.4 Developmental toxicity

In an embryotoxicity/teratogenicity study, pregnant rats received L-5-MTHF-Ca at daily doses of 0, 100, 300 and 1000 mg/kg bw from day 5-to-19 of pregnancy (Niederberger et al., 2019). On day 20, the dams were killed and dissected, and fetuses were removed from the uterus and examined for evidence of developmental toxicity. Out of 25, between 22-and-24 rats per group were found to be pregnant. The treatment was well tolerated and all animals survived until the end of the study. There were no differences between treated groups and controls in body weight gains or food consumption. The water intake was slightly increased in the high-dose group. Reproductive performance was not affected by the treatment. All pregnant females had litters with viable fetuses. None of the examined parameters (percent resorptions/litter, average number of live fetuses/litter, average fetal body weight/litter, and sex ratio/litter) was influenced by the treatment. Necropsy of the maternal rats did not reveal gross changes that could be attributed to the treatment. Examination of the fetuses for external, visceral and skeletal malformations and anomalies did not reveal any fetotoxic, embryotoxic or teratogenic effects associated with administration of L-5-MTHF-Ca to dams.

#### 6.4.3 Safety of calcium

The IOM has extensively reviewed biological and toxicological effects related to calcium deficiency and excess. IOM ULs for calcium are 1000 mg/d for 0-6-month-olds and 1500 mg/d for 6-12-month-



olds. As noted previously, in Part 3.2.3, the additional intake of 6 µg/d of calcium from inclusion of L-5-MTHF-Ca in infant formula would be considered insignificant.

## 6.5 Safety conclusion

The safety of use of L-5-MTHF-Ca in infant formula as a source of folate has been thoroughly assessed as described above. After ingestion, the product dissociates to its calcium and L-5-MTHF components. L-5-MTHF is the major naturally occurring form of folate in the human body, in food, and in breast milk and has been used around the world in food supplements and certain foods for many years. Consequently, there is a wealth of information available in the public domain on the absorption, distribution, metabolism, excretion, safety and efficacy of L-5-MTHF and L-5-MTHF-Ca. The scientific data, information and methods that form the technical element of this conclusion are:

- The establishment of the identity of L-5-MTHF-Ca;
- The method of manufacture and specifications demonstrating the safe production and high quality control of the process and final product which ensure purity of the product and prevention of contamination;
- The comprehensive body of literature available to describe ADME of folates;
- The suite of published genotoxicity and toxicity studies establishing the lack of genotoxic and toxic potential of L-5-MTHF-Ca, as well as low toxicity in the repeated dose and developmental toxicity studies in rats;
- The NOAEL of 400 mg/kg bw/d established in the 90-d toxicity study in rats provides a margin of safety of 13,289 at the highest 90<sup>th</sup> percentile intake of L-5-MTHS-Ca in the highest consuming infant age group, well above a required 100. In other infant age groups, margins of safety (MOS) are even higher. The NOAEL of 1000 mg/kg bw/d established in the developmental toxicity study provides even higher margins of safety; and
- The pivotal published and peer-reviewed infant feeding trial in which L-5-MTHF-Ca was included as the folate source demonstrating the equivalence of L-5-MTHF-Ca and folic acid and definitely establishing the safety of L-5-MTHF-Ca when provided in infant formula (Troesch et al., 2019).

The available information provide support for the safety of L-5-MTHF-Ca and demonstrate that, as its intended use is to replace folic acid in infant formula in equimolar amounts, the dietary intake of the vitamin folate would remain unchanged. L-5-MTHF-Ca is therefore Generally Recognized as Safe (GRAS) for this intended use in non-exempt and exempt infant formula based upon scientific procedures. The majority of the data used in the GRAS determination are publicly available and generally known from peer-reviewed scientific publications and therefore meet the “general recognition” standard under the FD&C Act. We therefore conclude that use of L-5-MTHF-Ca in non-exempt and exempt infant formula is GRAS under the intended conditions of use.

DSM is not aware of information that would be inconsistent with a finding that the proposed use of calcium L-methylfolate in non-exempt and exempt infant formula, meeting appropriate specifications and manufactured according to cGMP, is GRAS.

The scientific data and information that provide the basis for this GRAS conclusion by scientific procedures are published and in the public domain. Part 7 of this GRAS notice contains the citations for the published studies. This publicly available data and information fulfills the requirement for general availability of the scientific data and information. The peer-review of the published studies provides ample evidence of a consensus among qualified experts that there is reasonable certainty that use of L-5-MTHF-Ca in non-exempt and exempt infant formulas for infants as a replacement for folic acid is not harmful. The general availability and acceptance of the scientific data and information satisfies the common knowledge aspect of this GRAS conclusion.

## 7 LIST OF SUPPORTING DATA AND INFORMATION IN THE GRAS NOTICE

### 7.1 References

- Abrams S.A. (2010) Calcium absorption in infants and small children: methods of determination and recent findings. *Nutrients*, 2, 474-480. DOI: 10.3390/nu2040474.
- Abrams S.A., Griffin I.J., Davila P. & Liang L. (2001) Calcium fortification of breakfast cereal enhances calcium absorption in children without affecting iron absorption. *The Journal of pediatrics*, 139, 522-526. DOI: 10.1067/mpd.2001.116936.
- Abrams S.A., Griffin I.J. & Davila P.M. (2002) Calcium and zinc absorption from lactose-containing and lactose-free infant formulas. *The American journal of clinical nutrition*, 76, 442-446. DOI: 10.1093/ajcn/76.2.442.
- Abrams S.A., Wen J. & Stuff J.E. (1997) Absorption of calcium, zinc, and iron from breast milk by five- to seven-month-old infants. *Pediatric Research*, 41, 384 EP. DOI: 10.1203/00006450-199703000-00014.
- Bae Y.J. & Kratzsch J. (2018) Vitamin D and calcium in the human breast milk. *Best practice & research. Clinical endocrinology & metabolism*, 32, 39-45. DOI: 10.1016/j.beem.2018.01.007.
- Bhandari S.D. & Gregory J.F.I. (1992) Folic acid, 5-methyl-tetrahydrofolate and 5-formyl-tetrahydrofolate exhibit equivalent intestinal absorption, metabolism and in vivo kinetics in rats. *The Journal of nutrition*, 122, 1847-1854. DOI: 10.1093/jn/122.9.1847.
- Bostom A.G., Shemin D., Bagley P., Massy Z.A., Zanabli A., Christopher K., Spiegel P., Jacques P.F., Dworkin L. & Selhub J. (2000) Controlled comparison of L-5-methyltetrahydrofolate versus folic acid for the treatment of hyperhomocysteinemia in hemodialysis patients. *Circulation*, 101, 2829-2832.
- Bronner F. (1987) Intestinal calcium absorption: mechanisms and applications. *The Journal of nutrition*, 117, 1347-1352. DOI: 10.1093/jn/117.8.1347.
- Bronner F. (2003) Mechanisms of intestinal calcium absorption. *Journal of cellular biochemistry*, 88, 387-393. DOI: 10.1002/jcb.10330.
- Büttner B.E., Witthöft C.M., Domellöf M., Hernell O. & Öhlund I. (2014) Effect of type of heat treatment of breastmilk on folate content and pattern. *Breastfeeding medicine : the official journal of the Academy of Breastfeeding Medicine*, 9, 86-91. DOI: 10.1089/bfm.2013.0008.
- Clifford A., K Heid M., G Müller H. & Bills N. (1990) Tissue distribution and prediction of total body folate of rats. *The Journal of nutrition*, 120, 1633-1639. DOI: 10.1093/jn/120.12.1633.
- Codex Alimentarius (1981) International Food Standards. Standard for infant formula and formulas for special medical purposes intended for infants: CODEX STAN 72-1981.
- de Meer K., Smulders Y.M., Dainty J.R., Smith D.E.C., Kok R.M., Stehouwer C.D.A., Finglas P.M. & Jakobs C. (2005) 6S5-methyltetrahydrofolate or folic acid supplementation and absorption and initial elimination of folate in young and middle-aged adults. *European journal of clinical nutrition*, 59, 1409-1416. DOI: 10.1038/sj.ejcn.1602254.
- EFSA (2004) Opinion of the Scientific Panel on food additives, flavourings, processing aids and materials in contact with food (AFC) related to Calcium L-Methylfolate. *EFSA Journal*, 2, 135. DOI: 10.2903/j.efsa.2004.135.

- EFSA (2014) Scientific Opinion on Dietary Reference Values for folate. *EFSA Journal*, 12, 55. DOI: 10.2903/j.efsa.2014.3893.
- EFSA (2015) Scientific Opinion on Dietary Reference Values for calcium. *EFSA Journal*, 13, 469. DOI: 10.2903/j.efsa.2015.4101.
- Freeman M.P., Savella G.M., Church T.R., Góez-Mogollón L., Sosinsky A.Z., Noe O.B., Kaimal A. & Cohen L.S. (2019) A prenatal supplement with methylfolate for the treatment and prevention of depression in women trying to conceive and during pregnancy. *Annals of clinical psychiatry : official journal of the American Academy of Clinical Psychiatrists*, 31, 4-16.
- Gregory J.F., Bhandari S.D., Bailey L.B., Toth J.P., Baumgartner T.G. & Cerda J.J. (1992) Relative bioavailability of deuterium-labeled monoglutamyl tetrahydrofolates and folic acid in human subjects. *The American journal of clinical nutrition*, 55, 1147-1153. DOI: 10.1093/ajcn/55.6.1147.
- Grimes C.A., Szymlek-Gay E.A., Campbell K.J. & Nicklas T.A. (2015) Food sources of total energy and nutrients among U.S. infants and toddlers: national health and nutrition examination survey 2005-2012. *Nutrients*, 7, 6797-6836. DOI: 10.3390/nu7085310.
- Houghton L.A., Sherwood K.L., Pawlosky R., Ito S. & O'Connor D.L. (2006) 6S-5-Methyltetrahydrofolate is at least as effective as folic acid in preventing a decline in blood folate concentrations during lactation. *The American journal of clinical nutrition*, 83, 842-850. DOI: 10.1093/ajcn/83.4.842.
- Hunt C.D., Butte N.F. & Johnson L.K. (2005) Boron concentrations in milk from mothers of exclusively breast-fed healthy full-term infants are stable during the first four months of lactation. *The Journal of nutrition*, 135, 2383-2386. DOI: 10.1093/jn/135.10.2383.
- Hunt C.D., Friel J.K. & Johnson L.K. (2004) Boron concentrations in milk from mothers of full-term and premature infants. *The American journal of clinical nutrition*, 80, 1327-1333. DOI: 10.1093/ajcn/80.5.1327.
- Hunt C.D. & Johnson L.K. (2007) Calcium requirements: new estimations for men and women by cross-sectional statistical analyses of calcium balance data from metabolic studies. *The American journal of clinical nutrition*, 86, 1054-1063. DOI: 10.1093/ajcn/86.4.1054.
- Hunt C.D. & Meacham S.L. (2001) Aluminum, boron, calcium, copper, iron, magnesium, manganese, molybdenum, phosphorus, potassium, sodium, and zinc: Concentrations in common Western foods and estimated daily intakes by infants; toddlers; and male and female adolescents, adults and seniors in the United States. *Journal of the American Dietetic Association*, 101, 1058-1060.
- IOM (1998) Dietary reference intakes for thiamin, riboflavin, niacin, vitamin B<sub>6</sub>, folate, vitamin B<sub>12</sub>, pantothenic acid, biotin, and choline. The National Academies Press, Washington, DC.
- IOM (2011) Dietary reference intakes for calcium and vitamin D, Washington, DC.
- Jones M.L. & Nixon P.F. (2002) Tetrahydrofolates are greatly stabilized by binding to bovine milk folate-binding protein. *The Journal of nutrition*, 132, 2690-2694. DOI: 10.1093/jn/132.9.2690.
- Kobayashi A., Kawai S., Obe Y. & Nagashima Y. (1975) Effects of dietary lactose and lactase preparation on the intestinal absorption of calcium and magnesium in normal infants. *The American journal of clinical nutrition*, 28, 681-683. DOI: 10.1093/ajcn/28.7.681.
- Kocian J., Skala I. & Bakos K. (1973) Calcium absorption from milk and lactose-free milk in healthy subjects and patients with lactose intolerance. *Digestion*, 9, 317-324. DOI: 10.1159/000197457.

- Krumdieck C.L., Fukushima K., Fukushima T., Shiota T. & Butterworth C.E., JR (1978) A long-term study of the excretion of folate and pterins in a human subject after ingestion of <sup>14</sup>C folic acid, with observations on the effect of diphenylhydantoin administration. *The American journal of clinical nutrition*, 31, 88-93. DOI: 10.1093/ajcn/31.1.88.
- Lamers Y., Prinz-Langenohl R., Brämswig S. & Pietrzik K. (2006) Red blood cell folate concentrations increase more after supplementation with 6S-5-methyltetrahydrofolate than with folic acid in women of childbearing age. *The American journal of clinical nutrition*, 84, 156-161. DOI: 10.1093/ajcn/84.1.156.
- Lamers Y., Prinz-Langenohl R., Moser R. & Pietrzik K. (2004) Supplementation with 6S-5-methyltetrahydrofolate or folic acid equally reduces plasma total homocysteine concentrations in healthy women. *The American journal of clinical nutrition*, 79, 473-478. DOI: 10.1093/ajcn/79.3.473.
- Lowe K.E., Osborne C.B., Lin B.F., Kim J.S., Hsu J.C. & Shane B. (1993) Regulation of folate and one-carbon metabolism in mammalian cells. II. Effect of folylpoly-gamma-glutamate synthetase substrate specificity and level on folate metabolism and folylpoly-gamma-glutamate specificity of metabolic cycles of one-carbon metabolism. *The Journal of biological chemistry*, 268, 21665-21673.
- Lynch M.F., Griffin I.J., Hawthorne K.M., Chen Z., Hamzo M. & Abrams S.A. (2007) Calcium balance in 1-4-y-old children. *The American journal of clinical nutrition*, 85, 750-754. DOI: 10.1093/ajcn/85.3.750.
- Miraglia N., Agostinetti M., Bianchi D. & Valoti E. (2016) Enhanced oral bioavailability of a novel folate salt: comparison with folic acid and a calcium folate salt in a pharmacokinetic study in rats. *Minerva ginecologica*, 68, 99-105.
- Neal-Kluever A., Aungst J., Gu Y., Hatwell K., Muldoon-Jacobs K., Liem A., Ogungbesan A. & Shackelford M. (2014) Infant toxicology: state of the science and considerations in evaluation of safety. *Food and chemical toxicology : an international journal published for the British Industrial Biological Research Association*, 70, 68-83. DOI: 10.1016/j.fct.2014.05.003.
- Niederberger K.E., Dahms I., Broschard T.H., Boehni R. & Moser R. (2019) Safety evaluation of calcium L-methylfolate. *Toxicology Reports*. DOI: 10.1016/j.toxrep.2019.09.012.
- Papakostas G.I., Shelton R.C., Zajecka J.M., Etemad B., Rickels K., Clain A., Baer L., Dalton E.D., Sacco G.R., Schoenfeld D., Pencina M., Meisner A., Bottiglieri T., Nelson E., Mischoulon D., Alpert J.E., Barbee J.G., Zisook S. & Fava M. (2012) L-methylfolate as adjunctive therapy for SSRI-resistant major depression: results of two randomized, double-blind, parallel-sequential trials. *The American journal of psychiatry*, 169, 1267-1274. DOI: 10.1176/appi.ajp.2012.11071114.
- Pentieva K., McNulty H., Reichert R., Ward M., Strain J.J., McKillop D.J., McPartlin J.M., Connolly E., Molloy A., Krämer K. & Scott J.M. (2004) The short-term bioavailabilities of 6S-5-methyltetrahydrofolate and folic acid are equivalent in men. *The Journal of nutrition*, 134, 580-585. DOI: 10.1093/jn/134.3.580.
- Pérez-Conesa D., Haro Vicente J., Romero F. & Ros G. (2009) [6S]-5-Methyltetrahydrofolate enhances folate status in rats fed growing-up milk. *European journal of nutrition*, 48. DOI: 10.1007/s00394-009-0022-1.
- Perna A.F., Ingrosso D., Santo N.G. de, Galletti P., Brunone M. & Zappia V. (1997) Metabolic consequences of folate-induced reduction of hyperhomocysteinemia in uremia. *Journal of the American Society of Nephrology : JASN*, 8, 1899-1905.

- Picciano M.F., West S.G., Ruch A.L., Kris-Etherton P.M., Zhao G., Johnston K.E., Maddox D.H., Fishell V.K., Dirienzo D.B. & Tamura T. (2004) Effect of cow milk on food folate bioavailability in young women. *The American journal of clinical nutrition*, 80, 1565-1569. DOI: 10.1093/ajcn/80.6.1565.
- Scott J. (2001) Methyltetrahydrofolate: the superior alternative to folic acid. In: *Nutraceuticals in health and disease prevention* (ed. by K. Kramer, P.-P. Hoppe & L. Packer), pp. 90-104. CRC Press.
- Selhub J., Powell G.M. & Rosenberg I.H. (1984) Intestinal transport of 5-methyltetrahydrofolate. *The American journal of physiology*, 246, G515-20. DOI: 10.1152/ajpgi.1984.246.5.G515.
- Shane B. (2010) Folate chemistry and metabolism. In: *Folate in health and disease* (ed. by L.B. Bailey), 1-24. CRC Press, Boca Raton.
- Shelton R.C., Sloan Manning J., Barrentine L.W. & Tipton E.V. (2013) Assessing effects of L-methylfolate in depression management: results of a real-world patient experience trial. *The primary care companion for CNS disorders*, 15. DOI: 10.4088/PCC.13m01520.
- Sweeney M.R., McPartlin J. & Scott J. (2007) Folic acid fortification and public health: report on threshold doses above which unmetabolised folic acid appear in serum. *BMC public health*, 7, 41. DOI: 10.1186/1471-2458-7-41.
- Tamura T., Yoshimura Y. & Arakawa T. (1980) Human milk folate and folate status in lactating mothers and their infants. *The American journal of clinical nutrition*, 33, 193-197. DOI: 10.1093/ajcn/33.2.193.
- Thorand B., Pietrzik K., Prinz-Langenohl R., Hages M. & Holzgreve W. (1996) Maternal and fetal serum and red blood cell folate and vitamin B12 concentrations in pregnancies affected by neural tube defects. *Zeitschrift für Geburtshilfe und Neonatologie*, 200, 176-180.
- Troesch B., Demmelmair J., Gimpfl M., Hecht C., Lakovic G., Roehle R., Sipka L., Trisic B., Vusurovic M., Schoop R., Zdjelar S., Koletzko B. & on behalf of the MEFOLIN Study Group (2019) Suitability and safety of L-5-methyltetrahydrofolate as a folate source in infant formula: A randomized-controlled trial. *PLOS ONE*, 14, e0216790. DOI: 10.1371/journal.pone.0216790.
- Venn B.J., Green T.J., Moser R. & Mann J.I. (2003) Comparison of the effect of low-dose supplementation with L-5-methyltetrahydrofolate or folic acid on plasma homocysteine: a randomized placebo-controlled study. *The American journal of clinical nutrition*, 77, 658-662. DOI: 10.1093/ajcn/77.3.658.
- Venn B.J., Green T.J., Moser R., McKenzie J.E., Skeaff C.M. & Mann J. (2002) Increases in blood folate indices are similar in women of childbearing age supplemented with 6S-5-methyltetrahydrofolate and folic acid. *The Journal of nutrition*, 132, 3353-3355. DOI: 10.1093/jn/132.11.3353.
- Verwei M., Arkbåge K., Havenaar R., van den Berg H., Witthöft C. & Schaafsma G. (2003) Folic acid and 5-methyltetrahydrofolate in fortified milk are bioaccessible as determined in a dynamic in vitro gastrointestinal model. *The Journal of nutrition*, 133, 2377-2383.
- Visentin M., Diop-Bove N., Zhao R. & Goldman I.D. (2014) The intestinal absorption of folates. *Annual review of physiology*, 76, 251-274. DOI: 10.1146/annurev-physiol-020911-153251.
- WHO (2000) Air quality guidelines for Europe.
- Willems F.F., Boers G.H.J., Blom H.J., Aengevaeren W.R.M. & Verheugt F.W.A. (2004) Pharmacokinetic study on the utilisation of 5-methyltetrahydrofolate and folic acid in patients

with coronary artery disease. *British journal of pharmacology*, 141, 825-830. DOI: 10.1038/sj.bjp.0705446.

Wittsiepe J., Schrey P., Wilhelm M., Begerow J. & Dunemann L. (2003) Dietary intake of platinum and gold by children from Germany using duplicate portion sampling. *Journal of trace elements in medicine and biology*, 17, 117-122.

Wright A.J.A., King M.J., Wolfe C.A., Powers H.J. & Finglas P.M. (2010) Comparison of (6S)-5-methyltetrahydrofolic acid v. folic acid as the reference folate in longer-term human dietary intervention studies assessing the relative bioavailability of natural food folates: comparative changes in folate status following a 16-week placebo-controlled study in healthy adults. *The British journal of nutrition*, 103, 724-729. DOI: 10.1017/S0007114509992339.

Zajacka J.M., Fava M., Shelton R.C., Barrentine L.W., Young P. & Papakostas G.I. (2016) Long-term efficacy, safety, and tolerability of L-methylfolate calcium 15 mg as adjunctive therapy with selective serotonin reuptake inhibitors: a 12-month, open-label study following a placebo-controlled acute study. *The Journal of clinical psychiatry*, 77, 654-660. DOI: 10.4088/JCP.15m10181.

## 7.2 Appendices

Appendix A: United States Pharmacopeial Convention. Monograph for calcium L-5-methyltetrahydrofolate. Currently official as of 21-Oct-2019.

Appendix B: Merck & Cie (2015) Certificates of analysis for three batches of Metafolin®.

Appendix C: Specifications for calcium L-5methyltetrahydrofolate prepared at the 65<sup>th</sup> JECFA (2005)

Appendix D: Stability Reports

Appendix E: Self Affirmed GRAS Expert Panel Opinion

## Appendix A



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Currently Official as of: 21-Oct-2019

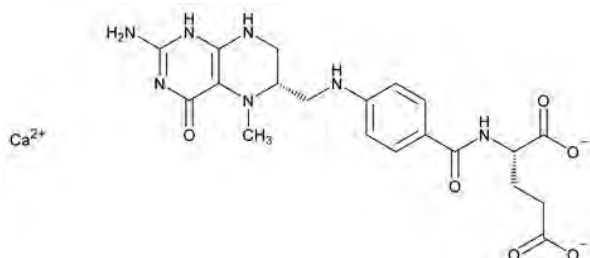
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## Calcium L- 5-Methyltetrahydrofolate

C<sub>20</sub>H<sub>23</sub>CaN<sub>7</sub>O<sub>6</sub> · xH<sub>2</sub>O C<sub>20</sub>H<sub>23</sub>CaN<sub>7</sub>O<sub>6</sub> (anhydrous)

497.52

N-[4-[[[(2-Amino-1,4,5,6,7,8-hexahydro-5-methyl-4-oxo-(6S)-pteridinyl)methyl]amino]benzoyl]-L-glutamic acid, calcium salt (1:1);

N-[4-[[[(6S)-2-Amino-1,4,5,6,7,8-hexahydro-5-methyl-4-oxo-6-pteridinyl)methyl]amino]-benzoyl]-L-glutamic acid, calcium salt (1:1) [151533-22-1].

### DEFINITION

Calcium L- 5-Methyltetrahydrofolate contains NLT 95.0% and NMT 102.0% of calcium 5-methyltetrahydrofolate (C<sub>20</sub>H<sub>23</sub>CaN<sub>7</sub>O<sub>6</sub>), the sum of the L- and D-diastereoisomers, calculated on the anhydrous and solvent-free basis, of which NMT 1.0% corresponds to calcium D- 5-methyltetrahydrofolate.

### IDENTIFICATION

#### A. INFRARED ABSORPTION (197K)

[NOTE—If the spectra obtained show differences, dissolve the substance to be examined and the [USP Calcium DL- 5-Methyltetrahydrofolate RS](#) separately in the minimum quantity of water, and add dropwise sufficient acetone to produce a precipitate. Allow to stand for 15 min, centrifuge to collect the precipitate, wash the precipitate twice with a minimum quantity of acetone, and dry. Record new spectra using the residues.]

#### B. IDENTIFICATION TESTS—GENERAL, CALCIUM (191):

A 5-mg/mL solution meets the requirements.

**C. HPLC:** The retention time of the major peak of the *Sample solution* corresponds to that of the *Standard solution*, as obtained in the *Assay*. It complies with the acceptance criteria of the test for *Enantiomeric Purity*.

### ASSAY

#### PROCEDURE

**Buffer:** 7.8 g/L of sodium dihydrogen phosphate dihydrate in water

**Solution A:** Adjust the *Buffer* with 32% (w/v) sodium hydroxide solution to a pH of 6.5.

**Solution B:** Methanol and *Buffer* (35:65). Adjust with 32% (w/v) sodium hydroxide solution to a pH of 8.0.

**Mobile phase:** Gradient elution. See [Table 1](#).

Table 1

Time (min)	Solution A (%)	Solution B (%)
0	100	0
14	45	55
17	0	100
24	0	100
24.01	100	0
33	100	0

[NOTE—After analysis the column should be flushed and stored in a mixture of methanol and water (85:15).]

**System suitability solution:** Transfer 25 mg of [USP Folic Acid RS](#) and 25 mg of [USP 4-Aminobenzoylglutamic Acid RS](#) to a 100-mL volumetric flask. Add about 15 mg each of sodium hydrogen carbonate and sodium carbonate to the flask, add sufficient water, sonicate to dissolve, and dilute with water to volume.

Transfer 1.0 mL of this solution to a second 100-mL volumetric flask containing 50 mg of [USP Calcium DL- 5-Methyltetrahydrofolate RS](#), dissolve, and dilute with water to volume.

[NOTE—The following Standard and Sample solutions must be injected immediately after preparation and injected only once.]

**Standard solution:** 0.5 mg/mL of [USP Calcium DL- 5-Methyltetrahydrofolate RS](#) in water

**Sample solution:** 0.5 mg/mL of Calcium L- 5-Methyltetrahydrofolate in water

**Chromatographic system**

(See [Chromatography \(621\), System Suitability](#).)

**Mode:** LC

**Detector:** UV 280 nm

**Column:** 4.6-mm × 25-cm; 5-μm packing L1

**Column temperature:** 32°

**Flow rate:** 1.1 mL/min

**Injection volume:** 10 μL

**System suitability**

**Samples:** *System suitability solution* and *Standard solution*

[NOTE—For the *System suitability solution* the relative retention times of the component peaks are listed in [Table 2](#). The L- and D-isomers of 5-methyltetrahydrofolate co-elute as a single peak. The 4a-hydroxy- 5-methyltetrahydrofolic acid, 5-methyltetrahydropteroic acid, and dimethyltetrahydrofolic acid are included as minor components in [USP Calcium D,L- 5-Methyltetrahydrofolate RS](#).]

**Suitability requirements**

**Resolution:** *System suitability solution*

NLT 6 between 4-aminobenzoylglutamic acid and 4a-hydroxy- 5-methyltetrahydrofolic acid

NLT 8 between folic acid and 5-methyltetrahydrofolic acid

NLT 15 between 5-methyltetrahydrofolic acid and dimethyltetrahydrofolic acid

**Relative standard deviation:** Prepare three separate *Standard solutions*, and inject each immediately and only one time. NMT 2.0%; peak response factor from three injections

**Analysis**

**Samples:** *Standard solution* and *Sample solution*

Calculate the percentage of calcium 5-methyltetrahydrofolate ( $C_{20}H_{23}CaN_7O_6$ ), the sum of the L- and D-diastereoisomers, in the portion of Calcium L- 5-Methyltetrahydrofolate taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times 100$$

$r_U$  = peak response from the *Sample solution*

$r_S$  = peak response from the *Standard solution*

$C_S$  = concentration of [USP Calcium D,L- 5-Methyltetrahydrofolate RS](#) in the *Standard solution* (mg/mL)

$C_U$  = concentration of Calcium L- 5-Methyltetra hydrofolate in the *Sample solution* (mg/mL)

**Acceptance criteria:** 95.0%–102.0% on the anhydrous and solvent-free basis

**IMPURITIES**

• **CHLORIDE**

**Sample:** 300 mg

**Blank:** Mix 1 mL of nitric acid with 75 mL of water.

**Titrimetric system**

(See [Titrimetry \(541\)](#).)

**Mode:** Direct titration

**Titrant:** 0.005 M silver nitrate VS

**Endpoint detection:** Potentiometric

**Analysis:** Dissolve the *Sample* in 75 mL of water (heat to maximum of 40°), add 1 mL of nitric acid, and titrate with the *Titrant*. Perform a *Blank* determination, and make any necessary correction.

Calculate the percentage of chloride (Cl) in the *Sample* taken:

$$\text{Result} = [(V_S - V_B) \times M \times F/W] \times 100$$

$V_S$  = volume of *Titrant* consumed by the *Sample* (mL)

$V_B$  = volume of *Titrant* consumed by the *Blank* (mL)

$M$  = actual molarity of the *Titrant* (mmol/mL)

$F$  = equivalency factor, 35.45 mg/mmol

$W$  = *Sample* weight (mg)

**Acceptance criteria:** NMT 0.5%

• **ELEMENTAL IMPURITIES—PROCEDURES (233)**

**Acceptance criteria**

**Boron:** NMT 50 μg/g

**Platinum:** NMT 10 μg/g

**Arsenic:** NMT 1.5 μg/g

**Cadmium:** NMT 0.5 μg/g

**Lead:** NMT 1.0 μg/g

**Mercury:** NMT 1.5 μg/g

• **RESIDUAL SOLVENTS (467)**

**Acceptance criteria**

**Ethanol:** NMT 0.5%

**2-Propanol:** NMT 0.5%

[NOTE—For acceptance criteria for any other residual solvents, see [Residual Solvents \(467\)](#).]

• **RELATED COMPOUNDS**

**Solution A, Solution B, Mobile phase, System suitability solution, Standard solution, Sample solution, Chromatographic system, System suitability, and Suitability requirements:** Proceed as directed in the Assay.

**Analysis**

**Samples:** Standard solution and Sample solution

[NOTE—The impurities are listed in [Table 2](#).]

Calculate the percentage of each impurity, as free acid, in the portion of Calcium L- 5-Methyltetrahydrofolate taken:

$$\text{Result} = (r_U/r_S) \times (C_S/C_U) \times F \times (M_{r1}/M_{r2}) \times 100$$

$r_U$  = peak response of the corresponding impurity from the Sample solution

$r_S$  = peak response of the principal peak from the Standard solution

$C_S$  = concentration of [USP Calcium DL- 5-Methyltetrahydrofolate RS](#) in the Standard solution (mg/mL)

$C_U$  = concentration of Calcium L- 5-Methyltetra hydrofolate in the Sample solution (mg/mL)

$F$  = relative response factor for the corresponding impurity peak (see [Table 2](#))

$M_{r1}$  = molecular weight of L- 5-methyltetrahydrofolic acid, 459.46

$M_{r2}$  = molecular weight of calcium L- 5-methyltetra hydrofolate, 497.52

**Acceptance criteria**

[NOTE—Disregard any impurity peak less than 0.05%.]

**Individual impurities:** See [Table 2](#).

**Table 2**

Name	Relative Retention Time	Relative Response Factor	Acceptance Criteria, NMT (%)
4-Aminobenzoylglutamic acid <sup>a</sup>	0.29	0.91	0.5
4a-Hydroxy- 5- methyl tetrahydrofolic acid <sup>b</sup>	0.37	1.09	1.0
(6R)-Mefox <sup>c,d</sup>	0.49	1.05	—
(6S)-Mefox <sup>c,d</sup>	0.50	1.05	1.0 (sum of 6R and 6S)
Tetrahydrofolic acid <sup>b</sup>	0.65	1.00 <sup>k</sup>	0.5
7,8-Dihydrofolic acid <sup>d</sup>	0.83	0.95	0.5
Folic acid <sup>d</sup>	0.85	0.83	0.5
5,10-Methylenetetrahydrofolic acid <sup>b</sup>	0.88	1.00 <sup>k</sup>	0.5
5-Methyltetrahydrop teroic acid <sup>l</sup>	1.10	0.67	0.5
Dimethyltetrahydrofolic acid <sup>d</sup>	1.25	1.00 <sup>k</sup>	0.15
Total impurities	—	—	2.5

<sup>a</sup> N-(4-Aminobenzoyl)-L-glutamic acid.

<sup>b</sup> N-[4-(((6S)-2-Amino-4a-hydroxy- 5- methyl-4-oxo-1,4,4a,5,6,7,8,8a-octahydropteridin-6-yl) methyl)amino]benzoyl]-L-glutamic acid.

<sup>c</sup> 2-Amino-8- methyl-4,9-dioxo-7- methyl-p-aminobenzoyl-glutamate-6,7,8,9-tetrahydro-4H-pyrazino-(1,2-a)-s-triazine.

<sup>d</sup> Report the impurity Mefox as the sum of 6S- and 6R-Mefox.

<sup>e</sup> N-[4-(((S)-2-Amino-4-oxo-1,4,5,6,7,8-hexahydropteridin-6-yl)methyl)amino]benzoyl]-L-glutamic acid.

<sup>k</sup> Estimated factor.

<sup>l</sup> N-(4-(((2-Amino-4-oxo-1,4,7,8-tetrahydropteridin-6-yl) methyl)amino)benzoyl)-L-glutamic acid.

<sup>m</sup> N-(4-(((2-Amino-4-oxo-1,4-dihydropteridin-6-yl) methyl)amino)benzoyl)-L-glutamic acid.

<sup>n</sup> N-(4-(3-Amino-1-oxo-5,6,6a,7-tetrahydroimidazo[1,5-f]pteridin-8(1H,4H,9H)-yl)benzoyl)-L-glutamic acid.

<sup>o</sup> (S)-4-(((2-Amino- 5- methyl-4-oxo-1,4,5,6,7,8-hexahydropteridin-6-yl) methyl)amino)benzoic acid.

<sup>p</sup> N-[4-(((S)- 5- Methyl-2-(methylamino)-4-oxo-1,4,5,6,7,8-hexahydropteridin-6-yl) methyl)amino]benzoyl]-L-glutamic acid.

• **ENANTIOMERIC PURITY**

**Buffer:** 4.54 g/L of sodium dihydrogen phosphate dihydrate in water

**Mobile phase:** Acetonitrile and *Buffer* (3:97). Adjust with 32% (w/v) sodium hydroxide to a pH of 6.8.

**Standard solution:** 0.5 mg/mL of [USP Calcium D,L-5-Methyltetrahydrofolate RS](#) in water

**Sample solution:** 0.5 mg/mL of Calcium L-5-Methyltetrahydrofolate in water

**System suitability solution:** Transfer 1.0 mL of *Standard solution* to a 50-mL volumetric flask, and dilute with *Sample solution* to volume.

#### Chromatographic system

(See [Chromatography \(621\)](#), [System Suitability](#).)

**Mode:** LC

**Detector:** UV 280 nm

**Column:** 4.0-mm × 15-cm; 5-µm packing L79

**Column temperature:** 40°

**Flow rate:** 1.0 mL/min

**Injection volume:** 10 µL

#### System suitability

**Sample:** *System suitability solution*

[NOTE—The relative retention times of L-5-methyltetrahydrofolate and D-5-methyltetrahydrofolate are about 1 and 1.5, respectively.]

#### Suitability requirements

**Resolution:** NLT 1.5 between L-5-methyltetrahydrofolate and D-5-methyltetrahydrofolate

#### Analysis

**Sample:** *Sample solution*

Calculate the percentage of D-5-methyltetrahydrofolate in the portion of Calcium L-5-Methyltetrahydrofolate taken:

$$\text{Result} = [(r_D / (r_D + r_L)) \times 100]$$

$r_D$  = peak response of D-5-methyltetrahydrofolate from the *Sample solution*

$r_L$  = peak response of L-5-methyltetrahydrofolate from the *Sample solution*

**Acceptance criteria:** NMT 1.0% of D-5-methyltetrahydrofolate

#### SPECIFIC TESTS

##### • CALCIUM

**Sample:** 250 mg

**Blank:** 150 mL of water, 15 mL of 1 N sodium hydroxide, and 300 mg of hydroxy naphthol blue

#### Titrimetric system

(See [Titrimetry \(541\)](#).)

**Mode:** Direct titration

**Titrant:** 0.05 M edetate disodium VS

**Endpoint detection:** Visual

**Analysis:** Dissolve the *Sample* in 150 mL of water, add 15 mL of 1 N sodium hydroxide and 300 mg of hydroxy naphthol blue, and titrate with the *Titrant* until the solution is deep blue in color. Perform a *Blank* determination, and make any necessary correction.

Calculate the percentage of calcium (Ca) in the *Sample* taken:

$$\text{Result} = [(V_S - V_B) \times M \times F / W] \times 100$$

$V_S$  = volume of *Titrant* consumed by the *Sample* (mL)

$V_B$  = volume of *Titrant* consumed by the *Blank* (mL)

$M$  = actual molarity of the *Titrant* (mmol/mL)

$F$  = equivalency factor, 40.08 mg/mmol

$W$  = *Sample* weight (mg)

**Acceptance criteria:** 7.0%–8.5% on the anhydrous and solvent-free basis

##### • WATER DETERMINATION, [METHOD I C \(921\)](#)

**Sample:** Transfer 40 mg of Calcium L-5-Methyltetrahydrofolate to a 20-mL headspace vial, and cap tightly. Heat the vial in a suitable Karl Fischer oven at 250°.

**Analysis:** The released and evaporated water is transferred into the titration-cell in a stream of dry nitrogen at a flow rate of about 40 mL/min as directed in [Water Determination, Method I C \(921\)](#).

**Acceptance criteria:** 6.0%–17.0%

#### ADDITIONAL REQUIREMENTS

• **PACKAGING AND STORAGE:** Store in a tight container, in a cool and dry place.

##### • [USP REFERENCE STANDARDS \(11\)](#)

[USP 4-Aminobenzoylglutamic Acid RS](#)

N-(4-Aminobenzoyl)-L-glutamic acid.

$C_{12}H_{14}N_2O_5$  266.25

[USP Calcium D,L-5-Methyltetrahydrofolate RS](#)

N[4-[[[2-Amino-1,4,5,6,7,8-hexahydro-5-methyl-4-oxo-6-pteridiny] methyl]amino]benzoyl]-L-glutamic acid, calcium salt (1:1).

$C_{20}H_{23}CaN_7O_6$  497.52

[USP Folic Acid RS](#)

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**Auxiliary Information-** Please [check for your question in the FAQs](#) before contacting USP.

Topic/Question	Contact	Expert Committee
CALCIUM L-5-METHYLTETRAHYDROFOLATE	<a href="#">Natalia Dawdova</a> Scientific Liaison +1 (301) 816-8328	NBDS2015 Non-botanical Dietary Supplements 2015

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**Chromatographic Columns Information:** [Chromatographic Columns](#)

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**Most Recently Appeared In:**

Pharmacopeial Forum: Volume No. 38(5)

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**Page Information:**

USP42-NF37 - 4794

USP41-NF36 - 4496

USP40-NF35 - 6858

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Previous DocID: [GUID-367663C9-FD49-40B2-8B50-2196AF9E71F6\\_1\\_en-US](#)

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## Appendix B

# Certificate of Analysis



**Material No.** CH2900019  
**Material Description** Metafolin® ground, (6S)-5-Methyltetrahydrofolic acid, calcium salt, L-Methylfolate, calcium  
**Chemical Formula** C<sub>20</sub>H<sub>23</sub>CaN<sub>7</sub>O<sub>6</sub>  
**Molecular Weight** 497.53 g/mol  
**Batch No.** LMSG045801  
**Inspection Lot No.** 040000130161  
**Retest Date** 31 May 2017  
**Storage Conditions** +2°C to +8°C  
**Manufacturing Date** 05 May 2015  
**Batch Size** 31.22 Kilogram

Test	Specification	Result
<b>Appearance</b> Appearance color Appearance texture	white to yellow or beige powder	light yellowish powder
<b>Identity (IR-Spectrum)</b> Identity / IR	conforms to reference	conforms to reference
<b>Identity Calcium (Wet Chemical)</b> Identity proof of calcium	positive	positive
<b>Water Content (KF, Coulometric)</b> Water content	6.0 to 17.0 %	13.0 %
<b>Residual Solvents (GC)</b> Assay Ethanol Assay Isopropanol	<=0.5 % <=0.5 %	< LOQ (0.04) % < LOD (0.003) %
<b>Chloride (Titration)</b> Chloride Content	<=0.5 %	0.21 %
<b>Assay Calcium (Titration)</b> Calcium Content on Dried Basis	7.0 to 8.5 %	8.0 %
<b>Specified Elemental Impurities</b> Assay Boron (ICP-OES) Assay Platinum (ICP-MS) Assay Arsenic (ICP-MS) Assay Cadmium (ICP-MS) Assay Lead (ICP-MS) Assay Mercury (ICP-MS)	<=10 ppm <=10 ppm <=1.5 ppm <=0.5 ppm <=1.0 ppm <=1.5 ppm	< LOQ (5) ppm < LOQ (5) ppm < LOQ (1.5) ppm < LOQ (0.5) ppm < LOQ (1.0) ppm < LOQ (1.5) ppm
<b>Assay &amp; Related Compounds (HPLC)</b> Identity retention time HPLC	conforms to reference	conforms to reference
Assay Calcium Mefolate, on dried basis	95.0 to 102.0 %	101.0 %
Assay Mefolate, acid as is		81.1 %
4-Aminobenzoylglutamic acid (ABGA)	<=0.5 %	0.09 %
Hydroxymethyl-THFA (HOMETHFA)	<=1.0 %	0.35 %
Mefox	<=1.0 %	0.16 %
Tetrahydrofolic acid (THFA)	<=0.5 %	< LOQ (0.01) %
7,8-Dihydrofolic acid (DHFA)	<=0.5 %	0.04 %
Folic acid (FA)	<=0.5 %	0.02 %

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 17 September 2015/14:17:27  
 CH41LMCGNS  
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# Certificate of Analysis



**Material No.** CH2900019  
**Batch No.** LMCG045801  
**Inspection Lot No.** 040000130161

Test	Specification	Result
Methylenetetrahydrofolic acid (CH <sub>2</sub> THFA)	<=0.5 %	0.03 %
Methyltetrahydroptericoic acid (MeTHPA)	<=0.5 %	0.03 %
Dimethyl-THFA (DiMeTHFA)	<=0.15 %	0.06 %
Sum of all related compounds	<=2.5 %	1.08 %
<b>Diastereomeric Purity (HPLC)</b> (6R)-Mefolate	<=1.0 % area	0.6 % area
<b>Microbial Enumeration Tests</b>		
Microbial Count (TAMC)	<=100 CFU/g	< LOQ (10) CFU/g
Microbial Count (TYMC)	<=100 CFU/g	< LOQ (10) CFU/g

Results reported "on dried basis" are calculated based on the water and residual solvent content.

The abbreviation LOQ represents the Limit of Quantitation or, if applicable, the Reporting Threshold. The Limit of Detection is abbreviated as LOD.

This Lot meets the requirements of the USP DS Monograph for Calcium L-5-Methyltetrahydrofolate and Merck & Cie's tighter specifications.

METAFOLIN® is a registered trademark of Merck KGaA, Germany.

Batch was released using electronic signature : Markus Richter, 12 June 2015  
HEAD OF QUALITY CONTROL

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# Certificate of Analysis



<b>Material No.</b>	CH2900019
<b>Material Description</b>	Metafolin® ground, (6S)-5-Methyltetrahydrofolic acid, calcium salt, L-Methylfolate, calcium
<b>Chemical Formula</b>	C <sub>20</sub> H <sub>23</sub> CaN <sub>7</sub> O <sub>6</sub>
<b>Molecular Weight</b>	497.53 g/mol
<b>Batch No.</b>	LMCG046801
<b>Inspection Lot No.</b>	040000137104
<b>Retest Date</b>	31 May 2017
<b>Storage Conditions</b>	+2°C to +8°C
<b>Manufacturing Date</b>	20 May 2015
<b>Batch Size</b>	31.97 Kilogram

Test	Specification	Result
<b>Appearance</b>		
Appearance color	white to yellow or beige powder	light yellowish powder
Appearance texture		
<b>Identity (IR-Spectrum)</b>		
Identity / IR	conforms to reference	conforms to reference
<b>Identity Calcium (Wet Chemical)</b>		
Identity proof of calcium	positive	positive
<b>Water Content (KF, Coulometric)</b>		
Water content	6.0 to 17.0 %	12.1 %
<b>Residual Solvents (GC)</b>		
Assay Ethanol	<=0.5 %	< LOQ (0.04) %
Assay Isopropanol	<=0.5 %	< LOD (0.003) %
<b>Chloride (Titration)</b>		
Chloride Content	<=0.5 %	< LOQ (0.10) %
<b>Assay Calcium (Titration)</b>		
Calcium Content on Dried Basis	7.0 to 8.5 %	8.0 %
<b>Specified Elemental Impurities</b>		
Assay Boron (ICP-OES)	<=10 ppm	< LOQ (5) ppm
Assay Platinum (ICP-MS)	<=10 ppm	< LOQ (5) ppm
Assay Arsenic (ICP-MS)	<=1.5 ppm	< LOQ (1.5) ppm
Assay Cadmium (ICP-MS)	<=0.5 ppm	< LOQ (0.5) ppm
Assay Lead (ICP-MS)	<=1.0 ppm	< LOQ (1.0) ppm
Assay Mercury (ICP-MS)	<=1.5 ppm	< LOQ (1.5) ppm
<b>Assay &amp; Related Compounds (HPLC)</b>		
Identity retention time HPLC	conforms to reference	conforms to reference
Assay Calcium Mefolate, on dried basis	95.0 to 102.0 %	100.6 %
Assay Mefolate, acid as is		81.7 %
4-Aminobenzoylglutamic acid (ABGA)	<=0.5 %	0.09 %
Hydroxymethyl-THFA (HOMETHFA)	<=1.0 %	0.28 %
Mefox	<=1.0 %	0.19 %
Tetrahydrofolic acid (THFA)	<=0.5 %	< LOQ (0.01) %
7,8-Dihydrofolic acid (DHFA)	<=0.5 %	0.03 %
Folic acid (FA)	<=0.5 %	0.02 %

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# Certificate of Analysis



**Material No.** CH2900019  
**Batch No.** LMCG046801  
**Inspection Lot No.** 040000137104

Test	Specification	Result
Methylenetetrahydrofolic acid (CH <sub>2</sub> THFA)	<=0.5 %	0.02 %
Methyltetrahydroptericoic acid (MeTHPA)	<=0.5 %	0.03 %
Dimethyl-THFA (DiMeTHFA)	<=0.15 %	0.07 %
Sum of all related compounds	<=2.5 %	1.10 %
<b>Diastereomeric Purity (HPLC)</b> (6R)-Mefolate	<=1.0 % area	0.5 % area
<b>Microbial Enumeration Tests</b>		
Microbial Count (TAMC)	<=100 CFU/g	< LOQ (10) CFU/g
Microbial Count (TYMC)	<=100 CFU/g	< LOQ (10) CFU/g

Results reported "on dried basis" are calculated based on the water and residual solvent content.

The abbreviation LOQ represents the Limit of Quantitation or, if applicable, the Reporting Threshold. The Limit of Detection is abbreviated as LOD.

This Lot meets the requirements of the USP DS Monograph for Calcium L-5-Methyltetrahydrofolate and Merck & Cie's tighter specifications.

METAFOLIN® is a registered trademark of Merck KGaA, Germany.

Batch was released using electronic signature : Markus Richter, 12 June 2015  
HEAD OF QUALITY CONTROL

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# Certificate of Analysis



**Material No.** CH2900020  
**Material Description** Metafolin® micronized, (6S)-5-Methyltetrahydrofolic acid, calcium salt, L-Methylfolate, calcium  
**Chemical Formula** C<sub>20</sub>H<sub>23</sub>CaN<sub>7</sub>O<sub>6</sub>  
**Molecular Weight** 497.53 g/mol  
**Batch No.** LCM048001  
**Inspection Lot No.** 010000345679  
**Retest Date** 31 March 2017  
**Storage Conditions** +2°C to +8°C  
**Manufacturing Date** 17 March 2015  
**Batch Size** 30.30 Kilogram

Test	Specification	Result
<b>Appearance</b> Appearance color Appearance texture	white to yellow or beige powder	light yellowish powder
<b>Identity (IR-Spectrum)</b> Identity / IR	conforms to reference	conforms to reference
<b>Identity Calcium (Wet Chemical)</b> Identity proof of calcium	positive	positive
<b>Water Content (KF, Coulometric)</b> Water content	6.0 to 17.0 %	12.3 %
<b>Residual Solvents (GC)</b> Assay Ethanol Assay Isopropanol	<=0.5 % <=0.5 %	< LOQ (0.04) % < LOD (0.003) %
<b>Chloride (Titration)</b> Chloride Content	<=0.5 %	< LOQ (0.10) %
<b>Assay Calcium (Titration)</b> Calcium Content on Dried Basis	7.0 to 8.5 %	8.0 %
<b>Specified Elemental Impurities</b> Assay Boron (ICP-OES) Assay Platinum (ICP-MS) Assay Arsenic (ICP-MS) Assay Cadmium (ICP-MS) Assay Lead (ICP-MS) Assay Mercury (ICP-MS)	<=10 ppm <=10 ppm <=1.5 ppm <=0.5 ppm <=1.0 ppm <=1.5 ppm	< LOQ (5) ppm < LOQ (5) ppm < LOQ (1.5) ppm < LOQ (0.5) ppm < LOQ (1.0) ppm < LOQ (1.5) ppm
<b>Assay &amp; Related Compounds (HPLC)</b> Identity retention time HPLC	conforms to reference	conforms to reference
Assay Calcium Mefolate, on dried basis	95.0 to 102.0 %	100.5 %
Assay Mefolate, acid as is		81.4 %
4-Aminobenzoylglutamic acid (ABGA)	<=0.5 %	0.05 %
Hydroxymethyl-THFA (HOMeTHFA)	<=1.0 %	0.29 %
Mefox	<=1.0 %	0.16 %
Tetrahydrofolic acid (THFA)	<=0.5 %	< LOQ (0.01) %
7,8-Dihydrofolic acid (DHFA)	<=0.5 %	0.04 %
Folic acid (FA)	<=0.5 %	0.01 %

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# Certificate of Analysis



**Material No.** CH2900020  
**Batch No.** LMCM048001  
**Inspection Lot No.** 010000345679

Test	Specification	Result
Methylenetetrahydrofolic acid (CH <sub>2</sub> THFA)	<=0.5 %	0.02 %
Methyltetrahydroptericoic acid (MeTHPA)	<=0.5 %	0.03 %
Dimethyl-THFA (DiMeTHFA)	<=0.15 %	0.07 %
Sum of all related compounds	<=2.5 %	0.91 %
<b>Diastereomeric Purity (HPLC)</b> (6R)-Mefolinate	<=1.0 % area	0.5 % area
<b>Microbial Enumeration Tests</b>		
Microbial Count (TAMC)	<=100 CFU/g	< LOQ (10) CFU/g
Microbial Count (TYMC)	<=100 CFU/g	< LOQ (10) CFU/g

Results reported "on dried basis" are calculated based on the water and residual solvent content.

The abbreviation LOQ represents the Limit of Quantitation or, if applicable, the Reporting Threshold. The Limit of Detection is abbreviated as LOD.

This Lot meets the requirements of the USP DS Monograph for Calcium L-5-Methyltetrahydrofolate and Merck & Cie's tighter specifications.

METAFOLIN® is a registered trademark of Merck KGaA, Germany.

Batch was released using electronic signature : Markus Richter, 05 June 2015  
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## Appendix C

# CALCIUM L-5-METHYLTETRAHYDROFOLATE

*New specifications prepared at the 65th JECFA (2005) and published in FNP 52 Add 13 (2005). At the 65th JECFA (2005) the Committee had no safety concerns for the use of the substance in dry crystalline or microencapsulated form as an alternative to folic acid used in dietary supplements, foods for special dietary uses and other foods.*

## SYNONYMS

L-5-Methyltetrahydrofolic acid, calcium salt  
L-Methyltetrahydrofolate, calcium salt  
L-Methylfolate, calcium  
L-5-MTHF-Ca

## DEFINITION

Calcium L-5-methyltetrahydrofolate (L-5-MTHF-Ca) is a synthetic derivative of folic acid, the predominant, naturally occurring form of folate. It is synthesized by reduction of folic acid to tetrahydrofolic acid followed by methylation and diastereoselective crystallization (in water) of L-5-MTHF as its calcium salt. The product contains variable amounts of water of crystallization.

Chemical name

N-{4-[[[(6S)-2-amino-3,4,5,6,7,8-hexahydro-5-methyl-4-oxo-6-pteridiny]methyl]amino]benzoyl}-L-glutamic acid, calcium salt

C.A.S. number

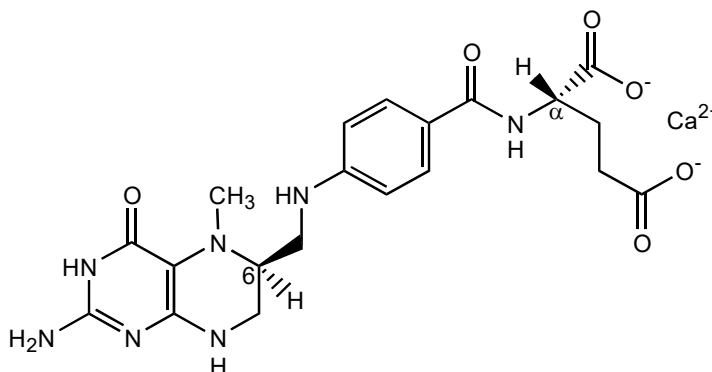
151533-22-1

Chemical formula

$C_{20}H_{23}CaN_7O_6$  (anhydrous form)

Structural formula

(anhydrous form)



Formula weight

497.5 (anhydrous form)

Assay

95.0 – 102.0% (anhydrous basis)

## DESCRIPTION

White to light yellowish, almost odourless, crystalline powder

## FUNCTIONAL USES

Nutrient supplement

## CHARACTERISTICS

### IDENTIFICATION

Solubility (Vol. 4)

Sparingly soluble in water and very slightly soluble or insoluble in most organic solvents; soluble in alkaline solutions

Infrared absorption

The infrared absorption spectrum of a potassium bromide dispersion of the

sample corresponds to that of a L-5-MTHF-Ca standard (see Appendix).

Calcium

Dilute 30 g of acetic acid (glacial) to 100 ml with water. Dissolve 5.3 g of  $K_4Fe(CN)_6$  in 100 ml of water. To 5 ml of the acetic acid solution, add 20 mg of the sample and then 0.5 ml of the potassium ferrocyanide solution. Mix and add 50 mg of ammonium chloride. A white crystalline precipitate is formed.

Liquid chromatography

Retention time matches that of a reference standard (see under TESTS)

PURITY

Water (Vol. 4)

Not more than 17.0% (Karl Fischer method)  
(Note: Allow sufficient time (15 min) for release of bound water.)

Calcium

7.0 - 8.5% (anhydrous basis)  
Accurately weigh 500 mg of sample and transfer to a 500-ml conical flask. Add 150 ml of water to dissolve the sample and 20 ml of a pH 10 buffer ( $NH_3/NH_4Cl$ ). Using eriochrome black T as indicator, titrate (continuous stirring) with standardized 0.1 M EDTA until the colour changes from violet to blue/green. Each 1.0 ml of 0.1 M EDTA corresponds to 4.008 mg of calcium. Calculate the calcium content on the anhydrous basis.

Other folates and related substances

Not more than 2.5%  
See description under TESTS

D-5-Methylfolate

Not more than 1.0%  
See description under TESTS

Total viable aerobic count  
(Vol. 4)

Not more than 1000 CFU/g

Lead (Vol. 4)

Not more than 2 mg/kg  
Determine using an atomic absorption technique appropriate to the specified level. The selection of sample size and method of sample preparation may be based on the principles of the methods described in Volume 4, "Instrumental methods".

**TESTS**

PURITY TESTS

Other folates and related substances

Using a L-5-MTHF-Ca reference standard, Quantitate other folates and related substances by HPLC. The suitability of the applied HPLC system is checked daily by a "system suitability test" (see below).

Reference standard solution: Accurately weigh 50 mg of L-5-MTHF-Ca (L-5-methyltetrahydrofolic acid, calcium salt (Merck Eprova AG, CH-8200 Schaffhausen, Switzerland) into a 100-ml volumetric flask. Dissolve in a small quantity of water and dilute to volume.

Sample solution: Prepare as for the reference standard using 50 mg of the sample.

Mobile phase solutions

A: Dissolve 7.80 g of  $NaH_2PO_4 \cdot 2H_2O$  (0.05 mol) in 1000 ml of water and adjust the pH to 6.5 with 32% NaOH. Filter and degas the solution.

B: Dissolve 5.07 g of  $NaH_2PO_4 \cdot 2H_2O$  (0.03 mol) in 650 ml of water and 350 ml of methanol (chromatography grade) and adjust the pH to 8.0 with 32%

NaOH. Filter and degas the solution.

**Chromatography Conditions**

Column: Hypersil-ODS, 5 µm; 250 x 4 mm (Thermo Hypersil Keystone or equivalent)

Flow rate: 1.1 ml/min

Gradient:

Time (min)	% Mobile phase A	% Mobile phase B	Remark
0	100	0	Start
0 - 14	100 – 45	0 – 55	Linear gradient
14 – 17	45 – 0	100	Linear gradient
17 – 22	0	100	Hold
22 – 31	100	0	Reconditioning

Temperature: Room temperature

Injection volume: 10 µl

Detection: UV (280 nm)

Run time: 22 min

Retention times given below are approximate:

Folates and related substances	Retention time (min)
4-Aminobenzoylglutamic acid (ABGA)	3.1
4α-Hydroxy-5-methyltetrahydrofolic acid (HOMeTHFA)	4.3
D-Pyrazino-s-triazine derivative (D-Mefox)	6.1
L-Pyrazino-s-triazine derivative (L-Mefox)	6.3
Tetrahydrofolic acid (THFA)	8.5
7,8-Dihydrofolic acid (DHFA)	11.2
Folic acid (FA)	11.4
5,10-Methylenetetrahydrofolic acid (CH <sub>2</sub> THFA)	11.7
5-Methyltetrahydrofolic acid (5-MTHF)	13.6
5-Methyltetrahydroptericoic acid (MeTHPA)	15.1
N <sup>2</sup> -Methylamino-5-methyltetrahydrofolic acid (DiMeTHFA)	17.6

**Sample analysis:** Inject the reference standard solution and the sample solutions immediately after preparation, using the conditions described above.

(Note: After analysis, flush the column with methanol/water 85:15 and store it under the same conditions.)

Calculate the content of each folate (other than 5-MTHF) and related substance, X<sub>i</sub> (%), listed in the above table according to the following formula:

$$X_i (\%) = A_i \times W_S \times S \times (RF)_i / A_S \times W$$

where

A<sub>i</sub> = the peak area for each folate (other than 5-MTHF) and related substance

A<sub>S</sub> = the peak area for the L-5-MTHF-Ca Standard

W<sub>S</sub> = the weight (mg) of L-5-MTHF-Ca Standard

W = the weight (mg) of the sample

S = the percent of L-5-MTHF in the L-5-MTHF-Ca Standard, calculated as



free acid

(RF)<sub>i</sub> = Response Factor for the i-th substance (absorbance at 280 nm in the applied eluent system relative to that of L-5-MTHF)

<i>Other folates and related substances</i>	<i>RF</i>
ABGA	0.93
HOMeTHFA	1.11
L-Mefox and D-Mefox	1.11
DHFA	0.98
FA	0.86
MeTHPA	0.68
THFA	1.00
CH <sub>2</sub> THFA	1.00
DiMeTHFA	1.00

If there are any unidentified impurities, apply a RF of 1.00.

Calculate the total amount of "Other folates and related substances" by summing the X<sub>i</sub> for all impurities.

#### System suitability test procedure

Mixed folates solution: Weigh 25 mg each of ABGA, HOMeTHFA, L-Mefox, DHFA, FA and MeTHPA (all available from Merck Eprova AG) into a 100-ml volumetric flask. Add a small quantity of water to dissolve the mixture; add some sodium hydrogen carbonate and sodium carbonate to aid the dissolution, and fill to the mark with water.

System suitability test solution (SST solution): Weigh accurately 50 mg of a L-5-MTHF-Ca sample containing DiMeTHFA into a 100-ml volumetric flask. (Available from Merck Eprova AG). Add 1 ml of the Mixed folates solution and a small quantity of water to dissolve, mix and dilute to volume with water.

System suitability test: Inject 10 µl of the SST solution immediately. The resolution between L-5-MTHF and MeTHPA must be at least 5.

#### D-5-Methylfolate

D-5-Methylfolate is quantitated by HPLC using a chromatographic system which allows separation of the D- from the L-stereoisomer. The suitability of the applied HPLC system is checked daily by a "system suitability test" (see below).

Sample preparation: Accurately weigh 50 mg of the sample into a 100 ml volumetric flask. Dissolve in water and dilute to volume with water.

Mobile phase: Mix 970 ml of 0.03 M NaH<sub>2</sub>PO<sub>4</sub> (obtained by dissolving 4.68 g of NaH<sub>2</sub>PO<sub>4</sub> · 2H<sub>2</sub>O in water and diluting with water to 1000 ml) with 30 ml of acetonitrile (chromatography grade) and adjust the pH to 6.8 with 32% NaOH. Filter and degas the solution.

#### Chromatography Conditions

Column: Chiral Protein HSA, 5 µm, 150 x 4 mm (ChromTech or equivalent)

Flow rate: 1 ml/min

Temperature: 40°

Injection volume: 10 µl

Detection: UV (280 nm)

Run time: 22 min

Solvent: Water

Sample analysis: Inject the sample solution immediately after preparation using the conditions described above. Determine the areas under peak for L-5-MTHF (retention time: ca. 11 min) and D-5-MTHF (retention time: ca. 15 min).

#### Calculation

Determine the ratio of the peak area for the D-isomer ( $A_D$ ) to the sum of the peak areas for the D- and L-isomers ( $A_T$ ), and calculate the D-5-MTHF content as follows:

$$\text{D-5-MTHF (\%)} = 100A_D/A_T$$

#### System suitability test procedure

System suitability test solution (SST solution): Weigh and transfer into a 200-ml volumetric flask the following: 1.0 mg of HOMeTHFA, 1.5 mg ABGA, 2.0 mg each of L-Mefox and MeTHPA, 3.0 mg of FA, 4.0 mg of DHFA, 10 mg of L,D-5-MTHF and D,D-5-MTHF (L-5-MTHF and D-5-MTHF carrying D-glutamic acid substitution), and 50 mg of racemic 5-MTHF-Ca (L-5-MTHF and D-5-MTHF carrying L-glutamic acid substitution) (all available from Merck Eprova AG). Add a small quantity of water to dissolve the mixture; add some sodium hydrogen carbonate to aid the dissolution, and fill to the mark with water. Immediately inject into the HPLC system.

The resolution between L-5-MTHF and D-5-MTHF must be at least 2.

**METHOD OF ASSAY** Calculate the percentage of L-5-MTHF-Ca in the sample from the content of 5-MTHF-Ca (L- and D-diastereoisomers), determined in the test for "Other folates and related substances", and the content of D-5-MTHF-Ca, determined in the test for D-5-Methylfolate, and correcting for water content, as follows:

$$\text{L-5-MTHF-Ca (\%)} = 100 \times A_T \times W_S \times S \times (100 - D) \times 1.083 / A_S \times W \times (100 - \%H_2O)$$

where

$A_T$  is taken from the calculation for the D-5-Methylfolate analysis

D = the percentage of D-5-Methylfolate in the sample

$A_S$ , W,  $W_S$ , and S are taken from the determination of Other folates and related substances

$\%H_2O$  = water content (%)

1.083 is the ratio of the formula weight of 5-MTHF-Ca to that of 5-MTHF.

## Appendix

Infrared spectra of  
Calcium L-5-Methyl-  
tetrahydrofolate

## Appendix D

## Product Information



# METAFOLIN<sup>®</sup>

## Stability Data

Lot No.: LMCA-G-0229  
Packaging Type: PE/aluminium bags  
Storage Condition: 5° C ± 3° C

Storage period in months	Retention of initial value [%]
3	98.4
6	98.0
9	99.0
12	98.5
18	97.6
24	98.1
36	98.0

## Product Information

# METAFOLIN<sup>®</sup>

## Stability Data

Lot No.: LMCA-G-0229  
Packaging Type: PE/aluminium bags  
Storage Condition: 25° C ± 2° C / 60% RH ± 5%

Storage period in months	Retention of initial value [%]
3	97.8
6	97.1
9	98.7
12	97.9

## METAFOLIN®

### Stability Data

Lot No.: LMCA-M-0180  
Packaging Type: PE/aluminium bags  
Storage Condition: 5° C ± 3° C

Storage period in months	Retention of initial value [%]
3	99.1
6	99.0
9	98.7
12	98.8
18	99.4
24	99.1
36	98.6

# Product Information



## METAFOLIN<sup>®</sup>

### Stability Data

Lot No.: LMCA-M-0180  
Packaging Type: PE/aluminium bags  
Storage Condition: 25° C ± 2° C / 60% RH ± 5%

Storage period in months	Retention of initial value [%]
3	98.1
6	98.0
9	98.0
12	98.0



## METAFOLIN®

### Stability Data

Lot No.: LMCA-M-0243  
Packaging Type: PE/aluminium bags  
Storage Condition: 5° C ± 3° C

Storage period in months	Retention of initial value [%]
3	100.7
6	100.6
9	99.8
12	99.1
18	100.3
24	99.4
36	98.8

## Product Information

# METAFOLIN<sup>®</sup>

## Stability Data

Lot No.: LMCA-M-0243  
Packaging Type: PE/aluminium bags  
Storage Condition: 25° C ± 2° C / 60% RH ± 5%

Storage period in months	Retention of initial value [%]
3	99.6
6	99.8
9	98.7
12	98.9

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## Appendix E

# GRAS Panel Consensus Statement on the Generally Recognized as Safe (GRAS) Determination for the Use of Calcium L-Methylfolate [L-5-MTHF-Ca] in Non-Exempt and Exempt Infant Formula

26 November 2019

## INTRODUCTION

DSM Nutritional Products (hereinafter “DSM”) convened a panel of independent scientists (GRAS Panel), qualified by their scientific training and relevant national and international experience in the safety evaluation of food ingredients, to conduct a critical and comprehensive assessment of data and information pertinent to the safety of the company’s Calcium L-Methylfolate (L-5-MTHF Calcium) and to determine whether its intended use in non-exempt and exempt infant formulas as a partial or complete replacement for folic acid as a source of the vitamin, folate would be Generally Recognized as Safe (GRAS) based on scientific procedures. The GRAS Panel consisted of the below-signed qualified scientific experts: Michael W. Pariza, Ph.D. (Emeritus Professor, University of Wisconsin-Madison, Food Research Institute); John A. Thomas, Ph.D., A.T.S. (Adjunct Professor, Indiana University School of Medicine); and Stanley M. Tarka, Jr., Ph.D., A.T.S., Panel Chair, (Tarka Group Inc., Adjunct Associate Professor, The Pennsylvania State University College of Medicine).

The GRAS Panel, independently and collectively, critically evaluated a summary of publicly available scientific data and information compiled from the literature in a dossier titled, “GRAS Notice for the Use of Calcium L-Methylfolate in Infant Formula” (28 October 2019), which included an evaluation of available scientific data and information, both favorable and unfavorable, relevant to the safety of the intended food-uses of Calcium L-Methylfolate. This information was prepared based on a comprehensive search of the scientific literature performed by DSM and included information characterizing the identity and purity of the ingredient, the manufacture of the ingredient, product specifications, supporting analytical data, intended conditions of use, estimated exposure under the intended uses, and the safety of Calcium L-Methylfolate.

Following its independent and collective critical evaluation, the GRAS Panel unanimously concluded that the proposed use of Calcium L-Methylfolate in non-exempt and exempt infant formulas as a partial or complete replacement for folic acid as a source of the vitamin, folate, meeting food-grade specifications and manufactured in accordance with current Good Manufacturing Practice (cGMP), is safe and suitable and GRAS based on scientific procedures.

## SUMMARY AND BASIS FOR GRAS

Metafolin® is a registered trademark for the calcium L-methylfolate manufactured by Merck KGaA. DSM Nutritional Products is the distributor of Metafolin®. The information provided in the supporting dossier referenced above is for Metafolin®. Calcium L-methylfolate (L-5-MTHF-Ca) has two chiral carbon atoms: the C-atom in position 6 of the pteroyl moiety and the  $\alpha$ -C atom in the L-glutamic acid moiety. During synthesis of Metafolin® (the L-5-MTHF-Ca described in this dossier) the chiral center at the C-atom in position 6 of the

tetrahydropteroyl moiety is formed by reduction of the starting material, folic acid. The  $\alpha$ -C atom in the L-glutamic acid moiety of L-5-MTHF-Ca stems from the starting material, folic acid, and its configuration ( $\alpha$ S or L) remains unchanged during synthesis. Thus, both chiral centers in L-5-MTHF-Ca have the natural L-configuration.

The GRAS Panel, individually and collectively, critically evaluated details of the manufacturing process as summarized below.

Metafolin® is synthesized from folic acid in a 3-step synthesis conducted under Good Manufacturing Practice (cGMP) conditions:

Step 1: Catalytic hydrogenation or sodium borohydride reduction (2 alternatives)

Step 2: Condensation of the resulting tetrahydrofolic acid benzenesulfonate intermediate with formaldehyde, reduction of the formed 5,10-methylenetetrahydrofolic acid to L-5-methyltetrahydrofolic acid with NaBH<sub>4</sub>

Step 3: Crystallization as the calcium salt of L-5-methyltetrahydrofolic acid

The GRAS panel noted that residues of starting materials, by-products, intermediates and reagents potentially present in the final material are listed and limited by the product specifications. The synthesis is performed in aqueous solution or in water/ethanol and the water soluble process chemicals are removed in the final steps of the manufacturing process, including the final washing steps in water/ethanol. Non water-soluble process materials, e.g. activated charcoal, are removed by filtration. All components of L-5-MTHF-Ca are common ingredients in the human diet with an extensive history of safe consumption.

The final product is prepared using either an impact mill or a jet mill depending on production capacity. The resulting products, called respectively "ground" or "micronized", comply with the provided specifications and have similar particle size distributions.

The molecular weight of L-5-MTHF-Ca is 497.5 Daltons and the molecular weight of L-5-MTHF is 459.5 Daltons.

The ingredient is a white-to-yellow or beige crystalline powder that is sparingly soluble in water, very slightly soluble or insoluble in most organic solvents and soluble in alkaline solutions that is slightly hygroscopic.

The purity of L-5-MTHF-Ca is not less than 95.0% and not more than 102.0% of calcium 5-methyltetrahydrofolate, the sum of the L- and D-diastereomers, calculated on the anhydrous and solvent-free basis, of which not more than 1.0% corresponds to calcium D-5-methyltetrahydrofolate.

Limits for the elemental impurities arsenic, cadmium, lead and mercury comply with limits set in the USP L-5-MTHF-Ca monograph. Sodium borohydride is used in the synthesis step 1 (sodium borohydride reduction) and in step 2 (reductive methylation). The USP specifies boron levels NMT 50 µg/g, however, manufacturer specifications of Metafolin® list boron as ≤10 mg/kg. Certificates of analysis for three batches of L-5-MTHF-Ca confirm that concentrations are below the LOQ of 5 mg/kg for the ICP-MS method. Platinum (Pt) is used as a catalyst in production step 1 (catalytic hydrogenation). Limits for platinum are set at ≤10 mg/kg in accordance with limits set in the USP L-5-MTHF-Ca monograph.

Organic impurities and their potential source in L-5-MTHF-Ca may be residues of folic acid (the starting material) and other organic by-products or degradation products. Residues of ethanol could potentially be present in L-5-MTHF-Ca because a mixture of ethanol and purified water is used to wash the isolated

product in the final synthesis step. Residues of ethanol in L-5-MTHF-Ca are specified by the USP and the manufacturer to be  $\leq 0.5\%$  in agreement with USP specifications. This is in agreement with ICH<sup>1</sup> Guidelines for residual solvents<sup>2</sup> which list ethanol as a class 3 solvent that should be limited by cGMP or other quality-based requirements. Solvents in this class may be regarded as less toxic and of lower risk to human health and it is considered that amounts of these residual solvents of 50 mg/day corresponding to 0.5% would be acceptable without justification.

The ethanol used in the final synthesis step is denatured with ca. 5% isopropanol and residues of isopropanol could also potentially be present in L-5-MTHF-Ca. Like ethanol, isopropanol is classified as a class 3 solvent; therefore, the manufacturer analyzes batches of L-5-MTHF-Ca to confirm that residues of isopropanol are also below the USP specified limit of  $\leq 0.5\%$ .

Certificates of analysis for three batches of L-5-MTHF-Ca confirm that concentrations of the two solvents are below 0.5%

While microbial specifications are not listed in the USP specifications for L-5-MTHF-Ca, the manufacturer tests every batch of Metafolin® for total aerobic microbial counts (TAMC) and total yeast/mold counts (TYMC) and specifies  $\leq 100$  CFU/g. Certificates of analysis of three batches of L-5-MTHF-Ca confirm that microbial counts are below manufacturer specified limits.

Food-grade specifications meet or exceed the U.S. Pharmacopeia (USP) specifications for L-5-MTHF-Ca to be used in dietary supplements. In addition to the USP specifications, food grade material specifications for L-5-MTHF-Ca have also been established by the Joint Food and Agricultural Organization of the United Nations (FAO)/World Health Organization (WHO) Expert Committee on Food Additives (JECFA) at their 65<sup>th</sup> meeting in 2005 for use in dietary supplements, foods for special dietary uses and other foods. L-5-MTHF-Ca to be used in infant formula also complies with the JECFA specifications.

All methods of analysis are internationally recognized standard procedures or internal methods that have been validated. The GRAS Panel reviewed the results of 3 non-consecutive batches of L-5-MTHF-Ca and concluded that the manufacturing process produces a consistent product that conforms to the established product specifications.

The GRAS Panel noted that the manufacturer of L-5-MTHF-Ca specifies a shelf-life of 24 months from the date of production when stored in the unopened original container at a temperature of 2-8°C.

The GRAS Panel reviewed information on the intended use and use levels in conventional infant formula for full-term infants of L-5-MTHF-Ca, and noted that the ingredient will be used to replace folic acid as a source of the vitamin folate in exempt infant formula insofar as exempt infant formulas may only deviate from the infant formula nutrient specifications listed in 21 CFR 107.100 under specific limited circumstances in which deviation is deemed necessary and will protect the public health.

Infant formula is a food which purports to be or is represented for special dietary use solely as a food for infants by reason of its simulation of human milk or its suitability as a complete or partial substitute for human milk<sup>3</sup>. The composition of infant formula should serve to meet the particular nutritional requirements and to promote normal growth and development of the infants for whom it is intended. Breast feeding is the ideal form of infant feeding, and data on the composition of human milk of healthy well-nourished women provides guidance for the composition of infant formula. Infant formula is a food

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<sup>1</sup> The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human use (ICH).

<sup>2</sup> [https://www.ich.org/fileadmin/Public\\_Web\\_Site/ICH\\_Products/Guidelines/Quality/Q3C/Q3C\\_R6\\_Step\\_4.pdf](https://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Quality/Q3C/Q3C_R6_Step_4.pdf).

<sup>3</sup> Federal Food, Drug and Cosmetic Act [as amended through P.L. 115-176, Enacted May 30, 2018] Chapter II – Definitions. Section 201.

with a standard of identity which specifically provides for the addition of folic acid. According to the nutrient requirements for infant formula (Section 412 of FD&C Act and 21 CFR 107.100), the minimum amount of folic acid in infant formula is 4.0 µg folic acid (folacin) per 100 kcal; no maximum amount is specified in the FD&C Act.

While the FDA has not set maximum upper levels for inclusion of folic acid in infant formula, the Codex Alimentarius provides a Guidance Upper Level (GUL) (Codex Alimentarius, 1981). GULs are set for nutrients without sufficient information for a science-based risk assessment and are derived on the basis of meeting the nutritional requirements of infants and an established history of apparent safe use. The purpose of GULs is to provide guidance to manufacturers; they should not be interpreted as goal values, and nutrient contents in infant formulas should usually not exceed the GULs. The Codex Alimentarius set the minimum amount of folic acid in infant formula to 10 µg/100 kcal and the GUL to 50 µg/100 kcal.

Dietary Reference Intakes for folate have been developed by the Food and Nutrition Board (FNB) at the Institute of Medicine (IOM) of the National Academies (IOM, 1998). The Adequate Intake (AI)<sup>4</sup> of folate per day, based on the mean intake of folate in healthy breastfed infants in the U.S., is 65 µg dietary folate equivalents (DFE)<sup>5</sup> for infants up to 6 months of age and 80 µg DFE from 7 to 12 months. Expressed in terms of folic acid, this would be 39 and 48 µg per day, respectively for each age group. Tolerable Upper Intake Levels (ULs)<sup>6</sup> for synthetic forms of folate in dietary supplements and fortified foods were established by the FNB to limit metabolic interactions between folate and vitamin B<sub>12</sub>, but no limits were set for infants from birth to 12 months of age as the FNB considered that breast milk, formula and food should be the only sources of folate for infants.

The amount of L-5-MTHF-Ca needed to supply the AI values of folate for infants set by the IOM, and the concentration needed to replace the amount of folate provided by folic acid when used at the legal minimum of 4.0 µg folic acid per 100 kcal, or to not exceed the Codex GUI levels that can be calculated based upon an understanding of the chemistry of L-5-MTHF-Ca and an assessment of the relative bioavailability of the two folate sources.

In aqueous media, e.g. in prepared infant formula, L-5-MTHF-Ca dissociates readily and completely to Ca and L-5-MTHF ions. Following consumption, L-5-MTHF is absorbed and enters the circulation and its fate becomes indistinguishable from that of other absorbed and metabolized natural folates or L-5-MTHF formed from synthetic folic acid.

The bioavailability of L-5-MTHF-Ca compared to folic acid is extensively reviewed in the dossier. Based upon publicly available literature, it can be concluded that L-5-MTHF-Ca is bioavailable to an extent similar or slightly higher than folic acid. This conclusion is supported by a recently published infant growth and development study that found no major differences in growth and tolerance among infants who consumed an infant formula with either L-5-MTHF-Ca or folic acid at equimolar doses (Troesch et al., 2019).

Because folic acid and L-5-MTHF-Ca have equimolar equivalence, the use level of L-5-MTHF-Ca to meet the minimum, adequate and upper intake levels can be calculated based upon the molecular weights of the folate sources. Folic acid use levels in several commercially available infant formulas in the marketplace are

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<sup>4</sup> Adequate Intake (AI): a recommended daily intake value based on observed or experimentally determined approximations of nutrient intake by a group (or groups) of healthy people that are assumed to be adequate used when an RDA cannot be determined.

<sup>5</sup> DFEs reflect the higher bioavailability of folic acid than that of food folate. At least 85% of folic acid is estimated to be bioavailable when taken with food, whereas only about 50% of folate naturally present in food is bioavailable. 1 µg DFE = 1 µg food folate = 0.6 µg folic acid from fortified food or as a supplement consumed with food = 0.5 µg of a folic acid supplement taken on an empty stomach.

<sup>6</sup> Tolerable Upper Intake Level (UL): the highest level of daily nutrient intake that is likely to pose no risk of adverse health effects to almost all individuals in the general population. As intake increases above the UL, the risk of adverse effects increases.

in the 15-16 µg per 100 kcal (5 fl oz, 147.9 ml). These commercially available formulas contain folic acid above the required minimum, but well below the Codex GUL. The amount of L-5-MTHF-Ca needed to replace folic acid in a typical commercially available infant formula would be 17 to 18 µg/100 kcal.

The daily dietary intake of L-5-MTHF-Ca was estimated using data available in the 2005-2012 cycle of the U.S. National Center for Health Statistics (NCHS)'s National Health and Nutrition Examination Survey (NHANES) (Grimes et al. 2015) to determine dietary intakes of energy and nutrients by US infants and toddlers. As reported, the mean calorie intake by infants aged 0-5.9 months was determined to be 612.5 ± 6.4 kcal/day while 6-to-11.9-month-olds consume about 40% more energy, 847.3 ± 13.3 kcal/day. Infant formula is the largest source of total energy intake, comprising 65.4% of daily energy intake in 0-5.9-month-old infants and 47.1% in 6-to-11.9-month-olds.

The contribution of infant formula to the mean total energy intake can be calculated for the two age groups from these data. As Table 1 show, daily energy intakes from infant formula for both age groups are almost identical (~400 kcal/day). The higher mean energy intake of older infants is due to increased consumption of baby food and introduced new foods such as milk, fruits, grain products and beverages, rather than an increase in infant formula intake.

Considering the energy intake from infant formula and typical folic acid use levels in commercial infant formulas (16 µg/100kcal), the amount of folic acid and the equivalent amount of L-5-MTHF-Ca ingested per day from consumption of a typical infant formula can be calculated.

Table 1: Folic acid and L-5-MTHF-Ca intake from infant formula calculated using data from Grimes et al. (2015)

Age (months)	Mean energy intake (kcal/day)	Energy intake from infant formula		Calculated intake (µg/day)	
		(%)	(kcal/day)	folic acid <sup>1</sup>	L-5-MTHF-Ca <sup>2</sup>
0 – 5.9	612.5 ± 6.4	65.4	401	64	72
6 – 11.9	847.3 ± 13.3	47.1	399	64	72

<sup>1</sup>Calculated assuming folic acid use level of 16 µg/100 kcal typical of commercial brands. <sup>2</sup>Calculated assuming L-5-MTHF-Ca use level of 18 µg/100 kcal to replace folic acid in typical commercial brands.

Replacement of folic acid in typical infant formulas by L-5-MTHF-Ca to provide an equivalent amount of folate would result in an estimated daily intake of 72 µg of L-5-MTHF-Ca by infants aged 0-to-12 months.

Infant intake of L-5-MTHF-Ca can also be calculated on a per kg body weight basis for each month of age using mean formula intake values for infants aged 0-to-11 months as described by Neal-Kluever et al. (2014). Considering that L-5-MTHF-Ca would be added at a concentration of 18 µg/100 kcal to replace folic acid in typical commercial brands, that the energy content of typical formulae on the market is 100 kcal per 150 mL of formula, and that the density of infant formula is 1.03 g/mL in the ready-to-drink form, infant formula would contain 0.12 µg/g L-5-MTHF-Ca. These data are shown in Table 2.



Table 2: Mean and 90<sup>th</sup> percentile formula and L-5-MTHF-Ca intake per kg body weight

Age (months)	Mean formula intake (g/kg bw/d)	Mean L-5-MTHF-Ca intake (µg/kg bw/d)	90 <sup>th</sup> percentile formula intake (g/kg bw/d)	90 <sup>th</sup> percentile L-5-MTHF-Ca intake (µg/kg bw/d)
0	170.4	20.4	250.5	30.1
1	150.0	18.0	240.6	28.9
2	140.0	16.8	199.3	23.9
3	123.1	14.8	183.0	22.0
4	113.1	13.6	177.9	21.3
5	98.5	11.8	157.9	18.9
6	103.9	12.5	158.4	19.0
7	89.2	10.7	144.7	17.4
8	79.9	9.6	122.0	14.6
9	76.8	9.2	114.8	13.8
10	66.2	7.9	99.5	11.9
11	59.6	7.2	101.7	12.2

At the intended use level to replace folic acid in infant formula, mean daily intake of L-5-MTHF-Ca would range from 7.2 to 20.4 µg/kg bw/d, whereas 90<sup>th</sup> percentile high level intake would range from 11.9 to 30.1 µg/kg bw/d for infants between the ages of 0-and-11 months.

The GRAS Panel reviewed absorption, distribution, metabolism and excretion studies on L-5-MTHF. In aqueous media or once ingested and exposed to the aqueous environment of the digestive tract, L-5-MTHF-Ca dissociates readily and completely into Ca and L-5-MTHF ions; L-5-MTHF is subsequently monoglutamated and absorbed mainly in the small intestine by carrier-mediated transport; and once absorbed, the fate of synthetic L-5-MTHF becomes indistinguishable from that of all other absorbed and metabolized natural folates, or L-5-MTHF formed from synthetic folic acid.

Similarly, the GRAS Panel reviewed data on the absorption, distribution, metabolism and excretion of calcium which can be summarized as follows: Intestinal calcium absorption occurs through an active, saturable, transcellular process and a non-saturable, passive process; Active transport is controlled by vitamin D and passive transport is paracellular; Calcium absorption varies considerably throughout the lifespan, being higher during periods of rapid growth and lower in old age; and the main routes of obligatory (endogenous) calcium loss are urine, feces, skin and sweat (dermal losses).

The GRAS Panel also reviewed data provided on the bioavailability of L-5-MTHF-Ca compared to folic acid in studies conducted in rats and humans. Dietary studies in humans have also been performed in healthy adults of both sexes and at different life stages and also in patients suffering from coronary artery disease as discussed in the dossier. Male rats divided into three groups and given a single capsule of folic acid, (6S)-MTHF calcium salt or Quatrefolic at a dose of 70 µg/Kg L-5-MTHF-Ca and monitored for 8 hours had numerically, but not significantly, higher maximum L-5-MTHF concentrations ( $C_{max}$ ) in plasma compared to animals given folic acid (Miraglia et al., 2016). The 8-hour area under the curve (AUC) for L-5-MTHF-Ca-treated rats was more than eight times higher than for the folic acid treatment, indicating higher overall absorption of L-5-MTHF; however, the speed of absorption (time to reach  $C_{max}$ ) was the same for both folate forms.

A longer study (4-weeks) in male rats receiving growing-up milk (milk for children between 1-and-3 years of age) as their sole food source found that, compared to folic acid, inclusion of L-5-MTHF increased folate concentration in erythrocytes and liver but not in plasma (Pérez-Conesa et al., 2009).

In healthy adult men, the short-term absorption kinetics of a single dose of 500 µg L-5-MTHF-Ca (given as a capsule) was found to be equivalent to that of folic acid (Pentieva et al., 2004).  $C_{max}$ , time to reach  $C_{max}$  and AUCs were similar for both folate sources. A single high oral dose (5g) of racemic 6[R,S] 5-MTHF-Ca administered to patients with coronary artery disease resulted in seven times higher plasma levels of L-5-MTHF within 1-3h of administration than patients given the same dose of folic acid indicating higher bioavailability of orally administered L-5-MTHF-Ca compared to folic acid (Willems et al., 2004).

Longer term dietary supplementation of healthy adults with 100 or 200 µg/d folic acid or equimolar amounts of L-5-MTHF-Ca for 16-or-24 weeks resulted in similar increases in plasma and erythrocyte folate levels (Venn et al., 2002; Venn et al., 2003; Wright et al., 2010). Supplements containing higher levels (400 µg/d folic acid or up to 416 µg/d L-5-MTHF-Ca) taken by healthy women of childbearing age for 24 weeks also resulted in similar or slightly higher increases in plasma and erythrocyte folate levels in women taking the L-5-MTHF-Ca supplements compared to those taking folic acid (Lamers et al., 2004; Lamers et al., 2006). In lactating women, 416 µg/d L-5-MTHF-Ca was at least as effective as 400 µg/d folic acid in preserving maternal plasma and erythrocyte folate concentrations in the first 16 weeks of lactation (Houghton et al., 2006). For middle-aged women, plasma folate levels did not differ between those taking folic acid or L-5-MTHF-Ca supplements for 5 weeks (de Meer et al., 2005). Interestingly, intestinal absorption of folate sources (folic acid and L-5-MTHF-Ca) may be age-dependent with middle-aged women having lower absorption rates compared to young adult women.

Similar to naturally occurring folates, the bioavailability of L-5-MTHF-Ca or folic acid used to fortify foodstuffs may be affected by the food matrix (EFSA, 2004). Naturally occurring folates in food such as broccoli, spinach and legumes are a mixture of mono- and polyglutamates that may not be completely released from the food matrix and may have some losses during digestion, leading to incomplete bioavailability (EFSA, 2014). In laboratory studies, FBP in cow milk was shown to protect L-5-MTHF (and other folates) against degradation (Jones & Nixon, 2002) and inclusion of cow milk in the diet of young women improved the bioavailability of folates naturally present in food, possibly due to the presence of FBP in the milk (Picciano et al., 2004). Both folic acid and L-5-MTHF are easily released from a milk matrix and made available for absorption (Verwei et al., 2003). The slightly lower binding affinity of milk FBP to L-5-MTHF compared to folic acid results in higher release of L-5-MTHF during gastric passage leading to slightly higher folate bioaccessibility from L-5-MTHF-Ca than folic acid.

The GRAS Panel also independently reviewed the specific and critical clinical study by Troesch et al., 2019 performed a feeding study in healthy term infants investigating the suitability and safety of L-5-MTHF (supplied as L-5-MTHF-Ca) as a substitute for folic acid as the folate source in infant formula. The growth and tolerance among infants receiving formula containing folic acid (10 µg/100ml-15.2 µg/100 Kcal) or the equimolar dose of 10.4 µg/100 ml L-5-MTHF which was added as 11.3 µg/100 ml of L-5-MTHF-Ca). This was compared to that of infants receiving formula containing the equimolar dose of L-5-MTHF-Ca. The study was performed as a randomized, double-blind, parallel, controlled trial with an additional group of breastfed infants as a reference group. Infants of parents who independently chose not to breastfeed and decided to start full formula-feeding within the first 28 days of life were randomly assigned to one of the two formula groups. Infants were examined and anthropometric data were collected at a baseline visit (age 1-27 days) and at four additional visits (Visits 1-4 at mean ages of 28, 56, 84 and 112 days). A total of 360 healthy term infants from singleton pregnancies were enrolled. Of the recruited infants, 120 were breastfed, 120 were allocated to the control formula (folic acid), and 120 were allocated to the intervention formula group (L-5-MTHF-Ca). A total of 315 infants completed the first visit and 298 completed the fourth visit. The number of dropouts did not differ between groups. Both formulae were well-accepted and no differences in acceptance and tolerability or consistency, color and smell of stool were reported. There were no adverse effects, or blood chemistry and hematology results that gave reason for safety concerns and all results were within the expected range and not different between the intervention and control groups. Most markers for folate status did not differ between the intervention and control groups; however, at visit 4, plasma level of unmetabolized folic acid was significantly higher in the control compared to the intervention group, with comparable concentrations of unmetabolized folic acid in the intervention and breastfed groups. Red cell folate levels were significantly higher in infants consuming the formula containing L-5-MTHF-Ca compared to control subjects (adjusted means of 907 nmol/L versus 839 nmol/L). The primary outcome, weight gain during the intervention period was within the predefined interval of  $\pm 3.5$ g/day, thus demonstrating equivalence. While there was not enough evidence to support equivalence for length growth, the gain in head circumference demonstrated equivalence of the two folate sources. It could be concluded that an infant formula with L-5-MTHF-Ca did not show significant differences in growth and tolerance compared to infants fed the same formula with folic acid at equimolar doses.

From the studies performed in rats and humans, the GRAS Panel agrees with the information provided that the bioavailability of L-5-MTHF-Ca, whether consumed as a supplement or as a folate source in milk or infant formula, is equivalent to or slightly higher than folic acid. As such, the study confirmed that L-5-MTHF-Ca is suitable for use in infant formula.

Besides the tolerability study in infants (Troesch et al., 2019) and the studies demonstrating the bioequivalence of the folate sources, additional studies in patients suffering from various illnesses provide evidence to support high tolerance for L-5-MTHF-Ca and L-5-MTHF.

The GRAS Panel also reviewed the results of a series of studies conducted under Good Laboratory Practice and according to the respective Organization for Economic Co-operation and Development (OECD) Guidance relating to safety of L-5-MTHF-Ca. These included genotoxicity, subchronic oral toxicity and developmental toxicity studies performed with L-5-MTHF-Ca (Niederberger et al. (2019)). The GRAS Panel concurred that the results from this suite of toxicology studies demonstrated safety and lack of toxicologic concern for the intended condition of use. The NOAEL of L-5-MTHF-Ca in the 90-day toxicity study in rats was established at 400 mg/kg bw/day which is 19,608 times above the highest mean intake (400 mg/kg bw/d; 20.4 µg/kg bw/day) and 13,289 times the highest 90<sup>th</sup> percentile intake of L-5-MTHF-Ca in the highest consuming infant age group (400 mg/kg bw/day; 30.1 µg/kg bw/day). In addition, the NOAEL in the prenatal developmental toxicity study in rats was established at 1000 mg/kg bw/day, providing an even higher margin of safety (MoS) for infants in both the mean and high intake groups:

Highest mean intake group MoS = 1000 mg/kg bw/day : 20.4 µg/kg bw/day = 49,020

Highest 90<sup>th</sup> percentile intake MoS = 1000 mg/kg bw/day: 30.1 µg/kg bw/day = 33,223

Guidance Upper levels of folic acid in infant formula set by the Codex Alimentarius would also apply to limit the amount of L-5-MTHF-Ca used in infant formulas to ensure that infant formulas are comparable in composition to breast milk.

Finally, use of L-5-MTHF-Ca at the intended use level does not result in any adverse effects in infants as demonstrated in the tolerance and safety study in infants by Troesch et al. 2019.

Infant formula has been estimated to contribute 79.2% of the 78.6 ± 1.7 µg/day folate consumed by 0-5.9 month-olds and 44.5% of the 136.2 ± 4.0 µg/day folate consumed by 6-11.9 month-olds (Grimes et al., 2015). Therefore, replacement of folic acid with L-5-MTHF-Ca, as DSM proposes, would not alter daily folate intake.

In terms of calcium exposure, in solution, L-5-MTHF-Ca readily dissociates into L-5-MTHF-Ca and calcium ion (Ca<sup>2+</sup>). Ca comprises about 8% of L-5-MTHF-Ca, therefore ingestion of 72 µg/day of L-5-MTHF-Ca would include intake of approximately 6 µg/day of Ca. Considering the mean Ca intake of infants reported by (Grimes et al., 2015):

- 0-5.9-month-olds: 469.7 ± 9.6 mg/day
- 6-11.9-month olds: 649.0 ± 12.4 mg/day

Inclusion of L-5-MTHF-Ca in infant formula would increase Ca intake by infants by 6 µg/day, which is an increase of <0.00001% of their normal daily Ca intake.

Infant formula is regulated to contain a minimum of 60 mg/100 kcal Ca with typical commercial formulas containing 67 to 82 mg/100 kcal. The additional Ca from inclusion of L-5-MTHF-Ca at 18 µg/100 kcal would be 1.4 µg Ca/100 kcal, i.e. 0.00002% of the typical Ca concentration in infant formulas.

It can therefore be concluded that, considering normal daily intake of Ca, the additional intake of Ca from replacement of folic acid with L-5-MTHF-Ca is insignificant.

Relative to impurities, all potential elemental and organic impurities in L-5-MTHF-Ca are limited by the conservative specifications. No other substances are expected to be formed in or on food under the intended use of L-5-MTHF-Ca in infant formula.

The safety of L-5-MTHF-Ca is further corroborated by the available data on the individual components, including folic acid and calcium. In addition, the safety of use of L-5-MTHF-Ca as a source of the nutrient folate has been assessed by several authoritative bodies. In the United States, in accordance with the requirements of Section 413(b) of the FD&C Act and Section 8 of the Dietary Supplement Health and Education Act, Merck KGaA filed a 75-day premarket notification with the FDA (Docket Number 95S-0316, filing date March 13, 2001) for the calcium salt of L-5-methyltetrahydrofolate (L-5-methyl-THF) as a new dietary ingredient for use in dietary supplements<sup>7</sup>. With no objections from the FDA, Merck KGA was able to lawfully use L-5-MTHF-Ca in dietary supplements in the United States after the 75-day period. In addition, a

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<sup>7</sup> <https://www.regulations.gov/document?D=FDA-2001-S-0575-0029>.

monograph for calcium L-5-methyltetrahydrofolate has been included in the USP Dietary Supplements Compendium. In the European Union in 2004, the European Food Safety Authority (EFSA) published the opinion of its Scientific Panel which concluded that the use of L-5-MTHF-Ca as a source of folate in foods for particular nutritional uses, food supplements and foods for the general population, with a tolerable upper level of 1 mg/adult person/day is not of concern from a safety point of view (EFSA, 2004).

On the basis of the EFSA opinion, L-5-MTHF-Ca is included in the list of vitamins authorized for use in food supplements (Directive 2002/46/EC as amended<sup>8</sup>) and in the list of vitamins for use in food for special medical purposes and in total diet replacement for weight control (Regulation (EU) 609/2013 as amended<sup>9</sup>).

To extend the use of L-5-MTHF-Ca to fortified foods, in 2007, Merck & Cie (formerly Merck Eprova AG), Switzerland submitted a novel food application to Ireland as the first assessing Member State. A favorable opinion of the Irish competent authority (FSAI) was issued and forwarded to all other Member States for comment. No 'reasoned objection' was received within the comment period. The Applicant received authorization for L-5-MTHF-Ca (Metafolin<sup>®</sup>) as a novel food on January 4<sup>th</sup> 2008<sup>10</sup>.

Following this approval, Merck & Cie requested the inclusion of L-5-MTHF-Ca in the EU positive list of vitamins that may be added to foods. As a result, Regulation (EC) 1925/2006 (with amendments) lists "Calcium-L-methylfolate"<sup>11</sup>.

At its 65<sup>th</sup> meeting in 2005, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) evaluated the safety of calcium L-5-methyltetrahydrofolate as an alternative to folic acid in food fortification and supplementation. The Committee had no concern about the safety of the proposed use of L-5-MTHF-Ca as an alternative to folic acid in food supplements, foods for special dietary uses and other foods<sup>12 13</sup>.

In 2005, an application was submitted on behalf of Merck & Cie (formerly Merck Eprova AG) to Food Standards Australia New Zealand (FSANZ) requesting the approval of L-5-methyltetrahydrofolate, calcium salt (L-MTHF) as a permitted form of the vitamin folate for use in specified foods where voluntary folate fortification is currently permitted in the FSANZ Code. The application submitted was based on and included the information provided in the JECFA and EFSA dossiers.

FSANZ performed a full scientific evaluation of L-MTHF to assess its safety for human consumption and suitability for fortification of certain foods and concluded in 2008 that the use of L-MTHF for voluntary fortification purposes would raise no public health and safety concerns. L-methyltetrahydrofolate, calcium is listed as a permitted form of folic acid in Schedule 15 of the Australia New Zealand Food Standards Code<sup>14</sup>.

Based on the evaluation of 5-L-MTHF by the Australian Therapeutic Goods Administration (TGA), levomefolate calcium is listed as a permitted ingredient for use in Complimentary Medicines, which is equivalent to food supplements<sup>15</sup>.

Finally, L-5-Methyltetrahydrofolate, calcium salt (L-5-MTHF-Ca) is listed as a chemical substance in the Natural Health Products Ingredients Database of Health Canada<sup>16</sup>.

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<sup>8</sup> <https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:02002L0046-20170726&qid=1535537044673&from=EN>.

<sup>9</sup> <https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:02013R0609-20170711&qid=1535537251102&from=EN>.

<sup>10</sup> [https://www.fsai.ie/uploadedFiles/Science\\_and\\_Health/Novel\\_Foods/Applications/2007%20%20L%20MTHF%20Ca.pdf](https://www.fsai.ie/uploadedFiles/Science_and_Health/Novel_Foods/Applications/2007%20%20L%20MTHF%20Ca.pdf).

<sup>11</sup> <https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:02006R1925-20170726&qid=1535537508786&from=EN>.

<sup>12</sup> [https://apps.who.int/iris/bitstream/handle/10665/43407/9241660562\\_eng.pdf;jsessionid=9E8B7C9CA98835C711492AF384706A18?sequence=1](https://apps.who.int/iris/bitstream/handle/10665/43407/9241660562_eng.pdf;jsessionid=9E8B7C9CA98835C711492AF384706A18?sequence=1).

<sup>13</sup> [https://apps.who.int/iris/bitstream/handle/10665/43408/WHO\\_TRS\\_934\\_eng.pdf?sequence=1](https://apps.who.int/iris/bitstream/handle/10665/43408/WHO_TRS_934_eng.pdf?sequence=1).

<sup>14</sup> <https://www.legislation.gov.au/Details/F2017C00328>.

<sup>15</sup> <https://www.legislation.gov.au/Details/F2017L00926>.

<sup>16</sup> <http://webprod.hc-sc.gc.ca/nhpdp-bdipn/ingredsReq.do?srchRchTxt=folate&srchRchRole=-1&mthd=Search&lang=eng>.

## CONCLUSION

We, the undersigned independent qualified members of the Generally Recognized as Safe (GRAS) Panel, have, independently and collectively, critically evaluated published and unpublished data and information pertinent to the safety of the intended uses of DSM's Calcium L-methylfolate (L-5-MTHF Calcium) and to determine whether its intended use in non-exempt and exempt infant formulas as a partial or complete replacement for folic acid as a source of the vitamin, folate. We unanimously conclude that the intended uses of DSM's Calcium L-methylfolate (L-5-MTHF Calcium), produced in a manner that is consistent with current Good Manufacturing Practice (cGMP) and meeting appropriate food-grade specifications as presented in the supporting dossier [*"GRAS Notice for the Use of Calcium L-Methylfolate in Infant Formula"*(28 October 2019)], is safe.

We further conclude that the intended uses of DSM's Calcium L-methylfolate (L-5-MTHF Calcium) as described above, produced in a manner that is consistent with current Good Manufacturing Practice (cGMP) and meeting appropriate food-grade specifications as presented in the supporting dossier is Generally Recognized as Safe (GRAS) based on scientific procedures.

It is our professional opinion that other qualified experts would concur with this conclusion.

November 26, 2019

Michael W. Pariza, Ph.D.  
Emeritus Professor, University of Wisconsin-Madison,  
Food Research institute

Date

John A. Thomas, Ph.D., A.T.S.  
Adjunct Professor, Indiana University School of  
Medicine

Date

Stanley M. Tarka, Jr., Ph.D., A.T.S.  
Panel Chair, President, Tarka Group Inc.  
Adjunct Associate Professor, The Pennsylvania State  
University College of Medicine

## CONCLUSION

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Nov. 26, 2019  
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Stanley M. Tarka, Jr., Ph.D., A.T.S.  
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Date



## CONCLUSION

We, the undersigned independent qualified members of the Generally Recognized as Safe (GRAS) Panel, have, independently and collectively, critically evaluated published and unpublished data and information pertinent to the safety of the intended uses of DSM's Calcium L-methylfolate (L-5-MTHF Calcium) and to determine whether its intended use in non-exempt and exempt infant formulas as a partial or complete replacement for folic acid as a source of the vitamin, folate. We unanimously conclude that the intended uses of DSM's Calcium L-methylfolate (L-5-MTHF Calcium), produced in a manner that is consistent with current Good Manufacturing Practice (cGMP) and meeting appropriate food-grade specifications as presented in the supporting dossier [*"GRAS Notice for the Use of Calcium L-Methylfolate in Infant Formula"* (28 October 2019)], is safe.

We further conclude that the intended uses of DSM's Calcium L-methylfolate (L-5-MTHF Calcium) as described above, produced in a manner that is consistent with current Good Manufacturing Practice (cGMP) and meeting appropriate food-grade specifications as presented in the supporting dossier is Generally Recognized as Safe (GRAS) based on scientific procedures.

It is our professional opinion that other qualified experts would concur with this conclusion.

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Michael W. Pariza, Ph.D.  
Emeritus Professor, University of Wisconsin-Madison,  
Food Research Institute

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Date

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John A. Thomas, Ph.D., A.T.S.  
Adjunct Professor, Indiana University School of  
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26 November 2019  
Date



## REFERENCES

- Codex Alimentarius (1981) International Food Standards. Standard for infant formula and formulas for special medical purposes intended for infants: CODEX STAN 72-1981.
- de Meer K., Smulders Y.M., Dainty J.R., Smith D.E.C., Kok R.M., Stehouwer C.D.A., Finglas P.M. & Jakobs C. (2005) 6S-5-methyltetrahydrofolate or folic acid supplementation and absorption and initial elimination of folate in young and middle-aged adults. *European journal of clinical nutrition*, 59, 1409-1416. DOI: 10.1038/sj.ejcn.1602254.
- EFSA (2004) Opinion of the Scientific Panel on food additives, flavourings, processing aids and materials in contact with food (AFC) related to Calcium L-Methylfolate. *EFSA Journal*, 2, 135. DOI: 10.2903/j.efsa.2004.135.
- EFSA (2014) Scientific Opinion on Dietary Reference Values for folate. *EFSA Journal*, 12, 55. DOI: 10.2903/j.efsa.2014.3893.
- EFSA (2015) Scientific Opinion on Dietary Reference Values for calcium. *EFSA Journal*, 13, 469. DOI: 10.2903/j.efsa.2015.4101.
- Grimes C.A., Szymlek-Gay E.A., Campbell K.J. & Nicklas T.A. (2015) Food sources of total energy and nutrients among U.S. infants and toddlers: national health and nutrition examination survey 2005-2012. *Nutrients*, 7, 6797-6836. DOI: 10.3390/nu7085310.
- Houghton L.A., Sherwood K.L., Pawlosky R., Ito S. & O'Connor D.L. (2006) 6S-5-Methyltetrahydrofolate is at least as effective as folic acid in preventing a decline in blood folate concentrations during lactation. *The American journal of clinical nutrition*, 83, 842-850. DOI: 10.1093/ajcn/83.4.842.
- IOM (1998) Dietary reference intakes for thiamin, riboflavin, niacin, vitamin B<sub>6</sub>, folate, vitamin B<sub>12</sub>, pantothenic acid, biotin, and choline. The National Academies Press, Washington, DC.
- IOM (2011) Dietary reference intakes for calcium and vitamin D, Washington, DC.
- Lamers Y., Prinz-Langenohl R., Brämwig S. & Pietrzik K. (2006) Red blood cell folate concentrations increase more after supplementation with 6S-5-methyltetrahydrofolate than with folic acid in women of childbearing age. *The American journal of clinical nutrition*, 84, 156-161. DOI: 10.1093/ajcn/84.1.156.
- Lamers Y., Prinz-Langenohl R., Moser R. & Pietrzik K. (2004) Supplementation with 6S-5-methyltetrahydrofolate or folic acid equally reduces plasma total homocysteine concentrations in healthy women. *The American journal of clinical nutrition*, 79, 473-478. DOI: 10.1093/ajcn/79.3.473.
- Miraglia N., Agostinetti M., Bianchi D. & Valoti E. (2016) Enhanced oral bioavailability of a novel folate salt: comparison with folic acid and a calcium folate salt in a pharmacokinetic study in rats. *Minerva ginecologica*, 68, 99-105.
- Niederberger K.E., Dahms I., Broschard T.H., Boehni R. & Moser R. (2019) Safety evaluation of calcium L-methylfolate. *Toxicology Reports*. DOI: 10.1016/j.toxrep.2019.09.012.
- Pentieva K., McNulty H., Reichert R., Ward M., Strain J.J., McKillop D.J., McPartlin J.M., Connolly E., Molloy A., Krämer K. & Scott J.M. (2004) The short-term bioavailabilities of 6S-5-methyltetrahydrofolate and folic acid are equivalent in men. *The Journal of nutrition*, 134, 580-585. DOI: 10.1093/jn/134.3.580.

Pérez-Conesa D., Haro Vicente J., Romero F. & Ros G. (2009) [6S]-5-Methyltetrahydrofolate enhances folate status in rats fed growing-up milk. *European journal of nutrition*, 48. DOI: 10.1007/s00394-009-0022-1.

Picciano M.F., West S.G., Ruch A.L., Kris-Etherton P.M., Zhao G., Johnston K.E., Maddox D.H., Fishell V.K., Dirienzo D.B. & Tamura T. (2004) Effect of cow milk on food folate bioavailability in young women. *The American journal of clinical nutrition*, 80, 1565-1569. DOI: 10.1093/ajcn/80.6.1565.

Troesch B., Demmelmair J., Gimpfl M., Hecht C., Lakovic G., Roehle R., Sipka L., Trisic B., Vusurovic M., Schoop R., Zdjelar S., Koletzko B. & on behalf of the MEFOLIN Study Group (2019) Suitability and safety of L-5-methyltetrahydrofolate as a folate source in infant formula: A randomized-controlled trial. *PLOS ONE*, 14, e0216790. DOI: 10.1371/journal.pone.0216790.

U.S. FDA 21 CFR 107.100 Infant Formula Nutrient Specifications [PART 107—INFANT FORMULA Subpart B—Labeling](#)

Venn B.J., Green T.J., Moser R. & Mann J.I. (2003) Comparison of the effect of low-dose supplementation with L-5-methyltetrahydrofolate or folic acid on plasma homocysteine: a randomized placebo-controlled study. *The American journal of clinical nutrition*, 77, 658-662. DOI: 10.1093/ajcn/77.3.658.

Venn B.J., Green T.J., Moser R., McKenzie J.E., Skeaff C.M. & Mann J. (2002) Increases in blood folate indices are similar in women of childbearing age supplemented with 6S-5-methyltetrahydrofolate and folic acid. *The Journal of nutrition*, 132, 3353-3355. DOI: 10.1093/jn/132.11.3353.

Verwei M., Arkbåge K., Havenaar R., van den Berg H., Witthöft C. & Schaafsma G. (2003) Folic acid and 5-methyltetrahydrofolate in fortified milk are bioaccessible as determined in a dynamic in vitro gastrointestinal model. *The Journal of nutrition*, 133, 2377-2383.

Willems F.F., Boers G.H.J., Blom H.J., Aengevaeren W.R.M. & Verheugt F.W.A. (2004) Pharmacokinetic study on the utilisation of 5-methyltetrahydrofolate and folic acid in patients with coronary artery disease. *British journal of pharmacology*, 141, 825-830. DOI: 10.1038/sj.bjp.0705446.

Wright A.J.A., King M.J., Wolfe C.A., Powers H.J. & Finglas P.M. (2010) Comparison of (6S)-5-methyltetrahydrofolic acid v. folic acid as the reference folate in longer-term human dietary intervention studies assessing the relative bioavailability of natural food folates: comparative changes in folate status following a 16-week placebo-controlled study in healthy adults. *The British journal of nutrition*, 103, 724-729. DOI: 10.1017/S0007114509992339.



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July 31<sup>st</sup>, 2020

**Re: Questions for GRN 915 -Calcium L-Methylfolate**

Dear Mr. Bonnette,

DSM Nutritional Products has thoroughly reviewed the questions posed by FDA for GRN 915 – Calcium L-methylfolate on July 14<sup>th</sup>, 2020 and offers the following responses elucidated below.

**Question 1. DSM provides two Chemical Abstracts Service (CAS) registry numbers for the notified substance (CAS Nos. 129025-21-4 and 151533-22-1). We consider the CAS number to be unique to each substance, and only one CAS number should be assigned to each chemical. Therefore, please clarify which CAS No. represents the notified substance, Calcium L-methylfolate.**

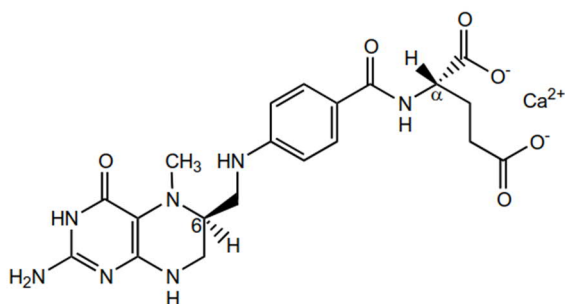
DSM Response:

While the molecular weight and description would be applicable to both CAS numbers 129025-21-4 (Calcium salt with an unspecified ratio of L-5-MTHF/Ca<sup>2+</sup>) and 151533-22-1 (Calcium salt with specified 1:1 ratio of L-5-MTHF/Ca<sup>2+</sup>). The CAS number which represents the notified substance is 151533-22-1. The notified substance is described by and is complying with USP monograph Calcium L-5-Methyltetrahydrofolate which references CAS No151533-22-1. Calcium content is specified for Metafolin and the specified range (7.0%-8.5% on anhydrous basis) corresponds with the 1:1 ratio (8.0%).

**Question 2. In the specifications (Table 2), DSM provided specification limits for (6R)-Mefolate ( $\leq 1.0\%$  area) and Mefox ( $\leq 1\%$ ). However, DSM didn't provide the chemical name for (6R)-Mefolate. Please clarify whether (6R)-Mefolate is an unnatural diastereomer of calcium L-methylfolate. Please also clarify whether the limit for Mefox ( $\leq 1\%$ ) is the sum of the 6-S and 6-R Mefox.**

DSM Response:

**Calcium L-methylfolate structural formula :**



***(6R)-Mefolinate :***

As per the structural formula above Calcium-L-methylfolate has two chiral carbon atoms: the C-atom in position 6 of the pteroyl moiety and the  $\alpha$ -C atom in the L-glutamic acid moiety. Consequently, there exists the possibility of four stereoisomers: (6S, $\alpha$ S), (6S, $\alpha$ R), (6R, $\alpha$ S), (6R, $\alpha$ R). The naturally occurring isomers of tetrahydrofolic acid and its 5-substituted derivatives are the (6S, $\alpha$ S) and (6R, $\alpha$ S) dia-stereoisomers. (6R)-Mefolinate represents the (6R, $\alpha$ S) configuration. The notified product has the 6S,  $\alpha$ S configuration. (6R, $\alpha$ R)- and (6S, $\alpha$ R)-diastereoisomers are not found in Metafolin® and not likely to be present considering the manufacturing process employed. (6R)-Mefolinate is specified and limited based on USP Calcium-L-5 methyltetrahydrofolate monograph (see enantiomeric purity, D-5-methyl tetrahydrofolate  $\leq 1.0$  % area).

***Mefox :***

The limit for Mefox ( $\leq 1.0$  %) is the sum of the 6-S and 6-R Mefox. Reference can be made to the USP monograph for Calcium L-5-methyltetrahydrofolate.

**Question 3. In addition, please clarify the specification for the “sum of all related compounds ( $\leq 2.5\%$ ).” Is this all potential impurities (by-product and degradation products) in the notified substance, or is this limited to only identified folate-related substances in the calcium L-methylfolate?**

DSM Response:

“Sum of all related compounds ( $\leq 2.5\%$ )” is meant for all potential impurities (by-product and degradation products) in the notified substance.

**Question 4. Additionally in the specifications, we note that the microbiological specifications did not include Cronobacter sakazakii or Salmonella spp. specifications. Please provide specifications for these organisms that are relevant for infant formula safety.**

DSM Response:

Please see below the revised specifications

Test parameter	DNP Microbiological Specification for use in infant formula	Method of reference
Total aerobic microbial count	Max. 10 <sup>3</sup> CFU/g or CFU/mL	Ph. Eur. 2.6.12
Total combined yeast/molds count	Max. 10 <sup>2</sup> CFU/g or CFU/mL	Ph. Eur. 2.6.12
<i>Enterobacteriaceae</i> (incl Cronobacter spp.) [Bile Tolerant Gram Negative Bacteria]	Negative in 100g	Ph.Eur. 2.6.13
<i>Salmonella</i> spp.	Negative in 125 g	Ph.Eur. 2.6.13
<i>Escherichia coli</i>	Negative in 10 g	Ph.Eur. 2.6.13
<i>Staphylococcus aureus</i>	Negative in 10 g	Ph.Eur. 2.6.13
<i>Pseudomonas aeruginosa</i>	Negative in 10 g	Ph.Eur. 2.6.13
<i>Bacillus cereus</i>	<100 CFU/g	ISO 7932: 2004
Sulphite reducing clostridia (SRC)	<100 CFU/g	ISO 15213:2003

**Question 5. The notice describes the stability data for calcium L-methylfolate per se at refrigerated and room temperatures (Appendix D). However, it did not discuss the stability of calcium L-methylfolate under the intended use conditions in infant formula formulated with the notified substance. Please provide information on the stability of calcium L-methylfolate in prepared liquid or powdered infant formulas.**

DSM Response:

As an ingredient supplier DSM typically does not typically conduct stability in finished product (infant formula) on behalf of our customers. Our customers who are the finished infant formula manufacturers are required to conduct stability on their specific formulations and then notify FDA when the formulation is changed. However, we do have data from the powdered form we used in the infant trial. Please find those result in the Annex 1 of this response for your consideration.

**Question 6. The notice indicates that the intended use is for non-exempt and exempt infant formula (IF) as a partial or complete replacement for folic acid as a source of the vitamin, folate: Please specify the specific exempt IF for the intended use and provide a brief statement that the replacement of folic acid with calcium L-methylfolate is not expected to be a safety issue for the subpopulation consuming those exempt IFs.**

DSM Response:

It is foreseen that calcium L-methylfolate could replace folic acid in infant formulas that could be fed to premature and low-birth-weight infants, infants with allergies, infants with inborn metabolic disorders or could be used in other exempt infant formulas. Folic acid must first be reduced to dihydrofolate and then to tetrahydrofolate to be able to enter the folate cycle and act as a co-factor and a source for methyl groups in the cell. Thus, tetrahydrofolate is a major metabolite of folic acid ultimately responsible for its efficacy. Calcium L-methylfolate is already a biologically active form, and it is absorbed and utilized at least as well as folic acid. Furthermore, calcium L-methylfolate efficacy is higher compare to folic acid in babies with polymorphism in the MTHFR and MTHFD1 genes. The safety profile of calcium L-

methylfolate is comparable to folic acid if not better as it does not cause unmetabolized folic acid in blood. Therefore, the substitution of folic acid with calcium L-methylfolate will have no impact on the efficacy or safety concerns for infants.

**Question 7. Please clarify what is meant by “partial” replacement**

DSM Response:

The term “partial” is meant to express that some Infant formula is formulated with folic acid and others with MTHF. It doesn't mean mixtures of both would be present in the infant formula.

**Question 8. Pg. 21 lists the search terms used for your updated literature search. Please provide the database used as well as the dates included in this search.**

DSM Response:

The literature search was performed in PubMed in October 2019. The database was searched using the names and synonyms for L-5-MTHF-Ca and the search terms, absorption, distribution, metabolism, excretion safety, toxicity, infant. Titles and abstracts of the resulting literature were visually scanned to select literature related to ADME and safety of L-5-MTHF-Ca in humans, or toxicity. Copies of the selected relevant literature were obtained and read. The reference lists of the obtained publications were also searched for additional relevant publications.

**Question 9. Please provide a statement or a narrative relating the actual levels of L-methylfolate detected in human milk [i.e. (Page et al., 2017)] to the proposed use levels to support your safety narrative. Reference: Page, R., Robichaud, A., Arbuckle, T.E., Fraser, W.D., and MacFarlane, A.J. (2017). Total folate and unmetabolized folic acid in the breast milk of a cross-section of Canadian women. *Am J Clin Nutr* 105, 1101-1109.**

DSM response:

Total folate concentrations in breast milk have been reported to range from 55 to 365 nmol/L (Büttner et al., 2014). According to the IOM, the average total folate concentration of breast milk is 193 nmol/L (IOM, 1998). Total folate concentrations in breast milk of 69 Canadian women at 4 to 16 weeks postpartum ranged from 159 to 207 nmol/L (Houghton et al., 2009). Brown et al., 1986 reported total folate levels in breast milk of US women up to 260 nmol/L (114.7 ng/ml) and Büttner et al., 2014 measured  $150 \pm 46$  nmol/L total folate in donated Swedish breast milk samples. Folate in breast milk is present in several different vitamers, reduced polyglutamate forms predominating, with L-5-MTHF making up the majority (Brown et al., 1986; Page et al., 2017). Concentrations of total folate, reduced folates, L-5-MTHF, and unmetabolized folic acid reported for in breast milk of 561 Canadian women were  $119 \pm 1.9$ ,  $72.0 \pm 1.4$ ,  $49.7 \pm 1.0$  and  $47.0 \pm 1.6$  nmol/L, respectively (Page et al., 2017). Similarly, total folate, L-5-MTHF and tetrahydrofolate in Swedish breast milk were  $150 \pm 46$ ,  $117 \pm 37$  and  $33 \pm 15$  nmol/L, respectively (Büttner et al., 2014).

Because of the important roles of folate in metabolic pathways, infant formula is supplemented with folic acid. It is added at levels greater than those of endogenous milk folates. The typical use level of folic acid in infant formula, 16 µg/100 kcal, provides approximately 240 nmol/L folic acid. Considering the equivalence of L-5-MTHF-Ca to folic acid (Troesh et al., 2019), an equimolar dose of L-5-MTHF-Ca would replace folic acid and provide approximately 246 nmol/L of L-5-MTHF in infant formula. According to the product specifications, almost all the

folate in infant formula made with L-5-MTHF-Ca would be in the form of L-5-MTHF and only very small residues of other forms of folate would be present.

L-5-MTHF concentrations of around 246 nmol/L in infant formula are higher than L-5-MTHF levels in breast milk reported by Page et al., 2017 and Büttner et al., 2014, but are comparable to total folate concentrations in breast milk (IOM, 1998; Page et al., 2017; Brown et al., 1986). The safety of L-5-MTHF-Ca at a concentration of 10.4 µg/100mL (226 nmol/L) as the sole folate source in infant formula has been confirmed (Troesh et al., 2019).

Inclusion of L-5-MTHF-Ca in infant formula has the advantage that it provides L-5-MTHF, the predominant natural form of folate in breast milk. Folic acid, in contrast, is not naturally present in breast milk but has been detected in breast milk samples as a result of maternal dietary folic acid supplementation (Page et al., 2017). Ingested unmetabolized folic acid may enter plasma via a diffusion-like process and could enter cells where it is converted to dihydrofolate or tetrahydrofolate and made into a polyglutamate, a process that is not subject to the usual control of cellular uptake to which circulating L-5-MTHF is subjected (Scott, 2001). Although the health consequences of unmetabolized folic acid for the mother and child are not yet clear, L-5-MTHF-Ca offers a choice that does not result in the circulation of unmetabolized folic acid.

Brown CM, Smith AM, Picciano MF. Forms of human milk folacin and variation patterns. *J Pediatr Gastroenterol Nutr.* 1986;5(2):278-282.

Büttner, B. E., C. M. Witthöft, M. Domellöf, O. Hernell, and I. Öhlund, 2014, "Effect of type of heat treatment of breastmilk on folate content and pattern," *Breastfeeding medicine : the official journal of the Academy of Breastfeeding Medicine* 9, 86.

Houghton LA, Yang J, O'Connor DL. Unmetabolized folic acid and total folate concentrations in breast milk are unaffected by low-dose folate supplements. *Am J Clin Nutr.* 2009;89(1):216-220. doi:10.3945/ajcn.2008.26564

IOM, 1998, Dietary reference intakes for thiamin, riboflavin, niacin, vitamin B6, folate, vitamin B12, pantothenic acid, biotin, and choline (The National Academies Press, Washington, DC).

Page, R., Robichaud, A., Arbuckle, T.E., Fraser, W.D., and MacFarlane, A.J. (2017). Total folate and unmetabolized folic acid in the breast milk of a cross-section of Canadian women. *Am J Clin Nutr* 105, 1101-1109.

Scott, J., 2001, in *Nutraceuticals in health and disease prevention*, edited by K. Kramer, P.-P. Hoppe, and L. Packer (CRC Press), p.90.

Troesch, B., J. Demmelmair, M. Gimpfl, C. Hecht, G. Lakovic, R. Roehle, L. Sipka, B. Trisic, M. Vusurovic, R. Schoop, et al., 2019, "Suitability and safety of L-5-methyltetrahydrofolate as a folate source in infant formula: A randomized-controlled trial," *PLOS ONE* 14, e0216790, <https://doi.org/10.1371/journal.pone.0216790>.

Sincerely,

**William  
Turney**

Digitally signed by William Turney  
DN: cn=William Turney, o=US, o=DSM  
Nutritional Products LLC, ou=Head of  
Regulatory Affairs North America,  
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**ANNEX 1**  
**Infant Formula Stability**



# Memo

DSM Nutritional Products  
DNP R&D Analytics  
Wurmisweg 576  
CH-4303 Kaiseraugst  
Switzerland

April 9, 2018

From: Stéphane Etheve

To: Mike Weiser  
Jerome Ravot  
Barbara Troesch  
Kate Niederberger (Saqual)

## Storage stability of calcium L-methylfolate (Metafolin®) in powdered infant formula

### 1. Objective:

Batches of powdered infant formula containing calcium L-methylfolate (Metafolin®) were prepared for a feeding trial in infants. The batches were stored for the duration of the infant feeding trial period and were analysed for folate concentration. Batches of infant formula containing folic acid instead of calcium L-methylfolate were used as the control group for comparison in the infant feeding trial and storage stability of folate from both sources can be compared. In addition to the investigation into the stability of calcium L-methylfolate in powdered infant formula, the stability of folate in the prepared product was also examined.

Study sponsor:  
DSM Nutritional Products (DNP)  
Wurmisweg 576  
CH-4303 Kaiseraugst  
Switzerland

### 2. Test material:

Metafolin® manufactured by Merck & Cie. The following batches of Metafolin® were used in the study:

- LMCG040602 Manufactured 05.12.2013
- LMCG045501 Manufactured 03.03.2015
- LMCG046508 Manufactured 13.05.2015

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**3. Test material use level:**

The powdered infant formula was prepared to contain calcium L-methylfolate or folic acid at concentrations of 78 µg/100g expressed as folic acid. The tolerances were -30 and +80 %.

**4. Powdered infant formula:**

The standard HiPP 1 Bio Combiotic low protein infant formula with folic acid was used as the control formula. The test formula was a modified HiPP 1 Bio Combiotic low protein infant formula with calcium L-methylfolate as the folate source instead of folic acid.

**5. Analytical Method:**

AOAC 992.05 Folic acid (Pteroylglutamic acid) in infant formula.

**6. Study start date:**

The following three batches of powdered infant formula containing calcium L-methylfolate (S055) were prepared:

- Batch 47103156 on 15.09.2014
- Batch 47180843 on 17. 07.2015
- Batch 47268441 on 19.08.2016

For comparison, the following batches of powdered infant formula containing folic acid (S056) were prepared:

- Batch 47072735 on 24.06.2014
- Batch 47180679 on 17. 07.2015
- Batch 47268636 on 08.08.2016

**7. Storage conditions:**

After production, the infant formula powders were packed in foil pouches. In each case two pouches were packed in a box. From each batch of powdered infant formula 20 boxes of the beginning, middle and end of the production were stored at room temperature (5-25 °C) in the dark.

**8. Sampling during storage:**

During storage samples were taken for quantification of folate immediately after production then after 1, 2, 3, 4, 7, 10, 12, 15 and 18 months.

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## 9. Results of the storage stability trial:

Intervention formula S055 "infant formula containing Metafolin in exchange of folic acid"			time of analysis											
			after production /beginning of shelf life	after 1 month	after 2 months	after 3 months	after 4 months	after 7 months	after 10 months	after 11 months	after 12 months	after 15 months	after 17 months	after 18 months
batch														
47103156	beginning	folic acid [µg/100g]	85,0	87,4	104,0	101,0	88,9	114	102,0	-	107,0	87,6	-	104,0
	middle		78,3	101,0	102,0	115,0	91,1	110	90,7	-	116,0	88,8	-	104,0
	end		84,7	87,2	104,0	98,1	91,1	116	94,1	-	109,0	97,7	-	92,3
	mean value		<b>82,7</b>	<b>91,9</b>	<b>103,3</b>	<b>104,7</b>	<b>90,4</b>	<b>113,3</b>	<b>95,6</b>	-	<b>110,7</b>	<b>91,4</b>	-	<b>100,1</b>
47180843	beginning	folic acid [µg/100g]	114,0	107,0	106,0	98,0	93,0	93,2	98,0	94,8	82,9	81,9	85,8	88,0
	middle		90,2	116,0	100,0	101,0	98,7	92,2	96,2	95,0	86,8	82,9	85,0	86,8
	end		109	116,0	96,9	93,8	92,9	88,6	100,0	103,0	92,7	88,3	86,5	94,4
	mean value		<b>104,4</b>	<b>113,0</b>	<b>101,0</b>	<b>97,6</b>	<b>94,9</b>	<b>91,3</b>	<b>98,1</b>	<b>97,6</b>	<b>87,5</b>	<b>84,4</b>	<b>85,8</b>	<b>89,7</b>
47268441	beginning	folic acid [µg/100g]	103,0	103,0	97,0	97,7	109,0	99,7	105,0	-	111,0	113,0	-	107,0
	middle		97,5	97,6	100,0	98,3	116,0	99,7	104,0	-	131,0	102,0	-	109,0
	end		107,0	110,0	107,0	98,8	114,0	96,4	104,0	-	112,0	114,0	-	101,0
	mean value		<b>102,5</b>	<b>103,5</b>	<b>101,3</b>	<b>98,3</b>	<b>113,0</b>	<b>98,6</b>	<b>104,3</b>	-	<b>118,0</b>	<b>109,7</b>	-	<b>105,7</b>

Control formula S056 "infant formula containing folic acid"			time of analysis											
			after production /beginning of shelf life	after 1 month	after 2 months	after 3 months	after 4 months	after 7 months	after 10 months	after 11 months	after 12 months	after 15 months	after 17 months	after 18 months
batch														
47072735	beginning	folic acid [µg/100g]	131,0	130,0	151,0	103,0	129,0	126,0	114,0	-	123,0	131,0	-	107,0
	middle		128,0	133,0	153,0	113,0	116,0	131,0	117,0	-	130,0	120,0	-	125,0
	end		133,0	134,0	154,0	116,0	111,0	136,0	117,0	-	117,0	115,0	-	129,0
	mean value		<b>130,7</b>	<b>132,3</b>	<b>152,7</b>	<b>110,7</b>	<b>118,7</b>	<b>131,0</b>	<b>116,0</b>	-	<b>123,3</b>	<b>122,0</b>	-	<b>120,3</b>
47180679	beginning	folic acid [µg/100g]	115,0	92,6	118,0	109,0	107,0	101,0	104,0	113,0	102,0	105,0	96,4	113,0
	middle		109,0	101,0	116,0	118,0	105,0	104,0	106,0	109,0	100,0	98,7	101,0	109,0
	end		124,0	133,0	112,0	133,0	104,0	102,0	108,0	113,0	104,0	107,0	98,3	112,0
	mean value		<b>116,0</b>	<b>108,9</b>	<b>115,3</b>	<b>120,0</b>	<b>105,3</b>	<b>102,3</b>	<b>106,0</b>	<b>111,7</b>	<b>102,0</b>	<b>103,6</b>	<b>98,6</b>	<b>111,3</b>
47268636	beginning	folic acid [µg/100g]	103,0	93,7	101,0	105,0	120,0	113,0	103,0	-	124,0	102,0	-	105,0
	middle		104,0	103,0	102,0	110,0	112,0	114,0	102,0	-	114,0	116,0	-	103,0
	end		101,0	108,0	103,0	103,0	113,0	116,0	103,0	-	109,0	110,0	-	104,0
	mean value		<b>102,7</b>	<b>101,6</b>	<b>102,0</b>	<b>106,0</b>	<b>115,0</b>	<b>114,3</b>	<b>102,7</b>	-	<b>115,7</b>	<b>109,3</b>	-	<b>104,0</b>

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## Storage stability of calcium L-methylfolate (Metafolin®) in powdered infant formula

### 10. Stability of folate in prepared formula:

In addition to the investigation into the stability of calcium L-methylfolate in powdered infant formula, the stability of folate in the prepared product was also examined. The formula was a standard solution of 13.0 g formula powder plus 90 mL water and was prepared as follows:

1. Boil fresh drinking water and leave it to cool to approx. 40-50°C
2. Pour 2/3 of the prepared water into the feeding bottle.
3. Fill the measuring spoon loosely and level the powder with the back of a knife. Put the recommended amount of powder into the feeding bottle.
4. Close the bottle and shake vigorously.
5. Add the remaining water and shake again several times.
6. Let it cool down to drinking temperature (approximately 37°C). Check the temperature.

#### Stability of folate in prepared product:

Intervention formula S055 "infant formula containing Metafolin in exchange of folic acid"	results in prepared product			
batch				
47268441	beginning		114	113
	middle		112	104
	end	folic acid [µg/100g]	99,2	107
	mean value		<u>108,2</u>	

### 11. Conclusion:

Batches of powdered infant formula containing calcium L-methylfolate (Metafolin®) were prepared and stored for the duration of the infant feeding trial period. A stability of calcium L-methylfolate (Metafolin®) in powdered infant formula for at least 18 months has been demonstrated as well as in prepared formula.