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June 30, 2021

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

Subject: GRAS Notification for the Use of Arcofolin®, a Monosodium Salt of L-5-Methyltetrahydro-folic acid, as a Source of Folate

Dear Sir/Madam:

In accordance with 21 CFR part 170, subpart E, Merck & Cie, hereby provides a notice of a claim that the food ingredient described in the enclosed notification document is excluded from the premarket approval requirement of the Federal Food, Drug, and Cosmetic Act because the notifier has concluded such use to be generally recognized as safe (GRAS), based on scientific procedures.

Three paper copies of the notification are provided as required; we also have provided a copy of the notification on the enclosed CD-ROM. If you have any questions or require additional information, please do not hesitate to contact me at 202-772-4915, or ntran@exponent.com.

Sincerely,

Nga Tran, DrPH, MPH
Principal Scientist

GRAS Conclusion for the Use of Arcofolin®, a Monosodium Salt of L-5-Methyltetrahydro-folic acid as a Source of Folate

SUBMITTED BY:

Merck & Cie
Im Laternenacker 5
8200 Schaffhausen
Switzerland

SUBMITTED TO:

U.S. Food and Drug Administration
Center for Food Safety and Applied Nutrition
Office of Food Additive Safety
HFS-200
5100 Paint Branch Parkway
College Park, MD 20740-3835

CONTACT FOR TECHNICAL OR OTHER INFORMATION:

Nga Tran
Principal Scientist
Exponent, Inc.
1150 Connecticut Avenue, NW, Suite 1100
Washington, DC 20036

June 29, 2021

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List of Acronyms

%	Percent, Percentage
°C	Degrees celsius
5,10-MTHF	5,10-Methylenetetrahydrofolate
5-FTHF	5-Formyltetrahydrofolate
5-MTHF	5-Methyltetrahydrofolate
[6S]-5-MTHF	Natural Diastereoisomer of 5-Methyltetrahydrofolate
ADI	Acceptable Daily Intake
AUC	Area Under the Curve
BMI	Body Mass Index
Bw	Body Weight
Ca	Calcium
CAS	Chemical Abstracts Service
CFR	Code of Federal Regulations
cGMP	Current Good Manufacturing Practices
C_{max}	Maximum Concentration
CRC	Colorectal Cancer
CSF	Cerebral Spinal Fluid
d	Day
DFE	Dietary Folate Equivalents
DHF	Dihydrofolate
DHFR	Dihydrofolate Reductase
DNA	Deoxyribonucleic Acid
DRI	Dietary Reference Intake
ECHA	European Chemicals Agency
EDI	Estimated daily intake
EFSA	European Food Safety Authority
EP	European Pharmacopeia
EPA	U.S. Environmental Protection Agency
EU	European Union
FAO	Food and Agricultural Organization of the United Nations
FCC	Food Chemical Codex
FDA	U.S. Food and Drug Administration
FOIA	Freedom of Information Act
FSANZ	Food Standards Australia New Zealand
g	Gram
GLP	Good Laboratory Practice
GRAS	Generally Recognized as Safe
h	Hour
HC1	Hydrogen chloride
HEM	Hydroxyethylmorpholine
HPLC	High-performance Liquid Chromatography
HR	Hazard ratio
IARC	International Agency for Research on Cancer
IBD	Inflammatory bowel disease
IOM	Institute of Medicine
IR	Infrared
JECFA	Joint FAO/WHO Expert Committee on Food Additives

Kg	Kilogram
L	Liter
LOAEL	Lowest-observed-adverse-effect level
m^2	Square meter
Mg	Milligram
mL	Milliliter
MOE	Margin of exposure
MTHFR	Methylenetetrahydrofolate reductase
NDIN	New Dietary Ingredient Notification
NHANES	National Health and Nutrition Examination Survey
NIH	National Institutes of Health
nmol	Nanomole
NOAEL	No-observed-adverse-effect level
NTP	National Toxicology Program
ODS	Office of Dietary Supplements
OECD	Organisation for Economic Co-operation and Development
PHA	Phytohemagglutinin
pmol	Picomole
RBC	Red blood cell
SAMe	S-adenosylmethionine
SOI	Standard of identity
THF	Tetrahydrofolate
UF	Uncertainty factor
UL	Tolerable upper intake level
U.S.	United States
USP	U.S. Pharmacopeia
Y	Year
Mg	Microgram

Part 1: Signed Statements and Certification

Merck & Cie submits to the U.S. Food and Drug Administration (FDA) this Generally Recognized as Safe (GRAS) notice in accordance with the 21 CFR part 170, subpart E.

Name and Address of Notifier

Merck & Cie
Im Laternenacker 5
8200 Schaffhausen
Switzerland

Name of GRAS Substance

The substance that is the subject of this GRAS notice is Arcofolin®, a Monosodium Salt of L-5-Methyltetrahydrofolic acid

Intended Use and Consumer Exposure

Arcofolin®, a monosodium salt of L-5-methyltetrahydrofolic acid (CASRN 2246974-96-7) is proposed for use as a source of folate. Arcofolin® is intended to be used as an alternative to folic acid for uses for which folic acid is an approved added nutrient under 21 CFR § 172.345:

> (d) Folic acid may be added, at levels not to exceed 400 micrograms ([micro]g) per serving, to breakfast cereals, . . . and to corn grits at a level such that each pound of corn grits contains not more than 1.0 milligram of folic acid.
>
> (e) Folic acid may be added to infant formula . . .
>
> (f) Folic acid may be added to a medical food . . . at levels not to exceed the amount necessary to meet the distinctive nutritional requirements of the disease or condition for which the food is formulated.
>
> (g) Folic acid may be added to food for special dietary use at levels not to exceed the amount necessary to meet the special dietary needs for which the food is formulated.
>
> (h) Folic acid may be added to foods represented as meal-replacement products, in amounts not to exceed:
>
> > (1) Four hundred [micro]g per serving if the food is a meal-replacement that is represented for use once per day; or
> >
> > (2) Two hundred [micro]g per serving if the food is a meal-replacement that is represented for use more than once per day.

Assuming Arcofolin® will substitute for all other sources of folic acid in foods (except for corn masa flour), the 95th percentile cumulative intake of folic acid is 919 μg/day among age group 51-70 years (FDA, 2016)

Basis for Conclusion of GRAS Status

Merck & Cie's conclusion of GRAS status for the intended use of Arcofolin®, a monosodium as a source of folate, substitutional to folic acid, is based on scientific procedures in accordance with 21 CFR §170.30(a) and (b).

Pre-Market Approval Exclusion Claim

Use of Arcofolin® is not subject to the pre-market approval requirements of the Federal Food, Drug, and Cosmetic Act because Merck & Cie has concluded that such use is GRAS through scientific procedures.

Availability of Information

The data and information that serve as the basis for this GRAS conclusion, as well as the information that has become available since the GRAS conclusion, will be sent to the FDA upon request, or are available for the FDA's review and copying during customary business hours at the office of Nga Tran at Exponent Inc., 1150 Connecticut Ave, NW, Suite 1100, Washington, DC 20036.

Exemptions From Disclosure

It is our view that none of the data and information in Parts 2 through 7 of the GRAS notice are exempt from disclosure under the Freedom of Information Act (FOIA).

Certification Statement

On behalf of Merck & Cie, I hereby certify that, to the best of my knowledge, this GRAS notice is a complete, representative, and balanced submission that includes unfavorable, as well as favorable information, known to me and pertinent to the evaluation of the safety and GRAS status of the use of the substance.

Name Miriam Wildt
Title: Strategic Marketing Manager
Company: Merck & Cie

Jun 30, 2021
Date

Martin Knüsel
Managing Director
Merck & Cie

Part 2. Identity, Method of Manufacture, Specifications, and Physical or Technical Effect

Identity

A Monosodium salt of L-5-Methyltetrahydrofolic acid

Chemical and Common Names

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

Arcofolin®

Monosodium L-Mefolinate

Monosodium L-Mefolate

Monosodium L-5-Methyltetrahydrofolate

L-Methylfolate

L-5-Methyltetrahydrofolic acid, Monosodium salt

L-5-MTHF-Na

(6S)-Methyltetrahydrofolic acid, Monosodium salt

(6S)-5-MethylTHF-Na

Chemical Abstracts Service (CAS) Registry Number

2246974-96-7

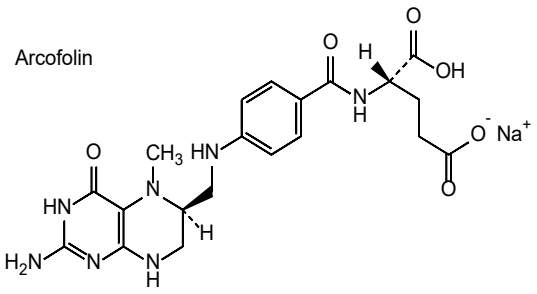
Molecular Weight and Empirical and Structural Formula

$C_{20}H_{24}N_7NaO_6$

Molecular weight: 481.44 g/mol

The structural formula of Arcofolin® is presented in **Figure 1** below.

Figure 1. Structural Formula of Arcofolin®



Chemical Formula: $C_{20}H_{24}N_7NaO_6$
Molecular Weight: 481.44

Method of Manufacture

Arcofolin® is manufactured from commercially available folic acid in accordance with current good manufacturing practices (cGMP) and preventive controls for foods. Standard reagents for both food grade and pharmaceutical grade manufacturing processes are used.

The synthesis route used to prepare Arcofolin® involves three steps (see **Figure 2**):

Step 1: Preparation of (6S)-tetrahydrofolic acid benzene-sulfonate (LTBH)

An aqueous solution of the starting material folic acid is hydrogenated in the presence of platinum (IV) oxide hydrate catalyst to give a mixture of (6S)- and (6R)-tetrahydrofolic acid. After removal of the catalyst an aqueous solution of benzenesulfonic acid is added and (6S)-tetrahydrofolic acid benzenesulfonate is crystallized. After reheating and subsequent cooling, LTBH is isolated.

Step 2: Preparation of (6S)-5-methyltetrahydrofolic acid (LMSR)

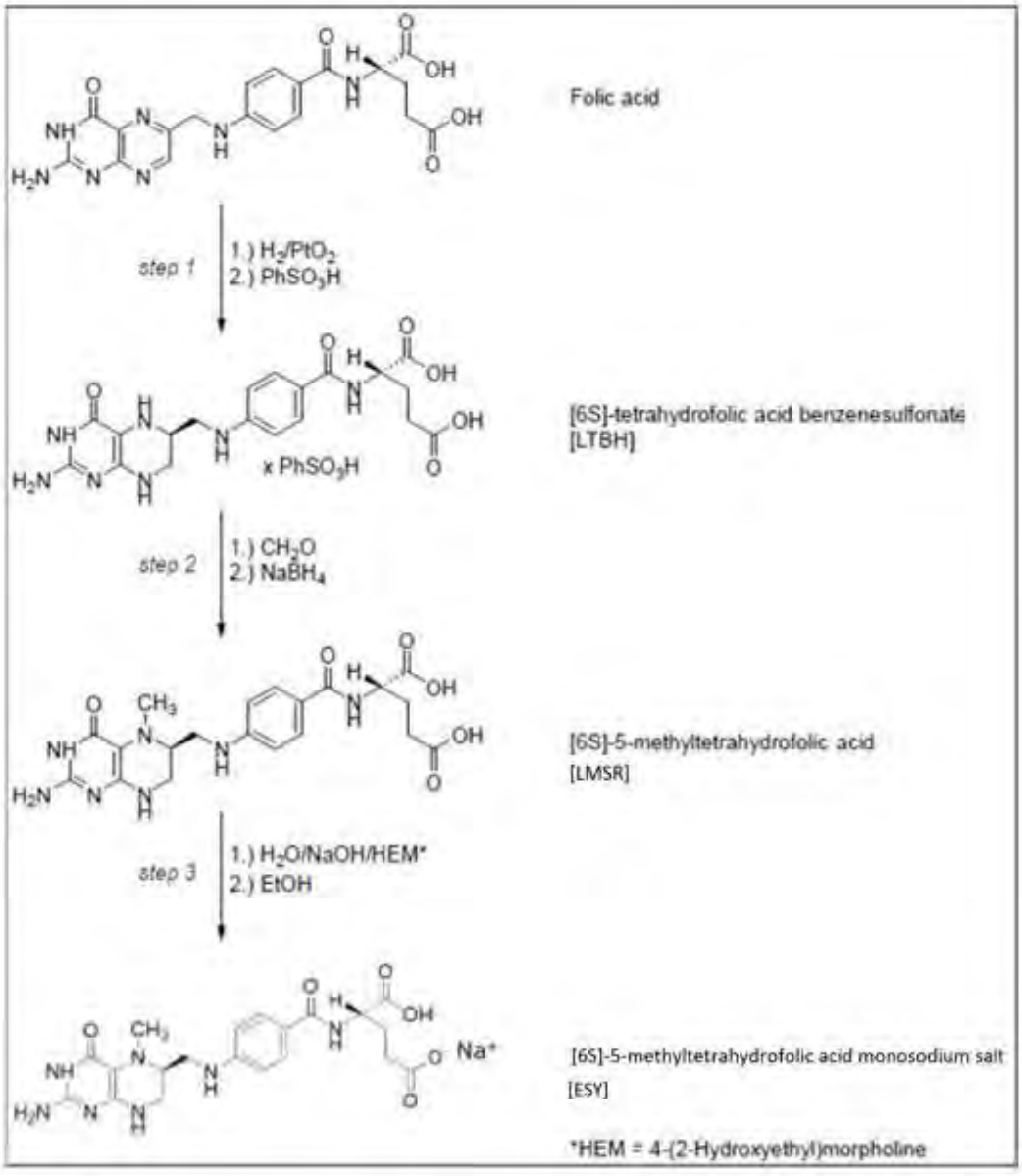
An aqueous solution of (6S)-tetrahydrofolic acid benzenesulfonate (LTBH) is treated with formaldehyde to give (6R)-5,10-methylenetetrahydrofolic acid which is subsequently reduced with sodium borohydride to give (6S)-5-methyltetrahydrofolic acid (LMSR). After destruction of the excess of sodium borohydride LMSR is crystallized and isolated in the heat.

Step 3: Preparation of L-Methylfolate, Sodium (ESY)

To a mixture of LMSR, water, ethanol and 4-(2-hydroxyethyl)morpholine) (HEM) an aqueous solution of sodium hydroxide is added. After filtration, the solution is heated to reflux and ethanol is added after seeding. The crystallized ESY is isolated.

The product is micronized or ground under nitrogen.

Figure 2. Synthesis Scheme for Arcofolin®



Materials Input:

The folic acid, hydrochloric acid, sodium hydroxide, sodium sulphite, and ethanol used to manufacture the finished product are either Generally Recognized as Safe (GRAS) ingredients / approved food additives/ meet Food Chemical Codex (FCC)/ United States Pharmacopeia (USP) standards/ or European Pharmacopeia (EP) standards. Benzenesulfonic acid, formaldehyde, α-monothioglycerol and sodium tetraborate decahydrate, used in Step 1 and Step 2 of the manufacturing process, are not present in the final product. Formaldehyde used in Step 2 also is not expected to be present in the final product. It is highly soluble in water with a boiling point of -19°C. In addition to its elimination through the multiple water-based washing steps,

formaldehyde is eliminated because the final product is dried at >40 °C, above the boiling point of formaldehyde.

Analysis of three commercial batches of Arcofolin® showed that Hydroxyethylmorpholine (HEM) , which is also used in the synthesis of the product, is below Merck's internal reporting threshold (< 0.05%). There are no published toxicology studies for HEM. However, based on the chemical structure and the morpholine moiety, a read-across to the safety database of morpholine (CAS 110-91-8) is reasonable. A search of databases (PubChem, European Food Safety Authority (EFSA), European Chemicals Agency (ECHA), U.S. Food and Drug Administration (FDA), Food Standards Australia New Zealand (FSANZ), Health Canada, the Joint Food and Agriculture Organization/World Health Organization (FAO/WHO), Expert Committee on Food Additives (JECFA), and Environmental Protection Agency (EPA)), found that Health Canada has established an acceptable daily intake (ADI) of 0.48 mg/kg bw/day for morpholine based on the no-observed-adverse-effect level (NOAEL) from a chronic oral toxicity study (no other information provided (HHA, Health Canada, modified 12/2002; accessed 04/2020)). Morpholine carcinogenic risks were evaluated by the International Agency for Research on Cancer (IARC) and it is not classifiable for its carcinogenicity to humans (IARC, 1989). Based on the 95th percentile cumulative intake of folic acid (i.e., age group 51 - 70 years, 95th estimated daily intake (EDI) of folic acid = 919 µg/day, see EDI section of the dossier), and assuming that Arcofolin® will substitute for all other sources of folic acid in foods (except for corn masa flour), the amount of HEM intake from the proposed use of Arcofolin® at the reporting limit of HEM of 0.05% and a default 60 kg bw is 0.008 µg/kg bw/day or 0.000008 mg/kg bw/day, orders of magnitude below the ADI established by Health Canada.

Importantly, all raw materials and processing aids, except HEM, are also used in the manufacture of Ca L-MTHF and their suitability and safety has been reviewed by FDA in the context of the New Dietary Ingredient Notification (NDIN) for Ca L-MTHF in 2001.

Product Specifications

Arcofolin® intended for use as an ingredient in foods meets specifications consistent with cGMP. The specifications and methods of analysis for Arcofolin® are listed in **Table 1**. Analytical data for three batch releases from a bulk production batch of Arcofolin® demonstrating compliance with specifications are provided in **Table 2**. Certificates of analysis for the batch data are located in **Appendix A**.

Table 1. Product Specification for Arcofolin®

Test	Specification	Method of Analysis	Compendial Method
Appearance	White to yellow or beige powder	Visual	
Identity / IR	Conforms to reference*	IR-Spectrometry	USP <197K>
Water	≤1.0%	Karl-Fisher Coulometer	USP <921> Method Ic
Residual Solvents			

Test	Specification	Method of Analysis	Compendial Method
Ethanol	NMT 0.5%	GC-Head space	USP <467>
Iso-propanol	NMT 0.5%		
IC (Cations)			
Identity retention time Sodium	conforms to reference	Ion chromatography	Validated Internal method
Assay Sodium	4.0 to 5.0 %		
Elemental Impurities**			
Boron	NMT 10 ppm	ICP-MS/OES	USP <233>
Platinum	NMT 10 ppm		
Arsenic	NMT 1.5 ppm		
Cadmium	NMT 0.5 ppm		
Lead	NMT 1.0 ppm		
Mercury	NMT 1.5 ppm		
Assay & Related Compounds			
Identity retention time Mefolinate	Conforms to reference	HPLC-UV	USP DS Ca-L-MeTHFA
Assay Mefolinate, acid as is	≥ 91.0 %		
4-Aminobenzoylglutamic acid (ABGA)	≤ 0.5 %		
Hydroxymethyl-THFA (HOMeTHFA)	≤ 1.0 %		
Mefox	≤ 1.0 %		
Tetrahydrofolic acid (THFA)	≤ 0.5 %		
7,8-Dihydrofolic acid (DHFA)	≤ 0.5 %		
Folic acid (FA)	≤ 0.5 %		
Methylenetetrahydrofolic acid (CH2THFA)	≤ 0.5 %		
Methyltetrahydropteroic acid (MeTHPA)	≤ 0.5 %		
Dimethyl-THFA (DiMeTHFA)	≤ 0.15 %		
Sum of all related compounds	≤ 2.5 %		
Diastereomeric Purity			
(6R)-Mefolinate	≤ 1.0 %area	HPLC-UV	USP DS Ca-MeTHFA
Microbiological Contaminants			
Total Aerobic Microbial Count	NMT 100 CFU/g	Enumeration	USP <61>
Total Combined Yeast/Molds Count	NMT 100 CFU/g		
Specified Microorganisms (SMO)			
Escherichia coli (in-house)	absent in 1 g	Enumeration	USP <62>

* IR reference standard was established after the COAs were issued. Originally, IR results were recorded for information. This is expressed on the COA as “OK”.

** The specification limits for metal impurities, including boron and platinum are identical to those of the related product Metafolin®

Table 2. Analytical Results of Non-Consecutive Batches of Arcofolin®

Batch	**ESY0008-XX**	**ESY0009-XX**	**ESY0010-XX**	**Specification**
Inspection Lot	**890000066482**	**890000066484**	**890000066483**	
Appearance				
Appearance color	beige	beige	beige	white to yellow or beige
Appearance texture	powder	powder	powder	Powder
Identity (IR-Spectrum)				
Identity / IR	ok	ok	ok	ok**
Water content (KF, Coulometric)				
Water content, %w/w	0.2	0.2	0.2	≤ 1.0 %*
IC (Cations)				
Identity retention time sodium	conforms to reference	conforms to reference	conforms to reference	conforms to reference*
Assay Sodium (IC), %w/w	4.5	4.7	4.5	4.0 to 5.0 %*
Residual Solvent (GC)				
Assay Ethanol, %w/w	0.06	0.07	0.06	≤ 0.5 %
Assay Isopropanol, %w/w	0.00	0.00	0.00	≤ 0.5 %
Specified Elemental Impurities				
Boron (ICP-OES), ppm	< LOQ (5)	< LOQ (5)	< LOQ (5)	≤ 10 ppm
Platinum (ICP-MS), ppm	< LOQ (5)	< LOQ (5)	< LOQ (5)	≤ 10 ppm
Arsenic (ICP-MS), ppm	< LOQ (1.5)	< LOQ (1.5)	< LOQ (1.5)	≤ 1.5 ppm
Cadmium (ICP-MS), ppm	< LOQ (0.5)	< LOQ (0.5)	< LOQ (0.5)	≤ 0.5 ppm
Lead (ICP-MS), ppm	< LOQ (1.0)	< LOQ (1.0)	< LOQ (1.0)	≤ 1.0 ppm
Mercury (ICP-MS), ppm	< LOQ (1.5)	< LOQ (1.5)	< LOQ (1.5)	≤ 1.5 ppm
Assay & Related Compounds (HPLC)				
Identity retention time HPLC	conforms to reference	conforms to reference	conforms to reference	conforms to reference
Assay Mefolinate, acid as is, %	95.2	95.4	94.3	≥ 91.0 %*
4-Aminobenzoylglutamic acid (ABGA), %	0.06	0.09	0.06	≤ 0.5 %
Hydroxymethyl-THFA (HOMeTHFA), %	0.11	0.29	0.11	≤ 1.0 %
Mefox, %	0.02	0.02	0.02	≤ 1.0 %
Tetrahydrofolic acid (THFA), %	0.07	0.07	0.10	≤ 0.5 %
7,8-Dihydrofolic acid (DHFA), %	0.01	0.01	0.01	≤ 0.5 %
Folic acid (FA) , %	< LOQ (0.01)	< LOQ (0.01)	< LOQ (0.01)	≤ 0.5 %
Methylenetetrahydrofolic acid (CH2THFA), %	0.02	0.02	0.02	≤ 0.5 %
Methyltetrahydropteroic acid (MeTHPA), %	0.11	0.12	0.13	≤ 0.5 %
Dimethyl-THFA (DiMeTHFA), %	0.05	0.05	0.06	≤ 0.15 %
Sum of all related compounds, %	0.62	0.85	0.65	≤ 2.5 %
Diasteriomeric Purity (HPLC) (6R)-Mefolinate	0.3	0.3	0.3	≤ 1.0 % area

Batch	ESY0008-XX	ESY0009-XX	ESY0010-XX	Specification
Inspection Lot	**890000066482**	**890000066484**	**890000066483**	
Microbial Enumeration Tests				
Microbial Count (TAMC), CFU/g	< LOQ (10)	< LOQ (10)	< LOQ (10)	≤ 100 CFU/g
Microbial Count (TYMC),CFU/g	< LOQ (10)	< LOQ (10)	< LOQ (10)	≤ 100 CFU/g
Specified Microorganisms (SMO)				
Escherichia coli [in.house test]	absent in 1 g	absent in 1 g	absent in 1 g	absent in 1 g

*Arcofolin® specific acceptance criteria
**at time of CoA issuing "report result". IR Reference is now being established and specification updated to "conform to reference"

Arcofolin® is Similar to Metafolin®

Arcofolin® and Metafolin® are related compounds. Both are salts of L-5-MTHF (Arcofolin®: L-5-MTHF-Na and Metafolin®: L-5-MTHF-Ca), produced in similar fashion, and have similar specifications.

The related product Metafolin® (L-5-MTHF-Ca) is similarly synthesized in three synthesis steps, with Steps 1 and 2 being identical to those of Arcofolin®. Step 3 synthesis of both salt forms starts with LMSR and follows similar processing steps with some differences, including the additive HEM being specific to Arcofolin® in the processing step, the pH-adjustment/ neutralization and carbon treatment steps being specific to Metafolin®, and the salt formation step being different as different salt form is being made. The comparison of Step 3 synthesis between Arcofolin® and Metafolin® is outlined in **Table 3**. The HEM component is unique to Arcofolin®, while HCl, activated carbon and the $CaCl_2$ solution are unique to Metafolin®.

Table 3. Step 3 Synthesis of Arcofolin® compared to Metafolin®

Arcofolin®		Metafolin®	
Processing Steps	**Material input, additives, processing Aids**	**Processing Step**	**Material input, additives, processing Aids**
Dissolving	LMSR	Dissolving	LMSR
	Water, ethanol		Water
	NaOH		NaOH
	HEM		
		pH-adjustment/ neutralization	HCl
		Carbon treatment	Activated Carbon
Filtration	Water	Filtration/washing	Water
Salt formation	Ethanol	Salt formation	$CaCl_2$ solution
Seeding crystal		Seeding crystal	
Crystallization	Ethanol	Crystallization	
Cooling		Cooling	
Centrifugation		Centrifugation	
Washing	Water, ethanol	Washing	Water, ethanol
Drying		Drying	

Given the similar manufacturing process for Arcofolin® and the related product Metafolin®, the specifications and methods of analysis for Arcofolin® and Metafolin® are similar (see **Table 4**), except for parameters specific to Arcofolin®, including identity/IR, cations, and assay "mefolinate, acid as is." It should be further noted that Metafolin® has an established USP-NF monograph, a copy is included in **Appendix B**.

Table 4. Arcofolin® and Metafolin® Similar Specifications

Test	**Arcofolin® Specification**	**Same as Metafolin®**	**Method of Analysis**
Appearance	White to yellow or beige powder	Yes	Visual
Identity / IR	conforms to reference*	Specific to Arcofolin®	IR-Spectrometry USP <197K>
Water	≤1.0%	No, Specific to Arcofolin®	K.F. Coulometer USP <921> Method Ic
Residual solvents			
Ethanol	NMT 0.5%	Yes	GC-Head space USP <467>
Iso-propanol	NMT 0.5%		
IC (Cations)			
Identity retention time Sodium	conforms to reference	No, Specific to Arcofolin®	Ion chromatography Validated internal method
Assay Sodium	4.0 to 5.0 %		
Elemental impurities			
Boron	NMT 10 ppm	Yes	ICP-MS/OES USP <233>
Platinum	NMT 10 ppm		
Arsenic	NMT 1.5 ppm		
Cadmium	NMT 0.5 ppm		
Lead	NMT 1.0 ppm		
Mercury	NMT 1.5 ppm		
Assay & Related Compounds			
Identity retention time Mefolinate	conforms to reference	Yes	HPLC-UV USP DS Ca-L-MeTHFA
Assay Mefolinate, acid as is	≥ 91.0 %	No, Specific to Arcofolin®	
4-Aminobenzoylglutamic acid (ABGA)	≤ 0.5 %	Yes	
Hydroxymethyl-THFA (HOMeTHFA)	≤ 1.0 %		
Mefox	≤ 1.0 %		
Tetrahydrofolic acid (THFA)	≤ 0.5 %		
7,8-Dihydrofolic acid (DHFA)	≤ 0.5 %		
Folic acid (FA)	≤ 0.5 %		
Methylenetetrahydrofolic acid (CH2THFA)	≤ 0.5 %		

Test	Arcofolin® Specification	Same as Metafolin®	Method of Analysis
Methyltetrahydropteroic acid (MeTHPA)	≤ 0.5 %		
Dimethyl-THFA (DiMeTHFA)	≤ 0.15 %		
Sum of all related compounds	≤ 2.5 %		
Diastereomeric Purity			
(6R)-Mefolinate	≤ 1.0 %area	Yes	HPLC-UV USP DS Ca-MeTHFA
Microbiological Contaminants			
Total Aerobic Microbial Count	NMT 100 CFU/g	Yes	USP <61>
Total Combined Yeast/Molds Count	NMT 100 CFU/g		
Specified Microorganisms (SMO)			
Escherichia coli (in-house)	absent in 1 g	Yes	USP <62>

***** IR reference standard was established after the COAs were issued. Originally, IR results were recorded for information. This is expressed on the COA as "OK".

Stability

Arcofolin®

Arcofolin® powder is stable in long-term storage for up to 36 months at 5 ºC and up to 25 ºC. The complete stability study report on Arcofolin® (packaged as a commercial supply) is located in **Appendix C**. Arcofolin® is as stable as the related product Metafolin®.

Metafolin®

Stability data of Metafolin® have been extensively reviewed by various authorities. According to the EFSA report on infant nutrition (EFSA, 2020) for L-5-MTHF, the stability of L-5-MTHF-Ca was tested in 14 batches including storage under refrigeration and at room temperature (25°C, 60% relative humidity) for up to 24 months and the product was determined to be stable based on compliance with specification for all tested parameters including water content, diastereomeric purity, and appearance.[1] Additionally, the concentration of folate provided as L-5-MTHF-Ca was reported to be stable in powdered infant formula and follow-on formula over an 18-month test period and stable in prepared liquid infant formula including no loss during preparation. FSANZ (2008) reported that L-5-MTHF demonstrated stability comparable to that of folic acid during heat treatment and concluded that the available information indicated that L-5-MTHF is stable in various processed foods.

[1]Stability data for up to 36 months are now available for Metafolin® since May 2020.

Part 3. Dietary Exposure

Proposed Use and Level

Arcofolin®, a monosodium salt of L-5-methyltetrahydrofolic acid, is proposed to be used as an alternative source of folate to folic acid in the following food categories for which folic acid is an approved added nutrient under 21 C.F.R. § 172.345:

> (d) Folic acid may be added, at levels not to exceed 400 micrograms ([micro]g) per serving, to breakfast cereals, . . . and to corn grits at a level such that each pound of corn grits contains not more than 1.0 milligram of folic acid.
>
> (e) Folic acid may be added to infant formula . . .
>
> (f) Folic acid may be added to a medical food . . . at levels not to exceed the amount necessary to meet the distinctive nutritional requirements of the disease or condition for which the food is formulated.
>
> (g) Folic acid may be added to food for special dietary use at levels not to exceed the amount necessary to meet the special dietary needs for which the food is formulated.
>
> (h) Folic acid may be added to foods represented as meal-replacement products, in amounts not to exceed:
>
> > (1) Four hundred [micro]g per serving if the food is a meal-replacement that is represented for use once per day; or
> >
> > (2) Two hundred [micro]g per serving if the food is a meal-replacement that is represented for use more than once per day.

Folic acid also may be added to foods defined by a standard of identity (SOI) that permits the addition of folic acid (e.g., enriched flour) but the proposed uses of Arcofolin® do not include use in foods defined by a SOI. The use of Arcofolin® is substitutional for select approved uses of folic acid, thus, is not expected to impact the dietary exposure to folate in the U.S. population.

The technical effect of adding Arcofolin® to foods is to provide a source of folate. Use of Arcofolin® in place of folic acid provides an alternate source of folate that may be particularly relevant for individuals with a polymorphism impacting folic acid metabolism and individuals using drugs that interact with folate metabolism. Use of Arcofolin® may also avoid the risk of masking vitamin B_{12} deficiency which is a potential result of use of folic acid.

Because genetic and other medical conditions may interfere with the body's ability to use food folate or folic acid, L-5-MTHF can serve to meet folate needs for populations that cannot efficiently metabolize typical folate sources. For example, a deficiency in or altered

methylenetetrahydrofolate reductase (MTHFR) activity leads to reduced conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate. Importantly, 5-methyltetrahydrofolate is essential for converting homocysteine to methionine (**Figure 4**), which is further used by the body for protein synthesis, DNA methylation reactions, and one-carbon metabolism, among many other physiological processes (Ducker and Rabinowitz, 2017).

Use of L-5-MTHF may have several benefits. First, in contrast to folic acid, L-5-MTHF does not require the MTHFR enzyme. Also, use of L-5-MTHF in place of folate or folic acid may reduce potential interactions with drugs that inhibit dihydrofolate reductase (DHFR) activity. Therefore, for individuals who are homozygous carriers of the 677T allele of the MTHFR gene and individuals on drugs targeted to DHFR as well as those with normal MTHFR function, L-5-MTHF can serve in meeting folate needs.

Estimated Daily Intake from Uses in Foods

Dietary intake of folate reflects intake of folate present naturally in food, folic acid added to enriched breads, cereals, flours, cornmeals, pastas, rice, corn masa flour, and other grain products; folic acid added to foods such as breakfast cereals as specified in 21 C.F.R. § 172.345; and use of dietary supplements. The proposed use of Arcofolin® is substitutional, thus, dietary exposure to total folate in the U.S. population is not expected to be impacted.

Based on nationally representative data from the National Health and Nutrition Examination Survey (NHANES) for the U.S. population in 2015 - 2016, the mean daily intake of folate from food and supplement sources was 696 μg DFE, and the intake of folic acid was 289 μg (USDA, 2019).

In 2016, as part of the safety evaluation for the petitioned use of folic acid in corn masa flour, the FDA developed estimates of cumulative folic acid intake from dietary sources (including foods and dietary supplements) and the intended use in corn masa flour (FDA, 2016b). Based on data from NHANES 2003-2008, FDA estimated median daily intake of 231 μg folic acid by the population ages 1 year and older. FDA estimated folic acid intake reflecting uses of folic acid at the time plus the maximum petitioned use of folic acid in corn masa flour to be 244 μg /day at the median level of intake and 775 μg/day at the 95th percentile of intake (**Table 5**). These modeled intake estimates assumed that all corn masa flour in the U.S. would be fortified with folic acid.

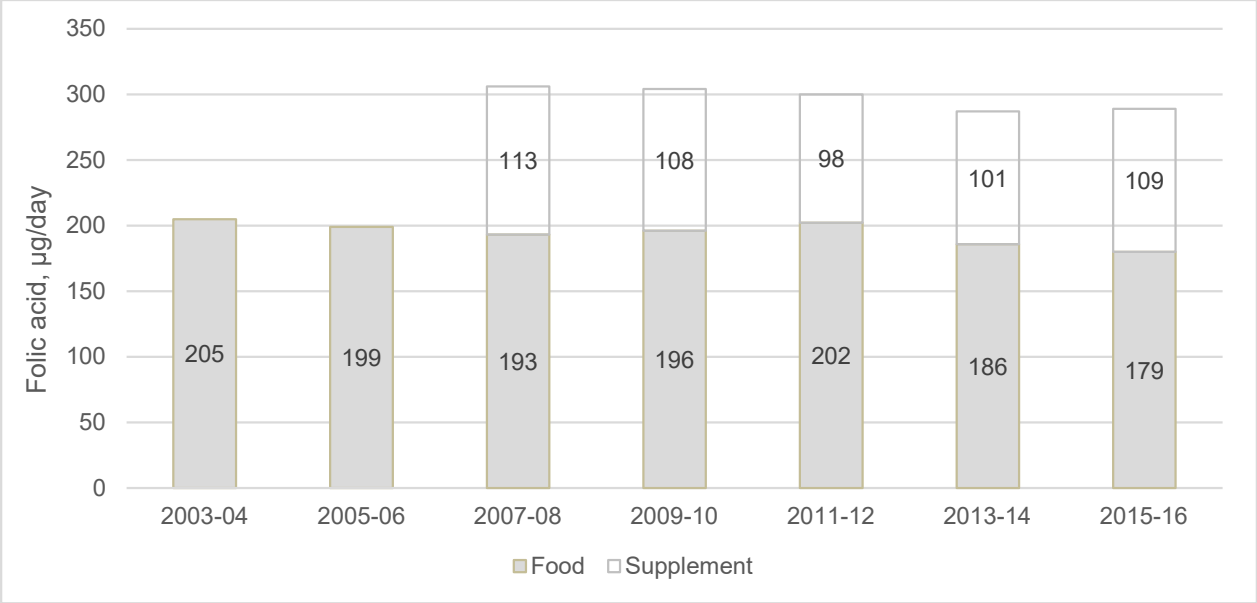
Table 5. Estimated Cumulative Intake of Folic Acid for the U.S. Population, NHANES 2003-2008 including Modeled use in Corn Masa Flour

Age, years	N	Median Intake µg/day		95th Percentile Intake µg/day	
		NHANES 2003-2008	Including modeled use in corn masa flour	NHANES 2003-2008	Including modeled use in corn masa flour
All, 1+	22,717	231	244	765	775
1-3 y	1911	156	160	493	504
4-8	2071	255	267	618	633
9-13	2608	240	257	622	328
14-18	3038	239	252	646	658
19-30	2608	229	247	744	758
31-50	4118	219	237	769	783
51-70	3861	266	271	919	927
71+	2302	255	258	836	840

Based on the highest 95th percentile intake of folic acid (i.e., age group 51 - 70 years, 95th EDI of folic acid = 919 µg/day, NHANES 2003 - 2008), and assuming that Arcofolin® will substitute for all other sources of folic acid in foods (except for corn masa flour), the amount of sodium intake from the proposed use of Arcofolin® is minimal (~0.05 mg/day, sodium: Arcofolin® molecular weight-basis).

Current estimates of folic acid intake from food and supplement sources by the U.S. population ages 2 years and older suggest that mean folic acid intake has not increased since 2003 - 2008, the time period considered in FDA's assessment for corn masa flour (**Figure 3**).

Figure 3. Estimated Cumulative Intake of Folic Acid for the U.S. Population, NHANES 2003 - 16, Day 1 Intake, Population 2 Year and Older



Source: What We Eat in America, NHANES 2007-2008, 2009-2010, 2011-2012, 2013-2014, 2015-2016, day 1 food and suppelement intake data, 2 years and over, and What We Eat in America, NHANES 2003-2004, 2005-2006, day 1 food intake data, 2 years and over. www.ars.usda.gov/nea/bhnrc/fsrg. Note: USDA did not report intake of nutrients from supplements prior to 2007-08.

Part 4. Self-Limiting Levels of Use

Arcofolin® is intended for use as a source of folate, substitutional to folic acid at levels not exceeding 400 μg per serving in select food categories for which folic acid has been approved, namely breakfast cereals, corn grits, infant formula, medical foods, food for special dietary use, and foods represented to be meal-replacement products, as specified in 21 C.F.R. § 172.345. We are not aware of technological or palatable issues associated with the proposed use levels. Self-limiting levels of use are not applicable to this notice.

Part 5. Experience Based on Common Use in Food before 1958

Arcofolin® is intended for use as a source of folate, substitutional to folic acid based on scientific procedures in accord with 21 CFR §170.30(a) and (b). Experience based on common use in food before 1958 is not applicable to this notice.

Part 6. Narrative

Introduction

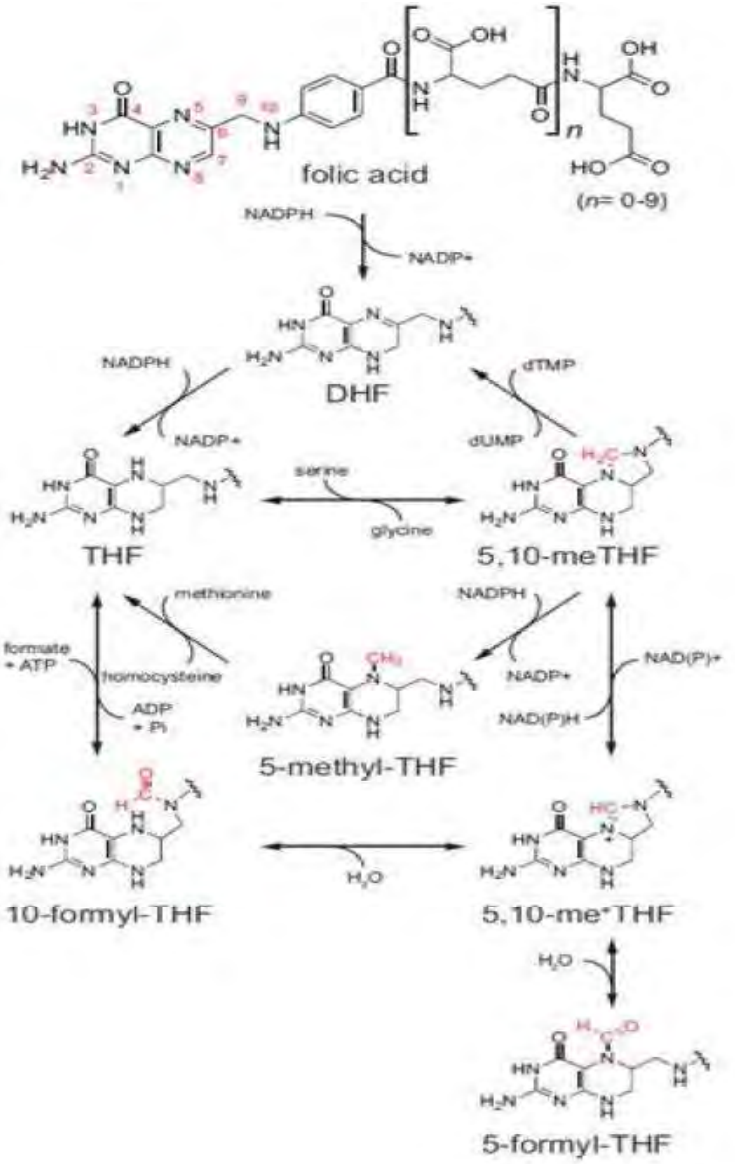
Folate exists in various forms (**Figure 4**) in the diet and in supplements. Food folate is intrinsic in foods, while folic acid is a synthetic food fortificant or supplement. L-5-MTHF is naturally found in food and also available commercially as a crystalline form of the calcium salt (Metafolin®). L-5-FTHF is also naturally found in food and available commercially as a calcium L-5-formyltetrahydrofolate. These various forms of folate share a common metabolic fate, conversion to L-5-MTHF. **Figure 5** shows a general scheme of the main reactions of folic acid conversion to L- 5-MTHF in humans. Once absorbed, circulating folates are indistinguishable.

As earlier described, Arcofolin® and Metafolin® are similar compounds. Both are salts of L-5-MTHF (Arcofolin® is L-5-MTHF-Na and Metafolin® is L-5-MTHF-Ca), produced in similar fashion, and have similar specifications. These salts are expected to completely dissociate *in vivo* and have comparable bioavailability. The comparable bioavailability of Arcofolin® and Metafolin® is supported by findings in published literature, as summarized herein. As such, publicly available safety data on Metafolin® can be relied upon to evaluate the safety of the related Arcofolin®.

Absorption, Distribution and Metabolism

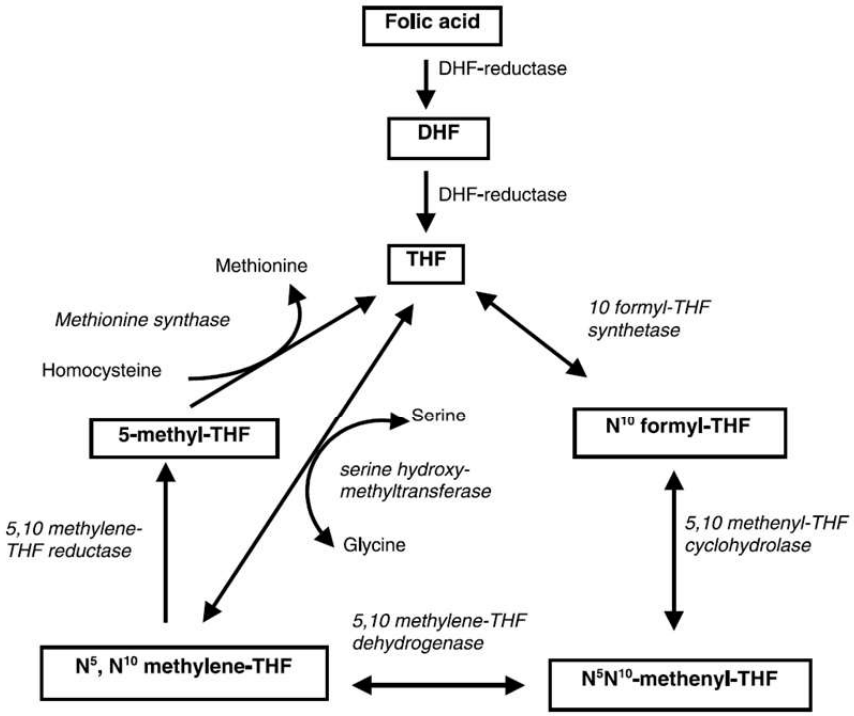
Folate, a water-soluble B vitamin, is the generic term for naturally occurring food folate and folic acid, which is a synthetic form of the vitamin that is only found in fortified foods, dietary supplements, and pharmaceuticals. Food folate consists of both monoglutamate and polyglutamate folate species, approximately 70% of which occurs as L-5-MTHF. Because folate must be in the monoglutamate form to be absorbed, dietary folate in the polyglutamate form must be deconjugated prior to absorption by intestinal mucosal cells (Melse-Boonstra et al., 2002). Dietary folate in the monoglutamate form, as well as folic acid, which exists only in the monoglutamate form, can be directly absorbed.

Figure 4. Chemical Transformations of Folates



DHF; dihydrofolate; THF, tetrahydrofolate; 5,10-meTHF, 5,10-methylene-THF; 5,10-me+THF, 5,10- methenyl-THF. Adapted from "One-Carbon Metabolism in Health and Disease," by G. S. Ducker and J. D. Rabinowitz, 2017, *Cell Metabolism*, *25,* p. 28.

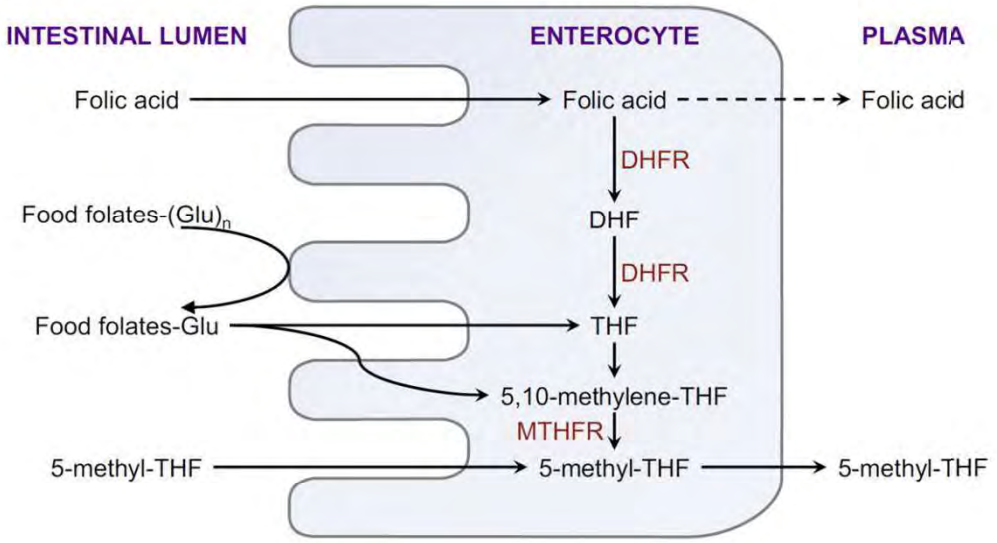
Figure 5. Main Reactions in Folic Acid Conversion to L-5-MTHF



DHF, dihydrofolic acid, THF, tetrahydrofolic acid. Adapted from "Effect of low doses of 5-methyltetrahydrofolate and folic acid on plasma homocysteine in healthy subjects with or without the 677C→T polymorphism of methylenetetrahydrofolate reductase," by P. Litynski et al., 2002, *European Journal of Clinical Investigation, 32,* p. 663.

Once absorbed, most dietary folate and folic acid added to the diet share a common metabolic fate, conversion to L-5-MTHF and other reduced forms of folate. After absorption from the brush border membrane into intestinal mucosal cells, the different chemical forms of folate must be converted to L-5-MTHF. The enzymatic conversion of folic acid in the small intestine to L-5-MTHF occurs in a multi-step process (**Figure 6**). Folic acid is first reduced to DHF and then to THF by DHFR. THF is the metabolically active form of folate within the cell. L-5,10-methylenetetrahydrofolate (5,10-MTHF) is subsequently formed from THF via acquisition of a carbon unit, and then reduced to L-5-MTHF, the active form of folic acid, by MTHFR. D-5-MTHF, the unnatural D-isomer of 5-MTHF appears in most cases to be metabolically inert. Folate from food is also converted to L-5-MTHF through the acquisition of a methyl group or groups. L-5-MTHF is subsequently absorbed through the mucosal cell into the peripheral circulation. L-5-MTHF is the predominant form of dietary folate and the principal form of circulating folate in the body, accounting for approximately 98% of folate in human plasma; all dietary and supplemental folate including folic acid and L-5-FTHF are converted to L-5-MTHF prior to transport into the peripheral circulation (Pietrzik et al., 2010). At intakes above 200 µg/day, folic acid can also appear, in small amounts, unchanged in circulation (Kelly et al., 1997).

Figure 6. Intestinal Absorption of Dietary Folates, Folic Acid and L-5-MTHF



Low doses of dietary folates and folic acid are converted in the small intestine to 5-methyl-THF. Folic acid at high oral doses enters the circulation through passive diffusion in unmodified form (dotted line). DHF: dihydrofolate; DHFR: dihydrofolate reductase; Glu: glutamate; (Glu)n: polyglutamate; MTHFR: 5, 10-methylene-THF reductase; THF: tetrahydrofolate. Adapted from "Safety and benefits of interventions to increase folate status in malaria-endemic areas," by H. Verhoef et al., 2017, *British Journal of Haematology,* p. 3 (originally adapted from *Clinical Pharmacokinetics, 49*, 2010, 535–548, Pietrzik, K., Bailey, L. & Shane, B. "Folic acid and L-5-methyltetrahydrofolate: comparison of clinical pharmacokinetics and pharmacodynamics." © 2010. With permission of Springer.)

Circulating folates are indistinguishable once consumed. L-5-MTHF, either from supplemental L-5-MTHF-Ca (Metafolin®) or formed in intestinal mucosal cells from dietary folate or folic acid added to the diet, circulates in the periphery as L-5-MTHF either in its free form or loosely bound to plasma proteins and is transported into most tissues via the reduced folate carrier (Pietrzik et al., 2010). This folate carrier has poor affinity for folic acid whereas a second distinct folate transporter, folate binding protein, has high affinity for both L-5-MTHF and folic acid (Zhao et al., 2009). Upon transport into cells, intracellular L-5-MTHF must be converted to its polyglutamate form; metabolism of folate to polyglutamates is required for cellular retention and subsequent biological activity.

Biologically active folate plays an essential role in one-carbon metabolism, facilitating the transfer of one-carbon units in reactions required for the synthesis of thymidine, which is incorporated into DNA, the synthesis of purines, which are building blocks for DNA and RNA, and the formation of methionine (Pietrzik et al., 2010). In these metabolic reactions, a single carbon unit from serine or glycine is transferred to L-THF to form L-5,10-MTHF (**Figure 4**). This is either used as such for the synthesis of thymidine (via conversion to DHF), oxidized to L-10-FTHF which is used for the synthesis of purines, or reduced to L-5-MTHF which is used to methylate homocysteine to form methionine (**Figure 4**), a reaction that is catalyzed by the B_{12}-dependent methionine synthase. Methionine is subsequently converted to S-adenosylmethionine (SAMe), which acts as the principal methyl donor in many reactions including the methylation of nucleic acids, proteins, lipids, and neurotransmitters.

Polymorphisms in the MTHFR Gene

MTHFR enzymatic activity may be altered due to common polymorphisms in folate genes (MTHFR). MTHFR is a key regulatory enzyme in folate and homocysteine metabolism. MTHFR catalyzes the irreversible conversion of L-5-MTHF, the methyl group donor for homocysteine, from 5,10-MTHF (**Figure 4**). Because MTHFR catalyzes the formation of L-5-MTHF, the principal form of circulating folate, a variant (677C → T) in the MTHFR gene has been demonstrated to significantly lower plasma folate concentrations in individuals with this polymorphism (de Bree et al., 2003; Deloughery et al., 1996; Harmon et al., 1996; Schwartz et al., 1997; van der Put et al., 1995; Yakub et al., 2012; Zittoun et al., 1998). Because MTHFR regulates the re-methylation of homocysteine to methionine, the 677 variant has also been shown to cause a mild elevation in plasma homocysteine concentration and is associated with decreased plasma folate concentration. Specifically, homozygous carriers of the 677T allele have reduced MTHFR activity, leading to reduced L-5-MTHF synthesis and elevated homocysteine concentrations, a known risk factor of vascular disease (ODS, 2020). Orally administered L-5-MTHF and its subsequent metabolism through the homocysteine/ methionine pathway is not as affected by the MTHFR polymorphism.

Drug Interactions

DHFR catalyzes the conversion of DHF, produced in thymidine synthesis, to THF. Several drugs, such as methotrexate, aminopterin, pyrimethamine, trimethoprim, and triamterene, inhibit DHFR activity and thereby interfere with folate metabolism and its utilization for DNA synthesis (Blakley, 1984). Orally administered L-5-MTHF is not as affected by DHFR inhibitors as is folic acid (**Figures 4** and **5**).

Masking Vitamin B_{12} Deficiency

Vitamin B_{12} deficiency, particularly in the elderly population, has been associated with neurological symptoms such as dementia, paresthesia, ataxia, spinal cord degeneration, and decline in cognitive function (Stabler et al., 1990; Smulders et al., 2005; Pietrzik et al., 2010). Using L-5-MTHF instead of folic acid reduces the potential for masking hematological symptoms and neurological pathologies of vitamin B_{12} deficiency until the consequences of neurological damage become irreversible (Ganeshagrur & Hoffbrand, 1978; Fava et al., 2009; ODS, 2020; Pietrzik et al., 2010).

Hematological sequela of vitamin B_{12} deficiency are attributed to disturbed DNA synthesis and explained by the methyltetrahydrofolate trap hypothesis (Herbert & Zalusky, 1962; Scott, 2001). In vitamin B_{12} deficiency, a block in the utilization of L-5-MTHF has been postulated to lead to a shortage of L-THF; L-5-MTHF cannot be metabolized via the B_{12}-dependent methionine synthase reaction, nor can it be reconverted to its precursor L-5,10-MTHF, the carbon donor necessary for DNA synthesis and RBC formation.

Supplementation with folic acid reverses the hematological symptoms of vitamin B_{12} deficiency by providing methyl donors necessary for DNA synthesis and RBC formation (THF, 5,10-MTHF). However, folic acid supplementation may also delay the timely diagnosis and treatment

of neurological dysfunction caused by vitamin B_{12} deficiency. In contrast, L-5-MTHF is not able to reverse the hematological symptoms of vitamin B_{12} deficiency due to the methyltetrahydrofolate trap hypothesis (Scott, 2001; Smulders et al, 2006). Under vitamin B_{12} deficient conditions, L-5-MTHF cannot be metabolized via the B_{12}-dependent methionine synthase pathway to methyl donors necessary for DNA synthesis. Adequate levels of vitamin B_{12} should be consumed along with L-5-MTHF.

Bioavailability of Chemical Forms of Folate

Folate bioavailability is defined as the fraction of folate that is absorbed and available for metabolic reactions and/or for storage (Caudill, 2010). The relative bioavailability of folates is commonly determined by comparing concentrations of plasma, serum, and/or red blood cell (RBC) folate and plasma homocysteine following repeated intake or changes in the incremental area-under-curve (AUC) levels of plasma folate after intake of single doses. Differences in the chemical forms of folate has implications for bioavailability. For example, it is well recognized that folic acid is more bioavailable than folate naturally occurring in food. In fact, these differences in bioavailability are accounted for in labeling the folate content of foods and dietary supplements and in evaluating adequacy of dietary intakes (FDA, 2016a; IOM, 1998). Since Arcofolin® is intended for use as a substitute for folic acid, it is important to understand the relative bioavailability of the salts of 5-MTHF chemical forms of folate.

A search of the literature identified studies comparing the bioavailability of folic acid and L-5-MTHF-Ca or L-5-MTHF of an unspecified form, which is presumably L-5-MTHF-Ca. Also, information comparing the bioavailability of folic acid and Arcofolin® and a comparative analysis of AUC plasma responses following ingestion of the L-5-MTHF salts (Arcofolin® and Metafolin®) relative to a folic acid control from separate studies are also located in the published literature. The bioavailability of L-5-MTHF-Ca and unspecified salts as reported in the published literature is reviewed below, followed by information specific to Arcofolin®.

Relative Bioavailability of L-5-MTHF Calcium Salt and Folic Acid

Bayes et al. (2019) conducted a systematic literature review of original research pertaining to the bioavailability of the different forms of folate in animal models and healthy adults. Folic acid and L-5-MTHF were the most commonly tested forms of folate. Bayes et al. (2019) identified two studies in animals and five studies in healthy adults that tested both folic acid and L-5-MTHF and reported outcome measures of plasma, serum, urinary, and/or RBC folate, and homocysteine. Bayes et al. (2019) evaluated studies published up to March 30, 2017; a PubMed search was conducted to capture any relevant original research published after this date. The search string is detailed in **Appendix D**.

Bioavailability in Animals

Perez-Conesa et al. (2009) evaluated the effect of L5-MTHF-Ca (Metafolin®)-fortified milk (reported as growing-up milk) and folic acid-fortified milk on folate status in folate-depleted weanling rats. Male Sprague-Dawley weanling rats (n=36) were divided into two groups and fed a folate-deficient diet (n=30) or a control diet supplemented with 1000 µg of folic acid (n=6).

Following a 28-day feeding period, six animals from each group were euthanized for baseline measurements. The remaining animals that received the folate-deficient diet were further divided into two separate groups (n=12/group) and received either 1000 µg /L of folic acid-fortified milk or 1041.91 µg/L of Metafolin®-fortified milk for 28 days. The primary outcome measures were plasma folate, RBC folate, and liver tissue folate. Rats fed with Metafolin® were reported to have significantly higher RBC and liver tissue folate levels than rats fed folic acid ($p<0.05$), though plasma folate did not differ between the groups.

In a pharmacokinetic study conducted by Miraglia et al. (2016), male Sprague-Dawley rats (54-56-d-old; n=6/group) were administered a single oral dose of 70 µg /kg bw of either folic acid, L-5-MTHF calcium salt, or L-5-MTHF glucosamine salt in capsule form. Plasma levels of L-5-MTHF were measured over eight hours along with pharmacokinetic parameters of C_{max}, T_{max}, and AUC_{8h}. The AUC_{8h} for L-5-MTHF glucosamine salt was 1123.9 ng/mL h, followed by 997.6 ng/mL h for L-5-MTHF calcium salt, and 114.7 ng/mL h for folic acid.

Bioavailability in Humans

Human bioavailability studies comparing folic acid to L-5-MTHF are summarized in **Table 6** and below. These studies were identified from Bayes et al. (2019), the PubMed literature search, the FSANZ (2008) assessment report on the calcium salt of L-5-MTHF, and the EFSA review of folate (EFSA 2014). The L-5-MTHF investigated in these studies was reported to be the calcium salt or an unspecified salt form.

In assessments of folate status following intake of a single dose of folic acid or L-5-MTHF, 48-h urinary folate was comparable between groups in a study of seven men (Gregory et al., 1992), as was the concentration of plasma folate concentration and plasma folate AUC in a study of 13 men (Pentieva et al., 2004). In a study of 24 women of child-bearing age, plasma folate AUC and C_{max} were higher after intake of L-5-MTHF compared to responses after intake of an equimolar dose of folic acid (Prinz-Langenohl et al., 2009).

In studies of folate status following repeat-intake of L-5-MTHF and folic acid, plasma and/or RBC folate concentrations were not different in several studies indicating comparable bioavailability (de Meer et al., 2005; Green et al., 2013; Litynski et al., 2002; Venn et al., 2002; Venn et al., 2003; Wright et al., 2010). However, some studies reported higher levels of RBC or blood folate following intake of L-5-MTHF compared with folic acid (Bailey and Ayling, 2018; Henderson et al., 2018; Houghton et al., 2006; Lamers et al., 2006). The amount of folates and the matrix in which folates were provided in these studies (e.g., in a food versus a supplement) varied across the studies, and observed differences in blood responses may relate to these factors.

Authoritative bodies in the U.S. and elsewhere also have reviewed data on the relative bioavailability of salts of L-5-MTHF (L-5-MTHF-Ca) and folic acid, and concluded that L-5-MTHF is equal or more bioavailable than folic acid. In the 2016 Final Rule on Food Labeling (FDA, 2016a), FDA acknowledged that the relative bioavailability of synthetic folate (e.g., a salt of 5-MTHF) and folic acid may differ and allowed manufacturers of synthetic folates to use their own established conversion factor for reporting levels of folate on supplement facts labels. However, until rulemaking on the issue is undertaken and completed, the highest permissible conversion factor a manufacturer may use is a factor of 1.7 to be comparable to folic acid. In a

2020 review of folate, the Office of Dietary Supplements (ODS) noted that the bioavailability of L-5-MTHF relative to folic acid is the same or greater based on the available clinical data (ODS, 2020).

EFSA (2004) previously concluded that the bioavailability of L-5-MTHF-Ca is at least as high as that of folic acid, and that the fate of L-5-MTHF-Ca is indistinguishable from that of other absorbed and metabolized natural folate forms. In a 2008 assessment, FSANZ similarly concluded that the available evidence suggests that L-5-MTHF and folic acid are "essentially bioequivalent." Overall, clinical data indicate that salts of L-5-MTHF including L-5-MTHF-Ca are at least as bioavailable as folic acid, while some data suggest that L-5-MTHF may be more bioavailable. Given the recent unequivocal clinical data and the statements from several authoritative bodies, it is reasonable to conclude that the bioavailability of L-5MTHF salts including L-5-MTHF-Ca is comparable to that of folic acid.

Relative Bioavailability of L-5-MTHF Calcium Salt and Arcofolin®

The bioavailability of Arcofolin® compared to the bioavailability of folic acid was examined (Obeid et al., 2020). As part of the same publication, the investigators compared results from the study of Arcofolin® to plasma L-5-MTHF and folate AUC responses to Arcofolin® and equimolar doses of Metafolin® as reported in a previously published study of Metafolin® compared to folic acid (Prinz-Langenohl et al., 2009); the 2020 published analysis therefore provides an approach to assess the relative bioavailability of Arcofolin® and Metafolin®.

In the study of Arcofolin® bioavailability compared to folic acid, healthy male and female subjects (n=12/sex; mean age 29.7±7.5 y) were enrolled in a randomized, double-blind, controlled cross-over study and received a single oral dose of 436 µg Arcofolin® or an equimolar amount of 400 µg folic acid in capsule form (Obeid et al., 2020). Inclusion criteria included healthy, nonsmoking men and women, aged 18 - 50 y, with a body mass index (BMI) between 18 and 30 kg/m^2, plasma folate in the range of 7 - 45 nmol/L, and RBC folate between 405 - 952 nmol/L. Exclusion criteria included anemia, vitamin B_{12} levels <148 pmol/L, homocysteine levels ≥15.0 µmol/L, pregnancy, breastfeeding, folic acid supplement intake in the three months prior to the study, and other supplement/drug intake (e.g., antacids, omeprazole, antifolate, vitamin C, biotin) three months prior to and during the study. The intervention consisted of two kinetic study days separated by a 14-day washout period. On each study day, plasma folate levels were examined for 8 h after subjects received a single dose of folic acid or Arcofolin®. The primary outcome measure was plasma concentrations of (6S)-5-MTHF (also referred to as L-5-MTHF in this document) that were further evaluated for differences in $AUC_{0\text{-}8\ h}$, C_{max}, and T_{max} between the substances. Secondary assessments were conducted for plasma concentrations of total folate and unmetabolized folic acid. Safety parameters of tolerability and adverse events were also monitored.

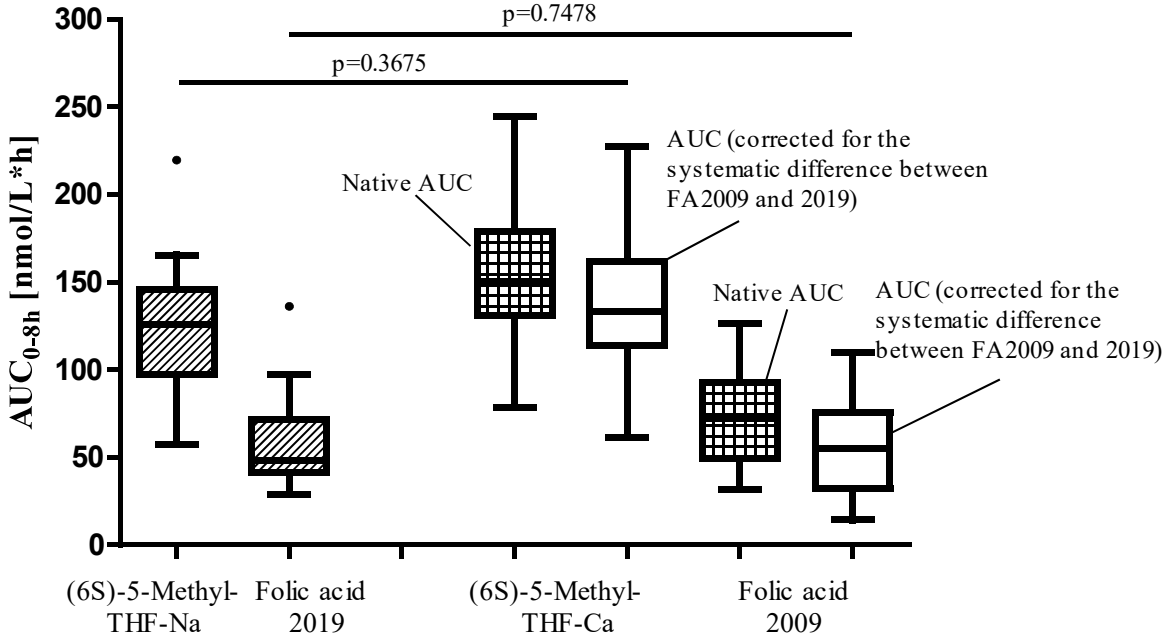
The $AUC_{0\text{-}8\ h}$ and C_{max} of (6S)-5-MTHF were significantly higher after Arcofolin® intake than after an equimolar amount of folic acid. Following a single oral dose of Arcofolin®, the $AUC_{0\text{-}8\ h}$ for plasma (6S)-5-MTHF was 126.0 ± 33.6 nmol/l·h versus 56.0 ± 25.3 nmol/l·h for folic acid ($p<0.0001$). Plasma levels of (6S)-5-MTHF after Arcofolin® and folic acid intake were nearly similar at 8 h. Total folate values for $AUC_{0\text{-}8\ h}$ also were higher after Arcofolin® intake. The time to reach C_{max} (measured parameter referred to as T_{max}) after Arcofolin® intake occurred 1 h

earlier than T_{max} for folic acid. Levels of circulating unmetabolized folic acid were undetectable after Arcofolin® intake. No adverse events or tolerability issues were reported. Overall, results from this study indicate that compared to folic acid, Arcofolin® results in a greater plasma (6S)-5-MTHF and plasma total folate AUC, a higher C_{max} of plasma (6S)-5-MTHF and plasma total folate, and a faster T_{max} for plasma (6S)-5-MTHF and plasma total folate. The study is only a single intake study and is limited by a small sample size (12/sex).

To understand the relative bioavailability of L-5-MTHF salts including Arcofolin® and Metafolin®, Obeid and colleagues compared the AUC responses of plasma (6S)-5-MTHF and plasma total folate following intake of a single dose of Arcofolin® and Metafolin® (Obeid et al., 2020). The comparison was based on data from the 2019 study of Arcofolin® versus folic acid in a population of healthy adults and data from a 2009 study of Metafolin® versus folic acid in a population of 24 women of child-bearing age (Prinz-Langenohl et al., 2009). Using raw data from each study, plasma (6S)-5-MTHF and plasma total folate AUCs were re-calculated from all 10 time points across the two studies (0, 15, 30, 60, 90, 120, 180, 240, 360, and 480 min). Values for a missing time point in each study were interpolated by calculating the mean of the concentrations at the two neighboring time points prior to recalculation of the AUC values.

As shown in **Figure 7**, the plasma (6S)-5-MTHF AUC response to Arcofolin® and Metafolin® relative to the concurrent folic acid control in each study was elevated. Among the participants, the AUCs for Arcofolin® and Metafolin® did not differ significantly (p = 0.7478). The study investigators concluded the Na- and Ca- salts of (6S)-5-MTHF likely do not differ in their pharmacokinetics. The comparable plasma folate responses to L-5-MTHF salts relative to folic acid support comparable bioavailability of Arcofolin® and Metafolin®.

Figure 7. Plasma (6S)-5-MTHF AUC Response to Arcofolin® and Metafolin® Versus Folic Acid



Source: Obeid et al., 2020

The AUC of plasma (6S)-5-Methyl-THF after intake of folic acid and (6S)-5-Methyl-THF salts from two independent studies. The $AUC_{0\text{-}8h}$ of (6S)-5-Methyl-THF-Ca is corrected for the systematic differences by subtracting 17.0 nmol/L*h [differences between the folic acid reference groups (2019 versus 2009)] from all AUCs. P-values are according to unpaired t-test after log-transformation. The original (6S)-5-Methyl-THF-Ca study is published by Prinz-Langenohl et al., 2009.

Summary

Arcofolin® and Metafolin® are related compounds. Both are salts of L-5-MTHF (Arcofolin®: L-5-MTHF-Na and Metafolin®: L-5-MTHF-Ca) produced in similar fashion and have similar specifications. These salts are expected to completely dissociate *in vivo* and have comparable bioavailability. The amount of calcium or sodium provided by either salt is negligible in the context of the diet (~0.1 mg calcium and ~0.05 mg sodium) and would not be expected to have any impact on health. The comparable bioavailability of Arcofolin® and Metafolin® is supported by findings in the published literature. In a single-dose study of the bioavailability of Arcofolin®, Arcofolin® produced a higher increase in plasma L-5-MTHF and total folate than folic acid (Obeid et al., 2020). Plasma levels of L-5-MTHF and total folate in response to Arcofolin® and Metafolin® relative to folic acid controls were compared and the evidence suggests that plasma folate responses to Arcofolin® and Metafolin® are comparable (Obeid et al., 2020).

Published studies comparing the bioavailability of L-5-MTHF-Ca or unspecified L-5-MTHF and folic acid have shown either increased or equivalent bioavailability of L-5-MTHF compared to folic acid. Collectively, clinical data indicate that salts of L-5-MTHF, including L-5-MTHF-Ca, are at least as bioavailable as folic acid. Based on the available evidence, authoritative bodies have recognized the bioavailability of L-5-MTHF salts including L-5-MTHF-Ca as comparable to that of folic acid. It is therefore reasonable to conclude that the overall bioavailability of L-MTHF salts, including both L-5-MTHF-Ca and the sodium salt form, is comparable to that of folic acid.

Table 6. Data Summary on Bioavailability Human Studies

Reference	Study Design	Study Population	Duration of Intake	Intervention/Dosage	Outcome Measures and Main Findings
Single intake studies					
Gregory et al., 1992	Bioavailability assessment with isotopically labeled folates	Males, age 20-30 y n=7	Single intake	Single oral dose of folate followed by single injection of folic acid control. 300 µg each of folic acid (two separately labeled forms) or [6S]-tetrahydrofolate; 320 µg each of [6S]-5-formyl tetrahydrofolate, [6R]-10-formyltetrahydrofolate, or [6S]-5-MTHF	48-h urinary folate as determined by the ratio of excreted folate to folic acid control was decreased compared to folic acid; statistically significant differences were noted for all folate forms compared to folic acid control except for L-5-MTHF
Pentieva et al., 2004	Double-blind, crossover RCT	Males, mean age 25.6 ± 5.5 y n=13	Single intake	Single oral dose Placebo capsule; 500 µg each of folic acid and [6*S*]-5-MTHF	Short-term bioavailability (monitored 10 hours) as determined by plasma folate concentration and AUC was equivalent between folic acid and (6S)-5-MTHF
Prinz-Langenohl et al., 2009	Double-blind, crossover RCT	Females, child-bearing age, TT- and cc-genotype of 677 CT mutation of MTHFR n=24	Single intake	Single oral dose 400 µg of folic acid or 416 µg [6*S*]-5-MTHF	Plasma folate AUC and C_{max} were higher with [6S]-5-MTHF compared to folic acid in both genotypes.
Repeat intake studies					
Litynski et al., 2002	Double-blind RCT	Healthy adults age 19-69 y n= 32 males n=8 females	7 wk	Single oral dose per day 400 µg folic acid or [*6RS*]-5-MTHF	Reduction in plasma homocysteine was equivalent between folic acid and 5-MTHF

Reference	Study Design	Study Population	Duration of Intake	Intervention/Dosage	Outcome Measures and Main Findings
Venn et al., 2002	Double-blind, RCT	Females, mean age 38 ± 8.4 y n=104	24 wk	Single oral dose per day Placebo, 100 µg folic acid, or 113 µg [*6S*]-5-methyltetrahydrofolic acid, calcium salt	Red cell folate and plasma folate were not different between folic acid and [*6S*]-5-methyltetrahydrofolic acid; no plateau was reached for either treatment by 24 wk
Venn et al., 2003	Double-blind, RCT	Healthy adults, age ≥ 18 y n=167	24 wk	Single oral dose per day Placebo, 100 µg folic acid, or 113 µg L-MTHF, calcium salt	Red cell folate and plasma folate were not different between folic acid and L-MTHF; greater reduction (p=0.045) in plasma total homocysteine in L-MTHF group (14.6%) compared to folic acid group (9.3%)
de Meer et al., 2005	Double-blind, RCT	Healthy young males and females, age <30 y n=12; healthy middle-aged males and females, age ≥50 y n=12	5 wk	Single oral dose per day 400 µg folic acid or 454 µg [*6S*]-5-MTHF	No significant differences in plasma folate concentrations were observed between interventions in either age group
Houghton et al., 2006	Double-blind, RCT	Healthy pregnant women, age 20-38 y n=72	16 wk	Single oral dose per day 400 µg folic acid, 416 µg [*6S*]-5-MTHF, or placebo	RBC folate was greater in [*6S*]-5-MTHF group (2178 nmol/d) compared to folic acid group (1967 nmol/d); p<0.05 Plasma folate and total homocysteine were not different between [*6S*]-5-MTHF group and folic acid group
Lamers et al., 2006	Double-blind, RCT	Healthy females, age 19-33 y n=144	24 wk	Single oral dose per day 400 µg folic acid, 416 µg [6*S*]-5-MTHF,208 µg [6*S*]-5-MTHF, or placebo	RBC folate increase over time was the highest in the 416 µg/d [6*S*]-5-MTHF group, followed by 400 µg/d folic acid and 208 µg/d [6*S*]-5-MTHF groups; p<0.001

Reference	Study Design	Study Population	Duration of Intake	Intervention/Dosage	Outcome Measures and Main Findings
					Plasma folate increase over time was the highest in the 416 µg/d [6*S*]-5-MTHF group, followed by 400 µg/d folic acid and 208 µg/d [6*S*]-5-MTHF groups; $p<0.05$ and $p<0.001$, respectively
Wright et al., 2010	Partially double-blind, RCT	Healthy adults age 18-65 y n=163	16 wk	Single oral dose per day 453 nmol of folic acid or L-MTHF, calcium salt (Metafolin®) or natural food folate	No significant differences in plasma or RBC folate levels between individuals supplemented with folic acid or Metafolin®
Green et al., 2013	Double-blind, RCT	Healthy females, age 18-45 y n=39	16 wk	Intake of one roll daily fortified with 400 µg folic acid or [6*S*]-5-MTHF	Comparable increase in erythrocyte folate and plasma folate
Sicińska et al., 2018	Double-blind, RCT	Healthy adults, age 50-65 y n=40	4 wk	Single oral dose per day 400 µg folic acid or [6*S*]-5-MTHF	Comparable reduction in serum homocysteine between both groups
Henderson et al., 2018	Double-blind, RCT	Healthy females, age 20-45 y n=142	12 wk	Single oral dose per day 1000 µg folic acid, 1130 µg L-5-MTHF calcium salt, or placebo	RBC folate was higher in L-5-MTHF group (1951 nmol/L) compared to folic acid group (1498 nmol/L); p=0.003 Plasma folate was higher in L-5-MTHF group (52 nmol/L) compared to folic acid group (40 nmol/L); p=0.023
Bailey and Ayling, 2018	Pharmacokinetics study	Females, mean age 28.1 ± 7 y n=61	2 wk	Oral administration Group A: 7500 µg [6*S*]-5-MTHF calcium salt every 24 h (three doses total), followed by 400 µg/d folic acid for two wk Group B: 7500 µg [6*S*]-5-MTHF every 12 h (five doses total), followed by 800 µg /d [6*S*]-5-MTHF calcium salt for two wk	Serum total folate levels in folate insufficient women were higher after 5-MTHF administration compared to folic acid

Reference	Study Design	Study Population	Duration of Intake	Intervention/Dosage	Outcome Measures and Main Findings
				Group C: 7500 µg folic acid every 24 h (three doses total), followed by 400 µg/d folic acid for two wk	

Dietary Reference Intakes of Folate

Authoritative bodies have established recommended intake and tolerable upper intake levels for folate from fortified foods and supplements. When L-5-MTHF was introduced as a new alternative for food folate and folic acid, it was established that the same tolerable upper intake established for folate could be applied to L-5-MTHF as well, and thus, that L-5-MTHF can be used interchangeably with folate/folic acid to meet dietary needs; no attempt was made to establish an independent UL for L-5-MTHF.

Tolerable Upper Intake Level (UL)

The IOM (1998) and EFSA (EFSA, 2014) established ULs for folate from fortified foods or supplements (folic acid form) as 1 mg/day for adults (**Table 7**). This UL was established based on the relationship of folate intake and neurological dysfunction; folate has the potential to reverse megaloblastic anemia due to vitamin B_{12} deficiency, thereby delaying the timely diagnosis and appropriate treatment of the disease and allowing the neurological dysfunction caused by B_{12} deficiency to progress. To derive the UL, an uncertainty factor (UF) of 5 was applied to the lowest-observed-adverse-effect level (LOAEL) of folate (5 mg/day) that was established based on a dose response assessment of case reports evaluating the relationship between folate/folic acid intake and adverse neurological effects. Data from 19 studies of vitamin B_{12}-deficient patients who experienced neurological complications while on folate therapy were not sufficient to set a NOAEL. A LOAEL of 5 mg/day was set based on "more than 100 reported cases of neurological progression" among patients on folate therapy at intakes of ≥ 5 mg/day and only eight cases of neurological progression among patients on less than 5 mg folate/day. The UF of 5 was established based on potential sources of uncertainty including the lack of controlled, dose response data, the use of a LOAEL instead of a NOAEL, and the severity of the neurological complications observed. Because no data were available to suggest that other life-stage groups have increased susceptibility to adverse effects, the ULs for children were extrapolated from the UL for adults on the basis of body weights.

The IOM (1998) and EFSA (2014) also reviewed other adverse effects relating to folate intake including carcinogenicity, intestinal zinc absorption, hypersensitivity, general toxicity, and un-metabolized folic acid in circulation. As noted by the IOM, the neuropathy that may be precipitated or exacerbated by excessive folate justified the selection of this outcome as the critical endpoint for establishing the folate UL. Applying an uncertainty factor of 5 to the NOAEL of 5 mg/day for neurological complications from folate therapy, the UL for folic acid was established at 1000 μg per day for adults, and lower levels for children based on extrapolation by body weight (**Table 7**).

Table 7. Tolerable Upper Intake Levels for Folate from Fortified Foods or Supplements

Institute of Medicine (IOM) 1998		European Food Safety Authority (EFSA) 2014	
Age groups	**Tolerable Upper Intake Level (UL) (µg/day)[a]**	**Age groups**	**Tolerable Upper Intake Level (UL) (µg/day)**
Children 1-3 y	300	Children 1-3 y	200
Children 4-8 y	400	Children 4-6y	300
Children 9-13 y	600	Children 7-10 y	400
Teens 14-18 y	800	Teens 11-14 y	600
		Teens 15-17 y	800
Adults 19+ y	1000	Adults 19+ y	1000

[a] Based on folic acid form of folate in fortified foods and supplements.

FDA (2016b) Review

In 2016, as part of its review of the petitioned use of folic acid in corn masa flour, FDA conducted a safety review and risk assessment on folic acid. FDA (2016b) considered several potential health effects of folic acid, including, masking of vitamin B_{12} deficiency, direct effect on vitamin B_{12} deficiency-related neurological effects, cancer, effects of prenatal exposure on childhood health, hypersensitivity, reproductive effects, and folic acid-drug interaction. Of these effects, the Agency found suggestive evidence for masking vitamin B_{12} deficiency and exacerbation of vitamin B_{12} deficiency-related neurological complications and cognitive decline, among the most at-risk population of 50 years and older. For the other health effects, the Agency found the overall evidence to be unclear and not substantiated based on the available evidence. Overall, FDA concluded that there was no definitive association of adverse effect of folic acid at the intake estimates derived by the Agency, as earlier summarized in **Table 5** (see EDI section). The Agency was not concerned that any of the intake estimates exceeding the UL would cause adverse health impact in any of the population subgroups for several reasons, including that the IOM ULs were calculated using a five-fold uncertainty factor, which is approximately twice what is used for other B vitamins, providing an additional margin of safety. In the review, FDA also noted that the risk of masking B_{12} vitamin deficiency and related neurological complications is low.

The proposed use of Arcofolin® is substitutional for select uses of folic acid, thus, dietary exposure to folate in the U.S. population is not expected to be impacted. Thus, it is reasonable to conclude that intake of a L-5-MTHF salt, namely Arcofolin®, in place of folic acid is also safe.

As previously noted, L-5-MTHF supplementation reduces the potential for masking the symptoms of vitamin B_{12} deficiency as well as the hematological manifestations of the deficiency, the complications of which formed the basis for the UL for folic acid. Thus, the current UL derived for supplemental folate in the form of folic acid is likely a conservatively low reference for safe intake of supplemental folate in the L-5-MTHF form.

Salts of L-5-MTHF

As previously described at the beginning of the safety section, Arcofolin® and Metafolin® are similar compounds. Both are salts of L-5-MTHF (Arcofolin® is L-5-MTHF-Na and Metafolin® is L-5-MTHF-Ca), produced in similar fashion, and have similar specifications. These salts are expected to completely dissociate *in vivo* and have comparable bioavailability. The comparable bioavailability of Arcofolin® and Metafolin® is further supported by findings from published literature, as summarized earlier. As such, publicly available safety data on Metafolin® can be relied upon to evaluate the safety of the related Arcofolin®.

Authoritative Safety Reviews of L-5-MTHF-Ca

EFSA (2004) and JECFA (2005)

In the late 1990s, L-5-MTHF-Ca was introduced as a new dietary ingredient intended as an alternative for food folate and folic acid. In 2004 and 2005, respectively, relying on the established UL for folate, EFSA and JECFA independently evaluated the safety of L-5-MTHF-Ca for use in dietary supplements, foods for special dietary uses, and other foods. Both committees concluded that L-5-MTHF-Ca, with a tolerable upper level of 1 mg/adult/day, is not a safety concern (EFSA, 2004; JECFA, 2005). Their conclusions were based on the assumption that the bioavailability of L-5-MTHF-Ca is at least as high as that of folic acid and that the fate of L-5-MTHF-Ca is indistinguishable from that of other absorbed and metabolized natural folate forms. Because L-5-MTHF supplementation reduces the potential for masking the symptoms of vitamin B_{12} deficiency as well as the hematological manifestations of the deficiency, the complications upon which the folate UL was based, L-5-MTHF may be tolerable at higher intakes than that established for folate/folic acid.

FSANZ Review (2008)

FSANZ (2008) conducted a dietary intake assessment to evaluate the impact of fortifying certain foods with L-5-MTHF-Ca as an alternative to folic acid. Assessments were based on food consumption data from Australia (1995) and New Zealand (1997) National Nutrition Surveys and from folic acid intake estimates from food alone (excluding dietary supplements). Assumptions included 100% folic acid replacement with L-5-MTHF-Ca for fortified foods and equivalent intakes between both folate forms. The authors determined that the major contributors to L-5-MTHF-Ca intake were breakfast cereals, yeast extracts, and breads, and children aged 2 - 3 y are most likely to exceed the folic acid UL. The authors also noted that the additional calcium intake from L-5-MTHF-Ca is less than 1 mg/d and is not considered to be a significant amount as part of the total diet. Thus, FSANZ (2008) concluded that L-5-MTHF used for the fortification of certain foods would not raise public health or safety concerns.

EFSA Scientific Opinion (2020) – Infant Nutrition

Recently, EFSA concluded that calcium L-5-methylfolate is “safe under the proposed uses and use levels for infants and young children,” and that the compound does not pose any concern for allergenicity (EFSA, 2020). The proposed uses of calcium L-5-methylfolate as a folate source

includes use in infant formula, follow-on formula, processed cereal-based food, and baby food for infants (<12 mo in age) and young children (12 - 36 mo in age). Supporting evidence for the safety or tolerability of calcium L-methylfolate in infant formula was based on human data from the Troesch et al. (2019) clinical study. Overall, the panel confirmed that the findings from Troesch et al. (2019) did not raise any concerns regarding the safety or tolerability of calcium L-5-methylfolate in infant formula, and that the study demonstrated calcium L-5-methylfolate bioavailability as an alternative folate source (EFSA, 2020).

Pre-Clinical Safety Data

Literature Search

To capture safety information relevant to this assessment, a literature search was conducted using the PubMed search engine for information related to the pre-clinical safety of L-5-MTHF. The search string is detailed in **Appendix D.** Search terms did not include specific reference to reproductive toxicity or mutation and instead used the more general strings "tox*" and "genotox*" to avoid capturing models of MTHFR gene mutations and reproductive toxicity information related to the well-studied area of folate deficiency during gestation. A single pre-clinical study of the calcium salt of L-5-MTHF (or L-5-MTHF-Ca) was identified in the PubMed search.

The search for regulatory information identified industry-sponsored pre-clinical safety studies summarized in an EFSA Scientific Panel opinion on the safe use of L-5-MTHF-Ca (EFSA, 2004). Pre-clinical studies of the racemic mixture of 5-MTHF were also summarized in a dietary ingredient notification to FDA (FDA,1998). However, since the 5-MTHF in the dietary ingredient notification is a mixture of the L and D isomers (1:1 mixture) and the 5-MTHF that is the subject of this GRAS review is principally the L-isomer form, with no more than 1% in the D-isomer form, the data reported in this dietary ingredient notification were excluded from further review.

There is no published pre-clinical study on Arcofolin®. Genotoxicity studies have been conducted for Arcofolin® and the unpublished study is summarized herein as corroborative evidence.

L-5-MTHF-Ca

Acute Studies

Eight-week-old fasted Hsd Cpb:WU strain rats (3 rats/sex) were administered 2000 mg/kg bw of 97.2% pure L-5-MTHF-Ca by gavage. Food was offered four hours after treatment, and rats were observed for a 15-day recovery period after administration of the single dose of test article. During this recovery observation period, all rats gained weight appropriately, and all survived until scheduled termination. There were no gross alterations of organs reported at necropsy and the LD_{50} was established as greater than 2000 mg/kg bw, the only dose tested. Other related substances also tested included the D-enantiomer, a racemic mixture, and the s-triazine oxidation product of L-5-MTHF. The LD_{50} values for these substances were also greater than 2000 mg/kg bw, the only dose tested (EFSA, 2004).

Short-term Studies

No short-term studies of calcium salt of L-5-MTHF were identified in the earlier EFSA review (EFSA, 2004) or in the PubMed search.

Subchronic Studies

Recently, a 13-week rat gavage study on L-5-MTHF-Ca that previously had been reviewed and reported by EFSA in 2004 as showing no toxic effects, was published in the literature (Niederberger et al., 2019). In this publication, authors reported conducting an Organisation for Economic Co-operation and Development (OECD) guideline 408 and GLP-compliant subchronic toxicity study in Hanlbm:Wistar (SPF) rats (n=10-15 rats/sex/group) that were administered L-5-MTHF-Ca via oral gavage at doses of 0, 25, 100, or 400 mg/kg bw/day for 13 weeks. Study parameters included clinical signs, behavior, body weight, food consumption, functional observational battery, locomotor activity, hematology, ophthalmology, clinical biochemistry, urinalysis, organ weights, and histopathology. Additional animals from the control and high-dose groups (n=5 rats/sex/dose) were observed during a treatment-free, 4-week recovery period. No treatment-related findings were observed for multiple parameters that included clinical signs, behavior, body weight, food consumption, functional observational battery, locomotor activity, hematology, ophthalmology, clinical biochemistry, urinalysis, organ weights, and histopathology. Observed changes in plasma levels of aspartate aminotransferase, lactate dehydrogenase, and creatine kinase in high-dose treated males were within the historical range and were not dose-dependent. Additionally, results from the 4-week recovery period did not reveal any differences between controls and high dose-treated males for plasma levels of aspartate aminotransferase, lactate dehydrogenase, and creatine kinase. Overall, the NOAEL for L-5-MTHF-Ca was selected by the authors to be 400 mg/kg bw/day based on the absence of any treatment-related effects up to the highest tested dose.

Chronic Studies

No chronic studies were located in the published literature.

Reproductive and Developmental Safety

Standard reproductive studies were not found in the PubMed search. However, a standard developmental toxicity study (teratology study) has been conducted and was previously summarized in a review by EFSA in 2004; EFSA stated that L-5-MTHF-Ca was not fetotoxic, embryotoxic, or teratogenic (EFSA, 2004). More recently, this developmental toxicity study was published by Niederberger et al., 2019. Niederberger et al. (2019) described the study as an OECD guideline 414 and GLP-compliant prenatal developmental toxicity study, in which virgin female Wistar rats (25 females/group) were administered L-5-MTHF-Ca from gestations day (GD) 5 to 19 at doses of 0, 100, 300, or 1000 mg/kg bw/day via oral gavage. Females were evaluated for behavior, clinical signs, body weight, and food and water consumption. After females were euthanized on GD 20, gravid uteri weight, number of corpora lutea, live and dead fetuses, resorptions, and implantations were evaluated. Fetuses were subsequently weighed and examined for any macroscopic abnormalities, sex distribution, skeletal abnormalities, and malformations. No maternal deaths occurred during the study and no treatment-related effects

were observed in body weight, food or water consumption, gravid uterus weight, corpora lutea number, or in the number of implantations. Similarly, no treatment-related effects were observed in resorptions, number of fetuses, fetus weight, sex distribution, skeletal variations, ossification, or soft tissue malformations. Based on the absence of treatment-related effects at all tested doses, authors set the NOAEL as the highest tested dose of 1000 mg/kg bw/day.

Genotoxicity

Genotoxicity studies on L-5-MTHF-Ca were recently published by Niederberger et al. (2019) and are summarized in **Table 8** below. Based on the study findings, authors concluded that L-5-MTHF was not mutagenic or genotoxic under the conditions tested. EFSA had previously reviewed the same studies (which were unpublished at the time) and also concluded that L-5-MTHF was not mutagenic or genotoxic (EFSA, 2004).

Table 8. Genotoxicity studies of L-5-MTHF-Ca

Source	Study Design	Strains, Species, or Cell Types	Results
Niederberger et al., 2019 EFSA, 2004	Ames test (bacterial reverse mutation)	Salmonella typhimurium TA 1535, 1537, 98, 100, 102 and E. coli WP2 uvr A pKM101	L-5-MTHF-Ca was negative in all strains and at all doses tested (up to 5000 µg/plate, suspension at highest doses) both with and without metabolic activation.
	Mutagenic potential in mammalian cells	L5178Y TK (+/-) mouse lymphoma cells	L-5-MTHF-Ca was non-mutagenic in mouse lymphoma cells at concentrations up to 5000 µg/mL.
	Unscheduled DNA synthesis	Male Wistar rats treated by gavage, followed by primary hepatocyte culture	No significant increase in unscheduled DNA synthesis.
	Micronucleus assay	Male Wistar rats treated by gavage	No increase in frequency of micronucleus formation in the bone marrow of treated rats.

L-5-MTHF-Na (Arcofolin®)

Genotoxicity Studies (Unpublished)

Three genetic toxicity studies have been conducted on Arcofolin® and are summarized below.

Merck's Study Report 18-DA0269-0 (2018): The mutagenic potential of Arcofolin® was evaluated according to OECD 471 guidelines in a bacterial reverse mutation test using *Salmonella typhimurim* tester strains TA98, TA100, TA1535, and TA1537 and *Escherichia coli* WP2 *uvrA*, ± S9 metabolic activation. Arcofolin® was tested in duplicate experiments (10 and

20% S9 were employed in the 1st and 2nd experiments, respectively) at concentrations that ranged from 5 to 5000 µg/plate. Overall results revealed that Arcofolin® is non-mutagenic under the stated experimental conditions.

Merck's Study Report 18-DA0271-0 (2019): In an OECD 473 guideline compliant *in vitro* chromosomal aberration study, human peripheral blood lymphocytes were exposed to Arcofolin® for five hours at concentrations that ranged from 250 - 2000 µg/ml ± S9 and separately for 29 hours at concentrations that ranged from 125 - 1000 µg/ml in the absence of S9 metabolic activation. Three independent experiments were conducted. Overall results indicated that exposure to Arcofolin® did not significantly induce chromosomal aberrations under the stated experimental conditions.

Merck's Study Report Number ICCR 2117200: In an OECD 487 guideline compliant *in vitro* micronucleus test, human peripheral blood lymphocytes were stimulated to divide for 48 hours in the presence of phytohemagglutinin (PHA). After PHA stimulation, lymphocyte cultures were exposed to Arcofolin® for four hours at concentrations that ranged from 13.8 - 2121 µg/ml ± S9. After a 16-hour recovery period the treated cells were cultured for a further 20 hours in the presence of cytochalasin B. In an independent experiment, lymphocyte cultures were exposed to Arcofolin® continuously for 20 hours to concentrations that ranged from 129 - 2121 µg/ml in the absence of S9 metabolic activation, after which they were cultured for a further 20 hours in the presence of cytochalasin B. In all exposures the maximum concentration tested was the maximum recommended by OECD 487. Concurrent negative (water), solvent (culture medium with 10.0% water containing 1 mg/mL Sodium L(+)-ascorbate) and appropriate positive controls were included in each exposure. Continuous treatment in the absence of S9 resulted in clearly negative findings for micronucleus induction. No evidence of cytotoxicity/cytostasis was observed in any exposure and therefore the three highest concentrations tested were examined for micronuclei. The short, 4-hour treatments induced small but biologically irrelevant increases in micronuclei following treatment at the highest concentration only. In the absence of S9, the increase was statistically significant, but with no significant linear trend and fell within the laboratory's 95% control limits of the historical solvent control data. Furthermore, the solvent control data demonstrated notable inter-replication variation, resulting in a low mean micronucleus frequency. In the presence of S9 no statistically significant increase in micronuclei or significant concentration relationship was observed. However, the micronucleus frequency at the highest concentration exceeded the laboratory's 95% control limits of the historical solvent control data, although it fell within the observed historical solvent control range. Overall, the results indicated that exposure to Arcofolin® did not induce biologically relevant increases in micronuclei under the stated experimental conditions.

Table 9. Genotoxicity Studies of Arcofolin®

Merck Study Report	Study Design	Strains, Species, or Cell Types	Treatment Concentration	Results
18-DA0269-0 (2018)	Ames test (bacterial reverse mutation) conducted according to OECD 471 testing guideline	*Salmonella typhimurim* tester strains TA98, TA100, TA1535, and TA1537 and *Escherichia coli* WP2 *uvrA*	5 to 5,000 µg/plate (± S9)	Arcofolin® is non-mutagenic at all doses tested
18-DA0271-0 (2019)	*In vitro* chromosomal aberration study conducted according to OECD 473 testing guideline	Human peripheral blood lymphocytes	1) 250-2,000 µg/ml (± S9) for 5 hours 2) 125-1,000 µg/ml (-S9) for 29 hours	Arcofolin® did not significantly induce chromosomal aberrations
ICCR 2117200	*In vitro* micronucleus test conducted according to OECD 487 testing guideline	Human peripheral blood lymphocytes	1) Following PHA (phytohaemagglutinin) stimulation: 13.8-2,121 µg/ml (± S9) for 4 hours 2) In an independent experiment: 129-2,121 µg/ml (-S9) for 20 hours	Arcofolin® did not induce biologically relevant increases in micronuclei

Pre-Clinical Data Summary

There are no published pre-clinical studies on monosodium L-5-MTHF (Arcofolin®). However, Arcofolin® and Metafolin® are related compounds. Both are salts of L-5-MTHF(Arcofolin®: L-5-MTHF-Na and Metafolin®: L-5-MTHF-Ca), produced in similar fashion, and have similar specifications. These salts are expected to completely dissociate *in vivo* and have comparable bioavailability. The comparable bioavailability of Arcofolin® and Metafolin® is supported based on findings from published studies as summarized earlier. Therefore, publicly available safety data on Metafolin® can be relied upon to evaluate the safety of the related Arcofolin®.

In a series of studies published by Niederberger et al. (2019), L-5-MTHF-Ca was found to be non-genotoxic, and developmental toxicity studies of standard design did not reveal any toxic potential to fetuses or pregnant dams. Previously, EFSA (2004) reviewed the same studies and reported the same conclusion. Unpublished genotoxicity studies on Arcofolin® were negative, providing corroborative evidence of non-genotoxicity. Specific reproductive and chronic safety studies have not been conducted. A 13-week rat gavage study that was previously reviewed by EFSA in 2004 was recently published by Niederberger et al. (2019). The authors established a NOAEL for L-5-MTHF-Ca of 400 mg/kg bw/day based on the absence of any treatment-related

effects at this highest tested dose. Arcofolin® is proposed to be used as an alternative source of folate to folic acid in food categories for which folic acid is an approved added nutrient. Assuming that Arcofolin® will substitute for all other sources of folic acid in foods (except for corn masa flour), the 95th percentile cumulative intake of folic acid is 919 µg/day among age group 51 - 70 years (FDA, 2016b). Using a default body weight of 60 kg, a cumulative intake of 15.3 µg/kg bw/day can be estimated, which is well below the NOAEL for Arcofolin®, with a margin of exposure (MOE) > 25,000.

Clinical Safety Data

Numerous clinical studies have been conducted with folic acid and folate derivatives as test articles. These studies, including systematic reviews and meta-analyses, largely focused on efficacy in areas pertaining to maternal health, depression, diabetes, metabolic disease, and cancer. The PubMed literature search for clinical studies with folic acid and the L-5-MTHF folate derivative yielded 119 papers (the search string is detailed in **Appendix D**). Many of the captured studies were based on topics such as efficacy, genetic polymorphisms, pharmacokinetics, and bioavailability.

Infant Clinical Study

The PubMed literature search produced a single infant clinical study from Troesch et al. (2019) which investigated the equivalency of infant formula containing either folic acid or L-5-MTHF on infant growth parameters, tolerance, and safety. Troesch et al. (2019) conducted a double-blind, RCT study in healthy infants of both sexes (n=360) that received folic acid control formula (10.0 µg/100 ml) or an equimolar amount of L-5-MTHF (10.4 µg/100 ml; Metafolin®, Merck & Cie, Schaffhausen, Switzerland) beginning in the first month of life through 16 weeks of age. Study participants were aged <28 days, were delivered between ≥37 to ≤41 weeks of gestation, and were 2500 - 4500 g in birth weight. A reference group of nonrandomized breastfed infants was also included. The primary outcome measure was weight gain which was evaluated in infants from the ages of 4 - 16 weeks. Secondary outcome measures were growth indices, dietary intake, feeding behavior, and stool characteristics. Additional measured parameters included folate status, blood chemistry, hematology analyses, and adverse events.

The study showed no differences in tolerability, adverse events, or in secondary outcome measures between both formulas, and no evidence for safety concerns. Equivalence between folic acid and L-5-MTHF was demonstrated by the comparable gains in body weight and head circumference in both groups. Inconclusive evidence for equivalency between both groups (differences were not statistically significant) was reported for body length gain and calorie intake. Statistically significant differences in folate status between folic acid and L-5-MTHF were observed in RCF (839.4 and 907.0 nmol/l, respectively; p=0.0095) and unmetabolized folic acid (1.15 and 0.73 nmol/l, respectively; p<0.0001) at the end of treatment. The authors determined that their findings are the first to demonstrate increased RCF from L-5-MTHF compared to folic acid administered to infants.

NTP Monograph (2015)

In response to concerns raised in published studies about the safe use of folic acid above 400 µg per day, the National Toxicology Program (NTP) and the National Institutes of Health (NIH) ODS convened an expert panel in May 2015 to evaluate the current body of science. Following a critical review of relevant literature, the panel identified four high-priority health effect categories, including cancer (due to numerous studies evaluating high folic acid intake among various populations that showed inconsistent results across cancer types), cognition in the presence of vitamin B_{12} deficiency, hypersensitivity-related outcomes, and thyroid- and diabetes-related disorders. The NTP expert panel excluded the following health effect categories due to either sufficient evidence demonstrating no adverse effect of high folic acid intake or lack of quality studies with consistent findings: cardiovascular outcomes, twinning and multiple births, autism, other neurological outcomes, other immunological outcomes, other endocrine and metabolic disease outcomes, other reproductive outcomes, and mortality. The panel reported possible adverse effects of high folic acid intake on cancer growth and cognition in the presence of vitamin B_{12} deficiency; however, underlying mechanisms remain to be elucidated and more research is necessary to establish a causal relationship. The panel also found a lack of consistent evidence concerning high folic acid intakes and asthma, thyroid disease, diabetes, and glucose/insulin metabolism and called for more research to better understand the relation between folic acid and these disease outcomes.

Colorectal Cancer

Low folate status has been associated with an increased risk in cancer, particularly colorectal cancer (CRC) (LPI, 2018). However, there has been concern regarding prevention of CRC with high doses of folic acid given the potential dual role of folic acid in the protection of normal cells while promoting the progression of existing neoplasia in the colon. There is a concern that high levels of circulating folic acid could increase the risk of colorectal neoplasia among susceptible populations, such as individuals with previously identified lesions, especially among those with genetic polymorphisms that allow for a high rate of folate metabolism (Kim, 2016). Mandated fortification of folic acid in uncooked cereal grains in the U.S. and Canada was initially attributed to an increase in CRC rates (Mason et al., 2007). However, a more recent ecological analysis argues that this relationship is spurious due to lagged effects of implementation and CRC incidence, and that folic acid fortification has likely contributed to the overall decline of CRC in the U.S. (Keum & Giovannucci, 2014).

As summarized earlier, cancer was identified as one of the four high-priority health categories by the NTP expert panel (NTP, 2015). A summary of the expert panel review related to folic acid and CRC outcomes presented in the NTP monograph along with a review of the safety literature published subsequent to the NTP monograph is presented below. In addition to concerns related to high folic acid intake and the risk of CRC, the potential for additional folate exposure within an individual resulting from the biosynthesis of B vitamins by the gut microbiome has been considered and is presented below.

CRC Data Reported in the NTP Monograph

In response to published studies reporting adverse health effects associated with high intakes (>400 μg) of folic acid, the NTP expert panel conducted a comprehensive systematic review of the literature to identify studies conducted in humans, animals, or *in vitro* models where the exposure included folate, folic acid, folacin, folinic acid, THF, MTHF, and 5-MTHF. Searches were conducted in 2011 and May 2013 with a final update conducted in December 2014. The results from the May 2013 literature review used to identify four high priority health effect categories were based on reporting of adverse outcomes associated with folic acid (including folate, folacin, or folinic acid) intakes greater than 400 ug/day or circulating blood levels above deficient range. Therefore, while THF, MTHF, and 5-MTHF were included in the search terms by the NTP expert panel, no literature was identified that focused on intake of these specific forms of folate. Further, the NTP monograph states that the majority of the studies did not report intakes in DFE.

The 2015 NTP monograph states that while folic acid was initially studied for cancer prevention, concerns about increased cancer risks were first raised in two Norwegian randomized controlled trials (RCTs) (Ebbing et al., 2008; Ebbing et al., 2009) along with mechanistic studies that demonstrate biological plausibility in folate both preventing and promoting cancer (Mason, 2009). Focusing their review on 43 pooled- or meta-analyses across 12 cancer types, the cancer sub-panel concluded that clinical research consistently showed that while inadequate dietary folate intake increases the risk of CRC in humans, there does not appear to be a benefit relative to CRC risk from supplementation among those with an adequate folate status at baseline. The sub-panel also identified that there is enough of a suggestion of folic acid supplementation having an adverse effect on cancer growth that it justifies further research to determine if a causal relationship can be established. The sub-panel determined there was a need to further clarify if the potential increased risk is solely among specific subgroups (i.e., by age, pre-existing neoplasia, and genetics), and to assess long-term outcomes from previous trials.

CRC Data Published Subsequent to the NTP Monograph

A review of the recent literature on risk of CRC from high folic acid intake was conducted to identify relevant studies that were published subsequent to the 2015 NTP monograph. PubMed searches were conducted to identify studies indexed since December 1, 2014 to identify systematic reviews of meta-analyses of any new clinical trials or observational studies related to CRC and folic acid intake. Given the large volume of published data on folic acid supplementation, when available, meta-analyses and systematic reviews were first selected for review. If there were no published meta-analyses or systematic reviews, then all identified individual clinical trials and epidemiologic studies within the specified timeframe were reviewed, with emphasis on higher quality studies (i.e., cohort studies those with a prospective design). The search was conducted using MeSH terms for folic acid and colorectal cancer. The search was conducted on April 6, 2020. The search strategies are outlined below.

Table 10. Summary of PubMed Literature Searches for Colorectal Cancer Safety Data on Folic Acid Published Subsequent to the NTP Monograph

Search	MeSH Search Terms	Limits	Hits (n)*
1	folic acid AND (cancer, colorectal)	Published since 12/1/2014, Systematic review or Meta-analysis, English language	33
2	folic acid AND (cancer, colorectal)	Published since 8/1/2016, Humans, Clinical trials or Observational study, English language	191

* The number of hits represents output from a search conducted on April 6, 2020.

The title and abstracts of the references identified in the literature searches were reviewed to identify potentially relevant papers. Abstracts that referred to folate and incidence of CRC were critically reviewed. In total, this safety review relies upon evidence from three meta-analyses (Moazzen et al., 2018; Qin et al., 2015; Burr et al., 2017) and one systematic review (Coletta et al., 2019). Two secondary analyses (Passarelli et al., 2019; Rees et al., 2017) of a previously conducted RCT (The Aspirin/Folate Polyp Prevention Study; Cole et al., 2007) provide additional evidence. In addition, based on a review of the current and planned registered clinical trials on clinicaltrials.gov, there are no new or ongoing folic acid trials on CRC being conducted. Thus, the most recent scientific literature consists mainly of secondary analyses of existing trials and observational studies. It is important to note that the results of the literature search yielded no studies that evaluated the association between folate supplementation or intake in the form of 5-TMHF and CRC risk. Information relevant to the safety of folic acid from these meta-analyses, systematic reviews, re-analyses of individual clinical trials, and observational studies is summarized herein.

Systematic Reviews and Meta-Analyses

Moazzen et al. (2018) published a systematic review of 44 RCTs, cohort studies, and case-control studies on the risk of CRC due to folic acid supplement intake or total folate intake from food and dietary supplements published between January 2000 and September 2016, 35 of which were included in stratified meta-analyses. RCTs were excluded if the dose of folic acid supplementation was less than 500 µg/day. Based on four RCTs reporting risk of CRC and six RCTs reporting risk for adenomas, the meta-analyses showed no significant adverse effects associated with folic acid supplementation (RR = 1.07; 95% CI: 0.86-1.43 and RR = 1.00; 95% CI: 0.86-1.51, respectively). Similarly, risk of CRC with folic acid supplementation among cohort studies was not statistically significant (RR = 0.96; 95% CI: 0.76-1.21). The meta-analyses also showed a reduced risk for CRC with increasing total folate intake in nine cohort studies with 13 data points (RR = 0.71; 95% CI: 0.59-0.86) and six case-control studies with 12 data points (RR = 0.77; 95% CI: 0.62-0.95). Both results are consistent with a previously pooled analyses of cohort studies by Kim et al. (2010). When exposure was measured as plasma or red blood cell folate content in nine case-control studies with 10 data points there was no association with risk of CRC (RR = 1.05; 95% CI: 0.85-1.30). When dietary folate intake from food alone

was evaluated, there also was no significant reduction in CRC risk (risk estimate not provided). In a sub-analysis, the authors reported a significant increased risk of CRC based on two studies that reported plasma folate levels. However, the authors raise concerns about this analysis given that these studies lack baseline plasma folate levels and the inferiority of the plasma folate biomarker compared to red blood cell folate as an indicator of long-term folate intake. The authors offer a biological explanation of this potentially adverse effect by suggesting that methylation in gene promoters such as CpG Island may trigger tumorigenesis in normal mucosal colorectal cells.

Qin et al. (2015) conducted a systematic review of RCTs published before October 2014 that evaluated the risk of CRC from folic acid supplementation. The review resulted in eight RCTs that met their inclusion criteria of providing details on CRC incidence and levels of folic acid supplementation. In a meta-analysis, Qin et al. (2015) reported no association of folic acid supplementation with risk of CRC in the total sample of participants (RR = 1.00; 95% CI:0.82–1.22) and the lack of association remained when the analysis was stratified by ethnicity, gender, body mass index, dose of folic acid, or duration of the study. Results were similar when limited to studies among individuals with prior colorectal adenomas (N=3 studies; RR = 0.81; 95% CI: 0.40-1.62).

Coletta et al. (2019) performed a systematic review of the evidence related to a variety of potential risk factors such as dietary habits including folic acid supplementation and the risk of CRC and endometrial cancer among individuals with Lynch syndrome, an inherited disorder that increases the risk of CRC and several other cancers. The authors identified one retrospective cohort study, which compared self-reported ≥1-month folic acid supplementation to less frequent or non-users, and a case-control study that compared folic acid supplement users to non-users. In their narrative findings, the authors report that neither study showed any association with folic acid supplementation and a risk of CRC.

Burr et al. (2017) performed a systematic review and meta-analysis of studies reporting the use of folic acid supplementation and incidence of CRC in patients with inflammatory bowel disease (IBD). CRC is noted to be a serious side effect of IBD, and the purpose of this study was to determine the role of folic acid supplementation as a chemo preventative agent for CRC among this diseased population. The meta-analysis, which pooled 10 cohort and case-control studies and resulted in a total of 4517 patients, suggested a protective effect for folic acid: pooled hazard ratio (HR) = 0.58 (95% CI: 0.37-0.80) with low to moderate heterogeneity among the studies included. They found this protective association to be accentuated in studies that were performed before the required folic acid fortification in the U.S.: pooled HR = 0.47 (95% CI: 0.20-0.75). The authors note that IBD patients are at an increased risk of folate deficiency due to inadequate nutritional intake, excessive intestinal loss, and reduced absorption due to competitive inhibition, and so were unlikely to identify studies displaying elevated levels of circulating folic acid in this subpopulation. Most of the studies also did not report dose of folic acid supplementation, instead only comparing users to non-users.

Secondary Analyses of The Aspirin/Folate Polyp Prevention Study

Passarelli et al. (2019) reanalyzed data from an RCT of folic acid supplementation (1000 µg/d) for the prevention of colorectal adenomas among individuals who had been recently diagnosed

with one or more adenomas (Cole et al., 2007). The RCT was first extended beyond its three-year treatment period due to a suggestion that longer exposure to folic acid might be necessary to observe an effect. Participants were given the option to discontinue treatment/placebo but continue to be followed observationally for health outcomes. During the second surveillance period, the RCT was terminated early on October 1, 2004, due to indications of increased risk of advanced and multiple adenomas (i.e., ≥3). All participants were unblinded to assignment on April 11, 2005. The original report used an intent-to-treat analysis for the second surveillance period, including both those who did and did not agree to extend treatment after the first surveillance period, and limiting follow-up only to colonoscopies that took place before study termination (Cole et al., 2007). The reanalysis conducted by Passarelli et al. (2019) instead focused only on those who agreed to extend treatment and included all colonoscopies that occurred until the intended end of the second period. The authors also performed a new analysis to evaluate the occurrence of neoplastic lesions during a post-treatment surveillance among all participants who agreed to follow-up. Post-treatment surveillance included the first or only colonoscopy observation after the first surveillance period for those who did not extend treatment, and first or only observation after the second surveillance period for those who did extend treatment, up until the conclusion of follow-up on May 31, 2012. Throughout the reanalysis, a new classification for sessile serrated adenomas/polyps (SSA/Ps) was used according to the currently accepted definition of including growths with cytological dysplasia. While the original study (Cole et al., 2007) identified elevated risks for advanced and multiple conventional CRC adenomas in the second surveillance period (RR = 1.67; 95% CI:1.00–2.80, RR = 2.32; 95% CI:1.23–4.35, respectively), the reanalysis showed an attenuated and no longer statistically significant relationship (RR = 1.20; 95% CI: 0.73- 1.97, RR = 1.58; 95% CI: 0.87-2.86, respectively). Increased risk of SSA/Ps, however, was statistically significant during the second surveillance period (RR= 1.94; 95% CI: 1.02- 3.68), although the increase in absolute risk was modest (8% compared with 5% in the folic acid and placebo treatment arms, respectively). There were also nonsignificant suggestions of an increased risk of any neoplasia during the second surveillance period among individuals who extended treatment and completed the second surveillance period (RR=1.21; 95% CI: 0.99- 1.47; P=0.06). There was no evidence of an elevated risk for any colorectal neoplasia (RR= 1.01; 95% CI: 0.80- 1.28) or for SSA/Ps (RR=1.38; 95% CI: 0.59- 3.19) during post-treatment surveillance.

Rees et al. (2017) also performed a reanalysis of the aforementioned RCT (Cole et al., 2007), focusing on methylated folates (sum of 5-MTHF and 4-α-hydroxy 5-methyl-THF) as well as unmetabolized folic acid in plasma samples taken at enrollment and the first surveillance period. The authors report no association between either methylated or unmetabolized folic acid and overall adenoma risk. However, during the second surveillance period, the risk of advanced or multiple adenomas was positively associated with methylated folate ($P_{\text{linear trend}} = 0.009$), with a 58% increased risk in participants in the highest versus lowest quartile of plasma methylated folate concentrations. The pattern of the risk of advanced or multiple adenomas across unmetabolized folic acid categories was irregular, although it suggested an inverse relationship ($P_{\text{linear trend}} = 0.049$). Furthermore, an inverse relationship was identified between methylated folates and serrated lesions ($P_{\text{linear trend}} = 0.03$). The authors reported there was no effect modification by sex or aspirin use during the study, although genetic polymorphisms that accelerate folate metabolism may have increased the occurrence of high-risk adenomas. The authors note that these findings only indirectly relate to the study of CRC, given that adenomas and serrated polyps are benign or precancerous. The authors also highlight potential imprecision

in exposure measurements given the blood draws were not timed to intake of food or study tablets.

Biosynthesis of Folate by the Microbiota and CRC Risk

In addition to concerns related to high folic acid intake and the risk of CRC, the potential for additional folate exposure within an individual resulting from the biosynthesis of B vitamins by the gut microbiome has been considered. There is evidence that bacteria present in the human colon may biosynthesize folate, which can be absorbed across the apical side of colonocytes and enter circulation. The end-product of *de novo* folate synthesis by the intestinal microbiota is THF or THF-polyglutamate, the forms of folate found in dietary sources (Rossi et al., 2011; Engevik et al., 2019). Recent evidence suggests that approximately 13% of 512 bacterial reference genomes contain all genes involved in THF production and 39% contain genes to synthesize intermediate metabolites of folate production, including chorismate, para-aminobenzoic acid, and dihydropterin pyrophosphate (Engevik et al., 2019). Specifically, bifidobacteria and lactobacilli are identified as producers of folate *in vivo* and have been extensively researched (Rossi et al., 2011). The biosynthesis of folate by the gut microbiome was recognized and considered during the development of dietary reference intakes of folate by the IOM and EFSA (IOM, 1998; EFSA, 2014). The clinical trials used in the determination of the DRIs were conducted among populations with an intact microbiome (as noted in Lakoff et al., 2014). Therefore, the IOM and EFSA reference intakes account for dietary intake of folate and folic acid in addition to that contributed by the biosynthesis of folate in the colon. A genome analysis of microorganisms found in the human gastrointestinal tract estimated that biosynthesized folate from the gut microbiome can contribute approximately 37% of the recommended dietary allowance for adult males and non-pregnant or lactating females (Magnusdottir et al., 2015), equivalent to approximately 148 μg/day. However, the level of folate produced by the gut microbiota has not been quantified in experimental trials (IOM, 1998; EFSA, 2014; ODS, 2020; Kok et al., 2020).

While the amount of folate produced via biosynthesis and absorbed has not been quantified, the fecal excretion of folate suggests that not all is absorbed in the colon. Recent research has identified factors that may affect folate biosynthesis and uptake of biosynthesized folate by colonocytes. These factors include composition of the diet, composition of the intestinal microbiota, and human genetic variation (summarized in Kok et al., 2020). There is little evidence to support an association between colonic folate levels and colorectal cancer and no critical upper bound of folate biosynthesis has been established (Kok et al., 2020). In fact, some evidence suggests a decrease in risk of advanced adenoma with increasing colonic folate levels (Flood et al., 2011).

Colorectal Cancer Summary

The NTP cancer sub-panel concluded in 2015 that clinical research consistently showed that while inadequate dietary folate intake increases the risk of CRC in humans, there does not appear to be a benefit from supplementation among those with an adequate folate status at baseline. The sub-panel also noted that folic acid supplementation may have an adverse effect on cancer growth though the available evidence was limited and a cause-and-effect relationship could not be confirmed.

Recently published meta-analyses have shown that folic acid supplementation does not increase the risk of CRC based on pooled results from numerous RCTs (Moazzen et al., 2018; Qin et al., 2015) and cohort studies (Moazzen et al., 2018). In the two published re-analyses of the Aspirin/Folate Polyp Prevention Study (Passarelli et al., 2019; Rees et al., 2017), the risks observed in the original analysis were attenuated and/or further explained. While the original analysis by Cole et al. (2007) identified a statistically significant elevated risk for advanced and multiple conventional CRC adenomas, the Passarelli et al. (2019) re-analysis showed an attenuated and no longer statistically significant relationship. Among individuals with previous CRC lesions, there is some suggestive evidence of an increased risk of CRC based on secondary analyses of a single RCT (Passarelli et al., 2019; Rees et al., 2017). However, the number of studies remains limited and the results are equivocal. Further, the current literature has not identified or quantified the potential additive effect of folic acid and natural (including biosynthesized) folate on CRC risk, nor is there a clear mechanism of how folate versus folic acid could differentially impact CRC risk (Kok et al., 2020). Overall, while there are reports of possible adverse effects of high folic acid intake on cancer growth, underlying mechanisms remain to be elucidated and more research is necessary to establish a causal relationship. This review of the potential adverse effect of folic acid or biosynthesized folate on CRC risk does not offer any conclusive evidence of cause and effect, and, consequently, does not appear to impact the conclusion on the safety of folate by the IOM, EFSA, and FDA at this time.

Summary

Folate exists in various forms in the diet and in supplements. Food folate is intrinsic in foods, while folic acid is a synthetic food fortificant or supplement, and L-5-MTHF is naturally found in food and commercially available as a crystalline form of the calcium salt, i.e., L-5-MTHF-Ca (Metafolin®). L-5-FTHF is also naturally found in food and available commercially as a calcium L-5-formyltetrahydrofolate. All folate forms share a common metabolic fate, namely, conversion to L-5-MTHF. Once absorbed, circulating folates are indistinguishable.

Arcofolin® and Metafolin® are related compounds. Both are salts of L-5-MTHF (Arcofolin®: L-5-MTHF-Na and Metafolin®: L-5-MTHF-Ca), produced in similar fashion, and have similar specifications. These salts are expected to completely dissociate *in vivo* and have comparable bioavailability. The comparable bioavailability of Arcofolin® and Metafolin® is supported by findings of similar plasma L-5-MTHF and total folate responses relative to folic acid controls. Therefore, publicly available relevant safety data on Metafolin® can be relied upon to evaluate the safety of the proposed use of Arcofolin®.

The publicly available data indicate that the bioavailability of the L-5-MTHF-Ca is at least as high as that of folic acid and that the fate of L-5-MTHF-Ca is indistinguishable from that of other absorbed and metabolized natural folate forms. In a 2008 assessment, FSANZ similarly concluded that the available evidence supports that L-MTHF and folic acid are “essentially bioequivalent.” Given the recent clinical data and the statements from several authoritative bodies, it is reasonable to conclude that the bioavailability of L-MTHF salts, including both the L-5-MTHF-Ca and sodium salt, is comparable to that of folic acid.

The IOM (1998) and EFSA (2014) established tolerable ULs for folate from fortified foods or supplements (folic acid form) as 1 mg /day for adults. This UL was established based on the relationship of folate intake and neurological dysfunction; folate has the potential to reverse megaloblastic anemia due to vitamin B_{12} deficiency, thereby delaying the timely diagnosis and appropriate treatment of the disease and allowing the neurological dysfunction caused by vitamin B_{12} deficiency to progress. L-5-MTHF supplementation reduces the potential for masking the symptoms of vitamin B_{12} deficiency as well as the hematological manifestations of the deficiency, the complications of which formed the basis for the UL. Thus, the current UL derived for supplemental folate in the form of folic acid is likely a conservatively low reference for safe intake of supplemental folate from the L-5-MTHFform.

In 2016, FDA conducted a safety review and risk assessment on folic acid and concluded that use of folic acid in foods is safe. The Agency was not concerned that any of the intake estimates exceeding the IOM UL for folate would cause adverse health impact in any of the population subgroups (FDA, 2016b). In its review, FDA noted that the risk of masking B_{12} vitamin deficiency and related neurological complications is low because the most at-risk population (50 years and older) would be taking multivitamins. The proposed use of Arcofolin® is substitutional for select uses of folic acid, thus, dietary exposure to total folate in the U.S. population is not expected to be impacted. Thus, it is reasonable to conclude that intake of a L-5-MTHF salt, namely Arcofolin®, in place of folic acid is also safe.

JECFA considered the safety of L-5-MTHF-Ca and reported no safety concern for its proposed use in dry crystalline or microencapsulated form as an alternative to folic acid used in dietary supplements, foods for special dietary uses, and other foods (JECFA, 2005). FSANZ also concluded that the use of L-5-MTHF-Ca for the fortification of certain foods would not raise public health or safety concerns (FSANZ, 2008). In the U.S., NDINs were filed for the use of L-5-MTHF in dietary supplements as a source of folate and the notifications were accepted for filing and acknowledged without objection by FDA. Recently, EFSA concluded that the proposed uses of L-5-MTHF-Ca as a folate source in infant formula, follow-on formula, processed cereal-based food and baby food for infants (<12 months in age) and young children (12 - 36 months in age) is safe and that the compound does not pose any concern for allergenicity (EFSA, 2020).

In a series of studies published by Niederberger et al. (2019), L-5-MTHF-Ca was found to be non-genotoxic, and developmental toxicity studies of standard design did not reveal any toxic potential to fetuses or pregnant dams. Previously, EFSA (2004) reviewed the same studies and reported the same conclusion. Unpublished genotoxicity studies on Arcofolin® were negative, providing corroborative evidence of non-genotoxicity. Specific reproductive and chronic safety studies have not been conducted. A 13-week rat gavage study that was previously reviewed by EFSA in 2004 was recently published by Niederberger et al. (2019). The authors established a NOAEL for L-5-MTHF-Ca of 400 mg/kg bw/day based on the absence of any treatment-related effects at this highest tested dose. Assuming that Arcofolin® will substitute for all other sources of folic acid in foods (except for corn masa flour), the 95th percentile cumulative intake of folic acid is 919 µg/day among age group 51 - 70 years (FDA, 2016b). Using a default body weight of 60 kg, a cumulative intake of 15.3 µg/kg bw/day can be estimated, which is well below the NOAEL for Arcofolin®, with a margin of exposure (MOE) > 25000.

Although there are reports of possible adverse effects of high folic acid intake on cancer growth, underlying mechanisms remain to be elucidated and more research is necessary to establish a causal relationship. A review of the potential adverse effect of folic acid or biosynthesized folate on colorectal cancer risk does not offer any conclusive evidence of cause and effect, and consequently, does not appear to impact the conclusion on the safety of folate by the IOM, EFSA, and FDA at this time.

Safety Conclusion

Arcofolin®, a monosodium salt of L-5-methyltetrahydrofolic acid is intended for use as a source of folate, substitutional to folic acid at levels not exceeding 400 micrograms ([micro]g) per serving in select food categories for which folic acid has been approved, namely breakfast cereals, corn grits, infant formula, medical foods, food for special dietary use, and foods represented to be meal-replacement products. L-5-MTHF, the biologically active form of folate, is naturally found in food and is commercially available as a crystalline form of the calcium salt, i.e., L-5-MTHF-Ca (Metafolin®). Arcofolin® and Metafolin® are essentially equivalent with respect to chemical composition. Furthermore, the publicly available data indicate that the bioavailability of the L-5-MTHF-Ca is at least as high as that of folic acid, and that the fate of L-5-MTHF-Ca is comparable to that of other absorbed and metabolized natural folate forms. The IOM (1998) and EFSA (2014) established a UL for folate from fortified foods or supplements (folic acid form) of 1 mg/day for adults, which, when applied to supplemental folate in the form of folic acid, is a conservative level of intake that has been applied to supplemental folate from the L-5-MTHF form. Assuming that Arcofolin® will substitute for all other sources of folic acid in foods (except for corn masa flour) consistent with the food additive regulation, the 95th percentile cumulative intake of folic acid is 919 µg/day among age group 51 - 70 years (FDA, 2016b), which is below the UL of 1 mg /day for adults. Further, using a default body weight of 60 kg, a cumulative intake of 15.3 µg/kg bw/day can be estimated, which is well below the NOAEL for Arcofolin®, with an MOE > 25000. A series of authoritative reviews have opined that the proposed use of folate salts such as Arcofolin®, when used as substitutional for select uses of folic acid, would have no impact on dietary exposure to total folate. Thus, it would reasonable to conclude that intake of a L-5-MTHF salt, namely Arcofolin®, in place of folic acid is safe. Additionally, published pre-clinical studies demonstrated that the bioequivalent Metafolin® is non-genotoxic, does not demonstrate reproductive or developmental toxicity, or cause any toxicologically significant effects at all tested doses in 13-week oral toxicity studies. Unpublished genotoxicity studies on Arcofolin® were negative, providing corroborative evidence of non-genotoxicity.

Therefore, it can be concluded that the proposed use of Arcofolin® as an alternative to folic acid in select food categories for which folic acid is an approved added nutrient, namely breakfast cereals, corn grits, infant formula, medical foods, food for special dietary use, and foods represented to be meal-replacement products is safe within the meaning of the FD&C Act, i.e., meets the standard of reasonable certainty of no harm under the conditions of intended use.

Discussion of Information Inconsistent with GRAS Determination

No information has been identified that would be inconsistent with a finding that the proposed use of Arcofolin® in select foods, meeting appropriate specifications specified herein and used according to cGMP, is safe and GRAS based on scientific procedures, under the conditions of intended use in foods.

Basis for Conclusion that there is Consensus Regarding Safety

General recognition of safety through scientific procedures requires common knowledge throughout the scientific community knowledgeable about the safety of food ingredients that there is a reasonable certainty that a substance is not harmful under the intended conditions of use in foods. The aforementioned regulatory and scientific reviews related to the consumption and safety of L-5-MTHF are published in the scientific literature, and therefore are generally available and generally known among the community of qualified food ingredient safety experts. There is broad-based and widely disseminated knowledge concerning L-5-MTHF and forms of folate with the same metabolic fate. The data and publicly available information supporting the safety of the proposed use of Arcofolin® as an alternative to folic acid in select food categories for which folic acid is an approved added nutrient, including breakfast cereals, corn grits, infant formula, medical foods, food for special dietary use, and foods represented to be meal-replacement products, as described herein are widely known and disseminated and are also commonly accepted among qualified food safety experts.

A GRAS Panel that consisted of the following individuals—Dr. Jesse Gregory (Professor Emeritus of Food Science and Human Nutrition University of Florida), Dr. Claire Kruger (Spherix Consulting Group), and Dr. Peter Pressman (The Daedalus Foundation)—critically evaluated Exponent's safety documentation (the dossier) and other available data and information that the members of the GRAS Panel believed to be pertinent to the safety of the proposed use of Arcofolin® intended as a source of folate, substitutional to folic acid. The GRAS Panel convened on April 1, 2020 via teleconference and independently, jointly, and unanimously concluded that Arcofolin®, a monosodium salt of L-5-methyltetrahydrofolic acid produced consistent with cGMP and meeting the stated specifications, is safe for use as an alternative to folic acid in select food categories for which folic acid is an approved added nutrient, including breakfast cereals, corn grits, infant formula, medical foods, food for special dietary use, and foods represented to be meal-replacement products. The GRAS Panel further concluded unanimously that the intended use of Arcofolin® in select foods is GRAS based on scientific procedures. It is also the unanimous consensus opinion of this GRAS Panel that other qualified experts would concur with these conclusions. The GRAS Panel Signed Consensus Statement is located in **Appendix E.**

The intended use of use of Arcofolin® in select foods has been determined to be safe through scientific procedures as set forth in 21 CFR§170.30(b), thus satisfying the so-called "technical" element of the GRAS determination. Because this safety evaluation was based on generally available and widely accepted data and information, it also satisfies the so-called "common knowledge" element of a GRAS determination.