

CALCIUM L-5-METHYLTETRAHYDROFOLATE (L-5-MTHF-Ca)

Chemical and Technical Assessment (CTA)

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1 Summary

Calcium L-5-methyltetrahydrofolate (L-5-MTHF-Ca; CAS No. 151533-22-1) is the calcium salt of L-5-methyltetrahydrofolic acid (L-5-MTHF), which is the predominant naturally occurring folate in foods. Folic acid (pteroyl glutamic acid, sometimes known as Vitamin B₉), which is widely used for food fortification and as an ingredient in dietary supplements, is the precursor of L-5-MTHF. L-5-MTHF-Ca is intended for use in dry crystalline or microencapsulated form as an alternative to folic acid in foods, including dietary supplements.

L-5-MTHF-Ca is a white to light yellowish, almost odourless, water-soluble crystalline powder. It is obtained by reduction of synthetic folic acid with sodium borohydride, condensation of the resulting tetrahydrofolic acid with formaldehyde, further reduction of the 5,10-MTHF with NaBH₄ to L-5-MTHF, and finally crystallization as the calcium salt.

Crystalline L-5-MTHF-Ca is stable on storage (48 months at 40 °C and up to 75% RH) after micronizing, and when compounded into vitamin and mineral tablets. It appears to be better suited than folic acid for the vitamin enrichment of foods. L-5-MTHF-Ca in microencapsulated form, preferably with ascorbate as an antioxidant, has also been shown to have long-term stability in a variety of foodstuffs.

Tests with L-5-MTHF-Ca have shown that L-5-MTHF has a similar or slightly higher bioavailability and bioefficacy than folic acid.

2 Description

Calcium L-5-methyltetrahydrofolate (L-5-MTHF-Ca; C₂₀H₂₃CaN₇O₆; formula weight 497.5; CAS No. 151533-22-1) is a white to light yellowish, almost odourless crystalline powder. The commercial product contains variable amounts of water of crystallization.

L-5-MTHF-Ca is sparingly soluble in water (1.07 g/100 g at 20 °C) and very slightly soluble or insoluble in most organic solvents. It is soluble in alkaline hydroxides.

L-5-MTHF-Ca decomposes above 300 °C and has a specific rotation of +45.0° to +50.0°. The pH of an aqueous solution (0.5 g/100 ml) is between 6.5 and 7.5.

The full chemical name for L-5-MTHF-Ca is N-{4-[[[(6S)-2-amino-3,4,5,6,7,8-hexahydro-5-methyl-4-oxo-6-pteridiny]methyl]amino]benzoyl}-L-glutamic acid, calcium salt. L-5-MTHF-Ca is also referred to in the literature as:

- L-Methylfolate, calcium;
- L-5-Methyltetrahydrofolic acid, calcium salt [(L-5-MTHF-Ca)];
- (6S)-5-Methyltetrahydrofolic acid, calcium salt [(6S)-5-MTHFCa];
- (6S)-5-Methyl-5,6,7,8-tetrahydropteroyl-L-glutamic acid, calcium salt;
- L-5-Methyl-tetrahydrofolic acid (L-5-MTHF), i.e. the cation is not specified.

3 Method of manufacture

L-5-MTHF-Ca is obtained by the reduction of synthetic folic acid with sodium borohydride, condensation of the resulting tetrahydrofolic acid with formaldehyde, further reduction of the 5,10-MTHF with NaBH₄ to L-5-MTHF, and finally crystallization as the calcium salt. An abbreviated reaction scheme is given in Appendix 1.

In the first step, reduction of folic acid with sodium borohydride proceeds under alkaline conditions to yield racemic tetrahydrofolic acid, i.e. (6R,S)-THF. A proprietary reagent is added which allows the 6S diastereoisomeric adduct to be selectively separated and recovered by crystallization. The adduct may be purified by further recrystallization. The (6S)-THF is then recovered from the adduct by treatment with base and reacted with formaldehyde to give (6R)-5,10-methylene-THF. Alkaline reduction with NaBH₄ yields (6S)-5-MTHF. Salts of boric acid are precipitated and filtered off. Acidification of the filtrate with HCl results in crystallization of (6S)-5-MTHF (free acid form), which is collected and redissolved in water. The solution is decolorized with activated charcoal, and filtered. Addition of calcium chloride to the filtrate results in crystalline L-5-MTHF-Ca.

4 Characterization

L-5-MTHF-Ca has two chiral carbon atoms at, respectively, position 6 of the pteroyl moiety and the α -C atom in the L-glutamic acid moiety (Groehn & Moser, 1999), and as a result there are four possible diastereoisomers. To avoid confusion about the stereochemistry, all naturally occurring diastereoisomers of reduced folates are defined as L-diastereoisomers and all others as D-diastereoisomers. The α -C atom in the glutamic acid moiety of L-5-MTHF-Ca comes from the starting material, folic acid, and its configuration remains unchanged (α S or L) during the synthesis. Therefore, both chiral centres in L-5-MTHF-Ca have the natural L-configuration. UV, IR, MS, and NMR spectra are available for L-5-MTHF-Ca.

Impurities in L-5-MTHF-Ca are of three classes (see Appendix 2 for structures):

1. Minor amounts of other folates:
 - Folic acid
 - Tetrahydrofolic acid
 - 5,10-Methylenetetrahydrofolic acid

The last two occur naturally in foodstuffs (Freisleben et al., 2003).

2. Breakdown and oxidation products of L-5-MTHF-Ca:
 - 4-Aminobenzoylglutamic acid
 - 5-Methyltetrahydroptericoic acid
 - 4 α -Hydroxy-5-methyltetrahydrofolic acid
 - 2-Amino-8-methyl-4,9-dioxo-7-methyl-p-aminobenzoylglutamate-6,7,8,9-tetrahydro-4H-pyrazino-(1,2-a)-s-triazine

The first three have been detected in food as a consequence of folate degradation (Blair et al., 1975; Ratanasthien et al., 1977; Gregory et al., 1984): 4 α -hydroxy-5-methyltetrahydrofolate has also been found in the urine of rats as a metabolic breakdown product of folic acid (Barford & Blair, 1975).

3. Other products: a diastereoisomer of 5-MTHF and the dimethylated form of tetrahydrofolic acid.

Impurities noted in 1 and 2 should not exceed 2.5% and the (6R)-diastereoisomer of 5-MTHF should not exceed 1% in L-5-MTHF-Ca.

L-5-MTHF-Ca has the (6S, α S) configuration, while the D-isomer has the (6R, α S) configuration. Since the configuration of the α -C atom of the L-glutamate moiety is conserved during the production process of L-5-MTHF-Ca, the corresponding (α R) stereoisomers do not occur in the product.

The purity of the L-5-MTHF-Ca being evaluated at the 65th JECFA is stated by the sponsor as 95% or above. The test products used in the 13-week oral toxicity and reproductive toxicity studies in rats evaluated by the European Food Safety Authority (EFSA) had reported purities of 97.1 and 99.9% respectively (EFSA, 2004). Specifications for identity and purity of the material intended to be added to

food will include limits for water content, residues of ethanol used in the manufacturing process as a solvent, calcium content, a limit for lead, and folate impurities and related substances.

5 Functional uses of L-5-MTHF-Ca

L-5-MTHF-Ca is intended for use in dry crystalline or microencapsulated form, as an alternative to folic acid (pteroyl glutamic acid, sometimes known as Vitamin B₉), in dietary supplements, in foods for particular special dietary uses and in other foods for fortification. L-5-MTHF-Ca is the calcium salt of naturally occurring L-5-methyltetrahydrofolic acid (L-5-MTHF), the latter acting in the body as an enzyme cofactor for transport of C₁ in methylation reactions in the biosynthesis of pyrimidines, purines, serine and glycine.

A panel of scientific experts in the USA has determined that L-5-MTHF-Ca is GRAS (Generally Recognized as Safe) for its intended use (Borzelleca *et al.*, 1999), and L-5-MTHF-Ca may be lawfully used as a dietary ingredient in the USA. EFSA adopted the favourable opinion of its Scientific Panel for L-5-MTHF-Ca as a source of folate in foods for specific nutritional uses (upper limit 1 mg/adult person per day (EFSA, 2004)). An advantage over folic acid is that it is unlikely to mask clinical symptoms of Vitamin B₁₂ deficiency (Scott, 2001).

Folic acid, although widespread in nature, is not abundant in quantity, and L-5-MTHF is the predominant natural form of folates. Tests with L-5-MTHF-Ca have shown that L-5-MTHF has a more consistent and similar or slightly higher bioavailability than folic acid (Prinz-Langenohl *et al.*, 1999). A 26% higher absorption of L-5-MTHF compared with folic acid has also been observed (Finglas, undated). It is also more highly bioeffective (bioefficacy is the efficiency with which a nutrient ingredient is absorbed and converted to its bioactive form) (Vanlieshout *et al.*, 2001) because folic acid must first be reduced in two enzymatic steps, whereas L-5-MTHF can be used directly in methylation reactions. L-5-MTHF-Ca, in contrast to folic acid, is also unlikely to mask clinical symptoms of Vitamin B₁₂ deficiency (Gutstein *et al.*, 1973; Hasselwander *et al.*, 2000; Scott, 2001).

Intakes of folates of more than the 1 mg/day set by the European Community Scientific Committee on Food in 2000 (SCF, 2000) (to avoid masking of B₁₂ deficiency) have been advocated for increased protection from neural tube defects (spina bifida) (Verhaar *et al.*, undated). However, in the absence of data on the relative influence of folic acid and L-5-MTHF on the progressive neuropathy caused by B₁₂ deficiency, EFSA has considered it prudent to apply the 1 mg/day level for L-5-MTHF-Ca, when used as a source of folate in foods for specific nutritional uses, food supplements and traditional foods (EFSA, 2004).

6 Reactions and fate in foods

For many years, the folate content of foods has been assessed by a time-consuming microbiological assay. HPLC methods have been developed more recently (Konings *et al.*, 1999; Freisleben *et al.*, 2003; Pawlosky *et al.*, 2003; Thomas *et al.*, 2003). Added L-5-MTHF-Ca can be quantified equally well by either method, and the same is true for naturally occurring L-5-MTHF or added folic acid.

Although folic acid is stable under most food processing and storage conditions, the reduced forms in solution are susceptible to oxidation (Blair *et al.*, 1975; Ratanasthien *et al.*, 1977; Williams *et al.*, 1995). Consequently, the folate content of foods may decrease substantially during cooking (Leichter *et al.*, 1978; Hawkes & Villota, 1989) or even on storage at ambient temperatures (Anderson & Öste, 1992). Crystalline L-5-MTHF-Ca is stable on storage (48 months at 40 °C and up to 75% RH) after micronizing, and when compounded into vitamin and mineral tablets. Micro-encapsulated L-5-MTHF-Ca (using different inert carriers) was found to be about 90% recoverable from baked bread, and 75% recoverable from breakfast cereals, in which it had been incorporated during the manufacturing process. The L-5-MTHF-Ca could be released completely from the encapsulation by incubation in an artificial digestive system at pH 6.8 for 3 hours (Biodar, 2000).

A loss of about 20% folic acid and natural folates in fortified flour (3 mg/kg) was measured during the baking of bread, but baking did not reduce the level of 5-MTHF (Osseyi *et al.*, 2001). Good stability of native L-5-MTHF was found in oven-baked chicken and fish, in cooked liver sausage, and during frozen storage of beef liver and strawberries (Vahteristo *et al.*, 1998). L-5-MTHF-Ca has been shown to be more stable in complex food systems than in simple aqueous buffer solutions. Folate-binding protein in

milk is particularly efficient in its stabilizing effect on L-5-MTHF-Ca (Jones & Nixon, 2002). It seems that L-5-MTHF-Ca in microencapsulated form, preferably with ascorbate as an antioxidant, has long-term stability in a variety of foodstuffs.

From a stability aspect, L-5-MTHF-Ca appears to be better suited than folic acid for the vitamin enrichment of foods (Hasselwander *et al.*, 2000).

7 References

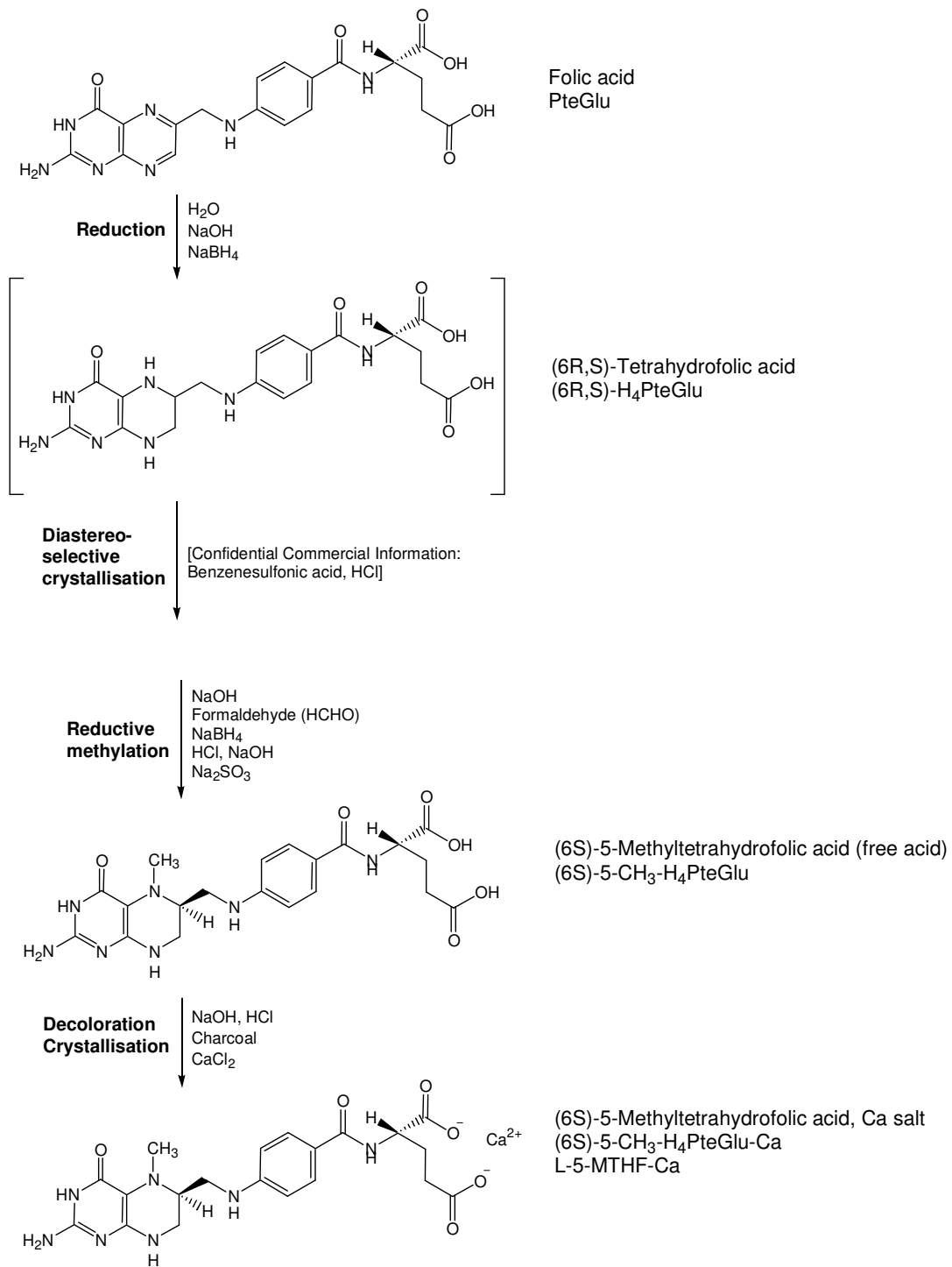
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Appendix 1: Flow chart of L-5-MTHF-Ca production



Appendix 2: By-products of the production process

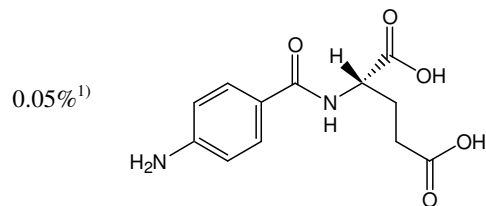
| Name | Structural formula | Typical levels found |
|-------------------------------------|--------------------|----------------------|
| <i>A. Other folates</i> | | |
| Folic acid | | 0.05% ¹⁾ |
| Tetrahydrofolic acid | | 0.01% ¹⁾ |
| 5,10-Methylene-tetrahydrofolic acid | | 0.03% ¹⁾ |
| | | |

¹⁾ Limit of detection: 0.01%

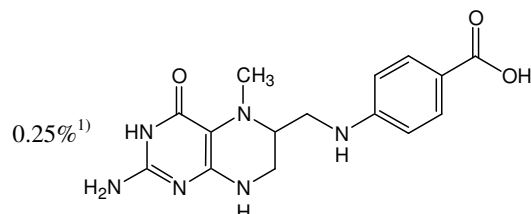
Appendix 2 (continued)

B. Oxidation and degradation products without folate activity

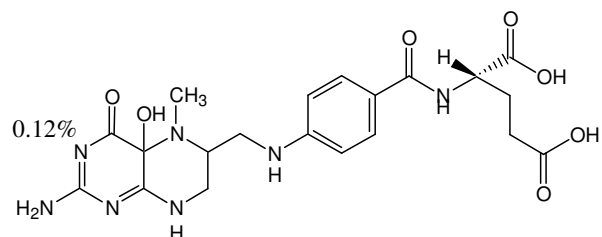
4-Aminobenzoylglutamic acid



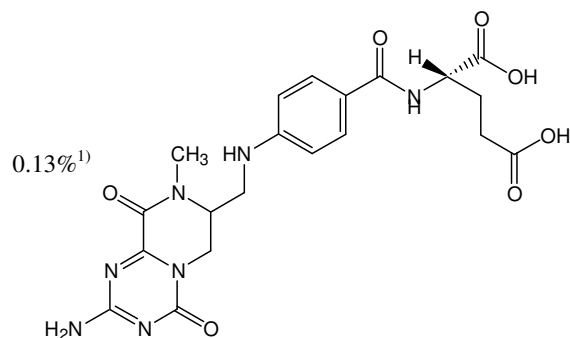
5-Methyltetrahydro
pterotic acid



4 α -Hydroxy-5-methyl-
tetrahydrofolic acid



Pyrazino-s-triazine
derivative²⁾



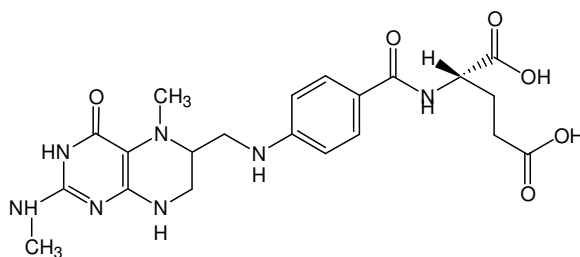
¹⁾ Limit of detection: 0.01%

²⁾ 2-Amino-8-methyl-4,9-dioxo-7-methyl-p-aminobenzoylglutamate-6,7,8,9-tetrahydro-4H-pyrazino-(1,2-a)-s-triazine

Appendix 2 (continued)

C. Others

Dimethyltetrahydro-
folic acid



0.1%¹⁾