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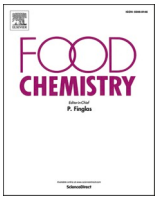

Short communication

Complete loss of 5-methyltetrahydrofolate in wheat during simulated gastric digestion

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ABSTRACT

Folate deficiency affects over one billion people globally, driving widespread cereal fortification programs. 5-methyltetrahydrofolate (5-MTHF), the predominant natural folate form, is emerging as a safer alternative to traditional folic acid for fortification. However, conflicting reports exist regarding its digestive stability. Here we demonstrate that 5-MTHF undergoes an apparent complete loss in wheat matrices during simulated gastric digestion. This loss occurred across all wheat fractions tested and exhibited concentration-dependent kinetics, with folate retention improving from undetectable levels to 80 % when wheat flour concentration was reduced fifty-fold. Ascorbic acid provided substantial protection, whereas gallic acid showed negligible effects. Nitrogen purging failed to prevent the phenomenon, and enzymatic analysis of digestion residues confirms that it was not attribute to simple physical absorption. These findings suggest endogenous wheat components promote a rapid, largely oxygen-insensitive loss of 5-MTHF, challenging existing fortification paradigms and underscoring the necessity for protective strategies in cereal-based folate delivery systems.

1. Introduction

Folate deficiency represents a critical global health challenge, affecting over one billion people and serving as a leading cause of neural tube defects, megaloblastic anemia, and cardiovascular disease (Balashova et al., 2024; Maynard et al., 2024; Stevens et al., 2022). To address this crisis, many countries have implemented mandatory folate fortification programs (mainly with folic acid, a synthetic form of folate), with cereals serving as primary vehicles due to widespread consumption (Crider et al., 2011).

However, growing concerns about folic acid, including potential masking of vitamin B12 deficiency and accumulation of unmetabolized folic acid in circulation (Cao et al., 2023; Patel & Sobczyńska-Malefora, 2017), have prompted interest in natural folate or alternative fortification strategies. 5-Methyltetrahydrofolate (5-MTHF), the predominant bioactive folate form in natural foods, is emerging as the preferred fortification agent due to its superior safety profile and bioavailability (Ferrazzi et al., 2020; Troesch et al., 2019). Unlike synthetic folic acid, 5-MTHF bypasses metabolic limitations and directly enters the folate cycle, making it particularly valuable for both populations relying on natural folate sources and those in regions considering new fortification approaches.

Current dietary guidelines and fortification policies assume that exogenously added 5-MTHF maintains superior bioavailability compared to natural dietary folate, with regulatory agencies typically assigning bioavailability factors of 1.7–2.0 for 5-MTHF relative to food folate (EFSA Panel on Nutrition, Novel Foods and Food Allergens (NDA Panel), et al., 2023). These assumptions form the basis for fortification dosing calculations, with the expectation that lower amounts of 5-MTHF can achieve equivalent nutritional outcomes compared to natural folate sources. However, these bioavailability estimates may not capture matrix-mediated protective effects or the accelerated pre-absorptive loss of 5-MTHF during digestion (Liu et al., 2024, Liu et al., 2025).

For example, milk- and/or soy-based matrix can stabilize 5-MTHF during storage and heat treatments, primarily through protective protein binding (Fang et al., 2025; Johns et al., 2017). Cereals, however, contain lower endogenous 5-MTHF (Ložnjak et al., 2020) and show reduced stability of both endogenous and fortified 5-MTHF during digestion (Liu et al., 2021, Liu et al., 2022a, Liu et al., 2022b; Ringling & Rychlik, 2017). This may, at least in part, reflect a comparatively pro-oxidative and weakly protective matrix microenvironment of cereal, indicating that bioavailability gains from cereal fortification could be

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partly overestimated.

To provide initial evidence of this critical phenomenon, the present study conducted a preliminary investigation of 5-MTHF stability in wheat-based matrices under standardized simulated digestion conditions. We assessed 5-MTHF stability in simulated gastric digestion using whole wheat flour and its anatomical fractions, across a range of pH values, matrix concentrations, and putative protective factors (antioxidant supplementation and nitrogen purging). While this study represents an initial exploration rather than a comprehensive mechanistic investigation, our findings aim to document this concerning phenomenon and highlight the urgent need for systematic research into cereal matrix effects on folate bioavailability in fortification applications.

2. Methods

2.1. Materials, calibrants and enzymes

Whole wheat flour, wheat bran, and wheat germ were obtained from a local grain mill (single wheat variety). 5-Methyltetrahydrofolate calcium salt were purchased from Schircks Laboratories (Jona, Switzerland), L-ascorbic acid, gallic acid, α-amylase from Aspergillus oryzae (A9857), and pepsin (P7125) were purchased from Sigma-Aldrich. Papain (S10011) was purchased from Yuanye Bio-Technology (Shanghai, China). Cellulase (9012–548) was purchased from NPEL Laboratory Technologies (Shanghai, China).

2.2. Wheat matrix preparation

The process of wheat matrix preparation was based on the protocol described by Van Wayenbergh et al. (2023). For water-insoluble fraction, 15 g of whole wheat flour was mixed with 150 mL distilled water, shaken for 30 min at room temperature, and centrifuged (4000 rpm, 10 min). The pellet was washed once and freeze-dried. For hexane-insoluble fraction, water-insoluble fraction obtained from the previous step was extracted with 150 mL hexane for 1 h, centrifuged, washed once, and air-dried to remove lipophilic components.

2.3. In vitro digestion model

Digestion followed the standardized INFOGEST protocol (Brodkorb et al., 2019) with modifications. Samples (5 g) were mixed with simulated oral fluid containing α-amylase (4 mL) to a final volume of 10 mL and incubated at 37 °C for 2 min. Subsequently, simulated gastric fluid containing pepsin (8 mL) was added, pH adjusted to 3.0, and a final volume of 20 mL mixture was incubated at 37 °C for 2 h with gentle rotation.

Post-digestion, pH was adjusted to 7.0, samples centrifuged (10,000 rpm, 10 min), and supernatants collected. Enzyme activity was terminated by boiling for 5 min, followed by filtration through 0.45 μm membranes.

2.4. 5-MTHF analysis

The analysis of 5-MTHF was carried out following the established protocol with modification (Liu et al., 2022a). The analysis started by folate extraction. For solid sample, 1 g of solid was combined with 15 mL of 2-(N-cyclohexylamino)-ethanesulfonic acid (CHES)/4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH 7.85) that contained 2 % sodium ascorbate and 10 mM 2-mercaptoethanol. The mixture was then heated to boiling water for 10 min. Subsequently, the pH was adjusted to 4.9, and α-amylase, cellulase and hog kidney conjugase were added. The extract was incubated for 3 h at 37 °C. Afterward, the pH was adjusted to 7 before the addition of papain. The solution was subjected to incubation at 37 °C for an hour. Finally, the reaction was terminated by heating the mixture in a boiling water bath (100 °C) for 5 min to inactivate the enzyme. The supernatant was collected after centrifugation (at 12,000 rpm for 10 min) and filtered through a 0.45 μm syringe filter. For the liquid sample collected after the *in vitro* digestion, folate extraction was not necessary, and the purification process can be carried out subsequently.

The purification of folate extracts was performed using affinity chromatography. Affinity agarose gel (Affi-Gel 10, Bio-Rad Laboratories, Richmond, CA, USA) was employed in conjunction with folate-binding protein (Scripps Laboratories, San Diego, CA, USA) for the purification of folates. The elution process involved the use of 0.02 M trifluoracetic acid and 0.01 M dithiothreitol in a 5 mL volumetric flask, which was subsequently filled with 10 mg of ascorbic acid and 5 μL of 2-mercaptoethanol. The eluent was then filtered through a 0.2-μm syringe filter, flushed with nitrogen, and stored at −20 °C for a maximum of 7 days.

5-MTHF was qualitatively and quantitatively analyzed using ultra-high performance liquid chromatography (UHPLC) coupled with a fluorescence detector (Waters, Milford, MA). The chromatographic column used for separation was a Waters HSS T3 column (100 × 2.1 mm, 1.8 μm). The column temperature was set at room temperature. Mobile phase A was acetonitrile containing 0.7 % formic acid, and mobile phase B was water containing 0.7 % formic acid, with a flow rate of 0.4 mL/min. The elution gradient was as follows: for the first 0.5 min, the proportion of mobile phase B was maintained at 95 %; by 3.8 min, the proportion of mobile phase B decreased to 50 % and was maintained until 5 min; at 5.2 min, the proportion of mobile phase B returned to 95 % and was maintained until 7.5 min. The excitation and emission wavelengths of the fluorescence detector were set at 290 nm and 356 nm, respectively. The external standard method was used for the qualitative and quantitative analysis of 5-MTHF.

2.5. Experimental design

The experimental design is available in the supplementary material.

2.6. Statistical analysis

Analysis of endogenous 5-MTHF in wheat matrices were carried out in triplicate, and the results are presented as mean ± standard deviation. All treatments to study 5-MTHF stability in this experiment were performed in duplicate. The experimental data presented are the average values of the two parallel experiments. The retention rate of 5-methyltetrahydrofolate (5-MTHF) was calculated using the following formula:

$$Retention\,rate\,(\%) = 100 \times \frac{Peak\,area\,of\,5-MTHF\,in\,the\,experimental\,group}{Peak\,area\,of\,5-MTHF\,in\,the\,reference\,group}$$

For the reference group, 1 μg of 5-MTHF was directly added to the affinity chromatography column during folate purification. After following the same procedures as the experimental group, the compound was eluted into 5 mL of solution and then analyzed by liquid chromatography. Data visualization was carried out using the R Studio platform.

3. Results and discussion

3.1. Complete 5-MTHF loss in wheat matrices during simulated gastric digestion

We first analyzed the endogenous 5-MTHF content in wheat matrices, which were negligible compared to exogenously added amounts, confirming that detected folate originated from supplemented sources (Fig. 1 A). During simulated gastric digestion, 5-MTHF retention was severely compromised, with complete loss observed in wholewheat flour (Fig. 1 B). Because essentially no detectable 5-MTHF remained after the gastric phase, subsequent intestinal digestion was not pursued. Analyses of post-gastric residues showed only trace 5-MTHF, and

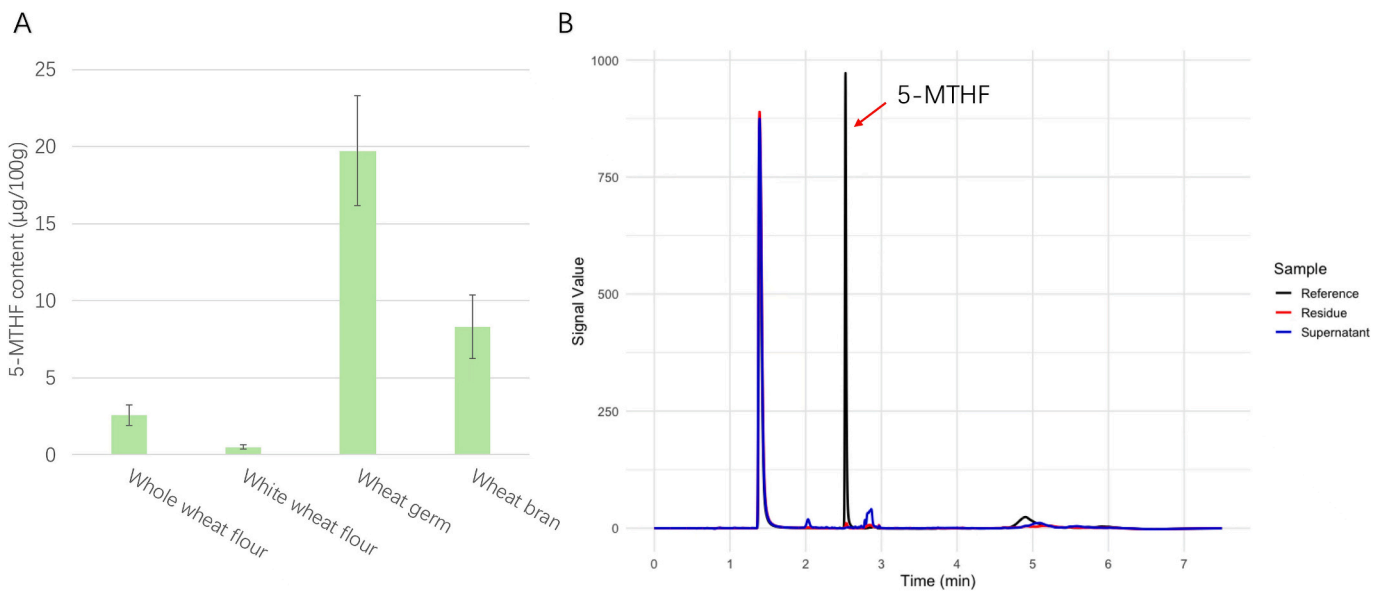

Fig. 1. Endogenous 5-MTHF content of different wheat matrices in this study (A) and a representative chromatograph to show the complete loss of 5-MTHF in wheat matrices during simulated digestion (B).

enzymatic treatment of the residual solids did not liberate additional folate (Fig. 1B), arguing against simple reversible physical adsorption as the dominant cause of the apparent loss. However, we did not characterize putative degradation products of 5-MTHF; therefore, the chemical fate of the missing 5-MTHF remains unresolved. Accordingly, we cannot yet discriminate whether the apparent disappearance is driven mainly by oxidative conversion, non-oxidative chemical transformation (*e.g.*, rearrangement or fragmentation), or a combination thereof. Similar observations have been reported in oat and rye matrices, indicating that this phenomenon may be widespread across cereal grains rather than being exclusive to wheat (Liu, 2022). Notably, cereals are known to contain high levels of MeFox, a pyrazino-*s*-triazine derivative of 4α-hydroxy-5-methyltetrahydrofolate and an oxidized, biologically inactive form of 5-MTHF (Shahid et al., 2020; Zamberlan, 2024). The prevalence of MeFox in these matrices suggests that cereal components may harbor specific factors or promote conditions that accelerate 5-MTHF degradation, representing a critical challenge for cereal-based folate fortification strategies.

3.2. *Effects of pH and matrix concentration on 5-MTHF stability*

To understand the mechanisms underlying the pronounced 5-MTHF losses in wheat matrices, we systematically evaluated pH and matrix concentration effects (Fig. 2 A). In buffer systems without wheat components, 5-MTHF demonstrated markedly different stability profiles: under neutral conditions (pH 7.0), retention reached 92 % in distilled water, while under acidic conditions (pH 3.0), retention dropped to 45 %, indicating its susceptibility to acid-mediated loss. When whole wheat flour was introduced, stability patterns changed dramatically—5-MTHF retention fell to 30 % under neutral conditions and became undetectable under acidic conditions, suggesting additional matrix-driven losses under both neutral and acidic conditions. Matrix concentration effects revealed a clear dose-dependent relationship: reducing whole wheat flour from 5 g to 0.1 g improved 5-MTHF retention from undetectable levels to 80 %.

The underlying processes appear to involve heat-stable, non-enzymatic constituents rather than active endogenous enzymes, as cereal matrices reportedly retain degradative capacity after thermal treatments that inactivate native enzymes (Liu, 2022). Metal-catalyzed oxidation is unlikely to be the sole driver because water-insoluble wheat fractions with reduced bioavailable metal content still promoted near-complete 5-MTHF loss. Although we did not characterize degradation products in this study—preventing definitive assignment of pathways—prior reports indicate that oxidative conversion of 5-MTHF to 5-methyldihydrofolate can yield intermediates exhibiting heightened pH sensitivity (Donaldson et al., 1962; Lucock et al., 1995). The concentration-dependent degradation pattern, combined with elimination of enzymatic and primary metal-catalyzed mechanisms, suggests that the loss likely involves heat-stable organic compounds or reactive functional groups in wheat proteins, fiber components, or secondary metabolites that operate through multiple concurrent pathways independent of traditional acid-catalyzed breakdown.

3.3. *Effects of antioxidants and nitrogen atmosphere on 5-MTHF stability*

To investigate potential protective strategies against 5-MTHF degradation in wheat matrices, we evaluated antioxidant supplementation and oxygen exclusion effects (Fig. 2 B). Consistent with previous studies (Chandra-Hioe et al., 2013; Yang et al., 2021), ascorbic acid (10 mM) demonstrated remarkable protective efficacy, significantly improving 5-MTHF retention from undetectable levels to 64 % in whole wheat flour and from 0 % to 59 % in hexane-insoluble fractions under simulated gastric conditions. In contrast, gallic acid supplementation (10 mM) showed minimal protective effects, with 5-MTHF retention remaining at only 3 % in whole wheat flour matrices, while nitrogen atmosphere treatment provided virtually no protection across tested wheat matrices, with most samples showing undetectable levels and only water-insoluble extract retaining minimal 5-MTHF (1 %).

These response patterns suggest that the principal degradation pathway(s) are not readily suppressed by either phenolic radical scavenging or simple removal of headspace oxygen. The comparatively greater effect of ascorbic acid could arise from physicochemical attributes distinct from gallic acid—such as redox potential, solubility, pH influence, metal interaction, or reactivity toward electrophilic intermediates—rather than from a single, specific competitive binding mechanism. The negligible benefit of nitrogen purging does not conclusively rule out oxidative participation, because residual dissolved oxygen, matrix-bound oxidants, or internally generated reactive species may persist. Absent structural identification of degradation products, the current findings should be interpreted as indicating differential intervention efficiency rather than definitive exclusion of particular mechanistic classes.

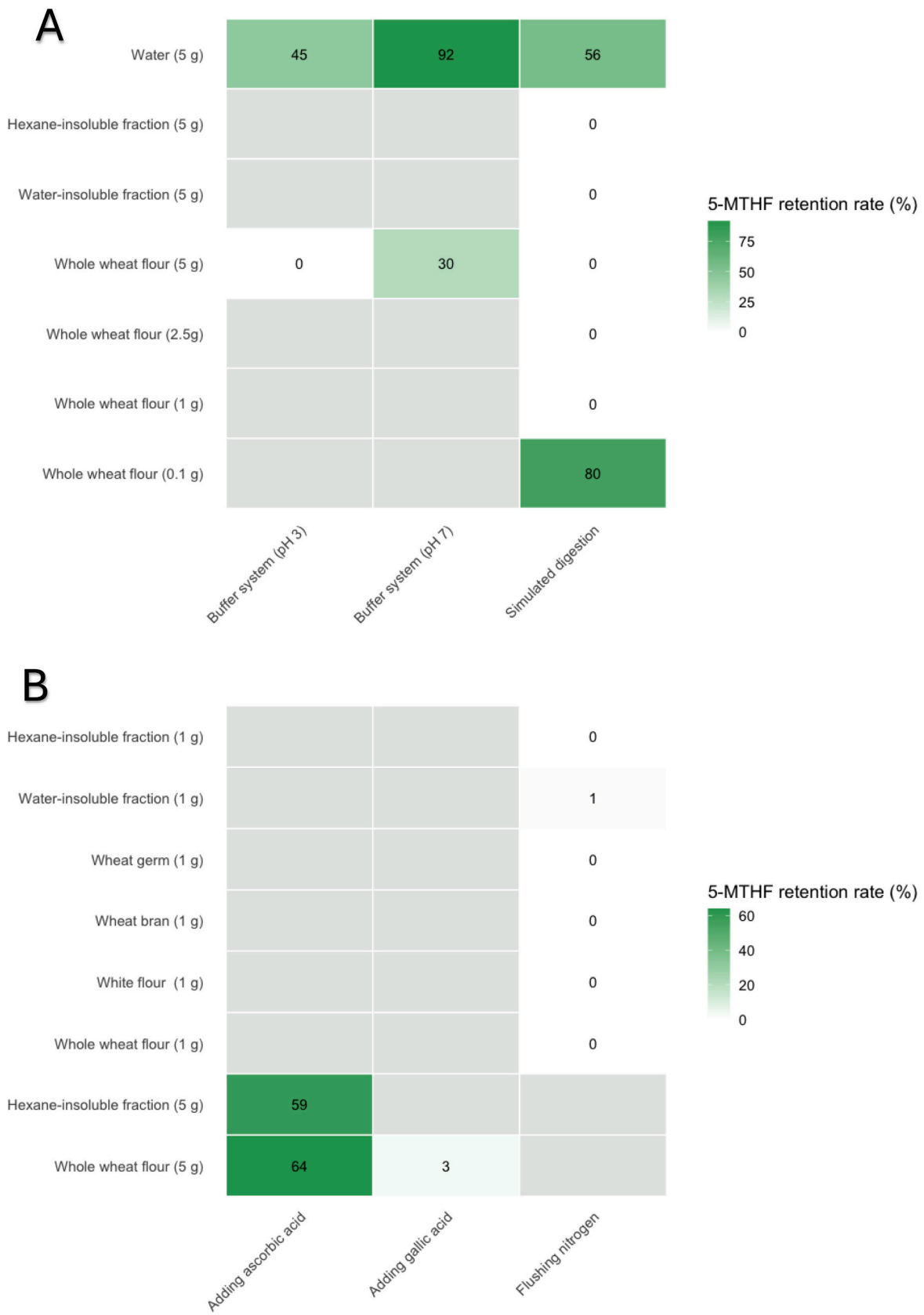

Fig. 2. A) Effects of pH and matrix concentration on 5-MTHF stability; B) effects of antioxidants and nitrogen atmosphere on 5-MTHF stability. The gray area indicates the data is not available.

4. Conclusion

This short communication reports a complete loss of 5-MTHF in wheat matrices during gastric digestion, suggesting that natural folate forms may be ineffective in wheat matrices, potentially leading to overestimation of bioavailable folate in fortified products—a particular concern given cereals' role as primary fortification vehicles worldwide. The concentration-dependent phenomenon indicates that processing conditions critically influence folate stability, suggesting that lower flour concentrations, dilution effects, or processing modifications that reduce wheat-folate interactions might preserve bioavailability, while the effectiveness of ascorbic acid protection points to co-fortification strategies as potential solutions. For manufacturers, these results highlight the need for careful folate form selection, with synthetic folic acid potentially offering better stability than natural forms in wheat matrices, requiring optimization of folate addition timing, storage conditions, and formulation modifications to ensure adequate folate delivery to consumers.

However, several limitations should be acknowledged. The simulated digestion model does not fully replicate human gastric conditions, including individual variations in pH, enzyme activity, and transit time. Since there are lacks *in vivo* study regarding the bioavailability of 5-MTHF in cereal matrices, future *in vivo* verification should be carried out. Additionally, identification of 5-MTHF degradation products would provide valuable mechanistic insights that were not explored in this study. While this short communication presents preliminary findings, the complete loss of 5-MTHF in wheat matrices represents a critical observation requiring immediate attention from the food fortification community. Comprehensive mechanistic studies using mass spectroscopy or other analytical methods are underway and will be reported separately.

CRediT authorship contribution statement

Fengyuan Liu: Writing – review & editing, Writing – original draft, Visualization, Supervision, Funding acquisition, Conceptualization. **Jinxian Zheng:** Investigation, Data curation. **Yunfei Mo:** Conceptualization. **Yaping Xiao:** Conceptualization. **Yun Wang:** Conceptualization. **Hua Zhang:** Conceptualization. **Jing-Kun Yan:** Funding acquisition. **Yaqing Liu:** Project administration, Conceptualization.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the authors used Monica AI in order to improve language and readability. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2025.146467.

Data availability

Data will be made available on request.

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