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ORIGINAL CONTRIBUTION

Folates in bread: retention during bread-making and in vitro bioaccessibility

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Abstract
Background Bread is an important folate source in several countries. However, bread-making was reported to cause losses of endogenous bread folates (~40%) as well as added synthetic folic acid (~30%). Furthermore, the bread matrix is suggested to inhibit absorption of folates.
Purpose To (1) estimate retention of both, endogenous folates and synthetic fortificants, during bread-making, (2) assess in vitro folate bioaccessibility from breads and a breakfast meal and (3) assess in vitro folate uptake.
Methods Retention of folate forms was assessed by preparing fortified (folic acid and [6*S*]-5-CH_3-H_4folate) wholemeal breads and collect samples from dough, proofed dough and the bread. In vitro folate bioaccessibility was assessed using the TNO gastrointestinal model TIM. In vitro folate uptake was assessed using a novel Caco-2 cell/stable isotope model. Folate content in samples was measured using LCMS.
Results Bread-making resulted in losses of 41% for endogenous folates and up to 25 and 65% for folic acid and [6*S*]-5-CH_3-H_4folate fortificant, respectively. 75% of endogenous bread folates and 94% of breakfast folates were bioaccessible as assessed by TIM. From [6*S*]-5-CH_3-H_4folate-fortified bread, relative folate uptake into Caco-2 cells was 71 ± 11% ($P < 0.05$) when compared with a standard solution.
Conclusion Retention of folic acid fortificant during bread-making was substantially higher compared to retention of [6*S*]-5-CH_3-H_4folate fortificant. Data from the TIM and Caco-2 cell trials suggest an inhibiting effect of the tested bread matrices on in vitro bioaccessibility of folates, whereas folate bioaccessibility from a breakfast meal is almost complete.

Keywords Folates · TNO gastrointestinal model TIM · Bread-making · In vitro bioaccessibility · Caco-2-cell/stable isotope model

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Introduction

The essential B-vitamin folate is required for DNA synthesis and a good folate status prevents against neural tube defects [1]. In countries without mandatory or voluntary folic acid fortification a gap of up to 45% between recommended and actual folate intake is observed [2], which might be linked to an increased risk for neural tube defects. In countries with fortification on the other hand, concerns regarding harmful effects from high intakes of the synthetic folic acid fortificant, e.g. increased rate of colorectal cancer [3], are discussed.

Bread is a good vehicle for folic acid fortification and thereby an important source of synthetic folic acid in countries with mandatory fortification but bread is also an important folate source in countries without fortification. For instance, in Sweden, bread contributes with 13% to folate intake and is a folate source almost as important as vegetables [4]. However, bread would provide even more folates if losses during bread-making, which were up to ~45% for endogenous bread folates [5, 6] and 33% for synthetic folic acid [7], could be reduced.

Table 1 Description of foods used in the trials

Food	Ingredients (% of total weight or g and mL) and brief description of bread-making	Trial	Endogenous folate content μg/100 g ($n = 3$) (fresh weight)
Wholemeal bread[a]	Flours: wholemeal wheat (13%), wheat (11%), rye (7%) and oat (3%), rye kernels (14%), flax seed (2%). Proofing 80 min, baking 200 °C for 50 min (weight 900 g) or 30 min (weight 600 g)	Bread-making and Caco-2 cell	43 ± 3
B3-bread	Flours: B3[b] (24%) and wheat (17%), barley flakes (7%). Proofing 30 min, baking 225 °C for 30 min (weight 600 g)	TIM	52 ± 3
Rye bran bread	Flour: wheat (33%), rye bran (18%). Proofing 30 min, baking 220 °C for 30 min (weight 600 g)	TIM	39 ± 4
Breakfast[c]	B3-bread (16 g), liver pâté (8 g), orange juice (104 mL) made from concentrate (1 + 4), sour milk (104 mL), bran flakes (16 g) and kiwifruit (52 g)	TIM	33 ± 1

[a] The wholemeal bread was fortified with either [6*S*]-5-CH_3-H_4folate or folic acid during individual bread-making occasions

[b] Flour from milling fraction B3 of rye wholegrain (78% extraction rate, reported protein content 14% and ash content 2%)

[c] Breakfast based on Swedish Nutrition Recommendations Objectified [15], portion size adjusted for TIM

In addition, the food matrix itself may affect the absorption of folates [8, 9]. Because in vivo absorption studies are tedious with respect to cost and labour, standardised in vitro models, e.g. TNO's computer-controlled dynamic gastrointestinal model TIM [10–13], are suitable to identify factors affecting bioavailability of folates. Because the TIM model has no enterocytes, bioaccessible folates are quantified instead of absorbed folates [11]; the latter may be accounted for by subsequent combination with, e.g. the Caco-2 cell model. By integrating data from in vitro TIM and Caco-2 cell trials [14] into a kinetic mathematical in silico model, Verwei et al. [10] successfully predicted plasma response in humans, both from a single dose and from long-term consumption of folic acid-fortified milk.

Our objectives were to (a) study the effects of bread-making on the content of endogenous folates and synthetic [6*S*]-5-CH_3-H_4folate and folic acid fortificant; (b) assess the in vitro bioaccessibility of endogenous folates from breads and a meal using the dynamic gastrointestinal model TIM; and (c) assess the effect of bread matrix on uptake and transport of the dominant food folate 5-CH_3-H_4folate, using a new Caco-2 cell/stable isotope model.

Methods

Food samples

Food samples used for all experiments are listed in Table 1. Breads and the flour from milling fraction B3 of rye wholegrain (B3-flour, 78% extraction rate, reported protein content 14% and ash content 2%) were prepared at the test bakery and pilot mill of Lantmännen (Järna, Sweden). The breakfast (Table 1) was composed according to Swedish Nutrition Recommendations Objectified (SNO) [15] and consisted of B3-bread (Table 1) and orange juice, donated by JO Bolaget (Stockholm, Sweden). The other breakfast ingredients, bran flakes (17% fibre, Coop Norden, Stockholm, Sweden), sour milk (0.5% fat, Arla, Stockholm, Sweden), liver pâté (41% liver, Scan, Johanneshov, Sweden) and kiwifruit, were purchased at a local store. All breads were kept stored at −20 °C and other foods at 5–8 °C during transport and storage.

Chemicals

Materials and chemicals of analytical grade were purchased from VWR (Stockholm, Sweden) unless otherwise stated. Folate standards; [6*S*]-H_4folate (sodium salt), [6*S*]-5-CH_3-H_4folate (sodium salt), [6*S*]-5-HCO-H_4folate (sodium salt) and folic acid (pteroylmonoglutamate) were gifts from Merck Eprova AG (Schaffhausen, Switzerland). [6*S*]-$^{13}C_5$-5-CH_3-H_4folate (calcium salt) was purchased from Merck Eprova AG. The isotopic enrichment was >90% and the purity 97.8% (data from Merck Eprova AG). 10-HCO-folic acid and [6*R*]-5,10-CH^+-H_4folate (chlorine hydrochloride) were purchased from Schircks Laboratories (Jona, Switzerland). [6*R*]-10-HCO-H_2folate was converted from [6*R*]-5,10-CH^+-H_4folate according to Pfeiffer et al. [16]. Folate standard stock solutions, prepared according to Jastrebova et al. [17] and with concentrations about 200 μg/mL, were stored under argon atmosphere at −80 °C and used within 3 months. Standard solutions were corrected for actual concentrations, as determined by spectroscopy [17]. A Milli-Q system (Millipore, Billerica, MA) was used to purify water.

Affinity chromatography columns for sample purification were prepared as described by Konings [18] using folate-binding protein isolated according to Nygren et al. [19]. To avoid overloading [16], columns were loaded to a maximum of 20% of total folate-binding capacity. Binding capacity was confirmed on each occasion ($n = 6$) by alternating application of a standard solution containing [6*S*]-5-CH_3-H_4folate (67.7 ng, recovery 97%) and 10-HCO-folic acid (68.6 ng, recovery 85%).

Bread-making

Effects of bread-making on content of endogenous folates and fortificant in the final product were studied using a wholemeal bread recipe and standardised bread-making procedures (Table 1). To fortify breads, folic acid or [6*S*]-5-CH_3-H_4folate was dissolved in drinking water (37 °C) containing 1% (w/v) NaCl and added to the dough fluid before mixing with flour. Fortificants were added by weight (2.8–12.4 mg/1,000 g final dough considering the actual concentration) aiming a folate content equivalent to 101–1,155 µg/100 g bread. Breads were baked at 200–225 °C until the inner temperature reached 96 °C, which was reached after 50 min for loaves with a weight of 900 g and after 30 min for loaves with a weight of 600 g (Table 1). The total bread-making time took 5 h including cooling in darkness at 8 °C for 2 h. Two (900 g loaf weight) or three (600 g loaf weight) loaves were baked from the dough at each occasion. Bread from each bread-making occasion was sampled by mixing six representative slices from two loaves into a composite sample, which was vacuum-packed and stored at −20 °C until folate determination.

Effects of bread-making on content of endogenous folates and fortificant were determined by collecting samples (~10 g) from dough, proofed dough and bread for folate quantification. Retention of folic acid fortificant (two bread-making occasions) and [6*S*]-5-CH_3-H_4folate fortificant (three occasions) were studied by preparing breads with 900 g loaf weight; and furthermore for [6*S*]-5-CH_3-H_4folate fortificant breads with 600 g loaf weight (one occasion).

Folate bioaccessibility in breads

The in vitro TNO gastrointestinal model TIM, mimicking the stomach, duodenum, jejunum and ileum [11], has been used to assess folate bioaccessibility from various foods [11–13]. All TIM trials were carried out in duplicate (at separate days). TIM food portions were prepared on the day of the trial by mixing the breakfast (223 g; Table 1), B3-bread (90 g + 100 g water) or rye bran bread (100 g + 100 g water) with a buffer solution (77–100 mL) containing amylase (8–15 mg) using a household mixer as described by others [11, 13]. Each food portion was placed in the gastric compartment of the TIM. Bioaccessible folate was quantified from dialysates from the jejunum and ileum compartments collected in four intervals (0–60, 60–120, 120–240 and 240–360 min) during transit through TIM. Ileal deliveries were collected representing the non-bioaccessible folate fraction. After each TIM run, residues from all compartments were collected to calculate folate recovery (mass balance). After addition of sodium ascorbate (1% w/v), aliquots of TIM samples (15–50 mL) were stored at −20 °C under argon atmosphere.

Effect of bread matrix on in vitro folate uptake

Differentiated Caco-2 cells expressing folate transporters have been used to assess factors affecting folic acid uptake [14, 20–22]. In this trial, stable isotope-labelled folate ([6*S*]-$^{13}C_5$-5-CH_3-H_4folate) was used to distinguish between the added folate and endogenous folates from the wholemeal bread matrix (about 5% of bread folate content) as well as the Caco-2 cells.

For the Caco-2 cell experiment, digests of the wholemeal bread fortified with [6*S*]-$^{13}C_5$-5-CH_3-H_4folate and the folate standard solution were prepared according to Salovaara et al. [23], as the extensively diluted (~5 L) TIM dialysates resulted in folate concentrations around quantification limit. In brief, bread (1.5 g containing 6.7 µg [6*S*]-$^{13}C_5$-5-CH_3-H_4folate) and the control folate standard solution (4.9 µg [6*S*]-$^{13}C_5$-5-CH_3-H_4folate) were incubated with a 'gastric' solution (containing pepsin, α-amylase, $NaHCO_3$, sodium ascorbate, NaCl and KCl) at 37 °C for 1 h (pH 2.1). After adjustment to pH 6.3, the digest was incubated with an 'intestinal' solution (containing porcine bile, pancreatine, $NaHCO_3$, sodium ascorbate, NaCl and KCl) at 37 °C for 1 h. The digest was centrifuged and filtered (0.2 µm) under sterile conditions.

The uptake and transport experiment using fully differentiated cells was performed under sterile conditions in six wells for each digest. Human intestinal Caco-2 cells, originally obtained from the American Cell Culture Collection (Rockville, USA), were kindly donated by P. Artursson (Department of Pharmacy, Uppsala University, Sweden) and cultured as previously described [24]. The net transepithelial electrical resistance for used monolayers was above 310 Ω cm^2 at 37 °C. Background resistance across filters in Hank's Buffered Salt Solution (HBSS, pH 7.4, 37 °C) without cells was about 80 Ω cm^2.

Before application of folate digests, cell monolayers were rinsed in phosphate-buffered saline, incubated in HBSS (pH 7.4, 37 °C, 20 min) and moved to new wells. The digests (0.5 mL, pH 6.3) of bread or folate standard solution, containing 0.4–0.5 µmol [6*S*]-$^{13}C_5$-5-CH_3-H_4folate (Table 2), were added to the apical chambers.

Table 2 Cellular uptake and transport (mean ± SD) of [6*S*]-$^{13}C_5$-5-CH_3-H_4folate

	[6*S*]-$^{13}C_5$-5-CH_3-H_4folate	
	Folate standard ($n = 5$)[a]	Bread matrix ($n = 6$)
Measured folate content in digest (nM)[b]	709	1,029[c]
Uptake as % of added amount (nM)	22 ± 3* (77 ± 11)	15 ± 2* (80 ± 13)
Transport as % of added amount (nM)	6 ± 2 (22 ± 6)	6 ± 1 (29 ± 4)
Recovery of Caco-2 cell model	99 ± 11%	88 ± 7%

* Wilcoxon's Mann–Whitney rank-sum test was used to compare uptake and transport of [6*S*]-$^{13}C_5$-5-CH_3-H_4folate between the digests of bread and standard solution, $P < 0.05$

[a] In one well no labelled folates were detected

[b] 0.5 mL was applied to each cell, digests were analysed in triplicates

[c] Bread matrix digest contained an additional 54 nM endogenous folates (sum expressed as folic acid)

Fresh HBSS (1.5 mL, pH 7.4) was added to the basolateral chambers. The uptake and transport experiment was carried out in the dark at 37 °C under gentle shaking (50 rpm) for 60 min. Samples from apical and basolateral chambers were collected in tubes containing 2% (w/v) sodium ascorbate and 0.1% (v/v) 2,3-dimercaptopropanol (BAL). Cell monolayers were washed in cold (4 °C) HBSS for 15 min and lysed in 1 M KOH. To avoid folate degradation of lysed cell samples, the pH was adjusted to 6 (within 15 min) and 2% (w/v) sodium ascorbate and 0.1% (v/v) BAL were added before storage at −20 °C under argon atmosphere.

Folate determination in foods, TIM and Caco-2 cell samples

Determination of folate content in bread and dough samples was carried out in triplicate. Composite bread and dough samples were thawed in the dark and mixed with 0.1 M phosphate buffer (10 mL/g sample, pH 6.1) containing 2% (w/v) sodium ascorbate and 0.1% (v/v) BAL [25]. Before extraction in boiling water [17], thermostable α-amylase (15 μL/g sample, 3,000 U/mL, Megazyme International, Bray, Ireland) was added. After homogenisation [16] and centrifugation [12], samples were deconjugated at 37 °C for 2 h using 3% (v/v) dialysed [25] rat serum (Scanbur, Sollentuna, Sweden) and 0.4% (v/v) α-amylase. Extracts were purified using strong anion exchange (SAX) solid phase extraction (SPE) columns as previously described [12, 17].

Folate content in bread (Table 1) and dough was quantified using an LC–MS system consisting of a quaternary gradient pump, an autosampler (8 °C), a column compartment (20 °C), a LCQ ion-trap MS and a multiwavelength detector (UV, 290 nm) (Agilent 1100, Agilent Technologies AB, Kista, Sweden). Folates were separated on an Ace 3 C_{18}, 150 × 4.6 mm i.d.; 3 μm column (Scantec lab, Partille, Sweden) using acetate buffer, acetonitrile and methanol [25]. [6*S*]-H_4folate, [6*S*]-5-CH_3-H_4folate, [6*S*]-$^{13}C_5$-5-CH_3-H_4folate, [6*R*]-10-HCO-H_2folate, [6*R*]-10-HCO-folic acid, [6*S*]-5-HCO-H_4folate and folic acid were quantified using a multilevel ($n \geq 6$) external calibration curve with a linear range over 4–200 ng/mL. Intra-assay CVs for quantification of endogenous folates and fortificants in the wholemeal bread were <15% ($n = 3$). Recovery of folates in wholemeal flour certified reference material (CRM 121, Institute of Reference Materials and Measurements, Geel, Belgium) was 74% ($n = 3$). A recovery lower than 100% was expected, since the reference value was obtained by microbiological assay (MA), which commonly results in ~25% higher folate content than HPLC methods [26].

The TIM samples were extracted, deconjugated, purified using affinity chromatography, filtered and quantified using HPLC with fluorescence detection (LC-FL, Agilent 1100) as previously described [12]. TIM food portions were heat extracted and purified using SAX SPE as described for bread samples prior to LC-FL quantification. Intra-assay CVs for quantification of endogenous folates in TIM dialysates were <10% ($n = 4$).

Samples from the Caco-2 cell trial were purified using SAX SPE [17], except for the basolateral samples, which were only centrifuged (10 min, 10,000*g*) prior to quantification using LC–MS. To determine the ratio of mono- to polyglutamated [6*S*]-$^{13}C_5$-5-CH_3-H_4folate in lysed Caco-2 cells, cell samples were analysed with and without deconjugation [25]. Intra-assay CVs for quantification of [6*S*]-$^{13}C_5$-5-CH_3-H_4folate in samples from the Caco-2 cell trial was <15% ($n = 3$).

Sample preparation was performed under subdued light and argon atmosphere to prevent oxidation during preparation and storage in the autosampler (8 °C, <15 h).

Calculations and statistics

Folate content (the sum of individual folate forms) in dough, proofed dough and bread was expressed as

microgram folic acid/100 g fresh weight (mean ± SD, analytical $n = 3$) according to Vahteristo et al. [27], using conversion factors 445.4 for H_4folate, 459.5 for 5-CH_3-H_4folate, 469.4 for 10-HCO-folic acid, 473.5 for 5-HCO-H_4folate and 441.4 for folic acid to account for differences in molecular weight.

Folate retention in the proofed dough and bread was calculated as:

$$\text{Retention}(\%) = 100 \times F_{\text{product}}/F_{\text{dough}}, \quad (1)$$

where F_{product} is the analysed folate content in proofed dough or bread and F_{dough} is the analysed folate content in dough. Data were corrected for water losses determined as weight losses (from dough to proofed dough: 2%; from dough to baked bread: 7%).

Folate bioaccessibility in TIM and the mass balance during the TIM trial were estimated according to Verwei et al. [11]. Folate bioaccessibility (mean ± SD) was calculated using data on [6*S*]-5-CH_3-H_4folate and 10-HCO-folic acid from two independent TIM runs, each analysed in duplicate as:

$$\text{Bioaccessibility}(\%) = 100 \times F_{\text{dialysate}}/F_{\text{food portion}}, \quad (2)$$

where $F_{\text{dialysate}}$ and $F_{\text{food portion}}$ were sum of [6*S*]-5-CH_3-H_4folate and 10-HCO-folic acid content in dialysates (jejunal and ileal) and the TIM food portion, respectively.

To account for the mass balance of the TIM model [11], folate recovery (mass balance, *R*%) was calculated for each trial as:

$$R(\%) = 100\left(F_{\text{dialysate}} + F_{\text{ileal delivery}} + F_{\text{residues}}\right)/F_{\text{foodportion}}, \quad (3)$$

where $F_{\text{dialysate}}$, $F_{\text{food portion}}$, $F_{\text{ileal delivery}}$ and F_{residues} were sum of [6*S*]-5-CH_3-H_4folate and 10-HCO-folic acid content in dialysates (jejunal and ileal), the TIM food portion, the ileal delivery and the residual samples, respectively.

In the Caco-2 cell trials, both folate uptake into the cell and transport across the cell were determined. Folate uptake (mean ± SD) was calculated based on data from six wells each as:

$$\text{Uptake}(\%) = 100\left(F_{\text{lysed Caco}-2\text{ cells}} + F_{\text{basolateral}}\right)/F_{\text{digest}}, \quad (4)$$

where $F_{\text{lysed Caco-2 cells}}$, $F_{\text{basolateral}}$ and F_{digest} were [6*S*]-$^{13}C_5$-5-CH_3-H_4folate content in lysed Caco-2 cells, basolateral samples and digests, respectively.

Folate transport across Caco-2 cells (mean ± SD) was calculated based on data from six wells each as:

$$\text{Transport}(\%) = 100 \times F_{\text{basolateral}}/F_{\text{digest}}, \quad (5)$$

Also here, recovery (mass balance) of the Caco-2 cell model was determined to account for folate losses during the trial. Recovery (*R*%) was calculated as:

$$R(\%) = 100\left(F_{\text{apical}} + F_{\text{lysed Caco}-2\text{ cells}} + F_{\text{basolateral}}\right)/F_{\text{digest}}, \quad (6)$$

where F_{apical} was [6*S*]-$^{13}C_5$-5-CH_3-H_4folate content in apical samples.

Data were not normally distributed so non-parametric methods were used. The Kruskal–Wallis test was used to compare retention of [6*S*]-5-CH_3-H_4folate fortificant in bread during bread-making as well as between different fortification levels. The Wilcoxon's Mann–Whitney rank-sum test was used to compare folate uptake and transport in Caco-2 cells between bread matrix and folate standard digests. Minitab® 15.1.20.0. was used for statistics.

Results

Folate retention during bread-making

The endogenous folate content in the three types of bread (Table 1) ranged from 39 to 52 μg/100 g. [6*S*]-5-HCO-H_4folate was the dominant folate form, ranging from 31 to 52%, followed by 10-HCO-folic acid (25–45%), [6*S*]-5-CH_3-H_4folate (11–30%) and [6*R*]-10-HCO-H_2folate (15–17%).

Retention of folic acid fortificant (Eq. 1) in wholemeal bread (Table 1) was 76 ± 5 and 76 ± 10% (for fortification levels of 295 and 568 μg/100 g bread). For [6*S*]-5-CH_3-H_4folate fortificant, mean retention (Eq. 1) was low and did not vary significantly between three different fortification levels: 31 ± 4% (fortification level 579 μg/100 g bread), 34 ± 6% (fortification level 744 μg/100 g) and 38 ± 5% (fortification level 1,155 μg/100 g). Reduced loaf weight from 900 to 600 g and thereby shortened baking time from 50 to 30 min doubled retention of [6*S*]-5-CH_3-H_4folate fortificant (Fig. 1), but did not affect texture of the final bread.

The content of endogenous folate (sum of individual folates) decreased from 59 ± 7 μg/100 g (dough) and 57 ± 6 μg/100 g (proofed dough) to 35 ± 6 μg/100 g (bread, $P < 0.005$) (two baking occasions, 900 g baked at 50 min). Both the content and ratio of individual endogenous folate forms were altered during baking (Fig. 2). While only ~35% [6*S*]-5-CH_3-H_4folate fortificant (Fig. 1) was retained, ~75% of endogenous [6*S*]-5-CH_3-H_4folate (Fig. 2) was retained during bread-making (900 g baked at 50 min).

Folate bioaccessibility in breads

Bioaccessibility (Eq. 2) of endogenous bread folates, as determined by the gastrointestinal model TIM, was 76 ± 3% from B3-bread, 77 ± 12% from rye bran bread

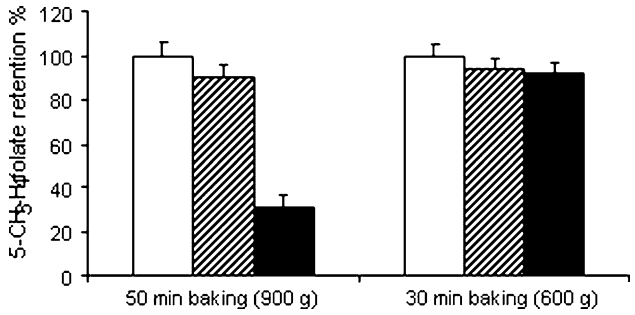

Fig. 1 Retention of [6*S*]-5-CH_3-H_4folate fortificant from dough (*white*), to proofed dough (*stripe*) and to bread (*black*). Mean ± SD ($n = 3$) of folate retention (%) during bread-making ($n = 1$), corrected for weight losses from dough to proofed dough (2%) and from dough to baked bread (7%)

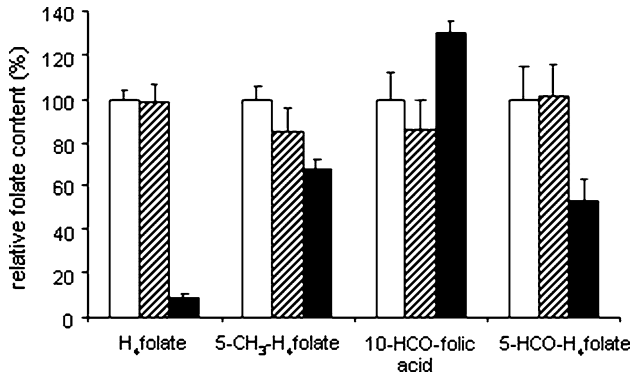

Fig. 2 Relative increase and decrease (%) of endogenous folate forms from dough (*white*), to proofed dough (*stripe*) and to bread (black). Mean ± SD ($n = 3$) of folate content (%) during bread-making ($n = 2$), corrected for weight losses from dough to proofed dough (2%) and from dough to baked bread (7%)

and 94 ± 12% from the breakfast. Presented bioaccessibility data were based solely on the sum of [6*S*]-5-CH_3-H_4folate (5-40 μg/TIM food portion) and 10-HCO-folic acid (5-15 μg/portion) because [6*S*]-5-HCO-H_4folate could not be quantified in the dialysates due to the lack of baseline separation and [6*S*]-H_4folate was not detected in any of the TIM samples. However, all TIM food portions and the breakfast contained minor amounts of [6*S*]-5-HCO-H_4folate (15–20 μg/portion) and [6*S*]-H_4folate (15 μg/portion). About 13% of folates (1.4 μg) in the breakfast and 20% (2.4 μg) in rye bran bread were not bioaccessible as estimated from folate content in the ileal delivery. For B3-bread, ileal delivery samples were missing, hence non-bioaccessible folate could not be calculated. Folate recovery (mass balance, Eq. 3) in TIM trials was 85 ± 9% ($n = 3$).

Effect of bread matrix on in vitro folate uptake

The relative uptake (Eq. 4) of labelled [6*S*]-$^{13}C_5$-5-CH_3-H_4folate fortificant from the bread matrix digest into Caco-2 cells was 71 ± 11%) of that from the folate standard digest (Table 2, $P < 0.05$). However, the bread matrix had no effect on relative transport (equation 5), which was 89 ± 11% (Table 2).

After enzymatic treatment of lysed cell extracts (using rat serum), the content of [6*S*]-$^{13}C_5$-5-CH_3-H_4folate monoglutamate increased with 15% (indicating intracellular presence of labelled folate polyglutamate).

After incubation with bread matrix and folate standard digest, also minor amounts of unlabelled [6*S*]-5-CH_3-H_4folate (29 ± 7 and 2 ± 1 nM, respectively) and [6*S*]-H_4folate (2 ± 0 nM in both) were detected in the apical, basolateral and lysed Caco-2 cell samples.

Discussion

Aim of the current trial was to study the retention of endogenous folates and folate fortificants during bread-making, and their subsequent in vitro bioavailability by combining studies using the gastrointestinal model TIM with a new Caco-2 cell/stable isotope model.

The high retention of folic acid during bread-making in this (~80%) and other trials [5, 7] confirms the suitability of using folic acid fortificant for bread. However, fortification with [6*S*]-5-CH_3-H_4folate has been suggested as an alternative because of possible harmful effects of high intakes of synthetic folic acid fortificant [3]. As shown in this trial (Fig. 1), retention of [6*S*]-5-CH_3-H_4folate fortificant during bread-making varied depending on the bread size (baking time) and was only 50% of that of folic acid in breads of the same size.

Folate bioaccessibility, as estimated by TIM, were in different wholemeal breads above 75% and in the breakfast 94%. The high folate bioaccessibility from the breakfast was probably due to orange juice contributing with 60% of the folate content in the breakfast. Folate bioaccessibility from orange juice was shown to be almost complete in previous TIM trials [11, 12], probably due to the stabilising effect from ascorbic acid on the vitamin.

Folate bioaccessibility from breads and the breakfast were in agreement with the estimated folate bioaccessibility from crisp bread of 87 ± 2% [11], whereby no literature data on folate bioaccessibility from soft bread are available. The performance of the TIM model with a folate recovery (mass balance, Eq. 3) of 85 ± 9% ($n = 3$) was in agreement with previous trials and not corrected for [11, 13].

Previous Caco-2 cell trials have focused on extrinsic [22] or intrinsic [20, 21] factors affecting only the uptake of folic acid. Verwei et al. [14] studied both uptake and transport. They [14] reported substantially lower uptake (3 vs. 22% in this trial; Table 2) and transport (2 vs. 6% in

this trial) of [6*S*]-5-CH_3-H_4folate from their folate standard solution. This discrepancy might be explained by the higher apical pH (7.0) used by Verwei et al. [14], as folate uptake is strongly pH-dependent [21]. Mason et al. [21] reported a K_m for folic acid of 0.7 μmol/L at pH 6.0 and 13.9 μmol/L at pH 7.1 using Caco-2 cells. Furthermore, Verwei et al. [14] used a standard containing a mixture of 5-CH_3-H_4folate with vitamin activity [6*S*] and without [6*R*], which most likely resulted in lower uptake.

We report for the first time the use of a new combined Caco-2 cell/stable isotope model. The use of stable isotope-labelled folate allowed to distinguish added [6*S*]-$^{13}C_5$-5-CH_3-H_4folate from unlabelled endogenous folates in the bread matrix (~5%) and Caco-2 cells. During the 1-h experiment, already a part of the labelled folate monoglutamate was retained in the cells as polyglutamate as indicated by the increased content of [6*S*]-$^{13}C_5$-5-CH_3-H_4folate monoglutamate in lysed cells after enzymatic treatment.

In this Caco-2 cell experiment, we attempted to simulate physiological conditions in the human gut and therefore digests of foods and standard solutions were used instead of isolated standard compounds. However, this trial was limited by the difference of folate content in the digests (0.35 vs. 0.5 μmol; Table 2) and by the use of the same seeding only in the six different wells.

The current mandatory fortification level of flour in USA increased average daily folic acid intake by around 75 μg [28] and significantly improved the folate status of the population [28]. However, findings from several short-term folate bioavailability studies [8, 9] indicate a reduced availability of folic acid fortificant from some cereal food matrices compared to supplemental folic acid. Entrapment of the vitamin within the cereal matrix was suggested to cause this reduced absorption [8], which would be in line with our in vitro TIM data, demonstrating a lower bioaccessibility of endogenous bread folates compared to a complete breakfast meal. In addition, our data from the in vitro Caco-2 cell experiment suggest that the uptake of [6*S*]-5-CH_3-H_4folate into the cells may be reduced by the cereal matrix.

Nevertheless, several intervention trials indicate that already smaller doses around 100 μg additional folic acid or endogenous food folate can improve folate status [29, 30] and we interpret the data from the TIM experiment that in vitro folate bioaccessibility from a mixed diet (the breakfast) is high.

To summarise, retention of folic acid fortificant during bread-making was substantially higher compared to the retention of added [6*S*]-5-CH_3-H_4folate, which could be improved by reducing the loaf weight and baking time. Data from the TIM and Caco-2 cell trials suggest an inhibiting effect on the in vitro bioaccessibility of [6*S*]-5-CH_3-H_4folate from the two tested bread matrices, but not from the complete breakfast meal.

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Conflict of interest statement The authors declare that they have no conflict of interest.

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