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ORIGINAL CONTRIBUTION

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Study of wheat breakfast rolls fortified with folic acid

The effect on folate status in women during a 3-month intervention

Summary *Background* Folate has come into focus due to its protective role against child birth defects such as neural tube defects (NTD). Swedish authorities recommend all fertile women to increase their folate intake to 400 µg/day by eating folate-rich foods. Because not all women follow these recommendations, there is a discussion today about whether Sweden should introduce folic acid fortification in wheat flour and sifted rye flour. This decision needs knowledge about the bioavailability of folic acid from fortified foods. *Aim of the study* To investigate effects of two folic acid fortification levels on folate status in healthy female volunteers and to study the folic acid stability during the baking procedure and storage of the fortified breakfast rolls. *Method* Twenty-nine healthy women were recruited. Folic acid-fortified wheat breakfast rolls were baked with the purpose to contain 200 µg folic acid/roll (roll L) and 400 µg folic acid/roll (roll H). Fourteen women were given one roll/day of roll L (group L) and 15 one roll/day of roll H (group H) during 12 weeks of intervention. Fasting venous blood samples were collected on days 0, 30, 60 and 90. Serum homocysteine concentrations were determined using an immunoassay. Serum and erythrocyte folate concentrations were analysed using a protein-binding assay with fluorescent quantification. The folic acid concentration in the breakfast rolls was analysed by HPLC on days 0, 30, 60 and 90. Total folate concentration was measured with microbiological assay on day 45. *Results Group L* Group L had initially an average erythrocyte folate concentration of 577 ± 93 nmol/L. After 90 days of intervention, an increase of 20 % ($p < 0.05$) was observed. At day 0, mean serum folate concentrations were 16.9 ± 4.3 nmol/L. The mean serum folate concentrations increased by 30 % ($p < 0.001$) after 90 days. At day 0, mean serum homocysteine concentrations were 9.1 ± 2.0 µmol/L, which decreased by 20 % ($p < 0.01$) after 30 days. *Group H* Group H had an initial erythrocyte folate concentration of 784 ± 238 nmol/L. After 90 days, an increase of 26 % ($p < 0.05$) was observed. Serum folate increased at least 22 % after 30 days, from a level of 18.7 ± 4.8 nmol/L at day 0. Thereafter, all women of group H had serum concentrations at or above the upper limit of quantification (23 nmol/L). At day 0, mean serum homocysteine concentrations were 8.4 ± 1.7 µmol/L, which decreased by 16 % ($p < 0.05$) after 30 days. The baking procedure resulted in 20–25 % loss of fortified folic acid in the rolls used in the present study. The size of the rolls affected the retention of folic acid during baking. No significant loss was seen in folic acid concentration in the rolls during the intervention period. *Conclusion* The present study showed that in healthy women, subjected to a 12-week intervention with breakfast rolls fortified with either 166 µg or 355 µg folic acid, serum homocysteine concentration decreased ($p < 0.05$) and erythrocyte folate increased ($p < 0.05$). The lower level of fortification seems to be sufficient to improve the folate status. Together with the average daily intake of natural folates, these women reach the recommended intake of 400 µg/day. Folic acid is stable in fortified bread for 90 days storage at –20 °C.

Key words folic acid – fortification – folate status – breakfast rolls – folate stability – Sweden

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Introduction

In recent years, folate, a water-soluble B vitamin, has come into focus due to its protective role against child birth defects such as neural tube defects (NTD), coronary heart diseases and certain forms of cancer. Folate is an important co-factor in the synthesis of DNA and RNA and the remethylation of homocysteine to methionine. A mildly elevated total plasma homocysteine concentration is therefore suggested to be a marker for a defect in folate metabolism or for folate deficiency. A high total plasma homocysteine concentration is considered a risk factor for cardiovascular disease [1, 2], neural tube defects [3, 4], Alzheimer's disease [5] and colon cancer [6]. In 1991, the MCR Vitamin Study group [7] confirmed that supplementary folic acid (the synthetic form of the vitamin folate) reduced the risk of recurrence of neural tube defects (NTD) in babies when taken during the periconceptional period. Berry et al. [8] implied 1999, based on data from a Chinese intervention study, that the NTD frequency can be decreased from 0.8 to 0.6 per 1000 pregnancies with a daily supplementation of 400 μg folic acid before conception. Since 1998, the Food and Drug Administration in USA has recommended the fortification of all cereal grain products produced from wheat, rice or maize with 140 μg per 100 g [9]. As a result of this mandatory folic acid fortification, a 19% reduction of NTD birth prevalence occurred [10].

Sweden has an NTD frequency of 0.7 cases per 1000 pregnancies (personal communication Göran Annerén), which is close to the American figures. According to dietary surveys performed during the last 15 years [11–13] only 10% of Swedish women and about one-third of Swedish men reach an intake of 300 μg per day. The Swedish Food Administration and National Board of Health and Welfare recommend that, from 1 January 2001, all fertile women increase their folate intake to 400 μg/day by eating folate-rich foods such as green vegetables, fruits, fermented milk products and coarse rye bread [14]. Because not all women follow these recommendations and some pregnancies are unplanned, the authorities are discussing whether Sweden should introduce folic acid fortification in wheat flour and sifted rye flour. The issue, to what concentration of folic acid the flour should be fortified, is important. This decision needs knowledge about folate bioavailability of folic acid-fortified foods. Today there are almost no folic acid-fortified products in Sweden. Therefore, the aim in our study was to investigate effects of two different folic acid-fortification levels on folate status in healthy women. The fortification levels of the rolls aimed to supply half of the women with an additional amount of 200 μg folic acid per day and the other half with an additional amount of 400 μg folic acid per day above their normal folate intake. As indicators for folate status, serum folate, erythrocyte folate and homocysteine levels were used [15, 16]. We also investigated the effect of the baking procedure and storage on folic acid stability in breakfast rolls to confirm that the subjects consumed the assumed amounts of folate from the fortified rolls throughout the 12-week intervention period.

Subjects and methods

Subjects

Thirty-three women (aged 23–50, mean body mass index 23.4) were recruited in January 2001 from the staff and student population of the Swedish University of Agricultural Sciences, Uppsala, and employees at the National Food Administration, Uppsala, Sweden. Ten women used contraceptive pills and four women were using intra-uterine contraceptive devices. Written consent was obtained from all volunteers after they had been given oral and written information on the study. Women who were pregnant, lactating, planning a pregnancy, smokers or consumers of excessive amounts of alcohol (corresponding to two bottles of wine/week) were excluded. Other exclusion criteria were use of folic acid, vitamin B_6 or vitamin B_{12} supplementation three months prior to and throughout the study, or medication that could interfere with folate metabolism. The women were asked not to donate blood two months prior to and during the intervention. The volunteers were screened for haemoglobin levels, serum ferritin, plasma glucose, liver functions (P-ALAT, P-ASAT, and P-GT) vitamin B_{12}, serum folate and erythrocyte folate status. Women were excluded if any of those parameters were out of normal range. However, erythrocyte and serum folate values above normal were accepted. Twenty-nine subjects qualified to participate in the study. The study design was approved by the Medical Ethical Committee of Uppsala University, Sweden.

Study design

Fasting venous blood samples (8 ml, but 15 ml at screening) were collected at baseline and then at day 30, 60, and 90 during the three-month intervention with folic acid-fortified breakfast rolls. On the same day, after collection of the baseline sample, the intervention began. Individual starting points were spread during three weeks. Participants were randomly assigned into two groups receiving either wheat breakfast rolls fortified with a low folic acid content (166 μg/roll) (group L) or a high folic acid content (355 μg/roll) (group H), with one roll to be eaten each day. The distributed breakfast rolls were kept in the freezer (–20 °C) until consumption. Participants were asked to thaw the rolls either in the refrigerator, in a dark cupboard overnight or by microwave (500 W,

maximum 1 min) to protect folic acid from light and heat. Participants were asked to replace any folic acid-fortified foods from their diets with non-fortified products during the intervention but otherwise continue with their normal dietary habits. Dietary habits were assessed at screening and again during the last week of intervention, using self-administrated food-frequency questionnaire (FFQ) focussing on the major folate contributors in the Swedish diet.

Blood samples

Blood samples were taken by authorised staff at the metabolic division at Samariterhemmet, Uppsala, Sweden. Samples were collected into gel-containing tubes for serum folate and serum homocysteine analysis, and into EDTA-containing tubes for erythrocyte folate analysis. Homocysteine samples were centrifuged (10 min) and the serum was transferred into plastic tubes within 30 min after sampling. All tubes were stored in the refrigerator and covered with aluminium foil to protect the folates. Serum folate, erythrocyte folate and serum homocysteine were analysed at the Clinical Chemistry and Pharmacology Centre for Laboratory Medicine, Uppsala University Hospital. Serum homocysteine concentrations were determined using a fluorescence polarisation immunoassay (Imx System, Homocysteine, Abbot Laboratories, Illinois, U. S.A.) according to kit instructions. The Imx Homocysteine assay quantifies total L-homocysteine in human serum resulting in intra-assay coefficients of variations of 1.4–2.2 % and inter-assay coefficients of variations of 3.7–5.2 %. Serum folate concentrations were analysed using a protein-binding assay with fluorescent quantification (Abbot Axsym System Folate, Abbot Laboratories, Illinois, U. S. A.). Sample treatment was performed according to kit instructions. The same protein-binding assay was used to measure erythrocyte folate concentrations. Erythrocyte folate samples were stored in a freezer (–20 °C) until all blood samples from the specific intervention day were taken, and then analysed in the same assay. Sample preparation procedures were performed according to the kit instructions. Due to initially high folate concentrations (> 23 nmol/L), four samples were diluted additionally two times with protein solution to fit within the standard curve. The total coefficient of variation was < 15 % at 5 nmol/L and < 6 % at 19 nmol/L for both serum folate and erythrocyte folate analysis.

Breakfast rolls

Baking

The folic acid-enriched wheat breakfast rolls were baked by a commercial bakery, Skogaholms, Stockholm. A test baking was first performed to estimate folic acid losses during the baking procedure and control the variation between different rolls. In the final baking, two types of breakfast roll (high and low folic acid content) were baked in different batches, each of 100 kg. To the first batch, 367.3 mg folic acid (Merck Eprova AG, Schaffhausen, Switzerland)/100 kg dough was added to give a folic acid concentration of 200 µg/roll (roll L). To the second batch, 733.4 mg folic acid/100 kg dough was added to give a concentration of 400 µg folic acid/roll (roll H). The rolls were immediately frozen and stored in a freezer (–20 °C) until distribution to the participants who stored the rolls in their home freezer until consumption.

Analysis of folic acid and endogenous folate in the breakfast rolls

Folic acid and endogenous folates in the breakfast rolls were analysed by HPLC on day 0, 30, 60 and 90 during the intervention to investigate the stability of the added folic acid during storage. Total folate concentrations in the rolls were analysed with microbiological assay.

Sample preparation

Prior to HPLC analysis, frozen rolls were freeze-dried (45 h, in a Labconco freeze-drier, abninolab, Upplands-Väsby, Sweden) and ground. A modified trienzyme method was used [17, 18]. Freeze-dried samples (2 g) were extracted in a boiling water bath for 10 minutes, in 40 ml of freshly prepared 0.1 M phosphate buffer pH 6.1, containing 1 % ascorbic acid and 0.1 % 2-mercaptoethanol together with 40 µl thermostable α-amylase (E-BLAAM, Megazyme International, Ireland). After cooling on ice to room temperature, 2 ml of protease (2 mg/ml 1 % sodium ascorbate solution) (P 5147 Sigma, Sweden) was added to the extracts and the samples were incubated for 3 hours at 37 °C in a water bath. The enzyme reaction was inhibited by boiling the extract for 5 minutes in a water bath. Most folates are present as poly-γ-glutamates. The HPLC method used requires cleavage to monoglutamates, by hog kidney conjugase. After cooling, pH was adjusted to 4.9 before adding 3 ml hog kidney conjugase (K 7250 Sigma, Sweden) (preparation modified from Gregory [19] and Phillips and Wright [20]) for another incubation for 3 hours at 37 °C. Thereafter, extracts were again placed in boiling water for 5 minutes to inactivate the enzymes and then cooled on

ice. The samples were filtered (Munktell filter, 120 H) and diluted with fresh extraction buffer (see above) to a final volume of 200 ml and stored at –20 °C until folate quantification. The whole sample treatment procedure was performed under subdued light to minimise photochemical degradation of folates and the extracts were flushed with nitrogen to prevent folate oxidation and degradation. Each roll type was extracted in triplicate on two different days.

Sample purification

On the day of HPLC quantification, the extracts were purified with strong anion-exchange (SAX) cartridges (Isolute, Sorbent) as described in detail by Jastrebova et al. [21]. The cartridges (3 ml, 500 mg) were conditioned by rinsing with 5 ml methanol, followed by 5 ml water. The cartridge was then loaded with 2.5 ml extract and then washed with 5 ml of water. Folates were eluted with 0.7 ml + 3 ml 10 % sodium chloride in 0.1 M acetate containing 1 % ascorbic acid and 0.1 % 2-mercaptoethanol. The first 0.7 ml fraction was discarded. Final volume was quantified by weight.

High performance liquid chromatography

HPLC analysis was performed using an Agilent 1100 series HPLC system equipped with a gradient quaternary pump, a cooled (+ 8 °C) autosampler, a thermostatted column compartment, a diode array (DAD) detector and a fluorescence detector. Agilent Chemstation software was used to control the HPLC system and data processing. An HPLC method of Jastrebova et al. [21] was slightly modified to achieve baseline separation of both folic acid and three native folate forms: tetrahydrofolate (H_4folate), 5-methyl-tetrahydrofolate (5-CH_3-H_4folate), 5-formyl-tetrahydrofolate (5-HCO-H_4folate) on a ZORBAX SB-C8 column (4.6x150 mm; 5 µm). The column temperature was maintained at 23 °C and the injection volume was 20 µl. The mobile phase was a binary gradient mixture of 30 mM acetic acid and acetonitrile. The gradient started at 6 % (v/v) acetonitrile maintained isocratically for the first 5 min; thereafter the acetonitrile content was raised linearly to 25 % within 20 min. The runtime was 33 min. Folic acid was detected with DAD at a wavelength of 290 nm. The native folates were detected with fluorescence detector; the excitation and emission wavelengths were set at 290 and 360 nm, respectively. Four folate standards, folic acid, (6S)–5-HCO-H_4folate, sodium salt, (6S)-5-CH_3-H_4folate, sodium salt and (6S)-H_4folate, sodium salt (Merck Eprova AG, Schaffhausen, Switzerland) were used for external calibration. The purity of the standards was checked according to the procedure of van den Berg et al. [22]. The detection limits were 0.5 ng/ml for H_4folate, 0.3 ng/ml for 5-CH_3-H_4folate, and 2 ng/ml for 5-HCO-H_4folate and folic acid.

Microbiological assay

The total folate concentrations in rolls were analysed with the microbiological assay one time, 45 days after the study started. Microbiological analysis (modified from AOAC Official method 992.05) [23] was carried out by the National Food Administration that is accredited for folate analysis in foods. All samples were extracted according to the in-house method at National Food Administration, which includes trienzyme treatment with α-amylase, protease and chicken pancreas conjugase treatment.

Statistics

To compare the characteristics of the two groups of women (Table 1), two-sample t, Minitab version 13 (2000) was used. To compare if the daily average intake of folate calculated from a food questionnaire changed before and after the intervention period a paired t-test was used, Minitab version 13 (2000). Statistical analysis concerning changes in the folate status parameters during the intervention period of 12 weeks (Table 2) was carried out using the procedure "Mixed", SAS (Statistical Analysis Systems Inc., Cary, North Carolina, USA). Procedure "Mixed" used two folate levels (H and L) as the main factors, subjects nested within levels and the factor day crossed with subjects. Changes in folate concentrations in the rolls when stored in a freezer were statistically analysed at days 0, 30, 60 and 90 using general linear modelling (GLM), SAS, according to a three factor factorial design (days; bread L and H; folate) (Table 3). For all statistical analyses, differences were considered significant at $p < 0.05$.

Table 1 Characteristics of the subjects[1]

	Group L 166 µg folic acid/roll (n = 14)	Group H 355 µg folic acid/roll (n = 15)
Age (y)	32.8 ± 8.5	35.9 ± 8.8
BMI (kg/m^2)	22.8 ± 2.3	24.0 ± 5.6
Contraceptive users (%)	43	47
Serum vitamin B_{12} (pmol/L)[2]	234 ± 62.4	253 ± 88.5
Erythrocyte folate (nmol/L)[3]	761 ± 180	885 ± 185
Serum folate (nmol/L)[4]	16.0 ± 4.6	18.8 ± 4.9
Serum homocysteine (µmol/L)[5]	9.1 ± 2.0	8.4 ± 1.8

[1] Mean ± SD; [2] ref. interval 100–420; [3] ref. interval 430–740; [4] ref. interval 5.8–22; [5] ref. interval < 15 mmol/L

Table 2 Erythrocyte folate, serum folate and serum homocysteine concentrations[1] during the 90-day intervention with folic acid-fortified breakfast rolls

	166 µg folic acid/roll (group L) (n = 14)	355 µg folic acid/roll (group H) (n = 15)
Erythrocyte folate (nmol/L)		
Day 0	577 ± 93[d]	784 ± 238[d]
Day 30	574 ± 82[d]	767 ± 160[d]
Day 60	591 ± 160	850 ± 150[d]
Day 90	694 ± 154[a, b]	987 ± 167[a, b, c]
Serum folate (nmol/L)		
Day 0	16.9 ± 4.3[d]	18.7 ± 4.8
Day 30	21.2 ± 2.4[a]	23[2]
Day 60	22.4 ± 1.3[a]	23[2]
Day 90	22.0 ± 2.1[a]	23[a]
Serum homocysteine (µmol/L)		
Day 0	9.1 ± 2.0[b, c, d]	8.4 ± 1.7[b, c, d]
Day 30	7.3 ± 1.2[a]	7.0 ± 1.3[a]
Day 60	7.4 ± 1.3[a]	7.0 ± 1.1[a]
Day 90	7.2 ± 1.3[a]	7.0 ± 1.2[a]

[1] Mean ± SD; [2] Upper limit of quantification was 23 nmol/L; [a, b, c, d] $p < 0.05$, significantly different from day 0 (a), day 30 (b), day 60 (c), day 90 (d)

Table 3 Folic acid, 5-CH_3-H_4folate, H_4folate and 5-HCO-H_4folate concentrations in breakfast rolls (µg/roll). Mean roll weight: 51.5 ± 0.7 g

	µg/roll
Roll L	
Folic acid	166 ± 16
5-CH_3-H_4folate	10.0 ± 0.8
H_4folate	2.5 ± 0.4
5-HCO-H_4folate	n. d.
Roll H	
Folic acid	355 ± 33
5-CH_3-H_4folate	10.2 ± 0.6
H_4folate	2.5 ± 0.3
5-HCO-H_4folate	n. d.

n. d. not detectable

Results

Subjects

Twenty-nine women completed the study; fourteen women in the group received breakfast rolls with the low folic acid concentration (group L) and 15 women in the group received the rolls with the high folic acid concentration (group H). Characteristics of the subjects at the beginning of the intervention are shown in Table 1.

After 90 days of intervention, the mean BMI of the women in group L was 22.9 ± 2.2 and in group H 23.9 ± 5.6, which indicates that the participants did not change their energy intake during the intervention. Estimates of folate intakes based on FFQ indicated no significant differences in folate intake from the diet between groups L and H on day 0, 221 µg/day and 209 µg/day, respectively. There were no significant changes in the folate intake between day 0 and 90 in the groups. Group L had an intake of 210 µg folate/day and group H had an intake of 215 µg/day on day 90 of the intervention.

Folate status

Clinical folate status parameters for the two groups receiving low and high folic acid-fortified breakfast rolls, respectively, are compiled in Table 2. At the start (day 0), two-thirds of the women had *erythrocyte folate* concentrations above the reference interval (430–740 nmol/L); therefore, four erythrocyte samples needed to be diluted to fit within the calibration range. Group L and group H had initially average erythrocyte concentrations of 577 ± 93 nmol/L and 784 ± 238 nmol/L, respectively, which did not differ significantly. After 90 days of intervention, a significant increase in erythrocyte folate of 20 % ($p < 0.05$, group L) and 26 % ($p < 0.05$, group H) was observed. Women receiving high folic acid-fortified breakfast rolls showed significantly higher ($p < 0.001$) mean erythrocyte folate concentrations than women consuming low folic acid-fortified breakfast rolls, 987 ± 167 nmol/L compared with 694 ± 154 nmol/L.

At day 0, mean *serum folate* concentrations were 16.9 ± 4.3 and 18.7 ± 4.8 nmol/L for the groups L and H, respectively. Serum folate concentrations were initially not significantly different between the two groups. The mean serum folate concentrations increased by 25 % ($p < 0.01$) and 30 % ($p < 0.001$) after 30 and 90 days, respectively, for group L. Between day 30 and 60, the mean serum folate concentration increased from 21.2 ± 2.4 to 22.4 ± 1.3 nmol/L, reaching a plateau, because at day 90 the mean serum folate remained the same (22.0 nmol/L). After 30 days, the serum folate levels for group H, which started with 18.7 ± 4.8 nmol/L, reached 23 nmol/L (the upper limit of quantification). This increase was significant ($p < 0.001$) amounting to at least 22 %. Since no dilutions of the serum samples were performed, all serum folate levels exceeded 23 nmol/L during the next 60 days for group H.

No significant difference in *serum homocysteine* concentrations between the two groups was observed initially. At day 0, mean total serum homocysteine concentrations were 9.1 ± 2.0 µmol/L and 8.4 ± 1.7 µmol/L for group L and group H, respectively. For women in group L, mean serum homocysteine decreased by 20 % ($p < 0.01$) after 30 days. Thereafter, the mean serum homocysteine concentration levelled off to a concentration close to 7 µmol/L. The women in group H decreased their serum homocysteine by 16 % ($p < 0.05$) after 30 days, which remained about 7.0 µmol/L for the next 60 days.

Breakfast rolls

The rolls in the *test baking* weighed 68.3 ± 7.5 g and contained 227 µg folic acid. We aimed to produce rolls with 200 µg folic acid/each. A 30 % excess to adjust for the losses during baking was used, but only 12 % of the added folic acid were lost. The folic acid recovery during the sample treatment and HPLC analysis was 76–82 %, and the variation in folic acid content between the rolls was 4.6 %.

In the *final baking* the roll weight was adjusted to 51.5 ± 0.7 g to produce rolls with 400 µg and 200 µg folic acid, respectively. A 10 % excess of folic acid was added to compensate for losses during baking. However, folic acid losses during baking were 25 % in bread L and 19 % in bread H. The concentrations of folic acid and natural folates in the rolls are shown in Table 3. The folic acid concentration was 166 ± 47 µg in roll L and 355 ± 63 µg/100 g in roll H. For roll H, no significant differences from day 0–90 were found ($p > 0.197$). For roll L, no significant differences in the folic acid concentration between day 30 and 90 were found ($p > 0.777$) except for day 0 when folic acid concentration was slightly lower.

Both L and H rolls contained similar amounts of native folate forms, amounting to about 10 µg and 2.5 µg of 5-CH_3-H_4folate and H_4folate, respectively. No detectable amounts of 5-HCO-H_4folate in the rolls were found. Total folate concentrations in the rolls, ready for consumption, quantified with the microbiological assay were 198 ± 80 µg/roll L and 365 ± 145 µg/roll H.

Discussion

Although all women had a good initial folate status with *erythrocyte folate* concentrations well within the reference interval (430–740 nmol/L), a significant increase of red cell folate was observed in both groups of women. The women had been totally randomised into two groups. The group H women had a higher base-line erythrocyte folate concentration than group L women, but it was not significantly higher. In both groups erythrocyte folate concentrations did not increase significantly after four weeks, but were significantly ($p < 0.05$) increased at day 90 of the intervention. These findings are in agreement with a normal red cell turnover of 3 months. Surprisingly, Brouwer et al. [1] observe a significant increase in red cell folate concentrations after as little as a four-week intervention both with 500 µg folic acid supplementation every day or every second day.

Unfortunately, the method to estimate serum folate concentrations had an upper limit of quantification at 23 nmol/L, which made it impossible to quantify the increase in serum folate concentrations during the intervention period. Yet, a significant increase in serum folate was observed as early as day 30 in both groups of women, which persisted during the intervention period.

Both fortification levels of 166 µg folic acid/day and 355 µg folic acid/day resulted in a significant decrease in *serum homocysteine* concentrations after the first four weeks of intervention (Table 2). This indicates that an intake of 166 µg folic acid/d is sufficient to decrease serum homocysteine in healthy women. Brouwer et al. [1] observe a 11.4 % decrease in total plasma homocysteine in 50 women receiving 500 µg folic acid supplementation every second day during four weeks. Volunteers in that study show similar mean homocysteine concentrations prior to intervention as those in the present study. The pronounced decrease in serum homocysteine in our study is difficult to explain, since folic acid bioavailability from fortified cereal products with a complex matrix is expected to be lower (30–85 %) than from pharmaceutical preparations [24–27]. Our results, however, support a study of Schorah et al. [28] investigating responses of plasma homocysteine to small increases in dietary folic acid. They report a significant ($p < 0.05$) decrease of 7.8 % in plasma homocysteine concentrations among 33 subjects with initial homocysteine of 10.2 µmol/L after an eight-week intervention with 200 µg folic acid-fortified breakfast cereals. In contrast to our results, Schorah and co-workers observed no significant decreases in homocysteine concentration after four weeks. However, the study of Schorah and co-workers shows that additional vitamin B_{12} or vitamin B_6 is not required to decrease plasma homocysteine concentrations, which is supported indirectly by our observations. For both of our regimes of folic acid-fortified breakfast rolls, a plateau in serum homocysteine seemed to be reached for the women within 30 days (Table 2). This is in agreement with several studies suggesting that a high dose of folic acid has no additional plasma total homocysteine-lowering effect after reaching this plateau [1, 29]. However, the lowest effective folate dose is not clear. Malinow et al. [30] observed a non-significant decrease of plasma homocysteine levels when they gave patients with coronary heart disease breakfast cereals fortified with 127 µg folic acid/d. The patients had a basal plasma homocysteine concentration of 9.9 ± 3.1 µmol/L which did not decrease. This indicates that this amount of folic acid fortification is too low to affect the homocysteine concentration.

According to the FFQ, the women in this study consumed about 200 µg dietary folate/day, which corresponds well with dietary surveys reporting a mean folate intake for Swedish women of 217 µg/day [13]. With an additional amount of 166 µg folic acid/day from fortified breakfast rolls, these women should have an average intake close to 400 µg, which is the recommended intake for fertile women. Fertile women in Sweden consume foods containing 100–150 g white wheat and sifted flour per day [31]. Thus, a folic acid fortification level of flour

corresponding to 140 µg per 100 g, as is mandatory in the US today, would supply the Swedish women with an additional folic acid amount between 140–210 µg per day. Daly et al. [32] show that a daily additional amount of 100 µg folic acid through supplementation increases the red cell folate levels by 67 µg/litre, whereas a daily additional amount of 200 µg folic acid increases the level by 130 µg/litre. We could see an increase in the red cell level of 53 µg/litre for women receiving 166 µg of additional folic acid/day. In a 3-month-long study by Cuskelly et al. [33], women were randomly assigned to one of the following five groups: 1) folic acid supplement (400 µg/d), 2) folic acid-fortified foods (400 µg/d), 3) dietary folate (400 µg/d), 4) dietary advice, or 5) control. Responses to intervention were assessed as changes in red cell folate concentrations after an intervention period of 3 months. Interestingly, only the groups consuming either folic acid supplements or food fortified with folic acid showed significant increases in red cell folate concentration, whereas the other groups had no significant change in their folate status. Kelly et al. [34] investigated unmetabolised folic acid in serum as an effect of an intake of 800 µg folic acid fortificant/day. However, they found no unmetabolised folic acid in subjects consuming 200 µg folic acid per day. A decision about folic acid fortification also needs to consider the risk that parts of the population might reach toxic levels or the increased risk for twin births [35]. Masking of vitamin B_{12} deficiency has been discussed but should not be a problem since clinical markers for vitamin B_{12} deficiency have been developed in numbers with sufficient sensitivity and specificity during recent years.

Folic acid losses during the test baking procedure were 12 %, which resulted in the decision to add a 10 % excess of folic acid during the main baking. Folic acid losses were, however, greater during the main baking (20 %). This could be explained by the smaller size of the final rolls, resulting in a relatively larger surface. Earlier studies report folic acid losses in fortified bread during baking corresponding to 11 % [36] and 30 % [29]. No folic acid, however, was lost during storage of the fortified breakfast rolls in a freezer (–20 °C) for 12 weeks, which ensured that the volunteers received the targeted daily folic acid dose during the entire intervention period. Both carefully checked data for folic acid losses during the baking procedure in relationship to bread roll size and the fact that the folic acid was stable in the freezer for three months might be an important information for the baking industry if fortification is introduced in Sweden.

In conclusion, the present study showed that in healthy women, subjected to a 12-week intervention with breakfast rolls fortified with either 166 µg or 355 µg folic acid, erythrocyte folate concentrations increased significantly ($p < 0.05$) after 12 weeks. Serum homocysteine concentrations decreased significantly ($p < 0.05$) after 30 days for both groups of women. Both investigated folic acid fortification levels positively affected the three status parameters. No negative effects on folic acid stability were observed during 3 months storage of the fortified rolls; and baking losses, which did not exceed 20 %, can be accounted for during bread production.

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