

EFFECT OF STORAGE CONDITIONS ON FOLIC ACID CONTENT IN FOLIC ACID-FORTIFIED BREAD – A MODEL STUDY*Renata Biezanowska-Kopec¹, Paweł M. Pisulewski¹, Magdalena Surma-Zadora², Teresa Leszczyńska¹**¹Department of Human Nutrition, ²The Malopolska Centre of Food Monitoring and Certification; Faculty of Food Technology, Agricultural University of Cracow, Cracow, Poland*

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Folic acid is a very unstable water-soluble vitamin which undergoes inactivation under the influence of such factors as UV radiation, temperature and air. In model studies an attempt was made to eliminate the above mentioned factors by using various storage methods. In view of the above, the objective of this study was to define the effect of storage conditions on folic acid content in folic acid-fortified wheat and rye bread.

The experimental material was wheat and rye bread fortified with folic acid (0.01 g/100 g flour) and stored for a customary shelf-life, *i.e.* 3 days. The bread samples were stored as follows: (a) without packaging, (b) in paper bags, and (c) in polypropylene bags (in light and without light). The samples of bread were collected after 1, 2 and 3 days of storage. The extraction of folic acid from the bread samples was conducted as described by Gujska & Kuncewicz [2005], and modified according to the Polish Standard [PN-EN 14131:2003], applying a trienzyme extraction (conjugase, α -amylase, protease) procedure. Folic acid was determined using HPLC (LaChromElite, Hitachi) with a UV/US detector.

The time of storage was found to have no effect on folic acid content in folic acid-fortified bread. Equally, the conditions of storage did not affect the folic acid content in the bread samples examined.

INTRODUCTION

Folic acid is one of B vitamins. It is found in liver, yeast, raw green vegetables and in legumes. Significant folate losses, *i.e.* reaching 50-90% of the initial content, occur at food processing and storage. A low intake of folic acid, vitamin B6, and vitamin B12 increase homocysteine levels in the population. An elevated plasma concentration of homocysteine is an independent risk factor for vascular disease [Chen *et al.*, 1999; Strain *et al.*, 2004; Voutilainen *et al.*, 2001]. Folate nutritive is also associated with neural tube defects [Jakubowski, 1997; Rosenquist *et al.*, 1996], neurodegenerative diseases (Alzheimer and Parkinson disease) [Muntjewerff *et al.*, 2006; Vastag, 2002], anaemia and carcinomas (especially colon carcinoma) [Akoglu *et al.*, 2004; Jennings, 1995]. Dietary improvement, providing abundant folic acid and B vitamins, may prevent vascular disease by lowering blood homocysteine.

Food enrichment, alias fortification, is in the last years the most willingly chosen solution to the problem of occurring deficiencies of B vitamins. Due to the great importance of folates in the proper functioning of the human body, some countries introduced obligatory food fortification in folic acid, the simplest synthetic form of folates for food enrichment [Final Assessment Report, 2006; Kunachowicz *et al.*, 2004]. In 1998, the Food and Drug Administration implemented legislation requiring the fortification of cereal products (bread, rusks, rice) with folic acid at the level of 1400 μ g/kg

of the product [Food and Drug Administration, 1996; Sobieraj, 2006].

In Poland, food fortification is implemented in accordance with the guidelines provided by the Regulation of the Minister of Health of 19 December 2002 on fortifying agents added to food and conditions for their use [Journal of Laws, No. 27, item. 237]. Polish and EU regulations do not define acceptable limits of variations between values specified in product labels and actual content of nutrients added to food. This regulation requires giving consideration to product diversity resulting from a different nature of raw materials or vitamin losses at production processes and storage. Various stability of added nutrients should also be taken into consideration. Taking into account all these aspects, food manufacturers often use high "technological excess", so that the actual value is not lower than the declared value, especially at the end of storage period. In order to eliminate excessive variations between the declared and actual value, storage tests should be carried out on fortified products [Jantarska *et al.*, 2007; Jurga, 2005]. Good manufacturing practice indicates that the excess should not be greater than 20%, unless the added component is relatively changeable and another value for the excess was determined analytically in the tests following foodstuff manufacture and in storage tests.

Folic acid is a compound sensitive to external conditions, such as: temperature, pH of an environment, UV rays or presence of oxygen [Jantarska *et al.*, 2007]. Significant folate losses, *i.e.* reaching 50-90% of the initial content, occur at cooking, food processing, storage and distribution [Food

Standards Australia New Zealand, 2006]. Efforts are made to reduce losses of this vitamin also during storage.

In this aspect the aim of this work was to determine the influence of storage of folic acid-enriched food on folic acid content, as affected by time and conditions of storage. To this end, four methods of bread storage were used: (a) unpacked at a room temperature, (b) in a paper bag, (c) in a polypropylene (PP) bag with and without access of light. The bread was stored for a customary shelf life, *i.e.* 3 days. Samples to be tested were collected after the first, second and third day of storage. The present data are a model study of storage of folate-fortified wheat and rye breads.

MATERIALS AND METHODS

Material

The samples included wheat light bread and rye light bread (90% of rye flour and 10% of wheat flour), enriched with folic acid in a quantity of 0.01 g per 100 g of flour. The proposed fortification level is aimed to preliminarily assess the changes in folic acid content at technological process and storage.

Laboratory baking was carried out twice using a one-phase method, according to the recipe presented in Table 1. Dough was prepared in a high-speed laboratory dough mixer made by Wiosna Company (Poland).

Sample preparation

The test was made so as to protect the test sample from light. Two samples of a bread pulp were taken from each variant of storage. A 2.5 g portion of the test sample (with accuracy to 0.001 g) was weighed into 50-mL bottles. Next, 20 L of a phosphate buffer (pH 6.1) with ascorbate (1%, according to the Polish Standard [PN-EN 14131:2003]) were added and the sample was homogenised for 30 s, at a speed of 13,500 rpm, using an Ultra Turrax T-25 homogeniser with a S 25-18 G dispersing element. The dispersing element was flushed with 5 mL of the phosphate buffer with ascorbate (pH 6.1). The homogenised samples were saturated with nitrogen, boiled for 10 min, cooled with ice and again homogenised under the same parameters (as above). The homogenised samples were treated with the following enzymes:

1 mL of α -amylase (20 mg/mL; Sigma, A-6211) and 0.5 mL of conjugase (5 mg/mL, Kidney acetone powder, Porcine Type II; Sigma, K-7250), saturated with nitrogen and incubated at a temperature of 37°C for 4 h. Then, the samples were boiled for 5 min and cooled with ice. The samples were treated with another enzyme: 2 mL of protease (2 mg/mL; Sigma, P-5380), saturated with nitrogen and incubated for 1 h at a temperature of 37°C. Following incubation the samples were boiled for 5 min and cooled with ice. The samples were placed in a High Speed Brushless Centrifuge MPW-350R and centrifuged at 6800 rpm at a temperature of 4°C for 15 min. Supernatant volume was measured and supernatants were filtered (Filtrak filters, type 289, ϕ =110 mm). The filtrate (1 mL) was drawn to Eppendorf tubes and again centrifuged at 18,000 rpm at a temperature of 4°C for 15 min. The collected supernatants were stored in Eppendorf tubes at a temperature of -80°C, until HPLC analysis.

Chromatographic analysis

The tests were made with a liquid chromatography method, with the use of an HPLC system (La ChromElite, Hitachi) with a UV/VIS detector. Separation was carried out on a LichroCART 250-4 Lichrosphere 100 RP-18 column (250 mm, 4 mm i.d., 5 μ m thickness of a stationary phase at a room temperature). A phosphate buffer of pH 5.2 (phase A) and acetonitrile (phase B) were used as eluents, with a flow rate of 1 mL/min. Records were made at a wavelength of λ =290 nm. The following elution was applied: 0 min (100% buffer), 0-10 min gradient to a 90% buffer, 10-17 min isocratically 90% buffer, in 18th min 100% buffer, 18-25 min 100% buffer. Folic acid was identified based on comparison of retention times of the test samples and reference standards (Figure 1). A qualitative analysis was based on an internal standard method. To perform the qualitative analysis, reference solutions of folic acid were prepared in the concentration range of 1-50 μ g/mL. Each of the standard solutions was analysed three times with HPLC – UV/VIS. On the basis of the received results, calibration curve equation $y=4.29 \cdot 10^{-6}x$ was determined with a linear regression method. A linear correlation coefficient, amounting to 0.999, was determined as well.

Statistical analysis

The data were subjected to ANOVA generated by the Statistica version 6.1 package, followed by *post-hoc* Duncan's multiple range test.

RESULTS AND DISCUSSION

In industrial processing of cereal products there are losses related to rye and wheat grain milling into flour. In wheat flour, which is popularly called "Wrocławska", folacin content is 35% related to its content in grain [Gawęcki, 2002]. As reported by Kunachowicz *et al.* [2005], folate content in wheat grain is 145 μ g/100 g, and after milling wheat flour type 1850 contains over 109 μ g, while type 500 only 54 μ g of folates. In wheat bread, their content reaches barely 30.7 μ g/100 g of the product. On the other hand, folate content in rye grain is 113 μ g/100 g, and in flour from 23 μ g (light rye flower, type

TABLE 1. Formula and baking conditions of wheat and rye breads.

Components	Wheat bread	Rye bread
Wheat flour type 550	100 g	10 g
Rye flour type 2000	-	90 g
Baker's yeast	3 g	3 g
Sodium chloride	2 g	3 g
Rye acid native concern Bionat	-	8 g
Water	63 g	76 g
Folic acid	0.01 g	0.01 g
Dough fermentation (temp.40°C, humidity 80%)	15 min	90 min
Final fermentation (temp.40°C, humidity 80%)	45 min	60 min
Wright pieces	250 g	250 g
Baking (temp. 230°C)	25 min	25 min

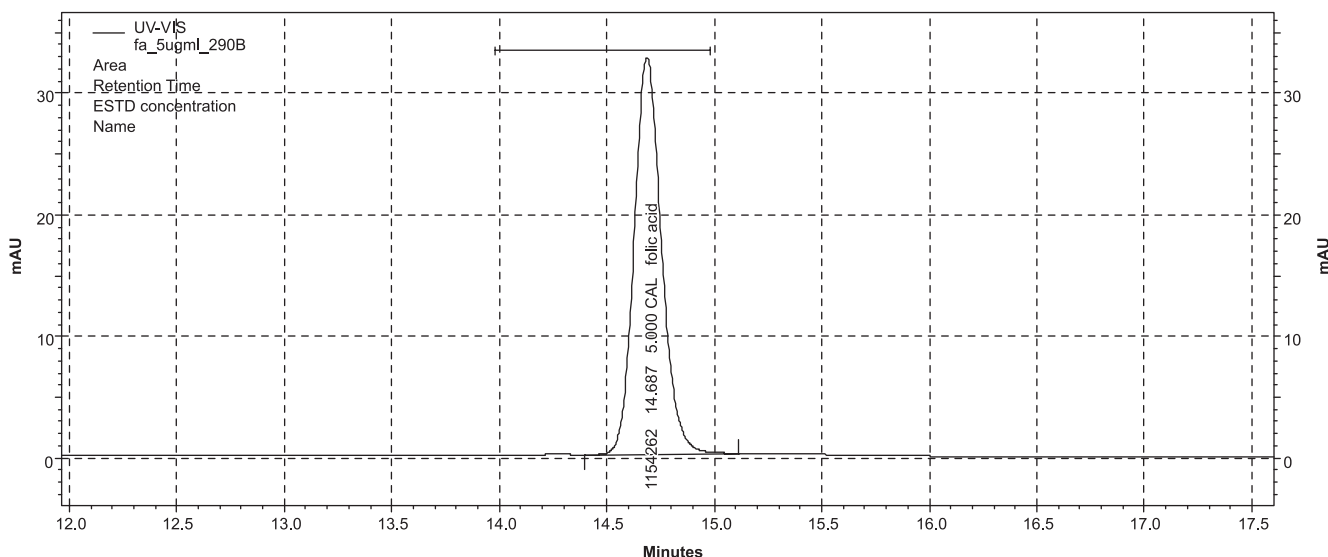


FIGURE 1. HPLC chromatogram of standard folic acid with UV/Vis detection (290 nm).

580) to 82 μg (whole rye flour, type 2000). Light rye bread contains just 11 μg folates per 100 kg of the product.

After bread baking a lower content of folic acid was determined compared to the added amount, which results from technological losses and the influence of high temperature, light and oxygen. As reported by Kariluoto *et al.* [2004], baker's yeast *Saccharomyces cerevisiae* clearly contribute to the final content of folates in bread, however it depends on yeast quantity and their capacity to synthesise folic acid at processing. According to Gawęcki & Hryniewiecki [2005], folacin content increases during dough fermentation as a result of synthesis performed by yeast, but simultaneously a similar quantity is destroyed in baking. This was confirmed also in other studies [Gregory, 2004; Final Assessment Report, 2006] which demonstrate that average losses of folic acid at baking of fortified bread may reach up to 40%.

Folate content in fresh wheat bread (Table 2) is higher than that in fresh rye bread (Table 3), since rye flour contains a lower level of folates than wheat flour.

The obtained results were analysed with reference to: folic acid content after the first, second and third day of storage of the folic acid-fortified bread and within the individual storage variants of the above-mentioned bread samples, compared with a fresh bread.

For the wheat bread folic acid content on specific days was referred to folic acid content in the fresh bread, *i.e.* 7447.5 $\mu\text{g}/100$ g of dry matter. In the bread stored unpacked folic acid content varied within the range of 7389 $\mu\text{g}/100$ g of dry matter (after the third day) to 7620 $\mu\text{g}/100$ g of dry matter (after the second day). On the last day of storage, the obtained result differed by approx. 1% of the initial value.

TABLE 2. Average content of folic acid in stored wheat bread ($\mu\text{g}/100$ g d.m. bread).

Storage \ Days	Fresh	I d	II d	III d
No packing	7447.5 \pm 129.0 ^a	7410.6 \pm 338.0 ^a	7620.8 \pm 170.0 ^b _a	7389.8 \pm 480.0 ^a
Paper bag	7447.5 \pm 129.0 ^a	7484.6 \pm 189.0 ^a	7197.4 \pm 352.0 ^{AB} _a	7423.9 \pm 176.0 ^a
PP bag (light)	7447.5 \pm 129.0 ^a _{ab}	7547.1 \pm 118.0 ^b	6837.5 \pm 37.0 ^a	7107.1 \pm 428.0 ^a _{ab}
PP bag (no light)	7447.5 \pm 129.0 ^a	7494.5 \pm 115.0 ^a	7229.5 \pm 556.0 ^{AB} _a	7079.1 \pm 695.0 ^a

\pm SEM – standard error of the mean; a, b – values in lines with different letters are significantly different, $p < 0.05$; A, B – values in columns with different letters are significantly different, $p < 0.05$.

TABLE 3. Average content of folic acid in stored rye bread ($\mu\text{g}/100$ g d.m. bread).

Storage \ Days	Fresh	I d	II d	III d
No packing	4336.5 \pm 40.0 ^a _{ab}	4205.3 \pm 382.0 ^a _{ab}	4508.5 \pm 357.0 ^a _b	4160.2 \pm 170.0 ^a
Paper bag	4336.5 \pm 40.0 ^a	4171.8 \pm 29.0 ^a	4216.4 \pm 170.0 ^a	4455.8 \pm 244.0 ^a
PP bag (light)	4336.5 \pm 40.0 ^a	4087.9 \pm 195.0 ^a	4255.4 \pm 108.0 ^a	4353.1 \pm 111.0 ^a
PP bag (no light)	4336.5 \pm 40.0 ^a	4370.2 \pm 60.0 ^a	4337.1 \pm 95.0 ^a	4327.2 \pm 98.0 ^a

\pm SEM – standard error of the mean; a, b – values in lines with different letters are significantly different, $p < 0.05$; A, B – values in columns with different letters are significantly different, $p < 0.05$.

The differences in this vitamin content on the subsequent three days appeared to be insignificant ($p > 0.05$). Wheat bread stored in a paper bag did not show any statistically significant changes ($p > 0.05$) in folic acid content. In the bread stored in a PP bag exposed to UV rays, the lowest content of folic acid was observed after the second day (-8%), and the highest after the first day (+1%), compared to the initial value. The differences, related to folic acid in the fresh bread, were not statistically significant ($p > 0.05$). Similarly, no statistical differences were shown after the third day of bread storage. In the bread stored in a PP bag without access of light successive reduction of folic acid content was observed. The differences were not statistically significant ($p > 0.05$). For the applied methods of bread storage, no statistically significant differences were shown after the first and third day ($p > 0.05$). After two days of storage the lowest result (-10% compared with the variant with no package) was obtained for the bread stored in a paper bag exposed to light.

In the rye bread folic acid content was compared with the value obtained for the fresh bread, *i.e.* 4336.5 $\mu\text{g}/100\text{ g}$ of dry matter (Table 4). In the bread stored unpacked folic acid content was by 3% lower after the first day of storage, compared with the fresh bread. On the next day this vitamin content was by 4% higher than in the fresh bread ($p > 0.05$). After three days folic acid content decreased by 4% compared with the initial value. The differences were not statistically significant ($p > 0.05$). In the bread stored in a paper bag after the first and second day lower folic acid content (by 4% and 3%, respectively) was observed, compared with the bread after baking. After the third day this vitamin content was by 3% higher, compared with the initial value. Statistical analysis did not show any significant differences in this respect ($p > 0.05$). Folic acid content in the bread stored in PP bags did not differ significantly ($p > 0.05$) after three days of storage. In the bread put in a PP bag with the access of light the content lower by 6% was observed after the first day, and by 2% after the second day, compared with the initial value. However, the final folic acid content remained at the comparable level. The determined folic acid content in the bread stored in a PP bag without the access of light remained at the constant level within the range of 4327.2 to 4370.2 $\mu\text{g}/100\text{ g}$ of dry matter. In the individual variants of rye bread storage folic acid content after the first, second and third day remained within the comparable range ($p > 0.05$).

The tests demonstrate, that storage of wheat or rye bread for three days did not considerably influence folic acid content in any of the storage variants. In the rye bread folic acid content was lower than for the wheat bread, after adding the same dose of this compound, *i.e.* 10,000 $\mu\text{g}/100\text{ g}$ of flour. Upon determination of folic acid content in the wheat bread

the differences between the quantity added to flour and determined in the wheat bread amounted to 34%, while for the rye bread – 61%. The tests carried out by Gujska & Majewska [2005] demonstrated, that after adding to the wheat and rye bread the equal dose of folic acid, *i.e.* 200 $\mu\text{g}/100\text{ g}$ of dry matter of bread, the difference between the determined folic acid in both types of bread was approx. 3% (in the wheat bread 19%, and in the rye bread 22%). Folate losses during production are more significant compared to the losses during storage. Folic acid losses are also lower in the food of a short shelf life, because of high stability of folic acid during storage [Final Assessment Report, 2006]. The tests carried out by the National Health and Medical Research Council demonstrate, that folic acid-enriched flour is 100% stable after 6 months of storage at a room temperature and for 4 months at 45°C. Even after 1-year storage at 45°C only slight losses are observed. Similarly, folic acid added to corn in form of a premix is stable in 90 -100% [NHMRC, 1995]. Morgan [1996] received comparable results after adding folic acid to flour in a quantity of 100 and 500 $\mu\text{g}/100\text{ g}$. Folic acid was retained in approx. 100% at the temperature range of -23 to 48.8°C for one year of storage [Final Assessment Report, 2006].

The analysis carried out for the bread storage method proves that the storage method did not significantly influence folic acid contained in the bread. Consequently, folic acid in the tested bread is stable. Limitations in access of light or air appeared to be insignificant. The conclusion is testified by the studies carried out by Louie & Stennett [1984]. They were determining the influence of various environmental conditions on folic acid stability. To this end, various concentrations of folic acid were mixed with a solution consisting of 25% of dextrose, 3.5% of amino acids and a vitamin mix. The samples were stored in various conditions, and duration of the experiment was modified. The obtained results imply that folic acid is stable for 48 h. Its stability does not depend on temperature or light, at which it is stored. In the executed test all the samples of bread were stored at a room temperature. Similar studies on storage temperature influence on folic acid-enriched rice were conducted by Williams [2001]. The obtained results demonstrate that after one year of storage of the folic acid-enriched rice at a room temperature the level of folic acid was above the required limit of 150 $\mu\text{g}/100\text{ g}$. During storage of rice at 35°C folic acid level decreased insignificantly only in some samples, in relation to the minimum required level. Therefore a suggestion was made to increase the folic acid dose to a level of 250-300 $\mu\text{g}/100\text{ g}$, especially in the hot and humid climate countries [Strain *et al.*, 2004]. When analysing the bread, samples were taken from the inside of a loaf, so very high temperature did not directly touch the bread pulp, which was significant in bread baking. In the middle of a loaf temperature is much lower and amounts to 80-90°C, therefore vitamin losses in the finished product amounted to 25-30% [Jurga, 2005]. This fact could be significant for the executed experiment, since temperature fluctuations during storage did not affect the bread pulp the more. Access of UV rays and air did not directly influence the bread pulp. Johansson *et al.* [2002] proved that folic acid was stable upon bread storage for 90 days at 20°C. As to folic acid losses in bread, Jantarska *et al.* [2007] recommend performing storage tests,

TABLE 4. Folic acid stability (%) as affected by the method of storage.

Storage	Wheat bread	Rye bread
No packing	100	99
Paper bag	100	99
PP bag (light)	96	98
PP bag (no light)	98	100

in which the losses determined upon storage would be averaged. In the case of the wheat bread, folic acid stability varied within the range of 96-100%, while for the rye bread it was 98-100% (Table 4).

CONCLUSIONS

1. Storage time does not have any influence on folic acid content in the wheat or rye bread enriched with this vitamin.

2. Storage method does not have any influence on folic acid content in the wheat or rye bread enriched with this vitamin.

3. When aiming to reach folate RDA, a consumer should not look at the type of folic acid fortified bread, but at the content of this vitamin in 100 g of a fresh product, considering that RDA for women and men ≥ 19 is 400 μg .

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