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Microencapsulation of L-5-Methyltetrahydrofolic Acid with Ascorbate Improves Stability in Baked Bread Products

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ABSTRACT: Fortification of foods with L-5-methyltetrahydrofolic acid (L-5-MTHF) is challenging due to low stability to environmental conditions that include exposure to pH, moisture, and temperature. The objective of the present study was to stabilize L-5-MTHF using microencapsulation technology. L-5-MTHF microcapsules constructed with different core-to-wall ratios of L-5-MTHF, both alone or in combination with sodium ascorbate, yielded high (>89%) recovery of L-5-MTHF. Time-of-flight secondary ion mass spectrometry (ToF-SIMS) analysis confirmed successful encapsulation of L-5-MTHF with high core-to-wall ratios. Microencapsulation of L-5-MTHF alone with a high core-to-wall ratio significantly ($p < 0.05$) improved the stability of L-5-MTHF over the course of bread baking, performed both in pilot plant and in commercial baking conditions. Breads made with fortified flour containing sodium ascorbate coencapsulated with L-5-MTHF had recoveries of L-5-MTHF that were 97% and 77%, respectively, for pilot plant and bakery breads. Co-encapsulating L-5-MTHF with ascorbate also significantly ($p < 0.05$) improved stability during storage, as compared to breads that contained free L-5-MTHF.

KEYWORDS: *L-5-methyltetrahydrofolic acid, microencapsulation, fortification, bread baking, stability, ToF-SIMS*

INTRODUCTION

Folic acid, pteroyl-L-glutamic acid, is a synthetic fully oxidized form of the naturally occurring B vitamin, folate. Folic acid, unlike naturally occurring folate, requires metabolic activation before it can function in an essential role in one-carbon transfer. However, due to superior stability and thus bioaccessibility as compared to natural folates, folic acid is preferentially used for vitamin supplementation and food fortification.[1,2] Mandatory fortification of grain products with folic acid has been adopted in 57 countries, and in 1997 was introduced in Canada and the United States to reduce the occurrence of neural tube defects (NTDs), such as spina bifida.[3] Although the incidence of NTDs dropped in both countries following fortification, there are concerns that folic acid might have negative health effects on the population. For example, folic acid intake greater than 1 mg/day can mask the hematologic signs of vitamin B_{12} deficiency[4,5] and was associated with cognitive impairment among seniors in one study;[6] also there are concerns that high folic acid intakes might increase the risk of certain types of cancer.[7−9] A potential alternative to synthetic folic acid presently used for fortifying foodstuffs is L-5-methyltetrahydrofolic acid (L-5-MTHF), a synthetic form of a naturally occurring folate, which is also commercially available.[10] L-5-MTHF should not mask vitamin B12 deficiency and thus poses less risk of causing neurological problems secondary to undiagnosed pernicious anemia. Moreover, fortification of foodstuffs with L-5-MTHF is preferable to supplementing with folic acid for some individuals that lack the enzyme methylenetetrahydrofolate reductase (5-MTHFR), which prevents the conversion of folic acid to L-5-MTHF. Previous studies have shown that L-5-MTHF in supplemental form fortification results in similar or even higher increases in blood folate indices in women of childbearing age[11,12] and lactating women.[13] The main limitation of using L-5-MTHF as a food fortificant is the challenge to retain stability in food products that are exposed to humidity conditions and thermal processing.[1,14,15] We have had some success, however, stabilizing L-5-MTHF with ascorbic acid for blending into skim milk powders that were used in baked bread products.[16] The aim of the present study was to further develop an effective encapsulation method for stabilizing L-5-MTHF for use in baked bread using a modified starch that provided greater resistance to loss of encapsulated material at high temperatures that are common to baking bread. Moreover, the success of this process was evaluated in both pilot plant and commercial baking conditions.

MATERIALS AND METHODS

Materials. Formic acid and acetonitrile were purchased from Fisher (New Jersey). Sodium ascorbate (NaAsc), β-mercaptoethanol (MCE), 2-(cyclohexylamino)ethanesulfonic acid (CHES), and 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES) were purchased from Sigma-Aldrich (St. Louis, Mo). Acetonitrile was isocratic grade for HPLC; the other chemicals were analytical grade. Water was purified using a Milli-Q system (Millipore, U.S.). All purpose flour (Robin Hood), granulated sugar (Rogers sugar), salt (Sifto), Fleischmann's yeast, and instant skim milk powder were purchased from Safeway Inc. (Vancouver, Canada).

Modified starch (HICAP 100) derived from waxy maize was a gift from National Starch (New Jersey). L-5-Methyltetrahydrofolic acid (L-5-MTHF, (6S)-5-methyltetrahydrofolic acid, calcium salt, Metafolin) was a kind gift from Merck & Cie (Schaffhausen, Switzerland). The standard stock solutions of folate (200 μg/mL) were prepared under reduced light in HEPES/CHES buffer (0.5 M, pH 7.85) containing 2%

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NaAsc and 0.2 M MCE. Aliquots of the standard solutions were placed in separate tubes, flushed with nitrogen, and stored at −80 °C.

Preparation of Encapsulated L-5-MTHF. Modified starch was dispersed with agitation in water at 60 °C for 30 min and then cooled. L-5-MTHF with or without NaAsc was then added with agitation (see Table 1 for ingredient proportions). Dispersions were immediately spray-dried using a lab-scale spray drier (VirTis, Gardiner, NY) with a 0.5 mm nozzle and inlet and outlet air temperatures of 135 and 90−95 °C, respectively. The encapsulated L-5-MTHF was collected and stored in laboratory desiccators, and the recovery of L-5-MTHF after the spray-drying encapsulation process was assessed.

Table 1. Microencapsulated L-5-MTHF Products

treatment	L-5-MTHF:NaAsc:modified starch weight ratio
Encap 1	1:0:9
Encap 2	1:0:99
Encap 3	0.1:1:99
Encap 4	0.01:1:99

Time-of-Flight Secondary Ion Mass Spectrometry Analysis. The ToF-SIMS spectra measurements were carried out using a PHI TRIFT V nanoTOF-SIMS spectrometer (Physical Electronics PHI Inc., Japan). A pulsed primary 30 keV Au^+ ion beam was rastered over a 400 μm × 400 μm area. To ensure static analysis conditions, the total ion dose per spectrum was kept at about 1.4×10^{11} ions/cm^2. Additionally, characteristic ion images 159 m/z, 162 m/z, and 175 m/z attributed to modified starch fragments, L-5-MTHF, and NaAsc were measured to monitor the degree of homogeneous capsulation across the sample surface, respectively.

Flour Fortification. L-5-MTHF fortification was performed by adding L-5-MTHF, free form or the encapsulated form, to a small amount of flour. This mixture was dispersed using a mortar and pestle before being added to a commercial grade mixer with additional flour that brought the final concentration of L-5-MTHF to ∼1.5 mg per kg flour. The folate fortification level was chosen on the basis of the Food and Drug Regulations of grain and bakery products in Canada (1.5 mg folic acid/kg flour). Twelve samples of the fortified flour were subsampled for quantifying L-5-MTHF content and to ensure even distribution. The standardized fortified flour was used for bread making in both pilot plant and a commercial bakery scale.

Pilot Plant Bread Baking Study. The stability of L-5-MTHF with different ratios of modified starch coating agent and NaAsc antioxidant was determined in the pilot plant bread baking study. Bread formula and baking conditions are given in Table 2. Dough was made using a bread maker (Black & Decker Corp., Towson, U.S.) and divided evenly to make six mini bread loaves. Two of the whole mini bread loaves were sampled after baking (0 days), and after storage in polyethylene bags for 3 and 7 days at room temperature. All samples (whole bread loaf) were freeze-dried, ground, and kept in desiccators before quantification of L-5-MTHF. The weights of fresh and freeze-dried mini bread loaves were recorded. Three independent bread baking processes were conducted.

Table 2. Formula and Baking Condition of White Bread in the Laboratory Pilot Plant and Commercial Bakery

formula and baking conditions	pilot plant (six mini loaves)	commercial bakery (one loaf)
flour	330 g	350 g
water	220 g	175 g
yeast	6 g	10 g
salt	4 g	4 g
sugar	10 g	10 g
skim milk powder	12 g	12 g
vegetable oil	18 g	
dough making	90 min	150 min
baking	185 °C, 25 min	230 °C, 30 min

Commercial Bakery Bread Making. The same L-5-MTHF fortified flours were also sent to a commercial bakery (Elite Bakery, Burnaby, Canada) to make regular bread loaves. Formula and baking condition are given in Table 3. The fresh loaves were sliced by a slicer immediately after baking. Different segments of the bread loaf (e.g., outside, inside (crumb) slices, and outside crust) were sampled immediately after baking (0 days), with the remaining bread slices stored in polyethylene bags for 3 days at room temperature. Three pieces of the loaf were sampled to represent each original segment of the bread. Segments of the bread loaf were also toasted. All samples were freeze-dried, ground, and kept in desiccators before quantification of L-5-MTHF. The weights of fresh and freeze-dried bread loaf samples were recorded. The bread-baking process was conducted in duplicate and repeated once.

Table 3. Variability and Recovery of Microencapsulated L-5-MTHF[a]

treatment	targeted L-5-MTHF concentration (mg/g)	measured mean L-5-MTHF concentration (mg/g)	95% confidence limits (mg/g)	mean % recovery
Encap 1	100	89.27	±4.31	89.27
Encap 2	10	9.10	±1.56	91.00
Encap 3	1.00	1.10	±0.03	110.00
Encap 4	0.10	0.12	±0.01	120.00

[a]Measured values are means for six samples per batch.

Measurement of Color. Color analyses on both fresh and toasted breads were performed using a HunterLab Labscan 600 spectrocolorimeter (Hunter Associates Lboratory Inc., Reston, VA). The instrument was calibrated with black and white tiles. Color was expressed in L^* ($L^* = 0$ yields black and $L^* = 100$ indicates diffuse white), a^* (negative values indicate green, and positive values indicate red), and b^* (negative values indicate blue, and positive values indicate yellow). The colorimetric difference ΔE^* was obtained through the equation: $\Delta E^* = [(L^*)^2 + (a^*)^2 + (b^*)^2]^{0.5}$. Five measurements were carried out on each sample of toasted bread.

Quantification of L-5-MTHF Using HPLC. Microencapsulated ingredients (0.05 g) were vigorously shaken in a 10 mL folate extraction buffer (0.5 M, pH 7.85 HEPES/CHES buffer with 0.2% NaAsc and 0.2 M MCE) using a vortex. The fortified flour and freeze-dried ground bread samples were suspended (0.5 g/10 mL) in the folate extraction buffer, vortexed three times for 1 min, and centrifuged at 3500g for 10 min. Supernatants were collected and filtered through a 0.22 μm nylon filter directly into HPLC vials for analysis.[15]

Reverse phase HPLC using a Zorbax SB-C18 column (4.6 mm × 50 mm, 1.8 μm particle size, Agilent, U.S.) was used to quantify L-5-MTHF according to Liu et al.[15] Samples were eluted using a gradient of acetonitrile (ACN) and 1.0% formic acid: 0−1 min, 5% ACN; 1−3 min, 5−15% ACN; 3−5 min, 15−70% ACN; 5−6 min, 70% ACN; 6−7 min, 70−5%, and maintained at 5% ACN for 1 min before the assay was terminated. L-5-MTHF was detected using a fluorescent detector (excitation at 290 nm and emission at 365 nm). Concentrations were calculated on the basis of the peak area in comparison to L-5-MTHF standard solutions. The concentrations of L-5-MTHF in the bread were expressed as mg L-5-MTHF/kg freeze-dried bread.

The expected L-5-MTHF concentration in the freeze-dried breads was determined on the basis of the following equation:

$$EC_B = MC_F \times W_F/W_{DB} \quad (1)$$

where EC_B is the expected L-5-MTHF concentration in the freeze-dried bread (mg/kg), MC_F is the measured L-5-MTHF concentration in the fortified flour (mg/kg), W_F is the weight of the fortified flour used to bake the bread, and W_{DB} is the weight of freeze-dried bread.

The % recovery of L-5-MTHF was then calculated as:

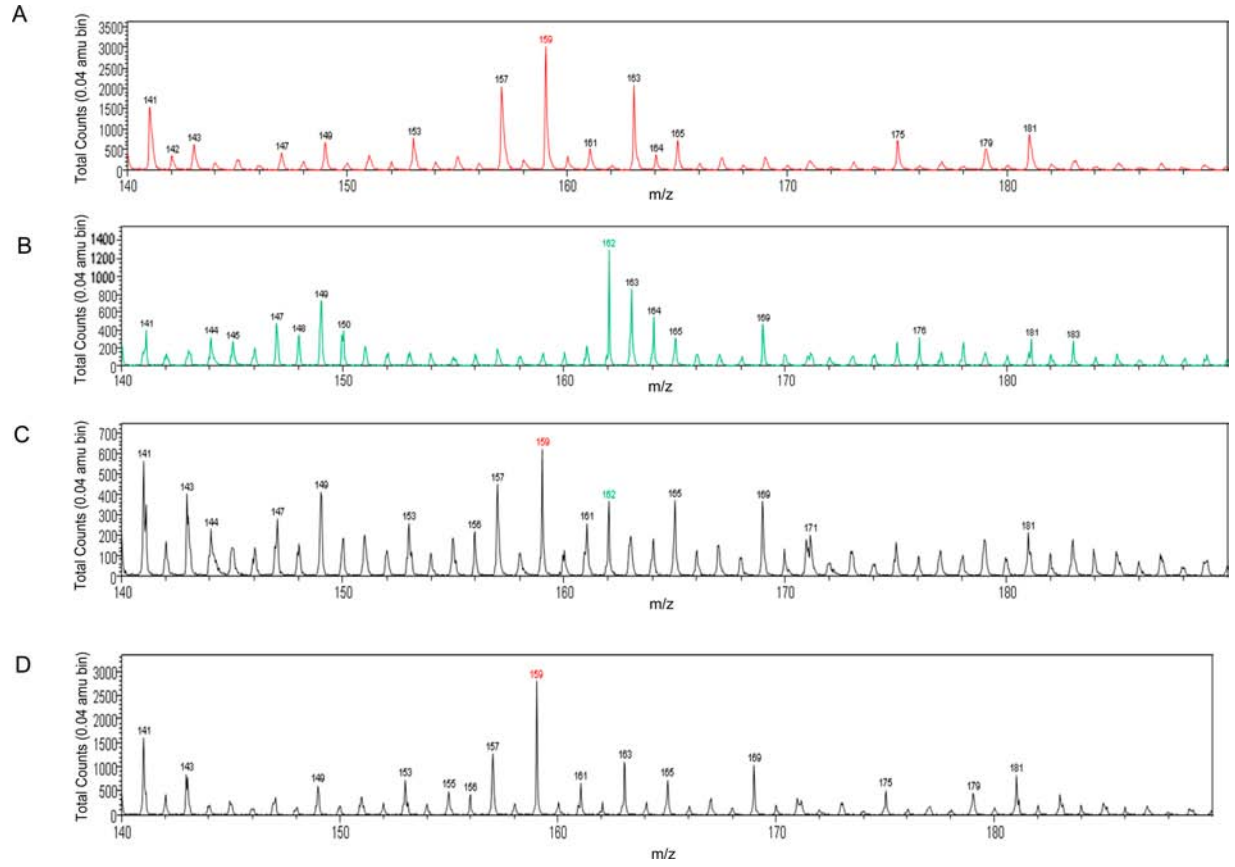

Figure 1. Negative ToF-SIMS spectra of the modified starch (A, red), L-5-MTHF (B, green), Encap 1 (C) and Encap 2 (D). *X*-axis is *m*/*z* (mass to charge ratio); *y*-axis is total counts of pixels (0.04 amu bin). Peak *m*/*z* 159 and peak *m*/*z* 162 are characteristic fragments that denote modified starch and L-5-MTHF, respectively. Peak *m*/*z* 159 and peak *m*/*z* 162 were detected in the outer layer of the Encap 1; Encap 2 spectrum only has an intensive peak (*m*/*z* 159). Encap1 (L-5-MTHF:starch = 1:9); Encap 2 (L-5-MTHF:starch = 1:99).

$$\% \text{ recovery} = (AC_B/EC_B) \times 100 \quad (2)$$

where AC_B is the actual L-5-MTHF concentration measured in the baked bread, and EC_B is the expected L-5-MTHF concentration in the bread.

Statistics. All results from the pilot plant baking study were presented as means of three independent experiments. All results from the commercial bakery study were means of two independent baking processes. Analyses for L-5-MTHF were done on duplicate samples with at least three samplings for the HPLC analysis. Statistic analysis of the data was carried out using two-way Analysis of Variance (ANOVA), followed by Bonferroni post-test. The level of confidence required for significance was selected at $p < 0.05$.

RESULTS AND DISCUSSION

Microencapsulation of L-5-MTHF. In our study, coating the active ingredients with modified starch yielded microcapsules that had a uniform high recovery of L-5-MTHF in all samples with different core-to-wall ratios (Table 3). Modified food starch has been successfully used to encapsulate many other food ingredients, such as volatile Caraway flavor extracts, and vitamins E and β-carotene against oxidation.[17−19] The modified starch used as the coating material in the present study was derived from waxy maise and is characterized by excellent resistance to oxidation.[18] The presence of NaAsc with L-5-MTHF further improved the recovery of L-5-MTHF during the microencapsulation process, showing that the noted reducing capacity of NaAsc is an important characteristic for stabilizing L-5-MTHF.[15,16,20]

Secondary ion mass spectrometry, operating in static mode, enabled surface characterization within 1−2 nm (e.g., a few topmost atomic layers are sampled) so that ToF-SIMS enabled us to confirm that the L-5-MTHF was indeed entrapped inside the modified starch granule and not just adsorbed to surface material. This result proved that a successful encapsulation occurred with the L-5-MTHF. Figure 1 shows negative ToF-SIMS spectra measured from modified starch only (Figure 1A), the L-5-MTHF standard alone (Figure 1B), and encapsulated L-5-MTHF with different starch:MTHF coating ratios (Encap 1, Figure 1C and Encap 2, Figure 1D). Characteristic fragment peaks denoting starch (*m*/*z* 159) and L-5-MTHF (*m*/*z* 162), respectively, were detected on the surface of the Encap 1 that consisted of a L-5-MTHF:starch ratio of 1:9. By contrast, the Encap 2, which had a relatively higher L-5-MTHF:starch ratio (1:99), was found to be devoid of L-5-MTHF and only showed the presence of starch. This result confirmed that L-5-MTHF was effectively entrapped (>2 nm in depth, which is about the length of two glucose molecule) inside the modified starch matrix, when using a high coating ratio (e.g., folate:starch = 1:99). Our study clearly showed that signal intensities measured by ToF-SIMS analysis were matrix dependent. Our results do not quantify the encapsulation efficiency with the ratio of the two secondary ion peaks. Alternatively, our use of the signature peaks for L-5-MTHF (mz 162) and starch (159) was useful to confirm effective entrapment inside the modified starch matrix.

To examine the degree of homogeneous capsulation across Encap 1 and 2 surfaces, similar ionization yields of characteristic

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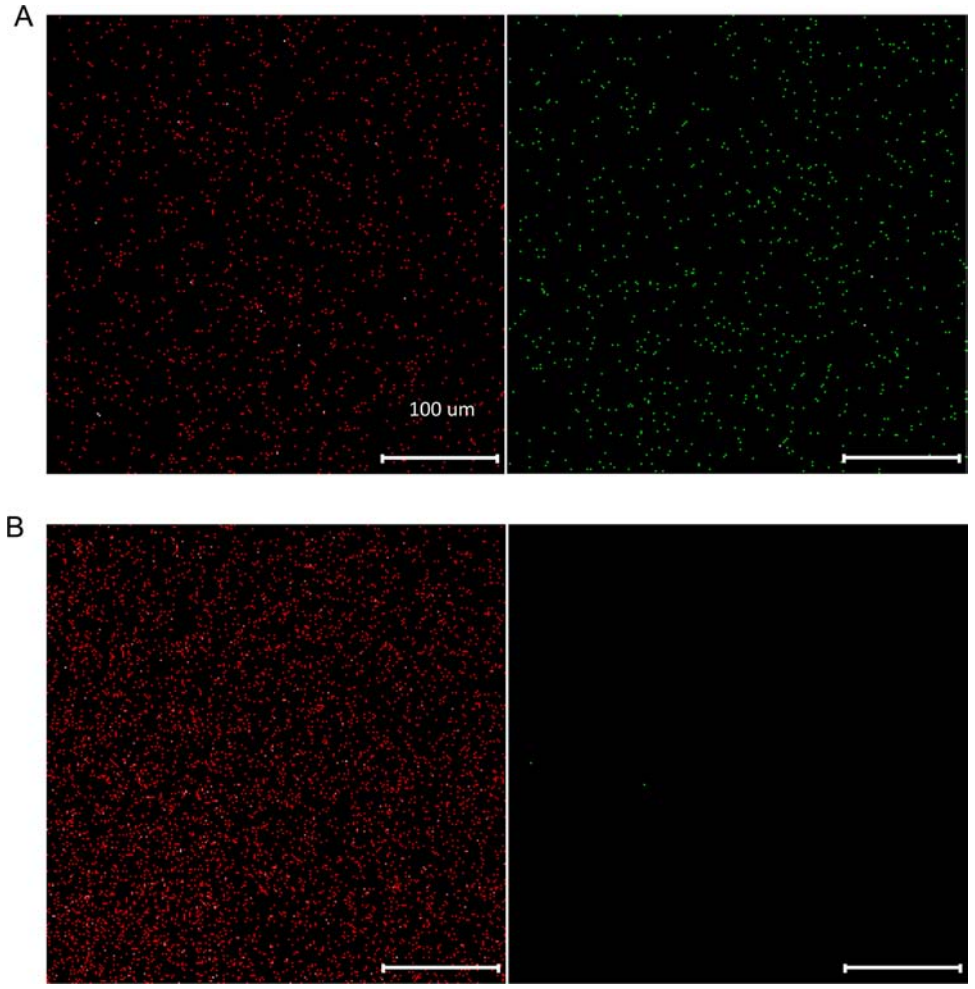

Figure 2. Mass resolved images (chemical maps) of Encap 1 (A) and Encap 2 (B). Red spots image (left) represents m/z 159 ToF-SIMS image, and green spots image (right) represents m/z 162 ToF-SIMS image. Peak m/z 159 and peak m/z 162 are characteristic fragments that denote modified starch and L-5-MTHF, respectively. Brighter color corresponds to higher intensity of fragments, blank to absence of fragments. Encap 1 (L-5-MTHF:starch = 1:9); Encap 2 (L-5-MTHF:starch = 1:99).

fragment peaks (m/z 159) and (m/z 162) attributed to modified starch and L-5-MTHF, respectively, were chosen. Ion images that were measured corresponded to starch and L-5-MTHF as shown in Figure 2. The luminated spots indicate the dispersion of modified starch (red spots on the left) and L-5-MTHF (green spots on the right) on the surface of the encapsulated products. Nonilluminated areas corresponded to an absence of the denoting fragments. Figure 2A shows the evenly distribution of L-5-MTHF on the surface of Encap 1, whereas Figure 2B shows the absence of L-5-MTHF in the outer layer of Encap 2. It is possible that the modified starch matrix was saturated with L-5-MTHF in Encap 1, thus showing L-5-MTHF on the surface layer.

Figure 3 shows the ToF-SIMS spectrum of Encap 3, which contained L-5-MTHF:NaAsc:starch at a relative ratio of 0.1:1:99. A strong signal for NaAsc (m/z 175) alone confirmed the presence of NaAsc in the outer layer of modified starch coating (Figure 3D). We could not confirm if NaAsc was encapsulated along with L-5-MTHF inside the granule, but suspect that its presence within the granule was an important contributing factor for the improved stability of L-5-MTHF obtained during both bread making and upon storage. It is possible that the *n*-octenyl succinic anhydride used to chemically modify the starch was more effective at interacting with the L-5-MTHF, rather than the hydrophilic NaAsc. This would have contributed to the observation that the NaAsc was detected on the outer layer of the microcapsules but L-5-MTHF was encapsulated.

Pilot Plant Bread Baking Study. Dispersion of microencapsulated L-5-MTHF products in flour was relatively uniform as shown by the low 95% confidence limits (Table 4). Blending of the microencapsulated vitamin product was found to be easier as compared to the free form of L-5-MTHF, an observation that likely was attributed to the greater mass of the encapsulated component product used to establish the same level of fortification.

Initial pilot plant experiments were conducted to compare the stability of L-5-MTHF, free, and encapsulated forms during bread baking and storage. Figure 4 shows the recovery of both free and microencapsulated L-5-MTHF in breads immediately following baking and after storage at room temperature for 3 and 7 days, respectively. Employing a high (1:99) core-to-wall ratio produced significantly ($P < 0.05$) greater stability as compared to low core-to-wall ratio and free L-5-MTHF, respectively, during both bread baking and following storage. Recovery of L-5-MTHF decreased significantly ($p < 0.05$) in all breads during storage. Using the higher coating ratio in this study to encapsulate the core material likely resulted in less L-5-

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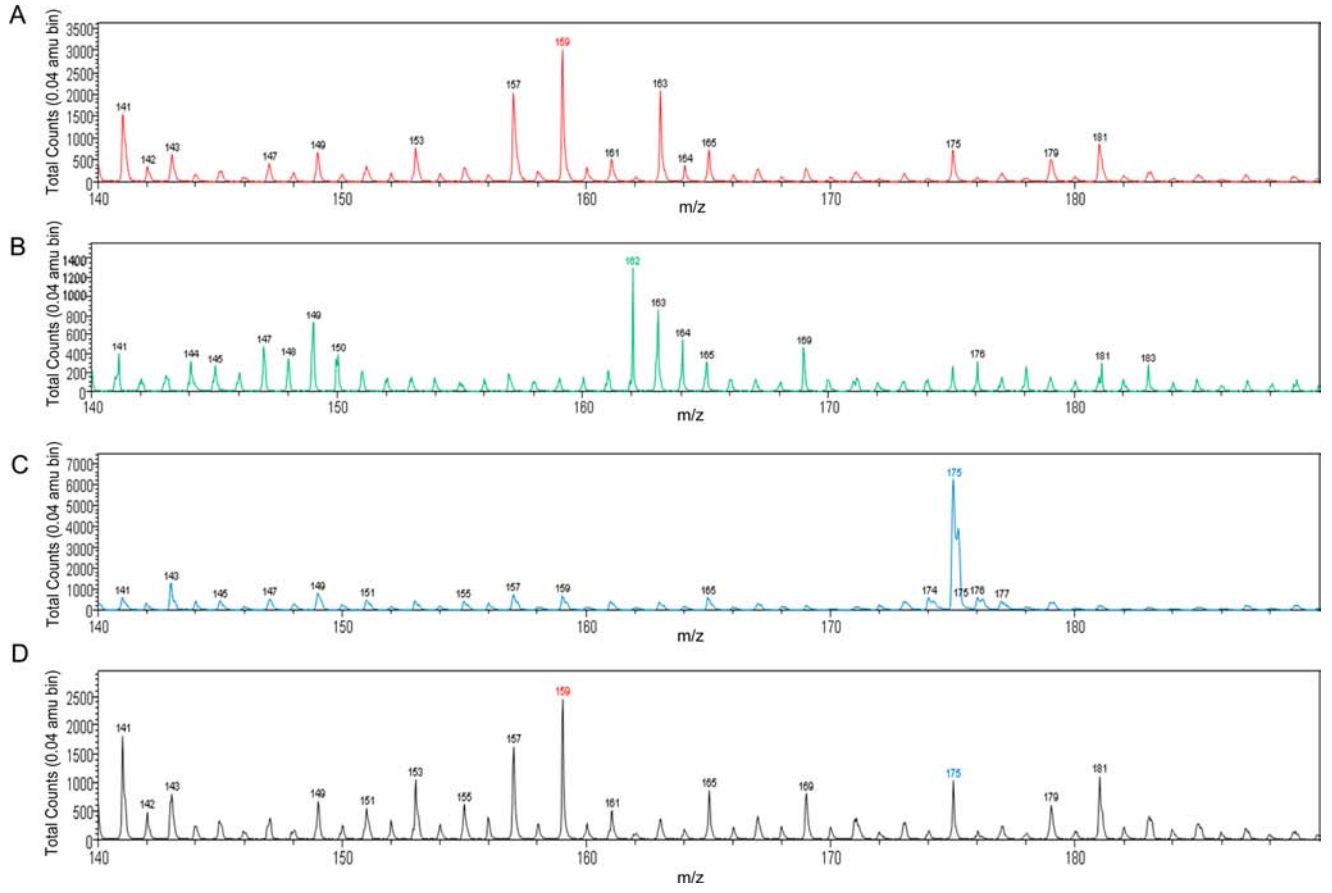

Figure 3. Negative ToF-SIMS spectra of the modified starch (A), L-5-MTHF (B), NaAsc (C), and Encap 3 (D). *X*-axis is *m*/*z* (mass to charge ratio); *y*-axis is total counts of pixels (0.04 amu bin). Peak *m*/*z* 159 and peak *m*/*z* 162 are characteristic fragments that denote modified starch and L-5-MTHF, respectively. Peak *m*/*z* 175 denotes NaAsc. Encap 3 (L-5-MTHF:NaAsc:starch = 0.1:1:99).

Table 4. Variability of L-5-MTHF Concentrations in Fortified Flour[a]

treatment	targeted L-5-MTHF concentration (mg/kg)	required weight of encapsulated products (g/kg)	measured mean L-5-MTHF concentration (mg/kg)	95% confidence limits (mg/kg)
free	1.50		1.92	±0.50
Encap 1	1.50	0.17	1.57	±0.06
Encap 2	1.50	0.16	1.57	±0.06
Encap 3	1.50	1.36	1.50	±0.05
Encap 4	1.50	12.5	1.56	±0.02

[a]Measured values are means for 12 samples per batch. Required weight of encapsulated products = targeted L-5-MTHF concentration/measured L-5-MTHF concentration in the encapsulated products.

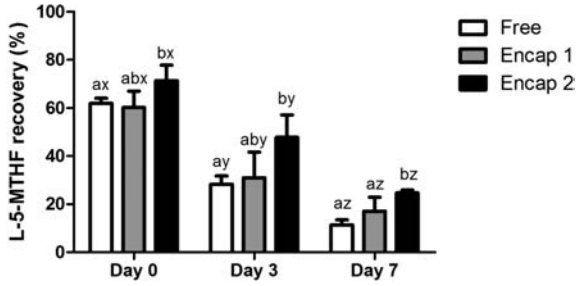

Figure 4. Recovery of L-5-MTHF in pilot plant breads fortified with free or microencapsulated L-5-MTHF after baking (day 0) and after 3 and 7 days of storage at room temperature. Encap 1 and Encap 2 are L-5-MTHF microcapsules with 90% and 99% coating, respectively. Values are means of three baking processes, and the error bars represent SD. Data are expressed as % of L-5-MTHF added to the bread. Superscripts "ab" indicate significant difference between free and microencapsulated L-5-MTHF on the same day; "xyz" indicate significant difference of the same type of L-5-MTHF on different days ($p < 0.05$).

MTHF being exposed to the risk of creating open structures in the coating material, a common occurrence with spray drying.[21,22] In this event, a reduced exposure of the vitamin to oxidation reactions would be expected to result in an increased stability of L-5-MTHF.

Several studies have reported increased stability of L-5-MTHF during processing when in the presence of ascorbate.[14,15,20] The addition of NaAsc in bread with free L-5-MTHF (MTHF+NaAsc) significantly ($P < 0.05$) improved the recovery of L-5-MTHF after baking, and also upon storage, when compared to the negative control that contained only free L-5-MTHF (Table 5). Microencapsulation of L-5-MTHF alone (e.g., Encap 2), improved the retention of L-5-MTHF immediately after baking, similar to that obtained by adding NaAsc. These breads, however, showed a greater ($p < 0.05$) loss of L-5-MTHF after 7 days storage as compared to breads that contained free L-5-MTHF and NaAsc, thus showing the important contribution of NaAsc to reduce L-5-MTHF degradation during storage. Indeed, microencapsulation of NaAsc along with L-5-MTHF in Encap 3 and 4, respectively, gave the greatest ($p < 0.05$) stability for L-5-MTHF observed.

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Table 5. Recovery of L-5-MTHF in Pilot Plant Breads after Baking (Day 0), and after 3 and 7 days of Storage at Room Temperature[a]

treatment	day 0	day 3	day 7
free	61.94 ± 1.68 ax	28.25 ± 2.85 ay	11.33 ± 1.74 az
MTHF+NaAsc	73.71 ± 1.65 bx	60.87 ± 5.15 by	44.58 ± 8.01 cz
Encap 2	71.40 ± 5.20 bx	47.82 ± 7.61 by	24.75 ± 0.90 bz
Encap 3	87.95 ± 1.56 cx	78.54 ± 1.73 cy	55.40 ± 5.78 cz
Encap 4	97.13 ± 1.95 dx	98.07 ± 1.27 dx	75.56 ± 4.38 dy

[a]Free: flour fortified with free L-5-MTHF. MTHF+NaAsc: flour fortified with free L-5-MTHF and NaAsc. Encap 2: flour fortified with microencapsulated L-5-MTHF (core-to-wall ratio = 1:99). Encap 3: flour fortified with NaAsc co-encapsulated L-5-MTHF (L-5-MTHF:NaAsc:starch = 0.1:1:99). Encap 4: flour fortified with NaAsc co-encapsulated L-5-MTHF (L-5-MTHF:NaAsc:starch = 0.01:1:99). Values are means ± SD of three baking processes. Data are expressed as % of L-5-MTHF added to the bread. Letters "abcd" indicate significant difference between different types of L-5-MTHF on the same day; Letters "xyz" indicate significant difference of the same type of L-5-MTHF on different days ($p < 0.05$).

For example, baked breads formulated with Encap 4, representing coencapuslation with NaAsc, had approximate recoveries of 97% and 98%, respectively, for L-5-MTHF after initial baking and a 3 day storage. We conclude that the coencapsulation of reducing agent, NaAsc, with L-5-MTHF provided another mechanism of protection for L-5-MTHF from oxidation.

Microencapsulated L-5-MTHF in Bakery Bread. The results of the pilot plant bread baking experiment that identified Encap 3 and 4 to be the most effective formulations to ensure maximum L-5-MTHF stability were extended to a commercial bread baking scale-up experiment. All bread loaves containing either free L-5-MTHF or microencapsulated L-5-MTHF showed no noticeable quality differences as compared to regular bread loaves. The $L^*a^*b^*$ values of bakery bread crusts from fresh bread slices and toasted bread slices are presented in Table 6. Bread containing NaAsc had a higher b^* value as compared to that of the control bread containing only free L-5-MTHF. Only small changes in the $L^*a^*b^*$ and ΔE values were found in the medium toasted bread slices. There is no significant change in the $L^*a^*b^*$ and ΔE values after storage for 3 days (data not shown).

Commercially baked bread loaves fortified with microencapsulated L-5-MTHF had a significantly ($p < 0.05$) higher recovery of L-5-MTHF immediately after baking and also after 3 days of storage, as compared to baked breads that contained fortified L-5-MTHF in the free form (Figure 5). Similar to the

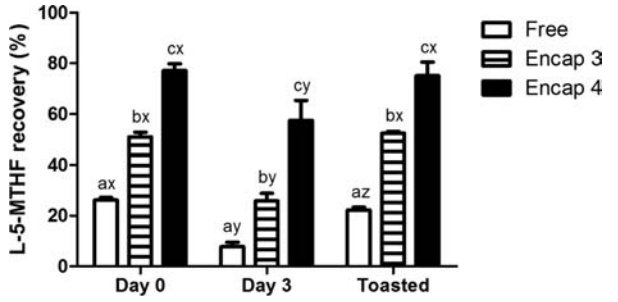

Figure 5. Recovery of L-5-MTHF in bakery breads after baking (day 0) and after 3 days of storage at room temperature. Free, flour fortified with free L-5-MTHF; Encap 3, flour fortified with NaAsc coencapsulated L-5-MTHF (L-5-MTHF:NaAsc:starch = 0.1:1:99); Encap 4, flour fortified with NaAsc coencapsulated L-5-MTHF (L-5-MTHF:NaAsc:starch = 0.01:1:99). Values are means of three baking processes, and the error bars represent SD. Data are expressed as % of L-5-MTHF added to the bread. Superscripts "abc" indicate significant difference between different types of L-5-MTHF on the same day; "xyz" indicate significant difference of the same type of L-5-MTHF on different days ($p < 0.05$).

results obtained from the pilot plant baking study, breads that contained more NaAsc also showed greater ($p < 0.05$) stability. Furthermore, no decreases were observed in the recovery of L-5-MTHF in breads that contained microencapsulated L-5-MTHF fortified flour when exposed to a medium toasting. Toasting baked bread was sufficient to produce a significant ($p < 0.05$) decrease in the recovery of free L-5-MTHF, however. This result again demonstrates that the importance of coencapsulation of L-5-MTHF with NaAsc to protect against vitamin losses derived from thermal oxidation reactions. It is important to note that, in general, greater losses were observed for both free and microencapsulated L-5-MTHF in the bakery breads, as compared to the corresponding breads produced from the pilot plant baking. For example, the baking loss of free L-5-MTHF in the commercial bakery breads was 74% and reached as high as 92% after 3 days of storage. This compared to only a 38% and 71% loss for free L-5-MTHF in the fresh and 3-day-old pilot plant breads, respectively. In the commercial baked breads, 77% and 58% retention of L-5-MTHF was found in fresh and 3 day old baked breads derived from Encap 4, respectively, as compared to the 97% and 98% retention obtained in breads produced in the pilot plant baking study.

Table 6. $L^*a^*b^*$ Color of Bakery Bread Crust[a]

treatment	L^*	a^*	b^*	ΔE^*
Fresh				
free	42.8 ± 3.09	10.89 ± 0.66	23.28 ± 1.90	50.06 ± 3.34
Encap 3	40.81 ± 4.55	7.73 ± 0.29	24.82 ± 1.22	48.45 ± 4.41
Encap 4	41.13 ± 1.05	9.03 ± 0.30	26.70 ± 0.25	49.87 ± 0.89
Toasted				
free	40.94 ± 1.49	8.61 ± 0.09	24.80 ± 0.58	48.66 ± 1.56
Encap 3	39.45 ± 2.86	8.52 ± 0.64	24.22 ± 1.18	47.10 ± 3.08
Encap 4	38.27 ± 1.06	10.05 ± 0.72	25.84 ± 0.85	47.29 ± 1.48

[a]Bread flours were fortified with free L-5-MTHF or microencapsulated L-5-MTHF. Encap 3: flour fortified with NaAsc co-encapsulated L-5-MTHF (L-5-MTHF:NaAsc:starch = 0.1:1:99). Encap 4: flour fortified with NaAsc co-encapsulated L-5-MTHF (L-5-MTHF:NaAsc:starch = 0.01:1:99). L^* indicates lightness, $-a^*$ to $+a^*$ indicates green to red, and $-b^*$ to b^* indicates blue to yellow. Total color difference $\Delta E^* = [(L^*)2 + (a^*)2 + (b^*)2]^{0.5}$.

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Although very little information exists on the stability of fortified L-5-MTHF during bread making and storage, there are reports of a wide range of significant folic acid degradation (e.g., 12−24%) occurring immediately after pilot plant scale baking in white bread loafs.[23,24] Many variables may contribute to this finding, including the proportion of the flour in the recipe, the type of the bread baked and baking conditions, water losses due to baking, and moreover the type of yeast used in the recipe.[23,24] It is therefore not unexpected that differences in physical properties of the baked bread product (e.g., larger size of the loaf and higher moisture content) as well as different bread making and baking conditions (e.g., longer dough making time and longer baking time together with higher baking temperature) could have accounted for the greater loss of L-5-MTHF in the commercial baked breads as compared to the pilot plant breads.

Finally, to determine the distribution of the fortified L-5-MTHF in the bread loaf, different parts of the bread loaf were sampled, and the L-5-MTHF contents are shown in Table 7.

Table 7. Distribution of L-5-MTHF in Different Parts of the Bakery Breads[a]

	free	Encap 3	Encap 4
expected	1.48	1.45	1.50
inside slice	0.39 ± 0.01 a	0.74 ± 0.02 a	1.16 ± 0.04 a
outside slice	0.46 ± 0.02 b	1.01 ± 0.09 b	1.29 ± 0.05 b
inner	0.41 ± 0.02 a	0.71 ± 0.05 a	1.10 ± 0.03 a
crust	0.62 ± 0.02 c	1.13 ± 0.06 b	1.42 ± 0.03 c

[a]Bread flours were fortified with free L-5-MTHF or microencapsulated L-5-MTHF. Encap 3: flour fortified with NaAsc co-encapsulated L-5-MTHF (L-5-MTHF:NaAsc:starch = 0.1:1:99). Encap 4: flour fortified with NaAsc co-encapsulated L-5-MTHF (L-5-MTHF:NaAsc:starch = 0.01:1:99). Measured values are means ± SD from two independent baking processes. Letters "abc" mean values within a column not sharing a common letter are significantly different ($p < 0.05$).

Interestingly, the content of L-5-MTHF from the outside slices and crust was significantly ($p < 0.05$) higher as compared to the inside slices obtained from the inner part of the same loaf. This was observed for both the free L-5-MTHF fortified breads as well as breads that contained the encapsulated L-5-MTHF. During the baking process, there is a transition of higher moisture present inside the loaf as compared to the outer crust,[25] which could influence the rate of L-5-MTHF degradation. In addition, Maillard reaction products (MRPs) are formed in the bread crust, which may prevent L-5-MTHF oxidation due to the antioxidant activity of the MRPs.[26] These two phenomena likely explain the higher retention of L-5-MTHF found in the bread crust versus the interior of the bread loaf.

In conclusion, the stability of L-5-MTHF was improved by microencapsulation using modified starch with a high core-to-wall ratio. The comicroencapsulation of NaAsc with L-5-MTHF greatly enhanced the recovery of L-5-MTHF during bread baking and storage. Future studies need to be conducted to determine the bioavailability of this fortified bread in humans.

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Notes

The authors declare no competing financial interest.

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REFERENCES

(1) Eitenmiller, R. R.; Landen, W. O. Folate. In *Vitamin Analysis for Health and Food Science*; Eitenmiller, R. R., Landen, W. O., Eds.; CRC Press: Boca Raton, FL, 1999; pp 411−465.

(2) Temple, C.; Montgomery, J. A. Chemical and physical properties of folic acid and reduced derivatives. In *Folates and Pterins*; Blekley, R. L., Benkovic, S. J., Eds.; John Wiley & Sons: New York, 1984; Vol. *1*, pp 61−120.

(3) Berry, R. J.; Mulinare, J.; Hamner, H. C. Folic acid fortification: neural tube defect risk reduction-a global perspective. In *Folate in Health and Disease*; Bailey, L. B., Ed.; CRC Press: Boca Raton, FL, 2010; pp 179−204.

(4) Hoffbrand, A. V.; Jackson, B. F. A. Correction of the DNA synthesis defect in vitamin B12 deficiency by tetrahydrofolate: evidence in favour of the methyl-folate trap hypothesis as the cause of megaloblastic anaemia in vitamin B12 deficiency. *Br. J. Hamaetol.* **1993**, *83*, 643−647.

(5) Wright, A. J. A.; Finglas, P. M.; Southon, S. Proposed mandatory fortification of the UK diet with folic acid: have potential risks been underestimated? *Trends Food Sci. Technol.* **2001**, *12*, 313−321.

(6) Morris, M. S.; Jacques, P. F.; Rosenberg, I. H.; Selhub, J. Circulating unmetabolized folic acid and 5-methyltetrahydrofolate in relation to anemia, macrocytosis, and cognitive test performance in American seniors. *Am. J. Clin. Nutr.* **2010**, *91*, 1733−1744.

(7) Van Guelpen, B.; Hultdin, J.; Johansson, I.; Hallmans, G.; Stenling, R.; Riboli, E.; Winkvist, A.; Palmqvist, R. Low folate levels may protect against colorectal cancer. *Gut* **2006**, 55.

(8) Smith, A. D.; Kim, Y.-I.; Refsum, H. Is folic acid good for everyone? *Am. J. Clin. Nutr.* **2008**, *87*, 517−533.

(9) Stolzenberg-Solomon, R. Z.; Chang, S. C.; Leitzmann, M. F.; Johnson, K. A.; Johnson, C.; Buys, S. S.; Hoover, R. N.; Ziegler, R. G. Folate intake, alcohol use, and postmenopausal breast cancer risk in the prostate, lung, colorectal, and ovarian cancer screening trial. *Am. J. Clin. Nutr.* **2006**, *83*, 895−904.

(10) Ginting, E.; Arcot, J. High-performance liquid chromatographic determination of naturally occurring folates during tempe preparation. *J. Agric. Food Chem.* **2004**, *52*, 7752−7758.

(11) Lamers, Y.; Prinz-Langenohl, R.; Bramswig, S.; Pietrzik, K. Red blood cell folate concentrations increase more after supplementation with 6S-5-methyltetrahydrofolate than with folic acid in women of childbearing age. *Am. J. Clin. Nutr.* **2006**, *84*, 156−161.

(12) Venn, B. J.; Green, T. J.; Moser, R.; McKenzie, J. E.; Skeaff, C. M.; Mann, J. Increases in blood folate indices are similar in women of childbearing age supplemented with 6S-5-methyltetrahydrofolate and folic acid. *J. Nutr.* **2002**, *132*, 3353−3355.

(13) Houghton, L. A.; Sherwood, K. L.; Pawlosky, R.; Ito, S.; O'Connor, D. L. 6S-5-Methyltetrahydrofolate is at least as effective as folic acid in preventing a decline in blood folate concentrations during lactation. *Am. J. Clin. Nutr.* **2006**, *83*, 842−850.

(14) Indrawati; Arroqui, C.; Messagie, I.; Nguyen, M. T.; Van Loey, A.; Hendrickx, M. Comparative study on pressure and temperature stability of 5-methyltetrahydrofolic acid in model systems and in food products. *J. Agric. Food Chem.* **2004**, *52*, 485−492.

(15) Liu, Y. Z.; Tomiuk, S.; Rozoy, E.; Simard, S.; Bazinet, L.; Green, T.; Kitts, D. D. Thermal oxidation studies on reduced folate, L-5-methyltetrahydrofolic acid (L-5-MTHF) and strategies for stabilization using food matrices. *J. Food Sci.* **2012**, *77*, 236−243.

(16) Tomiuk, S.; Liu, Y. Z.; Green, T. J.; King, M. J.; Finglas, P. M.; Kitts, D. D. Studies on the retention of microencapsulated L-5-methyltetrahydrofolic acid in baked bread using skim milk powder. *Food Chem.* **2012**, *133*, 249−255.
(17) Jarunee, L. Characteristics of microencapsulated *β*-carotene formed by spray drying with modified tapioca starch, native tapioca starch and maltodextrin. *Food Hydrocolloids* **2007**, *21*, 928−935.
(18) Partanen, R.; Ahro, M.; Hakala, M.; Kallio, H.; Forssell, P. Microencapsulation of caraway extract in ß-cyclodextrin and modified starches. *Eur. Food Res. Technol.* **2002**, *214*, 242−247.
(19) Tang, H. G.; Xia, W. S.; Yuan, S. L. Study on micro-encapsulation of vitamin E. *Food Mach.* **2005**, *21*, 4−6.
(20) Oley, I.; Verlinde, P.; Hendrickx, M.; Van Loey, A. Temperature and pressure stability of L-ascorbic acid and/or 6s 5-methyltetrahydrofolic acid: A kinetic study. *Eur. Food Res. Technol.* **2006**, *223*, 71−77.
(21) Dziezak, J. D. Microencapsulation and encapsulated ingredients. *Food Technol. (Chicago, IL, U.S.)* **1988**, *42*, 136−151.
(22) Gibbs, B. F.; Kermasha, S.; Alli, I.; Mulligan, C. N. Encapsulation in the food industry: a review. *Int. J. Food Sci. Nutr.* **1999**, *50*, 213−224.
(23) Anderson, W. A.; Slaughter, D.; Laffey, C.; Lardner, C. Reduction of folic acid during baking and implications for mandatory fortification of bread. *Int. J. Food Sci. Technol.* **2010**, *45*, 1104−1110.
(24) Gujska, E.; Majewska, K. Effect of baking process on added folic acid and endogenous folates stability in wheat and rye breads. *Plant Foods Hum. Nutr.* **2005**, *60*, 37−42.
(25) Ahrne, L.; Andersson, C. G.; Floberg, P.; Rosen, J.; Lingnert, H. Effect of crust temperature and water content on acrylamide formation during baking of white bread: Steam and falling temperature baking. *LWT-Food Sci. Technol.* **2007**, *40*, 1708−1715.
(26) Michalska, A.; Amigo-Benavent, M.; Zielinski, H.; del Castillo, M. D. Effect of bread making on formation of Maillard reaction products contributing to the overall antioxidant activity of rye bread. *J. Cereal Sci.* **2008**, *48*, 123−132.