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Development of a Simplified Method for the Determination of Folates in Baker's Yeast by HPLC with Ultraviolet and Fluorescence Detection

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A simplified HPLC method for rapid determination of folates in yeast with ultraviolet and fluorescence detection without sample purification has been developed. By use of the column Aquasil C_{18}, specially designed for polar analytes, and gradient elution, it was possible to separate and determine five folate derivatives: tetrahydrofolate, 5-methyltetrahydrofolate, and 5-formyltetrahydrofolate with fluorescence detection, and 10-formylfolic acid and folic acid with ultraviolet detection. The sample preparation required only a small amount of dry yeast (25−50 mg) and included an extraction of folates by heat treatment and deconjugation of folate polyglutamates to monoglutamates with the use of rat serum conjugase. Validation involved investigation of matrix effects, determination of recovery by standard addition method, repeatability, and stability tests. The dominating folate forms in commercial dry baker's yeast were found to be tetrahydrafolate and 5-methyltetrahydrofolate with a total folate content of 2890 μg/100 g (63.4 nmol/g). The simplicity of the method makes it suitable for folate screening studies of different yeast strains.

KEYWORDS: Folates; analysis; monoglutamates; yeast; food; deconjugation; HPLC; chromatography; validation

1. INTRODUCTION

Folates are a group of water-soluble B vitamins required for central cellular functions such as amino acid biosynthesis, replication, and growth. They also play a great role in preventing neural tube defects, cardiovascular disease, and certain cancers (*1*, *2*). Because of these health benefits, there is a trend to increase the daily recommended dietary folate intake either by fortification with synthetic folic acid or by biofortification, e.g., natural increases by fermentation/bioprocessing. Baker's yeast (*Saccharomyces cerevisiae*) is known to be a rich dietary source for native folate, containing between 1 and 4 mg of folate/100 g according to food tables (*3*). However, reliable data regarding the folate-producing capabilities of different yeast strains are lacking.

The accuracy and sensitivity of folate analysis is highly dependent on the merits of the preparative methods used, e.g., extraction method and the enzymatic deconjugation of the polyglutamyl folates as well as detection techniques. Total folate content in foods is usually determined by the microbiological assay (MA) based on the quantitative relationship between folate content and growth of *Lactobacillus rhamnosus* ATCC No. 7469 (*L. casei*). The method is highly sensitive and responds to nearly all tetrahydrofolate, dihydrofolate, and fully oxidized folate forms (*4*−*6*). There are, however, several limitations with MA including tedious laboratory work, lack of information on the individual folate forms, multiple interferences from the sample matrix, and significant growth response differences to various folate forms (*7*, *8*). In recent years high-performance liquid chromatographic (HPLC) methods for quantifying folates in different foodstuffs have become more common (*8*−*15*). The primary advantage of chromatography is the ability to separate and quantify the individual folate forms. However, additional purification of food extracts prior to HPLC analysis is necessary because of interference from the matrix, which increases the analysis time and makes these methods more complicated (*9*). Nevertheless, the HPLC methods have a great potential due to rapid progress of both separation and detection techniques as well as the possibility of automatizing the purification step.

The objective of this work was to develop and validate a simple HPLC method suitable for screening of different yeast strains for individual folate forms to evaluate differences in their inherent folate-producing capacity. A validated method will enable further investigations using yeast as a biofortificant, e.g., enhancing folate concentrations in foods by natural means.

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2. MATERIALS AND METHODS

2.1. Materials and Chemicals. Acetonitrile and methanol were of HPLC grade; other chemicals were of analytical quality and were purchased from Merck (Darmstadt, Germany). Water was purified using a Milli-Q system (Millipore, USA). Rat serum was obtained from Scanbur (Sollentuna, Sweden). Dry baker's yeast, *Saccharomyces cerevisae* (trademark: original kronjäst), was a gift from Jästbolaget, a Swedish yeast company in Rotebro, Sweden. It was vacuum-packed in plastic bags immediately after delivery and stored frozen at −80 °C until analysis.

Folic acid, (6*S*)-5,6,7,8-tetrahydrofolate, sodium salt (H_4folate), (6*S*)-5-formyl-5,6,7,8-tetrahydrofolate, sodium salt (5-HCO-H_4folate), and (6*S*)-5-methyl-5,6,7,8-tetrahydrofolate, sodium salt (5-CH_3-H_4folate), were all a kind gift from Merck Eprova AG, Schaffhausen, Switzerland. Pteroyltri-γ-L-glutamic acid ($PteGlu_3$) and 10-formylfolic acid, sodium salt (10-CHO-folic acid), were obtained from Dr. Schirck's Laboratories (Jona, Switzerland). The folate standards were stored at −80 °C until use. The purity of all standards was checked according to the procedure of van den Berg et al. (*16*) using molar extinction coefficients reported by Eitenmiller et al. (*6*). The standard stock solutions of folates of 200 μg/mL (purity corrected) were prepared under subdued light in 0.1 M phosphate buffer, pH 6.1, containing 1% sodium ascorbate and 0.1% 2-mercaptoethanol. Aliquots of the standard stock solutions were placed in separate tubes, flushed with nitrogen, and stored below −80 °C at most for 3 months. The calibration solutions were prepared immediately before use by dilution of the stock solution with extraction buffer (0.1 M phosphate buffer, pH 6.1, with 2% sodium ascorbate (w/v) and 0.1% 2-mercaptoethanol (v/v)).

2.2. Folate Conjugase Preparation. Rat serum (10 mL) was dialyzed in three steps (40 min each) by using 800 mL of 50 mM phosphate buffer, pH 6.1, containing 0.1% 2-mercaptoethanol in each step. The dialysis was performed with stirring at 4 °C. Folate conjugase activity was checked using $PteGlu_3$ as substrate in 0.1 M phosphate buffer, pH 6.1, containing 1% sodium ascorbate at 37 °C as described by Pfeiffer et al. (*11*). Concentrations of $PteGlu_3$ and produced folic acid were measured by means of UV detection at 290 nm using the HPLC method described later. The dialyzed rat serum was stored at −20 °C for a maximum of 1 month. To avoid the possible adverse effects of refreezing and rethawing on enzyme activity, rat serum was frozen in small portions (0.5 mL). The enzyme activity was always checked prior to use.

2.3. Sample Preparation. On the day for the sample preparation, an extraction buffer (0.1 M phosphate buffer, pH 6.1, with 2% sodium ascorbate (w/v) and 0.1% 2-mercaptoethanol (v/v)) was prepared. Then, 50 mg of yeast, in duplicates, was weighed exactly into plastic centrifuge tubes and made up with 20 mL of extraction buffer, flushed with nitrogen for 15 s, and thereafter capped and vortexed. Extractions were performed by placing the tubes in a boiling water bath for 12 min. Tubes were then rapidly cooled on ice and centrifuged at 27000*g* for 15 min at 4 °C. The supernatants were filled to an exact volume in 25-mL volumetric flasks with extraction buffer.

For deconjugation of folate polyglutamates to monoglutamates, 50 μL of rat serum was added to 1 mL of the yeast extract in a glass tube. Tubes were flushed with nitrogen gas for 15 s before capping, incubated on a shaking water bath at 37 °C for 3 h, and boiled for 5 min to inactivate enzymes, after which they were immediately cooled on ice. The samples were transferred to plastic Eppendorf tubes and centrifuged at 15500*g* for 10 min at room temperature. The obtained yeast extracts containing folate monoglutamates were analyzed by HPLC. The correction for dilution of yeast extracts due to addition of rat serum was made. Blank samples containing only rat serum and extraction buffer were prepared and treated in the same way as the yeast samples to check if the enzyme preparation contained any endogenous folates.

2.4. Optimization of Sample Preparation. *2.4.1. Folate Extraction Step.* To achieve complete extraction of folates from the yeast matrix, the optimal sample amount to buffer ratio was evaluated. A fixed volume of extraction buffer (20 mL) was added to different amounts of dry yeast (25, 50, 70, 100, 200, 300, and 400 mg). To ensure complete deconjugation of yeast folates, the volume of added rat serum conjugase was increased proportionally for the larger amounts of dry yeast. The obtained yeast extracts were analyzed by HPLC, and folate content in dry yeast (individual folate forms) was calculated and compared for different sample amount to buffer ratios. The optimized extraction procedure by heat treatment was compared with a conventional method for disrupting yeast cell wall by an aggressive and repeated vortex treatment of the yeast cells in a glass bead containing buffer (*17*). Yeast (50 mg) was weighed into a plastic centrifuge tube containing 1 g of clean glass beads and covered with 1.6 mL of extraction buffer, flushed with nitrogen for 15 s, and thereafter capped. The sample was vortexed for 30 s and put on ice for 30 s. This procedure was repeated until a total vortex time of 13 min was achieved. Supernatant was centrifuged and diluted to 25 mL with extraction buffer. Thereafter the yeast extract was deconjugated (1 mL of extract with 50 μL of rat serum) as described in section 2.3.

2.4.2. Folate Deconjugation Step. To check the deconjugation efficiency, different amounts of rat serum (25, 50, and 80 μL) were added to 1 and 3 mL of yeast extract spiked with $PteGlu_3$ as substrate in concentrations of 0.5 and 1 μg/mL (approximately 10 respectively 20 times higher than the expected folate concentration in yeast extract). The procedure was performed as described in section 2.3.

2.5. Purification of Yeast Extracts. To check the necessity for a purification step, the yeast extracts were purified by solid-phase extraction (SPE) on strong anion exchange (SAX) isolute cartridges (500 mg, 3 mL, International Sorbent Technology, UK) as described by Jastrebova et al. (*13*). Aliquots of yeast extracts of 0.5 mL were applied on SAX cartridges preconditioned with methanol (2 × 2.5 mL) and water (2 × 2.5 mL). The cartridges were washed with water (2 × 2.5 mL) to remove matrix interfering components. The retained folates were eluted slowly (flow rate not exceeding 1 drop/s) with 0.1 M sodium acetate containing 10% (w/v) sodium chloride, 1% (w/v) ascorbic acid, and 0.1% (v/v) 2-mercaptoethanol. The first portion (0.7 mL) of eluate was discarded, and the second portion (3.8 mL) was collected and weighed. A Visiprep SPE Vacuum Manifold (Supelco, USA) was used for elution under reduced pressure.

2.6. Chromatographic Equipment and Conditions. Analyses were performed using an HPLC system (Agilent 1100) consisting of a gradient quaternary pump, a thermostated autosampler, a thermostated column compartment, a diode array detector (DAD), and a fluorescence detector. The HPLC system was controlled by a personal computer running Agilent Chemstation software. The separation of folates was performed on an Aquasil C_{18} column, 150 × 4.6 mm, 3 μm (Thermo Electron Corporation, USA), with a guard column Opti-guard C_{18}, 1 mm (Optimize Technologies, Inc., USA), at 23 °C. The guard column was changed at regular intervals (after 80−100 samples). The analytical column life was 3−5 months. The flow rate was 0.4 mL/min; the injection volume was 20 μL; the temperature in the thermostated autosampler was 8 °C. For the detection and quantification of H_4folate, 5-CH_3-H_4folate, and 5-CHO-H_4folate, a fluorescence detector was used (excitation at 290 nm and emission at 360 nm), and for 10-CHO-folic acid and folic acid a DAD detector was used (the DAD channel was set at 290 nm). The mobile phase used was acetonitrile−30 mM phosphate buffer (pH 2.3) under linear gradient elution conditions. The gradient started at 6% (v/v) acetonitrile with a lag of 5 min, and then the gradient was raised linearly to 25% acetonitrile during 20 min and was kept constant for 2 min; thereafter it was decreased linearly to 6% acetonitrile during 1 min and was applied for 14 min to reequilibrate the column. Retention times of folate standards were used for peak identification; comparison of the ratio of sample peaks from fluorescence and diode array detectors to the ratio of standard peaks also from fluorescence and diode array spectra were used for verifying peaks if necessary.

2.7. Quantification. Quantification was based on an external standard method in which the peak area was plotted against concentration. A multilevel calibration curve was used ($n = 7$), and least-squares regression analysis was used to fit lines to the data. The amount of each folate form was calculated in its free acid form. The limit of detection (LOD) was defined as the lowest analyte concentration yielding a signal-to-noise ratio of 3. The limit of quantification (LOQ)

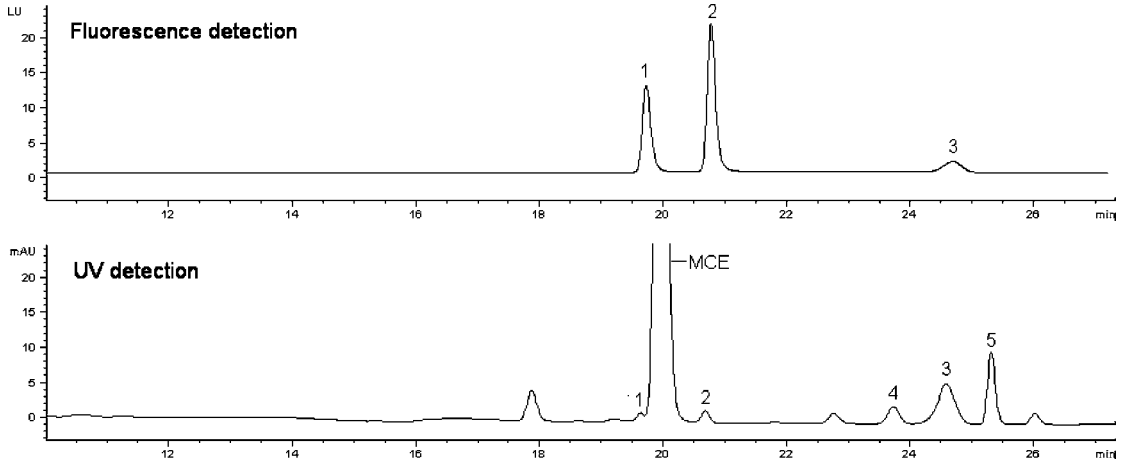

Figure 1. Chromatograms of folate monoglutamates in a standard mixture detected by fluorescence detector (λ_{ex} = 290 nm, λ_{em} = 360 nm) and DAD (set at 290 nm). Peaks: 1 = H_4folate (100 ng/mL), 2 = 5-CH_3-H_4folate (100 ng/mL), 3 = 5-HCO-H_4folate (600 ng/mL), 4 = 10-HCO-folic acid (600 ng/mL), and 5 = folic acid (600 ng/mL). MCE = 2-mercaptoethanol (used as antioxidant). For chromatographic parameters and conditions, see section 2.6.

was defined as the lowest analyte concentration yielding a signal-to-noise ratio of 10.

Because dialyzed rat serum contained small amounts of endogenous 5-CH_3-H_4folate, the concentration of 5-CH_3-H_4folate in yeast extracts was corrected for this endogenous 5-CH_3-H_4folate from serum by subtraction of the concentration of 5-CH_3-H_4folate in blank sample from the concentration of 5-CH_3-H_4folate in real yeast extracts. The concentration of endogenous 5-CH_3-H_4folate was determined by analyzing duplicates of blank samples and varied between 1.25 and 1.54 ng/mL when different batches of rat serum were used.

2.8. Method Validation. To estimate the accuracy of the method, recovery tests were performed by spiking triplicates of yeast samples with known amounts of 5-CH_3-H_4folate and H_4folate (folate forms actually found in yeast). The spiked samples were then treated according to section 2.3. The recovery (R) was calculated according to ref *18* as $R = (C_{found} - C_{sample})/C_{added}$, where C_{found} is the concentration in the spiked sample, C_{sample} is the concentration in the sample before spiking, and C_{added} is added concentration.

The intra- and interday precision of the method was estimated as the coefficients of variation (CV) for determination of folates in yeast extracts during three different days (six replicates each day).

The stability of folates in yeast extracts during storage in the thermostated autosampler (8 °C) was estimated after 24 and 48 h by reinjection of duplicate samples.

2.9. Statistics. To evaluate sample preparation and discover possible matrix effects, confidence intervals on the H_4folate and 5-CH_3-H_4folate mean were calculated based on the T distribution. The confidence intervals were based on folate values obtained when 50 mg of yeast was used in the extraction step (n = 7, e.g., seven independent series of duplicates). When other amounts of yeast were used in the extraction step, the folate content obtained (means based on duplicates) was compared to the confidence interval to determine if there were any significant differences in folate content due to matrix effects. A p value <0.05 was considered significant.

3. RESULTS AND DISCUSSION

3.1. Chromatographic Separation. A specially designed column (Aquasil C_{18}) for polar analytes, based on ultrapure silica with hydrophilic endcapping, was used for separation of folates. As seen in **Figure 1**, an excellent separation was achieved for all five folate monoglutamate standards selected for this study.

3.2. Optimization of Sample Preparation. *3.2.1. Folate Extraction Step.* For 5-CH_3-H_4folate there were no significant differences ($p > 0.05$) between values obtained for different

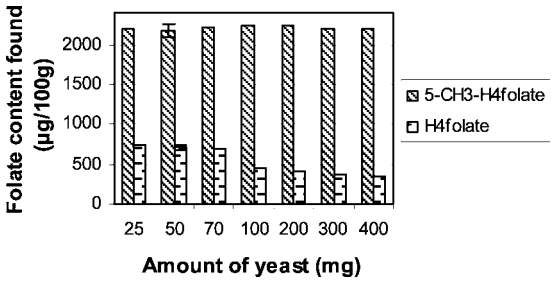

Figure 2. Evaluation of matrix effects for 5-CH_3-H_4folate and H_4folate by use of different amounts of yeast in the extraction step. Each bar is average values based on duplicates, but for 50 mg of yeast, the average values and standard deviations are based on duplicates in seven replicates.

sample amount to buffer ratios (from 25 mg/20 mL to 400 mg/20 mL), whereas for H_4folate there were significant differences ($p < 0.001$) between values obtained for smaller (25−70 mg/20 mL) and larger (100−400 mg/20 mL) sample amount to buffer ratios (**Figure 2**). The concentrations of H_4folate found in dry yeast when smaller sample amounts were used were significantly higher than those when larger sample amounts were used, which indicated matrix effects for H_4folate. These matrix effects may be caused by binding of H_4folate to the matrix; in such an event the use of larger sample amount to buffer ratio may hinder the breakdown of bindings. Therefore, sample amounts of 25−70 mg to 20 mL of buffer were found to be optimal.

The method for disrupting yeast cell wall was found to provide lower recovery of folates than heat treatment extraction (15% for H_4folate and 57% for 5-CH_3-H_4folate in comparison to recovery achieved by heat treatment).

3.2.2. Folate Deconjugation Step. According to Doherty et al. (*19*) folate conjugase from rat plasma provided the highest degree of conversion of $PteGlu_3$ to folic acid (99.4% compared with 81.1% for human plasma and 25.6% for hog kidney) at pH 6, 37 °C; therefore, rat serum was chosen as the source of folate conjugase in the present study. It was found that the deconjugation was incomplete when 25 μL of rat serum was used for 1 mL of yeast extract containing 2 mg of dry yeast per milliliter, but complete when both 50 and 80 μL of rat serum were used for both spiking concentrations of $PteGlu_3$. When

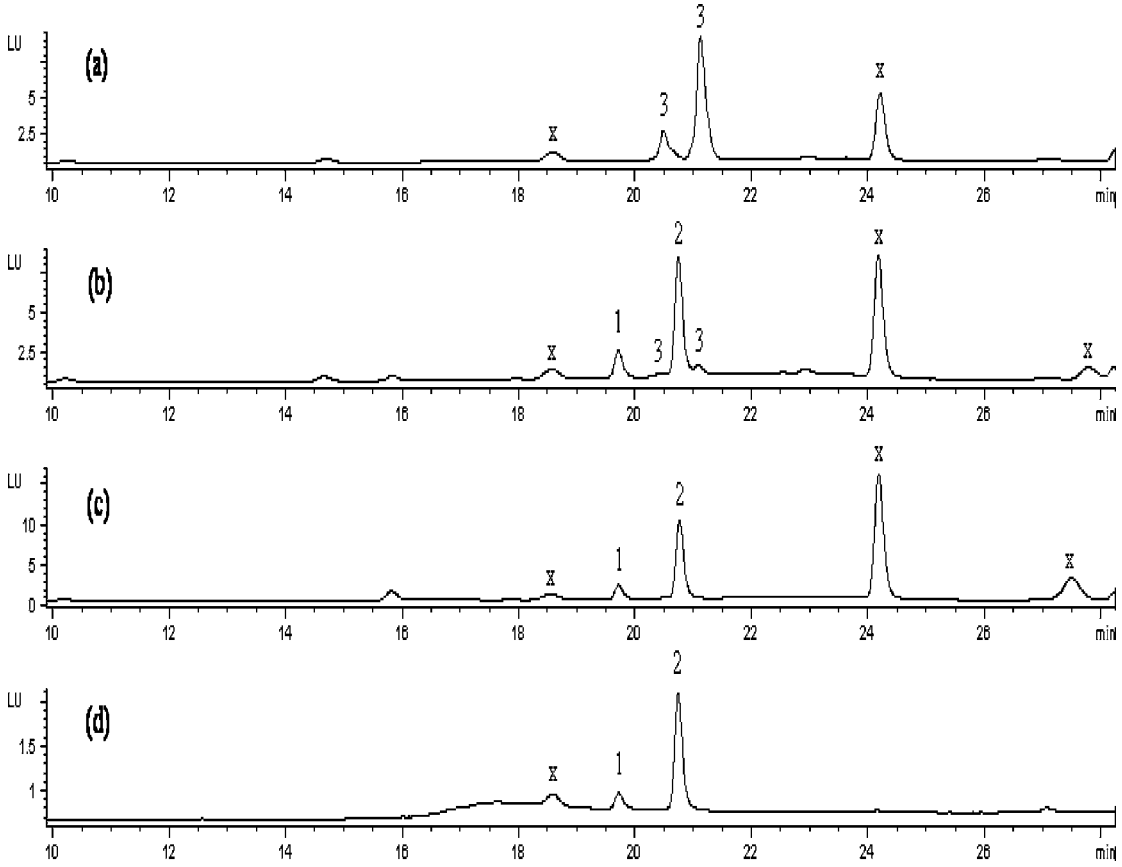

Figure 3. Chromatograms of yeast extracts. Peaks: 1 = H_4folate, 2 = 5-CH_3-H_4folate, 3 = folate polyglutamates, and x = interfering compounds from yeast matrix. (**a**) Yeast extract that had no addition of rat serum during deconjugation step; (**b**) partially deconjugated yeast extract with 50 μL of rat serum to 3 mL of yeast extract; (**c**) completely deconjugated yeast extract with 50 μL of rat serum to 1 mL of yeast extract; (**d**) yeast extract purified by SPE (0.5 mL of yeast extract was applied to SAX cartridge and eluated with 3.8 mL of elution buffer).

applying 3 mL of yeast extract with the same content of dry yeast (2 mg/mL), the addition of 80 μL of rat serum was necessary to achieve complete conversion of $PteGlu_3$ to folic acid and folate polyglutamates to monoglutamates, whereas 25 and 50 μL of rat serum were found to be insufficient for effective deconjugation. By comparing the chromatogram of nondeconjugated yeast extract (**Figure 3a**) with chromatograms of completely (**Figure 3c**) and partially (**Figure 3b**) deconjugated yeast extract, it was possible to detect peaks that were most likely of yeast folate polyglutamate origin. These peaks had retention times of 20.5 and 21.3 min (**Figure 3a**) and were comparable to peaks of H_4folate and 5-CH_3-H_4folate eluting at 19.7 and 20.7 min (**Figure 3c**). In partially deconjugated yeast extract (50 μL of rat serum to 3 mL of yeast extract) these polyglutamatic peaks were clearly decreased (**Figure 3b**), while in completely deconjugated yeast extract they were not detected (**Figure 3c**, 50 μL of rat serum to 1 mL of yeast extract). Our results indicate that the relation between amount of yeast and amount of rat serum plays a decisive role for achieving complete deconjugation. According to our results the use of rat serum in the ratio of 25−40 μL/mg dry yeast is sufficient to achieve complete deconjugation. By contrast, the use of smaller amounts of rat serum/plasma, such as 500 μL of rat plasma to 10 mL of yeast extract containing 33 mg of dry yeast per milliliter of extract, i.e., 1.5 μL rat plasma/mg dry yeast, may lead to a low ($\leq$25%) degree of deconjugation resulting in underestimation of folate content, as shown by Ndaw et al. (*20*). In addition, by our method a deconjugation time of 3 h was used compared to 2 h for Ndaw et al. (*20*). By this means the deconjugation time in combination with the rat serum ratio to yeast matrix can both contribute to the success in the conversion of folate polyglutamates to monoglutamates.

3.3. Purification Step: Check for Necessity. A chromatogram with fluorescence detection from real sample extract can be seen in **Figure 3c** (nonpurified extract) and **Figure 3d** (purified extract). The average differences between folate contents in dry yeast determined with and without a purification step were less than 2% with regard to both total folate content and individual folate forms (means of triplicates). However, for nonpurified samples, some disturbing peaks were found rather close to the retention time for 5-CHO-H_4folate and 10-CHO-folic acid at 290 nm, which could mask small amounts of these forms. In other words, it can be possible to analyze folates in yeast without purification in screening studies when rapid determination of main folate forms is of greatest interest; otherwise purification can be necessary to quantify all folate forms.

3.4. Validation of the Method. Linearity and sensitivity data are presented in **Table 1**. There was a linear relationship between the peak area and the concentration of each folate form over the ranges tested. The squared correlation coefficients for the seven-point calibration curves determined for the five folate standards were in the range 0.99986−1.00000.

Accuracy of the HPLC method was determined by recovery tests. The samples of dry yeast were spiked with a standard solution of H_4folate and 5-CH_3-H_4folate (folate forms detected in the yeast) at three different concentrations corresponding to 25, 50, and 100% of their respective content in yeast. The mean

Table 1. Linearity and Sensitivity of the HPLC Method[a]

folate form	linearity range (ng/mL)	correln coeff, R^2	LOD (ng/mL)	LOQ (ng/mL)
H_4folate	0.5–100	0.999 97	0.15	0.5
5-CH_3-H_4folate	0.3–100	1.000 00	0.1	0.3
10-HCO-folic acid	15–600	0.999 86	4.0	15.0
5-HCO-H_4folate	8–600	0.999 93	2.0	8.0
folic acid	4–600	0.999 96	1.0	4.0

[a] Abbreviations: LOD, limit of detection; LOQ, limit of quantification.

Table 2. Recoveries of H_4folate and 5-CH_3-H_4folate Added to Yeast Sample (μg/100 g Dry Weight)

folate form	amt in sample	added	recovery (%) ($n = 3$)
H_4folate	709	737	97 ± 3
		369	98 ± 1
		184	97 ± 1
5-CH_3-H_4folate	2180	2312	97 ± 1
		1156	97 ± 1
		578	98 ± 4

Table 3. Repeatability within Days[a] and between Three Different Days by the Use of Fluorescence Detector (RSD %)[b]

	within day			between
folate form	1	2	3	days 1 and 3
H_4folate	2.8	2.8	3.3	3.6
5-CH_3-H_4folate	2.5	2.2	1.9	4

[a] Repeatability within day are means of six samples. [b] RSD % means percent relative standard deviation.

recoveries ($n = 3$) of H_4folate and 5-CH_3-H_4folate were in the range of 97−98% (**Table 2**), which indicates that the method is accurate.

The intra- and interday repeatability of the entire analytical procedure was evaluated by analyzing six replicates of yeast samples on three separate occasions and showed a relative standard deviation <4% for both folate forms in both cases (**Table 3**).

The stability of yeast extracts in the autosampler (+8 °C) was tested by reinjection of duplicate samples. The difference between initial (0 h) and replicate (24 h) values was less than 5%, which indicated that the samples were stable for at least 24 h in the autosampler.

3.5. Folates in Baker's Yeast. In this study, the main folate forms found in dry baker's yeast, prepared by a Swedish yeast company, were found to be 5-CH_3-H_4folate and H_4folate, corresponding to 2180 ± 80 μg/100 g (47.4 ± 1.7 nmol/g) and 709 ± 25 μg/100 g (16.0 ± 0.5 nmol/g), respectively, which gives a total folate content of 2890 ± 90 μg/100 g (63.4 ± 1.9 nmol/g) (the recovery rates obtained were not taken into account for the quantification). These results are in agreement with previously published data for dry baker's yeast. The total folate content in dry baker's yeast analyzed by HPLC methods was found to be 49.5 ± 1.4 nmol/g (*20*) and 55.5 ± 3.5 nmol/g (*21*) with 5-CH_3-H_4folate and H_4folate as dominating folate forms (*21*).

A simple and rapid HPLC method for determination of the main folate forms in yeast was developed. The exclusion of a sample purification step makes this method time efficient, relatively cheap, and suitable for screening studies of different yeast strains.

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