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High-Performance Liquid Chromatographic Determination of Naturally Occurring Folates during Tempe Preparation

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A trienzyme treatment (protease, α-amylase, and human plasma conjugase), followed by purification using SPE with SAX cartridges and reversed-phase HPLC with UV-PDA detection, was performed for determination of the distribution of various folate forms and content at various stages of tempe preparation. The major folate form in soybean identified was 5-formyl tetrahydrofolate (5-CHO-H_4-folate), followed by 10-formyl tetrahydrofolate (10-CHO-PGA), and 5-methyl tetrahydrofolate (5-CH_3-H_4folate), whereas folic acid was not detected and tetrahydrofolic acid (H_4folate) was not detectable. The most predominant form in tempe was also 5-CHO-H_4folate, followed by 10-CHO-PGA, whereas the quantities of 5-CH_3-H_4folate and folic acid were negligible. Quantities and retention of folate significantly decreased during the first boiling, dehulling, soaking, and second boiling procedures, yielding folate retention of 32%. A remarkable increase in folate content was found after fermentation, 5.2-fold higher than that of the boiled soybean. This may be due to de novo formation of folate by *Rhizopus oligosporus*, the principal mold in tempe fermentation. HPLC results were ∼38−55% lower than the values obtained from the microbiological assay using *Lactobacillus casei*.

KEYWORDS: Folates; tempe; trienzyme treatment; HPLC; microbiological assay

INTRODUCTION

There is enough evidence in the literature to suggest that folate deficiency is linked to neural tube defects (NTDs), coronary heart disease, and megaloblastic anemia (*1*, *2*). Therefore, increasing dietary folate is important, particularly through the consumption of foods that are rich in folates. However, measurement of naturally occurring folates in foods is found to be difficult due to the presence of various chemical forms of folate, which differ in stability and bioavailability, and their existence in relatively low concentrations (*3*).

The determination of different folate forms in foods is possible through the HPLC technique, whereas the microbiological assay (MA), the most widely used method for folate analysis, can measure only the total folate content (*3*, *4*). Nearly 80% of natural folates in foods exist as polyglutamates. Using HPLC methods, it was found that 5-methyl tetrahydrofolate (5-CH_3-H_4folate) was the major folate form in fruits and vegetables (*5*), 5-CH_3-H_4folate and tetrahydrofolate (H_4folate) were dominant in animal products (*6*), and H_4folate was the predominant form in livers (*6*, *8*). 5-Formyl tetrahydrofolate (5-CHO-H_4folate), 10-formyl dihydrofolate (10-CHO-H_2-folate), and 10-formylfolic acid (10-CHO-PGA) together with 5-CH_3-H_4folate and folic acid were the major forms in cereals (*9*).

Legumes are good sources of folate, which may range from 100 to 600 μg/100 g (*10*). A few folate studies on legumes showed that 5-CHO-H_4folate was the predominant form in soybean (*11*), lima beans (*7*), and peanuts (*12*) and 10-CHO-H_2folate and 5-CHO-H_4folate in cooked chickpeas and cooked common beans, respectively (*13*). In addition, dried legumes are always processed prior to consumption. This may affect folate availability in legume foods as they are water soluble and sensitive to light, air, heat, and pH changes. Losses of folates in processed legumes during soaking, boiling, and cooking have been reported (*14*−*16*), which varied from 34 to 81%. Conversely, increased folate content was found after fermentation, such as in yogurt (*17*) and bread (*18*). However, there is no available literature on folate distribution, content, and retention in fermented legume products, such as tempe, which originates from Indonesia. According to the Indonesian Central Bureau of Statistics (ICBS) (*19*), about 24 and 46% of the households consumed tempe every day and two to five times per week, respectively, reflecting the importance of tempe in the Indonesian diet.

This study accomplished a modified trienzyme treatment (protease, α-amylase, and human plasma conjugase) (*13*), followed by a purification using solid-phase extraction (SPE) with SAX cartridges (*22*) and a reversed-phase HPLC with UV-PDA detection for the determination of folate distribution, content, and retention in samples collected from each step of tempe preparation. A comparison using the MA method with *Lactobacillus casei* was also performed.

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MATERIALS AND METHODS

Chemicals. All reagents were of analytical grade, and Milli-Q grade, distilled and deionized water was used. In addition, all of the glassware used was covered with aluminum foil to prevent exposure to sunlight.

Folate Standards. Folic acid, 10-formylfolic acid, (6*R*,*S*)-5-formyl-5,6,7,8-tetrahydrofolic acid, calcium salt (5-CHO-THF), (6*R*,*S*)-5-methyl-5,6,7,8-tetrahydrofolic acid, calcium salt (5-CH_3-THF), (6*R*,*S*)-5,6,7,8-tetrahydrofolic acid trihydrochloride (THF), 7,8-dihydrofolic acid (DHF), and pteroyltriglutamate ($PteGlu_3$) were obtained from Dr. B. Schircks Laboratories, Jona, Switzerland. Preparation of standard solutions was performed under subdued light conditions (*22*), and the concentration of each folate standard was checked using a spectrophotometer at appropriate wavelengths (*20*) with a pH 7.0 phosphate buffer as the blank. Standard solutions were flushed with nitrogen and stored at −80 °C for no more than 3 months. For calibration purposes, standard mixtures were prepared within the range of 5−200 ng/mL in 0.1 M acetate buffer/elution buffer. The standard mixtures were prepared fresh on the day of use.

Sample Preparation. Tempe was prepared once from an imported variety of soybean [*Glycine max* (L.) Merr.], using the common domestic method of preparation used in Indonesia (*21*) as follows: soybean seeds were boiled in water (1:3 w/v) for 20 min. The hulls were removed using a dehulling machine, followed by soaking of the dehulled seeds overnight (18 h). Subsequently, they were boiled for 25 min, drained, and cooled at room temperature for 4−5 h prior to inoculation with tempe starter (dried culture of *Rhizopus oligosporus*) that was obtained from LIPI, Bandung, Indonesia, at a level of 1 g/kg of dry seeds. The inoculated seeds were wrapped with banana and hibiscus leaves and fermented for 44 h at room temperature to obtain fresh tempe (2 kg of tempe could be prepared from 1 kg of raw soybean).

Samples were taken from each stage of tempe preparation, and prior to analysis, they were freeze-dried and stored at −20 °C. The preparation of tempe was performed in the Indonesian Legume and Tuber Crops Research Institute (ILETRI), Malang, Indonesia, whereas folate analysis was done in the Food Science and Technology Department, University of New South Wales (UNSW), Sydney, Australia.

Sample Extraction. Sample extraction was performed under protection from direct daylight (*13*) with a minor modification to the trienzyme treatment. Freeze-dried samples were first milled using a Fritsch Mill/Pulverisette (Fritsch, Oberstein, Germany). A 5-g sample was suspended in 40 mL of 0.05 M CHES−HEPES buffer, pH 7.85, containing 2% (w/v) sodium ascorbate and 0.01 M 2-mercaptoethanol flushed with nitrogen, covered/capped, and mixed well using a vortex stirrer. The mixture was heated for 10 min in a boiling water bath and swirled occasionally and then immediately cooled in an ice bath. Two milliliters (2 mg/mL) of protease (P-5147, Sigma Chemical Co., St. Louis, MO) was added and incubated in a shaking water bath at 37 °C for 2 h. The enzyme was deactivated by heating the samples for 3 min in a boiling water bath and cooling on ice. The pH was then adjusted to 6.0 with 4 M chloric acid, followed by the addition of 5 mL of crude human plasma (obtained from the Prince of Wales Hospital, Sydney, Australia) and 1 mL (20 mg/mL) of α-amylase (EC 3.2.1.1, type IV-B from porcine pancreas, no. 9002090-2, Sigma Chemical Co.), incubated for 4 h in a shaking water bath at 37 °C. Finally, the samples were heated again in a boiling water bath for 5 min, cooled on ice, and centrifuged for 15 min at 10000 rpm and 4 °C (Beckman J2-MC with JA 4 fixed-angle rotator). The residue was suspended in 10 mL of extraction buffer and recentrifuged for 15 min. The supernatants were combined, made up to exact volume (50 mL) with the extraction buffer, and filtered with a Minisart CE syringe filter (0.45 μm pore size; 25 mm i.d.; no. 17598, Sartorius). The filtrates were flushed with nitrogen and stored at −20 °C until purification (within a few days). All samples were analyzed in duplicate. An enzyme blank (without sample) was also included to examine folate content in the enzymes used. This value was subtracted from the folate content in the samples to give actual folate concentrations.

Prior to being used in the trienzyme treatment, the conjugase activity of human plasma was assayed using 0.34 mmol of $PteGlu_3$ as a substrate at pH 6.0 in 0.05 M CHES−HEPES buffer containing 2% sodium ascorbate and 0.01 M 2-mercaptoethanol at 37 °C for 4 h. The mixture was filtered after incubation and heat denaturation and injected without any purification. The conjugase activity was considered to be adequate if the area of the folic acid peak was >90% of the total area of $PteGlu_3$ and folic acid.

Sample Cleanup or Purification. Purification and concentration of the sample extracts were done using solid-phase extraction (SPE) (*22*) with a strong anion exchange (SAX) cartridge (3 mL/500 mg of quaternary amine N^+, counterion Cl^-, no. 57017, Supelco, Inc.) and applied using a Supelco 24-port Visirep vacuum manifold. First, the cartridges were conditioned with 3 mL of hexane, methanol, and water and then equilibrated with 10 mL of 0.01 M phosphate buffer/conditioning buffer. Second, the sample [5 mL of the sample extract was diluted with 10 mL of distilled water, and ∼15 μL (0.1% v/v) of 2-mercaptoethanol was added] was slowly loaded onto the cartridge at a rate of <1 mL/min. This was followed by washing the cartridge twice with 1.5 mL of conditioning buffer. Finally, folate compounds were eluted with 2.5 mL of 0.1 M acetate buffer/elution buffer with a flow rate of <0.3 mL/min. The eluted sample was weighed, and the exact volume was calculated using the value of 1.074 g/mL for the density. Purified extracts were immediately analyzed without any further freeze-storage.

HPLC Analysis. A Waters liquid chromatograph (Waters, Rydalmere, Australia) was equipped with a model 717 plus autosampler, a model 600 controller pump (gradient pump), and a UV−photodiode array (PDA) detector (model 996). Recording of the chromatograms and evaluation of the peak areas were performed using the Millenium 32 data acquisition system (Waters). The folate compounds were separated with a Phenomenex C_{18} (2) column (5 μm; 150 mm L × 4.6 mm i.d.; Luna), which was protected with a guard column (Phenomenex C_{18} ODS, octadecyl, 4 mm L × 3.0 mm i.d.; Luna). UV absorbance at a wavelength of 290 nm was used to detect the elution of folate compounds.

Gradient elution with acetonitrile and 30 mM phosphate buffer, pH 2.2, was performed to separate folate compounds at a flow rate of 0.8 mL/min. The gradient was started at 5% acetonitrile, which was maintained isocratically for the first 8 min, and then the acetonitrile concentration was raised linearly to 24% within 23 min and decreased back to 5% after 5 min. The injection volume was 100 μL. The running time was 36 min, and the time between injections was 46 min.

A calibration curve for each folate standard was obtained by the external standard method in which peak area was plotted against six concentrations of folate standards injected (0.5−20 ng/injection). These calibration plots were prepared on the day of use. Quantification of folate contents in the samples was performed using linear regression procedures. The values were then adjusted for the folate content in the enzyme blank treatment. Peak identification was based on the retention time and spiking (addition of standard compounds into the purified sample extract) to confirm peaks for any samples in which identification using the retention time was considered to be inadequate.

Quality Control. Recovery studies were also performed either by carrying known concentrations of folate standard mixture in CHES−HEPES buffer through the entire procedure or by adding them to a selected sample before extraction and carrying them through the trienzyme treatment and purification. One microgram of each folate standard was added per gram of freeze-dried sample for recovery studies. Recoveries of folate (percent) were calculated as follows: (concentration of folate measured in spiked sample − concentration of folate measured in unspiked sample)/(concentration of folate added in spiked sample) × 100 (*23*).

Microbiological Assay (MA). Sample extracts prepared for HPLC analysis were also subjected to microbiological assay using glycerol-cryoprotected culture of *L. casei* ATCC 7469 (*24*) to determine their total folate contents. The culture of *L. casei* was obtained from the School of Microbiology and Immunology, UNSW, Sydney, Australia, and the assay employed was according to previous methods (*25*, *26*). A standard reference material (wholemeal flour, CRM 121) supplied by the Institute for Reference Materials and Measurements (IRMM, Geel, Belgium) was also analyzed to ensure the accuracy of the data. The value obtained was 36.6 ± 3.3 μg/100 g of dry mass ($n = 3$),

Table 1. Recoveries of Folates after Heat Extraction, Trienzyme Treatment, and SPE-SAX Purification

standards	folate form	amount in sample[a] (μg/100 g)	added (μg/100 g)	recovered[a] (μg/100 g)	recovery[a] (%)
carried in extraction buffer	5-CH_3-H_4folate	0	4	3.8 ± 0.2	96 ± 6
	5-CHO-H_4folate	0	4	4.3 ± 0.4	108 ± 10
	folic acid (PGA)	0	4	4.4 ± 0.2	109 ± 5
	10-CHO-PGA	0	4	3.6 ± 0.6	90 ± 15
added to tempe	5-CH_3-H_4folate	nd	100	79.6 ± 4.5	80 ± 5
	5-CHO-H_4folate	227.6 ± 3.6	100	315.6 ± 9.6	88 ± 6
	folic acid (PGA)	nd	100	86.2 ± 3.4	86 ± 4
	10-CHO-PGA	425.5 ± 11.8	100	507.9 ± 9.3	83 ± 3

[a] Values are means ± SD of duplicate analyses. nd = not detected.

within the range of the certified value (33.92−70.18 μg/100 g of dry mass) for MA (*27*).

Statistical Analysis. Total folate content during the preparation of tempe was evaluated by one-way analysis of variance using the Statsgraphic package (Statistical Graphics Corp., 1993, Manguistics Inc.). Differences in total folate content between processing steps were analyzed using the least significant difference (LSD) test with a significance level of 0.05.

RESULTS AND DISCUSSION

Extraction and Purification. Folate standards (5-CH_3-H_4-folate, 5-CHO-H_4folate, 10-CHO-PGA, and folic acid) carried in CHES−HEPES buffer gave high recovery values (90−109%) as presented in **Table 1**, showing that the extraction buffer containing combined antioxidants of 2-mercaptoethanol and ascorbate were effective in preventing folate losses during the whole analysis. The SPE-SAX procedure for purification was also shown to be efficient for folate standards. Other studies (*9*, *13*, *28*, *29*) using similar extraction buffer have reported recovery values of 95−107% for five different folate forms, stressing the superiority of CHES−HEPES buffer in maintaining the stability of folate compounds during the extraction.

Recoveries of folate standards added to tempe samples were accepted (80−88%), even though the values were lower when compared to standards in the extraction buffer alone. Other studies (*9*, *13*) have reported folate recoveries of about 85−107 and 78−113% when added to cereal foods and salami, respectively (using affinity chromatography for purification). In another study (*22*) using SPE-SAX for purification purposes, folate recoveries of 56−77, 75−90, and 70−90% were observed in baked fish, milk powder, and lyophilized pig liver, respectively. This indicates that food matrix interference may cause some losses of folate during analysis as well as purification procedures.

A minor modification to the sequence of the trienzyme treatment, length of incubation, and source of conjugase was performed in this study in comparison to an earlier study (*13*). In fact, no systematic experiments on the sequence of addition of the enzymes are evident from the literature. Protease was added first as an earlier study (*23*) observed a significant increase of folate extraction in enriched cereal grain products when protease was added prior to the addition of α-amylase and conjugase. Also, no specific information on incubation time of protease is available for individual food items. One hour of incubation for protease treatment was used (*9*) as overnight incubation caused a 20% decrease of the measured total folate relative to 1 h of treatment, whereas another study (*23*) used 3 h of incubation. Therefore, 2 h of incubation for protease was chosen in this study given the relatively high protein content in soybean and tempe samples.

Human plasma was used as a source of conjugase because it is easily available and produces monoglutamates as the end products, which are essential for HPLC analysis. However, relatively few studies have been published regarding the use of human plasma during HPLC analysis of folate (*22*, *30*, *31*). In this study, pH 6.0 was used for deconjugation purposes as no difference in the human plasma activity was observed between pH 4.5 and 6.0 (data not shown). A lack of folate recovery in the pH range of 4.5−6.0 was also reported (*32*), even though an optimum activity of human plasma was found at pH 4.5. Another study (*33*) observed a substantial loss of folate (32%) when deconjugation was performed at pH 4.5 as compared with pH 6.0 and 7.0. One study (*26*) also reported a significant lowering of total folate detected in samples treated with human plasma at pH 4.5 in comparison with chicken pancreas at pH 7.2. Losses of folate may relate to lower stability of folate derivatives at acidic pH (*25*).

Preliminary trials showed that 6 h of incubation of human plasma gave 88% conversion of $PteGlu_3$ to folic acid. However, 4 h of incubation was chosen (81% conversion level) to be used for further analysis in order to accomplish the sample extraction, including the trienzyme treatment, within one working day with respect to minimizing folate losses.

Normally, ∼50 μL of human plasma was used for testing of deconjugation activity and 0.5 mL for actual sample extraction (*22*). However, optimum deconjugation was obtained only when 10-fold the volume of human plasma (500 μL) was used for testing its activity with the human plasma used in this study. Therefore, 5 mL was used for extraction of all samples. A lower folate conjugase activity of human plasma compared to that of other studies (*32*, *33*) may relate to the initial condition of human plasma, which had already been stored for 2 months at −20 °C. Other studies (*22*, *32*) reported losses of enzyme activity by 20 and 30% when human plasma was stored at −20 °C for 2 and 4 months and also found unexpectedly low activity of human plasma conjugase in both commercial (Sigma) and in-house preparations (fresh and outdated plasma).

Chromatographic Performance The adapted gradient elution using a Phenomenex Luna C_{18} (2) column (5 μm; 150 × 4.6 mm) showed a good separation of folate compounds (5-CH_3-H_4folate, 10-CHO-PGA, 5-CHO-H_4folate, and folic acid) within 31 min with capacity factors (k') of 9.0, 11.3, 11.6, and 12.0, respectively. However, the peak of H_4folate was likely to have been masked by 2-mercaptoethanol, an antioxidant used in the preparation of H_4folate standard, particularly at low concentrations. Using a Hypersil ODS column (3 μm; 50 × 4.6 mm) along with 2-mercaptoethanol and ascorbic acid as antioxidants did not interfere with the chromatographic separation of folate compounds, including H_4folate using both UV

Table 2. Detection Limits for the HPLC System Used

folate compounds	limit of detection (ng per injection)
5-CH_3-H_4folate	0.5[a,b]
5-CHO-H_4folate	0.9[a,c]
10-CHO-PGA	1.3[a,b]
folic acid (PGA)	0.3[a,b]

[a] UV detection at 290 nm. [b] Signal-to-noise ≥ 3. [c] Signal-to-noise ≥ 2.

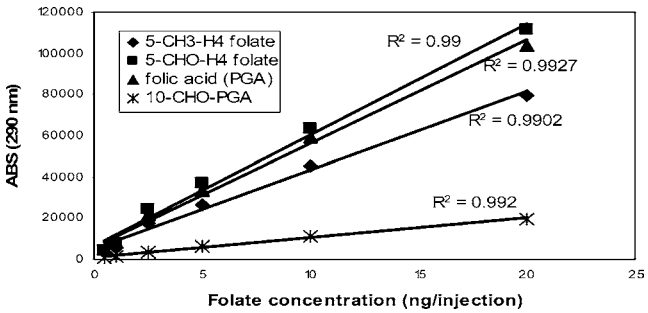

Figure 1. UV calibration curves for four folate compounds detected at 290 nm.

and fluorescence detection (*13*, *22*). A column filled with 3 μm diameter particles used in their studies may have produced better separation of H_4folate. H_4folate is highly labile and naturally found in small amounts only in biological materials, except in livers (*22*, *34*). Considering that trace amounts only of H_4folate might also be present in the samples studied, it was decided not to determine this particular derivative, as actual quantification would be difficult with the column used in this study.

The detection limits for the HPLC system used in this study for four folate compounds are presented in **Table 2**. Calibration curves showed a linear response ($R^2 > 0.99$) of peak area versus concentration as shown in **Figure 1**. The retention times of individual folate compounds were repeatable with CV < 1% ($n = 4$ injections) as well as for quantitative analysis (peak area) with CV < 2% (**Figures 3** and **4**).

Distribution and Retention of Folate during Tempe Preparation. Table 3 shows that 5-CHO-H_4folate was the major folate form in soybean seeds (63%), followed by 10-CHO-PGA (28%) and 5-CH_3-H_4folate (8%), whereas folic acid was not detected. These findings agreed with an earlier study (*11*) which found 65−70% of 5-CHO-H_4folate, 15% of 5-CH_3-H_4folate, 10% of 10-CHO-H_4folate, and 10% of 10-CHO-PGA in soybean. However, a larger proportion of 10-CHO-PGA was obtained in this study. In the soybean chromatogram (not shown), the peak elution at 25.4 min (as 10-CHO-PGA) did not increase in size by spiking, but was only partially resolved, suggesting that 10-CHO-PGA might be present at a very low concentration in the analyte, thus masked by the compound with

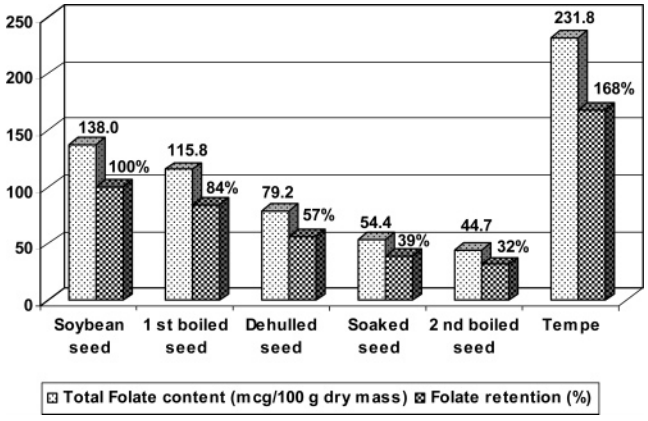

Figure 2. Total folate content and retention in samples collected during tempe preparation.

similar retention time as also reported (*13*) in cooked chickpea and common bean. In their UV chromatogram, the peak size increased with spiking; however, when it was further detected using a fluorescence detector at its specific wavelengths, they found that the large amounts of 10-CHO-PGA in the samples had to be disregarded. Nevertheless, dual detection of UV and fluorescence was not possible using the HPLC system used in this study, so it was difficult to confirm the actual quantity of 10-CHO-PGA. Hence, data for total folate are presented both including and excluding 10-CHO-PGA (**Table 3**), and for the calculation of folate retention, 10-CHO-PGA was excluded (**Figure 2**).

The total folate contents in soybean, including and excluding 10-CHO-PGA, were 177 and 136.7 mg/100 g of dry mass, respectively, as shown in **Table 3**. No comparison could be made with other studies (*11*) because current data available in the literature were obtained mostly using MA and folate distribution using DEAE-cellulose column chromatography.

Prior to fermentation, total folate contents in the first boiled and dehulled soybean significantly decreased ($P < 0.05$), giving folate retentions of 84 and 57%, respectively (**Figure 2**). The presence of 5-CH_3-H_4folate was not seen after dehulling, whereas 5-CHO-H_4folate was gradually reduced. A decreased retention of total folate was also noted during soaking overnight (39%) and subsequent boiling (32%). Another study of tempe preparation (*35*) reported a higher retention of total folate (69%) during overnight soaking of whole soybean. A slightly different method of tempe processing, which omitted the first boiling and performed dehulling after soaking, might contribute to such differences. Losses of total folate during soaking and boiling were mainly due to leaching into the water, followed by heat destruction (*15*, *16*, *35*). The presence of lactic acid bacteria, including *L. casei* and *Streptococcus faecium* (*36*), may be

Table 3. Distribution of Folate Derivatives and Contents (Micrograms per 100 g of Dry Mass) in Samples Collected during Tempe Preparation

					total folate[b]		
sample	5-CH_3-H_4folate[a]	5-CHO-H_4folate [a]	10-CHO-PGA[a]	folic acid	including 10-CHO-PGA	excluding 10-CHO-PGA[c]	total folate using MA[d]
soybean seed	16.9 ± 1.4	121 ± 0.4	53.8 ± 2.4	nd[e]	191.8 ± 0.7	138.0 ± 1.7 b	248.7± 15.3
seeds (first boiled)	2.5 ± 0.2	113.3 ± 2.7	60.5 ± 0.2	nd	176.3 ± 2.7	115.8 ± 2.5 c	212.5 ± 17.0
dehulled seed	nd	79.2 ± 2.6	17.1 ± 4.6	nd	96.3 ± 2.0	79.2 ± 2.6 d	150.0 ± 7.0
soaked seed	nd	54.4 ± 3.0	244.7 ± 2.8	nd	299.1± 5.7	54.4 ± 2.9 e	121.4 ± 14.6
seeds (second boiled)	nd	44.7 ± 1.7	441.4 ± 4.7	nd	486.1 ± 3.0	44.7 ± 1.7 f	71.6 ± 5.4
tempe	nd	231.8 ± 3.4	402.3 ± 1.6	nd	634.1 ± 2.1	231.8 ± 3.7 a	416.4 ± 68.7

[a] Values are means ± SD of duplicate analyses. [b] Sum of the individual forms. [c] Values followed by different letters are significantly different at $P < 0.05$. [d] Values are means ± SD of triplicate analyses. [e] nd = not detected.

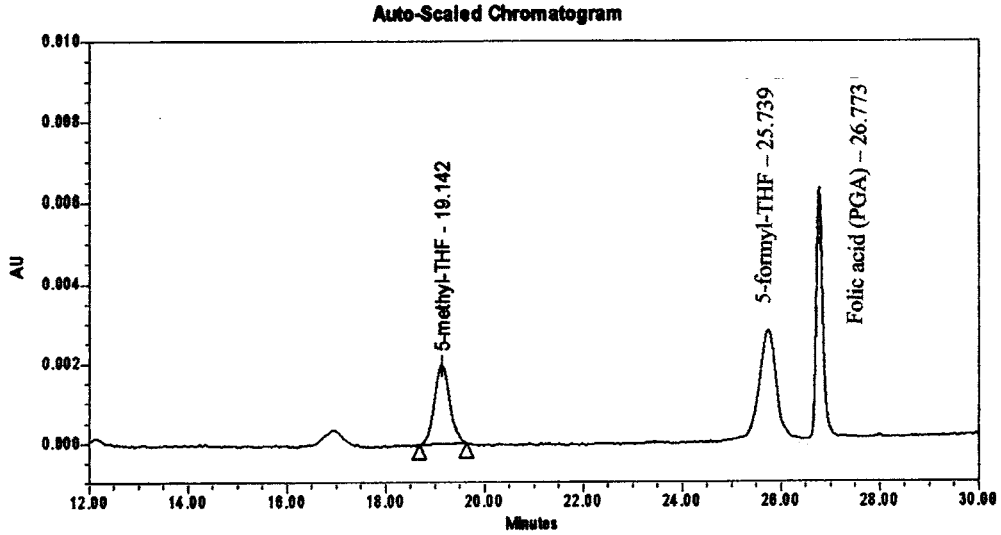

Figure 3. Chromatogram of standard mixtures of 5-CH_3-H_4folate, 5-CHO-H_4folate, and folic acid (PGA).

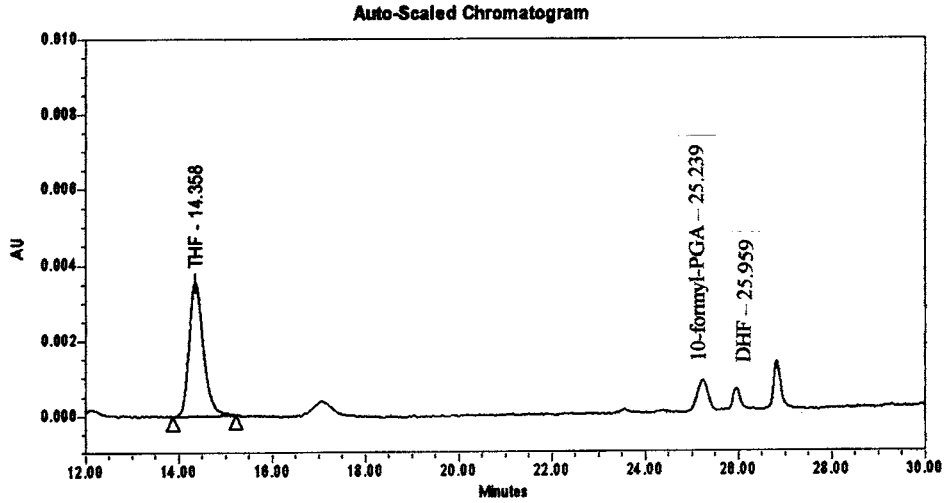

Figure 4. Chromatogram of standard mixtures of H_4 folate, 10-CHO-PGA and H_2 folate.

another cause for the decreased total folate content during soaking as these microorganisms are known to utilize folate (*2*).

Like soybean, 5-CHO-H_4folate was also predominant in tempe (**Table 3**), whereas neither 5-CH_3-H_4 folate nor folic acid was detected. Similarly, 10-CHO-PGA elution was also observed in the tempe chromatogram (**Figure 5**). However, the apparent large quantity of this particular compound was not ascertained due to masking by another compound as discussed previously. The total folate content in tempe (excluding 10-CHO-PGA) was remarkably high (231.8 mg/100 g of dry mass), ~5.2-fold higher than that of the boiled seed before fermentation. This seemed to be close to the observation of an earlier study (*37*) that noted the total folate in tempe was 4−5 times higher after 48 h of fermentation using MA, which is the first study on tempe found in the literature. Interestingly, these authors found no significant difference in total folate values between using *L. casei* and *S. faecalis*, reflecting the fact that 5-CH_3-H_4folate was not present in tempe, as *S. faecalis* does not respond to 5-CH_3-H_4folate, whereas *L. casei* responds to almost all folate forms up to triglutamates (*2*). Their finding was supported by this study, which found negligible amounts of 5-CH_3-H_4folate in tempe.

Increased amounts of total folate in tempe may be due to the liberation of folate compounds by the actions of enzymes (protease, lipase, and amylase) produced by *R. oligosporus* during fermentation. It might be also due to de novo formation of folate compounds as another study (*38*) showed the synthesis of N-CHO-5,6,7,8-tetrahydropteroylglutamic acid and rhizopterin by *R. oligosporus* after culture in folate-free medium. The present study found that 5-CHO-H_4folate was the predominant folate form in tempe, supporting the latter evidence. Increased amounts of folate were also reported in yogurt (*17*), kefir (*39*), cheese (*40*), and bread (*2*), highlighting the important role of microorganisms as folate producers during fermentation.

It seems that the conditions of fermentation and tempe starter used are more important in dictating the final folate content in tempe than initial folate content in soybean. A dried culture of *R. oligosporus* as used in this study is currently available on the market. However, some tempe processors prefer to use traditional starters (usar), which may contain other species of *Rhizopus*, such as *R. arrhizus*, *R. oryzae*, and *R. stolonifer* (*41*). Different species of *Rhizopus* were reported to yield various levels of riboflavin, nicotinic acid, nicotinamide, and vitamin B_6 during tempe fermentation, and *R. oligosporus* was likely to be the best vitamin producer (*41*). This may also be true for folate synthesis; however, no studies have been reported. Length of fermentation may also influence total folate content in tempe as it would gradually decline after 48 h of fermentation (*37*).

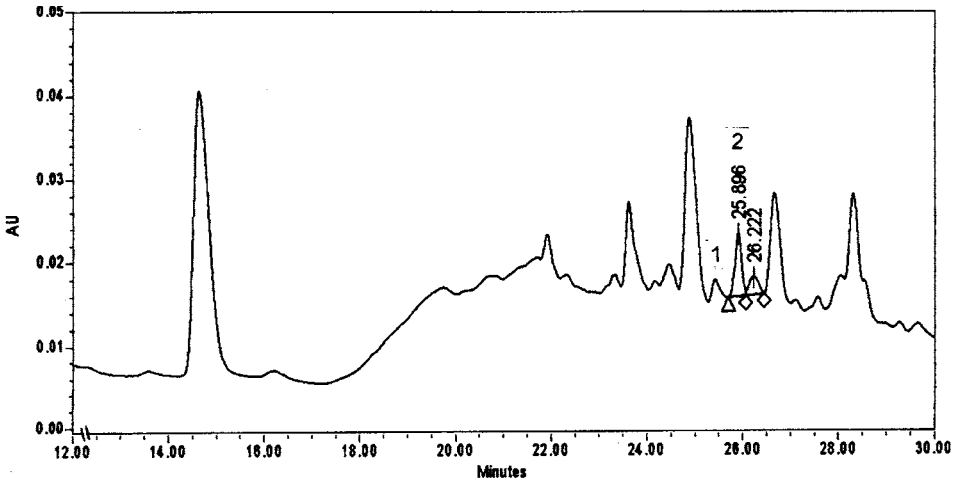

Figure 5. Reversed-phase chromatograms of major folate forms present in tempe. (1) 10-CHO-PGA; (2) 5-CHO-H_4 folate. A Phenomenex C_{18} (2) column (5 μm; 150 mm L × 4.6 mm I.D; Luna), a mobile phase of acetonitrile - 30 mM phosphate buffer, pH 2.2 with increasing acetonitrile concentration and a UV-PDA detection at 290 nm were used.

Similarly, shorter fermentation (24 h) gave only a 2-fold increase of total folate as noted in an earlier study (*35*).

The total folate values obtained by HPLC analysis were 38−55% lower than the values determined by MA (**Table 3**). Excluding some folate compounds, such as H_4folate and 10-CHO-PGA, the presence of folic acid and 5-CH_3-H_4folate at undetectable levels (**Table 3**) may be attributed to these differences as they can be utilized by *L. casei* used in MA. Another study (*13*) also found that total folate was 24 and 52% lower relative to MA in cooked chickpea and common bean, respectively. This was likely due to the presence of some unidentified folate compounds, even though a specific purification method (affinity chromatography) was used. In the present study, a less specific purification method (SPE-SAX) was used and some unidentified peaks were observed (**Figure 5**), contributing to the low recovery of folate from food samples. A relatively low activity of the human plasma conjugase used (81%) may have also contributed to a higher total folate obtained through MA as *L. casei* may utilize folate up to triglutamates (*2*). HPLC results of 30−40 and 20−35% relative to the MA were also reported by others (*12*, *34*), in four certified reference materials (wholemeal flour, milk powder, mixed vegetables, and pig liver), stressing the need for further studies using the HPLC analysis.

However, other studies (*9*, *43*) reported that total folate contents in fortified cereal products were in agreement with the values obtained from MA. This suggested that added folate is not embedded in the matrix and can be extracted easily. The predominant folate derivatives determined in their studies were folic acid and 5-CH_3-H_4folate, which seemed to give values of total folate close to that of the MA (*42*). However, 5-CH_3-H_4-folate and folic acid were present in only trace amounts in soybean and tempe.

The MA results showed that fresh tempe is a good source of folate in the Indonesian diet. Deep-frying, a common processing method, resulted in 79% folate retention (*35*). It was estimated that the consumption of 50−100 g of fried tempe/serving would provide 45−90% of the recommended dietary intake for folate as recommended by the NHMRC (*44*).

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