



## The use of a plant enzyme for rapid and sensitive analysis of naturally-occurring folates in food by liquid chromatography-tandem mass spectrometry

Ložnjak, Petra; García-Salinas, Carolina; Díaz de la Garza, Rocío Isabel; Bysted, Anette; Jakobsen, Jette

*Published in:*  
Journal of Chromatography A

*Link to article, DOI:*  
[10.1016/j.chroma.2019.02.037](https://doi.org/10.1016/j.chroma.2019.02.037)

*Publication date:*  
2019

*Document Version*  
Peer reviewed version

[Link back to DTU Orbit](#)

*Citation (APA):*  
Ložnjak, P., García-Salinas, C., Díaz de la Garza, R. I., Bysted, A., & Jakobsen, J. (2019). The use of a plant enzyme for rapid and sensitive analysis of naturally-occurring folates in food by liquid chromatography-tandem mass spectrometry. *Journal of Chromatography A*, 1594, 34-44. <https://doi.org/10.1016/j.chroma.2019.02.037>

---

### General rights

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

## Accepted Manuscript

Title: The use of a plant enzyme for rapid and sensitive analysis of naturally-occurring folates in food by liquid chromatography-tandem mass spectrometry

Authors: Petra Ložnjak, Carolina García-Salinas, Rocío Isabel Díaz de la Garza, Anette Bysted, Jette Jakobsen



PII: S0021-9673(19)30185-2  
DOI: <https://doi.org/10.1016/j.chroma.2019.02.037>  
Reference: CHROMA 360046

To appear in: *Journal of Chromatography A*

Received date: 3 October 2018  
Revised date: 12 February 2019  
Accepted date: 16 February 2019

Please cite this article as: Ložnjak P, García-Salinas C, de la Garza RID, Bysted A, Jakobsen J, The use of a plant enzyme for rapid and sensitive analysis of naturally-occurring folates in food by liquid chromatography-tandem mass spectrometry, *Journal of Chromatography A* (2019), <https://doi.org/10.1016/j.chroma.2019.02.037>

This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

**Title:**

The use of a plant enzyme for rapid and sensitive analysis of naturally-occurring folates in food by liquid chromatography-tandem mass spectrometry

**Authors:**

Petra Ložnjak<sup>a\*</sup>, Carolina García-Salinas<sup>b</sup>, Rocío Isabel Díaz de la Garza<sup>b</sup>, Anette Bysted<sup>a</sup>, Jette Jakobsen<sup>a</sup>

<sup>a</sup>Research Group for Bioactives – Analysis and Application, National Food Institute, Technical University of Denmark, Kemitorvet, DK-2800 Kgs. Lyngby, Denmark

<sup>b</sup>School of Engineering and Science, Institute of Technology and Higher Education, Ave. Eugenio Garza Sada 2501, MX-64849 Monterrey, N.L., México

\*Corresponding author: Petra Ložnjak, Research Group for Bioactives – Analysis and Application, National Food Institute, Technical University of Denmark, Kemitorvet, DK-2800 Kgs. Lyngby, Denmark, Telephone: + 45 93518869, E-mail: [petlon@food.dtu.dk](mailto:petlon@food.dtu.dk)

**Highlights:**

- Single-laboratory validation of a new method for folate quantification in food
- The single-enzymatic extraction step is used for folate analyses in food
- A plant origin hydrolase enables folate deglutamylation within 1 h
- Conversion between 10-HCO-PteGlu, 5,10-CH<sup>+</sup>=H<sub>4</sub>folate and 5-HCO-H<sub>4</sub>folate is studied
- 50% of 5,10-CH<sup>+</sup>=H<sub>4</sub>folate is converted into 5-HCO-H<sub>4</sub>folate during folate analysis

## Abstract

A rapid, sensitive and reproducible method for analysis of naturally-occurring folates and folic acid in food has been developed and validated. A single-enzyme extraction step, in which a pure recombinant enzyme of plant origin (*Arabidopsis thaliana*) was used, enabled fast and reproducible deglutamylation during folate extraction within the incubation time of 1 h. Six commonly occurring folate forms (tetrahydrofolate, 5,10-methenyltetrahydrofolate, 10-formylfolic acid, 5-formyltetrahydrofolate, folic acid and 5-methyltetrahydrofolate) were detected and quantified in 9 min using liquid chromatography-tandem mass spectrometry (LC-MS/MS).  $^{13}\text{C}_5$ -labeled 5-formyltetrahydrofolate,  $^{13}\text{C}_5$ -labeled folic acid and  $^{13}\text{C}_5$ -labeled 5-methyltetrahydrofolate were used as internal standards for the quantification. The method is described by a calibration curve ( $R^2 > 0.99$  and trueness 85-115%), a limit of quantification at 0.1  $\mu\text{g}/100\text{ g}$ , trueness at 80-120% in spiked samples and certified reference materials, and a precision  $< 10\%$ . However, the precision in quantification of tetrahydrofolate was not within the acceptable limits due to the lack of use of the corresponding internal standard. An interconversion study of unstable formyl forms was performed which showed that 50% of 5,10-methenyltetrahydrofolate is converted to 5-formyltetrahydrofolate during the analysis. The developed LC-MS/MS method is a candidate for a future standard method for folate analysis in food.

## Keywords

Folates; Plant enzyme; LC-MS/MS; Extraction; Food; Interconversion

### 1. Introduction

Folate or vitamin B<sub>9</sub> is a generic term for a group of water-soluble compounds that are essential cofactors for humans since folate biosynthetic pathways in mammals are absent [1]. Folates play a key role in one carbon transfer reactions in all living organisms, being important for synthesis,

reparation, and methylation of DNA and consequently for cell division and production of red blood cells. They are involved in the prevalence of neural tube defects (NTDs) and in the methylation metabolisms [2,3]. There are strong evidences for a relationship between adequate folate status, reduced homocysteine concentration and reduced risk of coronary heart diseases [4,5]. The main food sources of folates are green vegetables, legumes, liver, and certain fruits such as tropical fruits and berries, whereas the folate content in commonly consumed fruits such as bananas and apples is insignificant [6]. The daily dietary intake from food among population is lower than the recommended 300 µg/day in Nordic countries [7] and 400 µg/day in the USA [8]. A comparison of standardized dietary folate intakes across different European populations showed that daily dietary folate intake is the lowest in Nordic countries, whereas it ranged from 201 µg/day in Swedish women to 304 µg/day in Danish men. In general, women population in Nordic countries which took part in this study had daily dietary folate intake <259 µg/day [9]. High prevalence of inadequate dietary folate intake resulted in introducing mandatory food fortification by folic acid, a synthetic form of vitamin B<sub>9</sub> that can act as cofactor after its reduction in the intestine. In 1998, the mandatory fortification of enriched cereal grain products with folic acid was implemented in the United States, Canada, and Costa Rica, followed by 53 other countries which decreased the prevalence of NTDs and vascular diseases [10]. The current strategy of the European Union (EU) is to set recommendations in order to reduce the prevalence of NTDs [11,12]. Therefore, women, who are planning to become pregnant, are advised to take supplements additionally to food rich in folates. Up to now, the EU did not introduce mandatory folic acid fortification, due to the connection between the chronic excessive intake of folic acid and some adverse health effects [13], among the others, a positive association between increased risk of breast cancer in postmenopausal women [14]. Since 44% of the pregnancies worldwide are unintended [15], these women could be less motivated to take folic acid supplementation, which results in a failure of prevention despite the

recommendation. As health benefits from an adequate natural folate intake are numerous for the whole population, and concerns about folic acid excessive intake do not apply to natural folates, it is important to increase the intake of naturally occurring folates from food.

Folates are labile compounds, very sensitive to different environmental conditions such as exposure to light, oxygen or temperature, which affects their stability during food processing [16–18]. The chemical structure of folates consists of a pteridine ring attached by a methylene bridge to *p*-aminobenzoic acid, which is coupled to one or more glutamyl residues. Natural folates are a mixture of reduced tetrahydrofolates (H<sub>4</sub>folate), substituted by methyl (CH<sub>3</sub>), formyl (HCO), methylene (CH<sub>2</sub>), methenyl (CH<sup>+</sup>) or formimino (CH=NH) groups [19]. Assuming that at least 3 states of reduction of the pteridine ring can occur, that the length of the polyglutamyl chain can include more than 6 residues, and that 6 different one-carbon substituents can be present on N<sub>5</sub> and N<sub>10</sub> position of the molecule (Fig. 1), there are more than 150 different folate forms in nature.

The most abundant natural folate form in food is 5-methyltetrahydrofolate (5-CH<sub>3</sub>-H<sub>4</sub>folate), followed by the formyl forms; 5-formyltetrahydrofolate (5-HCO-H<sub>4</sub>folate) and 10-formylfolic acid (10-HCO-PteGlu). Furthermore, folate forms are formed by interconversion and oxidation such as 5,10-methenyltetrahydrofolate (5,10-CH<sup>+</sup>=H<sub>4</sub>folate), 10-formyldihydrofolate (10-HCO-H<sub>2</sub>folate), and 10-formylfolic acid (10-HCO-PteGlu)[19]. Tetrahydrofolate (H<sub>4</sub>folate) is one of the most sensitive folate forms, but it occurs in minor concentrations in food. However, in liver H<sub>4</sub>folate provides the second largest folate contribution following 5-CH<sub>3</sub>-H<sub>4</sub>folate [20,21].

Folate analysis in foods have been challenging, due to the structural differences and multiplicity of folate forms, their low stability and low concentrations in food, but also because of the complexity of sample matrices. The analytical steps involve the liberation of folates from cellular matrix, removal of the polyglutamyl chain and formation of reduced mono- and diglutamate forms followed

by detection by the microbiological or chemical assays. Microbiological assay, which utilizes estimation of the growth of bacteria such as *Lactobacillus sp*, was the only method for folate analysis recommended by Association of Official Analytical Communities International [22]. Even though microbiological assay is a very sensitive method, it is very tedious, time-consuming, and the results depend a lot on the folate form used as a calibration compound [23]. Furthermore, it is not possible to distinguish between the folate forms present in food samples which is necessary due to the presumed different bioavailability of various folate forms [24,25]. Chemical assays have been introduced using chromatographic techniques combined with different detectors such as; ultra-violet (UV), diode array (PDA) and fluorescence [26–28], electrochemical [29,30], mass spectrometry or tandem mass spectrometry [31–34]. These methods are able to quantify each of the folate forms separately. Among the detection principle, mass spectrometric detection in combination with the use of isotopically labeled internal standards is the optimal choice in folate analysis due to sensitivity, selectivity and applicability to various folate forms.

For chemical assays, polyglutamate folates present in food samples are enzymatically hydrolyzed to monoglutamate forms by the use of  $\gamma$ -glutamyl-hydrolases (GGHs). The most commonly used GGHs are of animal origin such as rat plasma/serum, human plasma/serum and the extracts of hog kidney and chicken pancreas. Chicken pancreas is only able to remove polyglutamyl tail until diglutamate forms and it is, therefore, combined with some other deconjugases in LC-MS/MS analysis. Rat plasma/serum are the most commonly used, probably due to the commercial availability and very low amounts of endogenous folates [32,33,35–37]. In 1990, Martin et al. [38] introduced tri-enzyme treatment which included the use of  $\alpha$ -amylase and protease prior the GGH in order to release folates from food matrix and improve their extraction. Puwastien et al. [39] conducted an inter-laboratory study for three food materials (soybean flour, fish powder, and breakfast cereal) in 26 laboratories worldwide. Even though tri-enzyme extraction was

recommended for folate extraction, only 9 laboratories used that method, while 8 laboratories used only GGH. Also, among 17 laboratories where *L. casei* microbiological assay was performed, the inter-laboratory coefficient of variation between the test materials was 24%, 35% and 24% for soybean flour, fish powder and breakfast cereal, respectively. Due to the numerous possibilities of folate extraction, it has become a bottle-neck in folate analysis.

Recombinant plant GGHs have been produced in engineered *E. coli* [40,41], which enabled faster and more accurate folate analysis in papaya, tomato, banana and avocado fruit by the use of HPLC coupled to a four-channel electrochemical detector [42]. Progress in mass spectrometry resulted in worldwide use of various MS/MS detectors, which makes them suitable for development of a chromatographic standard method. Although both of these detectors are highly sensitive, the better selectivity, the unaltered capacity of detection with the time and easier handling with the system makes LC-MS/MS a system of choice for folate analysis. Recently, a LC-MS/MS method for quantification of PteGlu, 5-CH<sub>3</sub>-H<sub>4</sub>folate and 5-HCO-H<sub>4</sub>folate has been successfully tested in infant formula and adult nutritionals by the use of tri-enzyme extraction [43]. The aim of this study was to develop a rapid, sensitive and reproducible LC-MS/MS method for folate analysis in foodstuffs such as: dairy products, cereals, legumes, fruit, vegetables, offal and meat, using a GGH of plant origin (*Arabidopsis thaliana*).

## **2. Materials and methods**

### **2.1. Chemicals and reagents**

#### **2.1.1. Enzyme preparation**

Kanamycin and chloramphenicol (Tropigen, Montreal, QC, Canada),  $\beta$ -mercaptoethanol, imidazole, and LB medium (Sigma Aldrich, Missouri, USA), isopropyl-D-thiogalactopyranoside (IPTG)



(Promega, Wisconsin, USA), potassium phosphate, sodium chloride, and hydrogen chloride (Fisher Scientific, New Jersey, USA).

### 2.1.2. Folate extraction and quantification

Ammonium acetate, ascorbic acid (Asc), sodium dihydrogen phosphate, sodium hydroxide solution, and ortho-phosphoric acid, 85% (Merck, Darmstadt, Germany), glacial acetic acid, and sodium phosphate dibasic anhydrous (Fisher BioReagents, Geel, Belgium), ammonium hydroxide, 28-30 wt% solution in water, and ammonium formate (Acros Organics, Geel, Belgium),  $\beta$ -mercaptoethanol, and formic acid (Sigma-Aldrich, Steinheim, Germany), methanol (Honeywell Riedel-de Haën, Seelze, Germany), and acetonitrile (VWR International, Fontenay-sous-Bois, France). Ultra-pure water (18.2 M $\Omega$ cm) was obtained from Milli-Q water purification system (Millipore, Bradford, USA).

## 2.2. Buffer solutions

Acetate buffer I: 50 mM acetate buffer with 2% Asc and 0.1%  $\beta$ -mercaptoethanol, pH 7.0

Acetate buffer II: 50 mM acetate buffer with 1% Asc and 0.1%  $\beta$ -mercaptoethanol, pH 6.0

Phosphate buffer: 50 mM phosphate buffer with 1% Asc and 0.1%  $\beta$ -mercaptoethanol, pH 6.0

Elution buffer: Methanol with 1% Asc and 10% formic acid (v/v)

## 2.3. Folate standards and internal standards

The following monoglutamyl folate standards were used; (6S)-5,6,7,8-tetrahydrofolic acid (H<sub>4</sub>folate), (6R,S)-5,10-methenyl-5,6,7,8-tetrahydrofolic acid chloride (5,10-CH<sup>+</sup>=H<sub>4</sub>folate), 10-formylfolic acid (10-HCO-PteGlu), (6S)-5-formyl-5,6,7,8-tetrahydrofolic acid, calcium salt (5-HCO-H<sub>4</sub>folate), folic acid (PteGlu) and (6R,S)-5-methyl-5,6,7,8-tetrahydrofolic acid, calcium salt

(5-CH<sub>3</sub>-H<sub>4</sub>folate) from Schircks, Jona, Switzerland. Furthermore, polyglutamyl forms such as: pteroyltri- $\gamma$ -L-glutamic acid, pteroylpenta- $\gamma$ -L-glutamic acid and pteroylhepta- $\gamma$ -L-glutamic acid were also from Schircks, Jona, Switzerland. Internal standards ( [<sup>13</sup>C<sub>5</sub>]-labeled-5-HCO-H<sub>4</sub>folate, [<sup>13</sup>C<sub>5</sub>]-labeled PteGlu and [<sup>13</sup>C<sub>5</sub>]-labeled-5-CH<sub>3</sub>-H<sub>4</sub>folate) were from Merck Eprova, Schaffhausen, Switzerland. The folate standards were stored at -80°C until use.

Standard stock solutions (400  $\mu$ g/mL) were prepared by dissolving each folate standard in acetate buffer I. Each of the stock solutions was aliquoted into 1 mL Eppendorf tube in order to minimize freeze-thaw cycles, and stored at -80°C. The concentration for each of the standard stock solutions was corrected by the content of salt and moisture provided by supplier. Folate stock solutions were used for 6 months.

Working solutions were prepared on the day of analysis by serial dilution of stock solutions in acetate buffer II ranging from 1-1000 ng/mL. The concentrations of [<sup>13</sup>C<sub>5</sub>]-labeled internal standards in calibration curve were 20 ng/mL ([<sup>13</sup>C<sub>5</sub>]-labeled 5-CH<sub>3</sub>-H<sub>4</sub>folate) and 40 ng/mL ([<sup>13</sup>C<sub>5</sub>]-labeled PteGlu and [<sup>13</sup>C<sub>5</sub>]-labeled 5-HCO-H<sub>4</sub>folate). For establishment of a calibration curve each of the labeled forms were added to unlabeled standards.

#### **2.4. Food samples used in method validation**

To examine matrix effect during method validation, various in-house reference materials were used: oat flour, milk-based infant formula, freeze-dried broccoli, freeze-dried pig liver, fruit puree, green peas, and hard fat-cheese, which were all flushed with nitrogen and stored in either bags (polyester/aluminium/polyethylene) or plastic containers. Furthermore, white rice sample cooked in extensive amount of water and passed through three freeze-thaw cycles in order to remove endogenous folates was used for recovery tests, together with white cabbage samples.

Commercially available certified reference materials (CRM); BCR 487 Pig Liver (Sigma-Aldrich, Darmstadt, Germany), NIST 1546a Meat Homogenate, NIST 1549a Whole Milk Powder, NIST 1846 Infant Formula, and NIST 1849 Infant Formula, (NIST, USA) were used to check the trueness of the method.

## 2.5. Enzyme preparations

Expression constructs harboring a short version of Arabidopsis  $\gamma$ -glutamyl-hydrolase, AtGGH2 (At1g78680) [40] was kindly provided from Dr. Andrew Hanson (University of Florida). The procedure of enzyme preparation was described by Ramos-Parra et al. [44], but in this study slight modifications have been applied in terms of the use of high pressure homogenizer for lysis of the bacteria cells, which enabled higher yield in enzyme production.

In short, gene sequences with a 5' end nucleotide addition coding for a histidine tag were contained in a pET28b expression vector (Novagen, Wisconsin, USA). *Escherichia coli* BL21 (DE3) pLysS was transformed and selected in kanamycin-chloramphenicol plates (50 and 20  $\mu\text{g/mL}$ , respectively). One-liter LB medium cultures were grown at 27°C until they reached  $A_{600}$  of 0.5-0.6. The expression of the recombinant protein was induced by the addition of IPTG to a final concentration of 1 mM. Induced cultures were incubated until they reached an  $A_{600}$  of 1.6-1.8. Bacteria were pelleted by centrifugation (5.000xg, 5 min). The pellets were lysed in 10 mL of lysis buffer (50 mM potassium phosphate, pH 8.0, 1.5 M NaCl, 10 mM  $\beta$ -mercaptoethanol) [40] using a high pressure homogenizer (Lysis cell EmulsiFlex C-3, AVESTIN, Canada). The pressure of 5000 psi and 15000-20000 psi was used for 2 and 5 min, respectively. The obtained solution was centrifuged (16.000xg, 4°C, 10 min), and soluble protein was recovered from the supernatant. The recombinant protein was purified using Ni-NTA Agarose column (Qiagen, California, USA) following the manufacturer's protocol and as described by Ramos-Parra et al. [44]. Recovered

protein was desalted by means of a PD-10 column (GE Healthcare, Buckinghamshire, UK) using 100 mM potassium phosphate, pH 6.0, 10% glycerol, and 10 mM  $\beta$ -mercaptoethanol. In this elution buffer GGH was stored. Purified protein was quantified by Bradford method [45] and verified by SDS-page acrylamide gel. The obtained enzyme was stored at 4°C. The yield of this procedure ranged from 9-17 mg of purified protein for 1 L of *E.coli*, which was significantly higher than the 3-6 mg of purified protein for 1 L of *E.coli* using the former procedure [44].

## 2.6. Method optimization

### 2.6.1. Optimization of extraction conditions

We tested different antioxidants in standard solution of H<sub>4</sub>folate, as the least stabile folate form. H<sub>4</sub>folate and internal standard were added to phosphate buffer and aliquots were treated with: 1% Asc, mixture of 1% Asc and 0.1%  $\beta$ -mercaptoethanol, and a mixture of 1% Asc and 0.1% thiobarbituric acid.

### 2.6.2. Optimization of LC-MS/MS conditions

To ensure the best performance, chromatographic conditions such as; column type, column oven temperature, mobile phase composition, flow rate and injection volume were optimized. The addition of commonly used modifiers such as acetic acid (0.1% v/v), formic acid (0.1% v/v) and ammonium formate (2.5 mM) to aqueous (A) and organic eluent (B) was examined. Acetonitrile and methanol were tested as the organic eluent. Multiple reaction monitoring (MRM) transitions and source parameters were optimized for the six folate vitamers and three internal standards in order to obtain the highest selectivity and the lowest limit of quantification. The source parameters, such as; drying gas temperature (200-300°C), drying gas flow (4-12 mL/min), nebulizer gas (10-50 psi), capillary voltage (2500-5000 V), sheath gas temperature (200-400 °C) and sheath gas flow

(10-12 mL/min) optimized in the positive electrospray ionization mode and optimal MRM conditions (see Section 2.8.) were used in further analysis.

### 2.7. Sample preparation: homogenization, enzyme treatments and purification

Food samples were frozen in liquid nitrogen and immediately homogenized for 1 min in a coffee grinder (EGK 200, Rommelsbacher, Germany). 0.5–1 g of sample was weighted in a Pyrex glass tube. 10 mL of phosphate buffer and internal standards, 20 ng/mL of [ $^{13}\text{C}_5$ ]-labeled 5-CH<sub>3</sub>-H<sub>4</sub>folate and 40 ng/mL of [ $^{13}\text{C}_5$ ]-labeled PteGlu and [ $^{13}\text{C}_5$ ]-labeled 5-HCO-H<sub>4</sub>folate, were added. Atmospheric oxygen was replaced with nitrogen and samples were shaken (Multi Reax, Heidolph, Germany) for 15 min at room temperature. Samples were heated for 10 min in boiling water bath (100°C) in order to denature proteins and release natural folates from food matrix. After heat treatment, samples were immediately cooled on ice. 100 µg of  $\gamma$ -glutamyl-hydrolase enzyme was added per g of sample, and atmospheric oxygen was replaced by nitrogen, followed by 1 hour in a shaking waterbath (VWR International, Belgium) at 37°C. Thereafter, samples were heated in boiling water for 10 min to stop the enzyme activity, followed by cooling on ice for 10 min. 10 mL acetonitrile was added and mixed, and finally centrifuged for 15 min (4000xg, 4°C). Then cleanup on a strong anion exchange solid-phase extraction (SPE, SAX, quaternary amine, 55 µm, 70 Å, 500 mg/3mL, Phenomenex) was performed. Beforehand the SPE cartridge was conditioned by 6 mL methanol, and equilibrated with 6 mL of phosphate buffer. The sample was loaded, followed by washing with 6 mL phosphate buffer and 3 ml methanol. Then folates were eluted by 2 mL elution buffer, and an aliquote of the eluate, 250 µL, was dried under nitrogen at 30 °C and reconstituted in 250 µL of acetate buffer II. Finally, the samples were centrifuged at 10.000xg for 10 min at 4°C and the supernatants were transferred into LC-MS/MS glass vials, followed by LC-MS/MS analysis. The whole extraction procedure was performed under yellow light, using brown glass flasks or by wrapping the transparent glass with aluminum foil in order to protect folates from light.

## 2.8. Liquid chromatography and mass spectrometry conditions

The liquid chromatography analysis was done on an Agilent 1200 Series HPLC system including degasser, binary pump, autosampler and thermostated column compartment (Agilent Technologies, Santa Clara, CA) equipped with an Ascentis Express C18 (2.1 mm x 10 cm, 2.7  $\mu$ m) column fitted with Ascentis Express C18 (2.1 mm x 5 mm, 2.7  $\mu$ m) guard column from Supelco Analytical (Bellefonte, PA). The eluent A was 2.5 mM ammonium formate in water:methanol (95:5) and the eluent B was 2.5 mM ammonium formate in methanol. The gradient elution started with 5% eluent B, which linearly increased to 15% from 1 to 2.5 min, and to 35% from 2.5 to 5 min. The next 4 min were used to re-equilibrate the system to 5% eluent B. The flow rate was 0.4 mL/min, the column temperature 30°C, the autosampler temperature 5°C, and the injection volume 5  $\mu$ L. Prior the injection, the needle was washed in the flush port with 2-propanol for 10 s.

The LC-system was connected to an Agilent 6470 series Triple Quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA) equipped with electrospray ionization (ESI) source and jet stream. Quantification was done in MRM mode, which provided the best selectivity and sensitivity. Nitrogen was used as a collision gas. Source parameters were optimized for each of the analyzed folate form: drying gas temperature at 225°C, drying gas flow at 11 mL/min, nebulizer at 40 psi, capillary voltage at 3000 V, sheath gas temperature at 400 °C, sheath gas flow at 12 mL/min. Dwell time was optimized to 25 ms in order to obtain the acceptable chromatographic peak statistics of at least 20 data-points across one peak. Specific MS parameters for each folate and their MRM transitions and retention time are given in Table 1. Mass Hunter Workstation Software (version B.08.00, Agilent Technologies, Santa Clara, CA) was used for instrument control and data acquisition. Furthermore, comparison of the retention time and relative abundance of the quantifier and qualifier ions were used for identification of each of the analytes.

**Table 1** MRM transitions and corresponding parameters for analyzed folate forms

### 2.9. Determination of enzyme activity

The polyglutamylated forms of folic acid; pteroyltri- $\gamma$ -L-glutamic acid, pteroylpenta- $\gamma$ -L-glutamic acid and pteroylhepta- $\gamma$ -L-glutamic acid were used to determine the activity of AtGGH2. Three different set-ups were used to test the enzyme activity: a) Different amounts of enzyme were used in one polyglutamylated standard during the same incubation time; b) Different amounts of enzyme were used in the mixture of polyglutamylated standards during the same incubation time; c) The same amount of enzyme was used in one polyglutamylated standard added to green peas extract in different incubation times.

To perform the first two tests, 1  $\mu$ M concentration of pteroylpenta- $\gamma$ -L-glutamic acid standard and an equimolar mixture (0.34  $\mu$ M of each polyglutamylated standard) were prepared in 5 mL of phosphate buffer and divided into aliquots of 1 mL. To each aliquot internal standards were added. Thereafter, 10, 20 and 30  $\mu$ g of AtGGH2 enzyme per reaction was added and the samples were incubated at 37°C for 1 h. Reactions were stopped by boiling for 10 min, followed by the centrifugation. The supernatants were cleaned up by SPE (section 2.7.) and analyzed by LC-MS/MS (section 2.8.).

To perform the third set up, ten gram of green peas in-house reference material were frozen by liquid nitrogen, pulverized, added internal standard, and processed as previously described in 100 mL of phosphate buffer. After first cooling step, the sample was centrifuged and supernatant was divided in two aliquots; of which one was spiked with 26.5  $\mu$ g of PteGlu. Spiked and non-spiked extracts were divided into 4 mL aliquots before deconjugation. 100  $\mu$ g of AtGGH2 was added to the

samples, which were then incubated for 30 min, 1 h, 2 h, and 3 h at 37°C and processed as described in section 2.7 and 2.8.

Extent of deglutamylation was calculated by subtracting the endogenous folate amounts from the non-spiked samples that were fully deglutamylated with AtGGH2. Also, spiked and non-spiked aliquot were analyzed without addition of enzyme in order to check the enzyme activity.

## **2.10. Method validation**

To evaluate the method performance, the following criteria were used: limit of quantification, calibration curve, intra- and inter-day precision, trueness and matrix effect [46].

### 2.10.1. Limits of detection and quantification

The specificity of the method was determined by carrying out the whole procedure with blank samples containing phosphate extraction buffer. The acceptable limit of quantification (LOQ) was based on the nutritional relevant level, which was decided to be 0.5 µg of each folate form per 100 g of food.

### 2.10.2. Calibration curve

Calibration curve was based on a mixture of unlabeled standards at the following levels; 1, 5, 10, 50, 100, 500 and 1000 ng/mL, as these were the concentration levels of folate expected in food samples. It was extended for the test of enzymatic activity to 5000 ng/mL. Three independent injections of the standard mixture were performed at each level, and polynomial regression was determined between the analyte/internal standard area ratio and the analyte/internal standard concentration ratio. All calibration curves were of second order,  $1/x^2$  weighed and not forced through curve origin (0,0). Correlation coefficients ( $R^2$ ) determined for all folate forms were >0.99, and trueness of 85-115%, which confirmed a satisfactory calibration curve within examined



concentration range. The stability of the calibration curve in terms of unchanged slope was checked on the daily basis in order to ensure that standards did not degrade.

#### 2.10.3. Precision and trueness

Intra- and inter-day precision were expressed as relative standard deviation (RSD) obtained from three spiking levels analyzed in triplicate and on three different days. Rice free of folates was spiked with 10 ng, 500 ng and 900 ng standard mixtures of the six folates and analyzed (section 2.7.). Also, the analysis of endogenous folate was performed in triplicate and it was confirmed that the level was <LOQ. Spiking experiments were also performed in white cabbage samples, where endogenous folates were taken into account during the calculation of recovery.

Certified reference materials (BCR and NIST) representing various matrices were analyzed by the developed method to document trueness of the method. Certified reference materials were analyzed in duplicate on 2 different days (n=4), except BCR 487, which was analyzed on 1 day. Total folate content was expressed as folic acid equivalent.

#### 2.10.4. Matrix effect studies

To examine the matrix effect 400  $\mu$ L of a mixture of the six folate forms and the 3 internal standards (50 ng/mL) was mixed with 400  $\mu$ L of phosphate extraction buffer or with 400  $\mu$ L of different food extract solution after the sample purification. Food extract solutions were prepared from the house reference materials which represented different groups of food with various content of folate i.e. oat flour, milk-based infant formula, freeze-dried broccoli, freeze-dried pig liver, fruit puree, green peas, and hard fat-cheese.

#### 2.10.5. Interconversion study

To test the interconversion of three formyl folate forms, 500 ng/mL standard solutions of 5-HCO-H<sub>4</sub>folate, 5,10-CH<sup>+</sup>=H<sub>4</sub>folate and 10-HCO-PteGlu were prepared each in phosphate extraction buffer. Each standard was processed as a sample (described in section 2.7.) and all analyses were done in triplicate. The analyzed concentration of each folate compound was compared with expected concentration.

#### 2.10.6. Calculation of the total folate content

The total folate content was expressed as folic acid equivalent. The concentration of each folate form was multiplied by the ratio of molecular weights of PteGlu and the folate form of interest. Thus, the concentration of each folate form obtained by LC-MS/MS analysis was multiplied by 0.99, 0.97, 0.94, 0.93, 1.00, and 0.96 for H<sub>4</sub>folate, 5,10-CH<sup>+</sup>=H<sub>4</sub>folate, 10-HCO-PteGlu, 5-HCO-H<sub>4</sub>folate, PteGlu, and 5-CH<sub>3</sub>-H<sub>4</sub>folate, respectively. The sum of obtained values represented total folate content as PteGlu equivalent. When assessing trueness of the method, content of the individual folate forms was also given.

### 2.11. Statistical analyses

All folate analysis was conducted in three or more independent determinations and the means were compared using ANOVA and Tukey-Kramer tests ( $p \leq 0.05$ ). Statistical analyses were performed using JMP<sup>®</sup> Statistical Discovery software version 13.0 (SAS Institute Inc. Cary, NC, USA). The results of the total folate content were given as mean  $\pm$  standard deviation (SD).

## 3. Results and discussion

### 3.1. Optimization of folate extraction and sample cleanup

Folates' stability is highly affected by the pH value of the extraction buffer, the level of antioxidant and the combination of antioxidants. De Brouwer et al. [47] studied the stability during heat

treatment of all the folates included in this method. Without heating any stability issues were shown for pH around 6.0 in a phosphate buffer containing 1% ascorbic acid and 2%  $\beta$ -mercaptoethanol. The results from our optimization showed the highest stability when using a combination of ascorbic acid and  $\beta$ -mercaptoethanol. For the most labile folate, H<sub>4</sub>folate, the relative recovery was 100±16%. Therefore, the combination of those antioxidants was used in method (see SOM, Section S1 Figure S1). This combination of antioxidants was in accordance with former studies where enzymes of animal origin were used for deglutamylation [32,33,47]. It was also applied in analysis with plant AtGGH2 where 50 mM Na-Hepes/50 mM CHES buffer (pH 7.9, 1% Na-ascorbate, 10 mM  $\beta$ -mercaptoethanol) was used [44]. Since the maximum activity of AtGGH2 is at slightly acidic pH [40], Ramos-Parra et al. [44] adjusted the pH of extraction buffer from 7.9 to 6.0 during the deglutamylation step. We aimed to shorten the time of analysis by running the extraction in a phosphate buffer of pH 6.0; thereby the pH adjustment step was excluded. Furthermore, the lower pH has been shown to improve the stability of H<sub>4</sub>folate [48].

In order to ensure an optimal cleanup, SAX and weak anion exchange (WAX) SPE columns (Strata-X-AW, 33  $\mu$ m, 500 mg/6 mL) were tested, where the second washing fraction containing methanol and the two elution fractions containing 1% Asc and 10% formic acid in methanol were analyzed and compared. The use of SAX enabled elution of all vitamers in the first elution fraction, whereas various folate forms were distributed throughout the two elution fractions from the WAX column. Therefore, SPE similar to the one reported by Ringling and Rychlik [32] was used to purify the samples. A slight modification in elution step was made, as we used a methanol solution containing 1% Asc and 10% formic acid instead of 5% sodium chloride solution containing 1% Asc, 0.1 M sodium acetate, and 0.2 M  $\beta$ -mercaptoethanol. It should be mentioned that we did not test other cleanup procedures for plant matrices like 10kDa cut-off and 0.22 micron membrane filters [48,49].

### 3.2. Optimization of HPLC and MS/MS conditions

Due to the small differences in ionic character of various folates, it is difficult to separate all folate forms by chromatographic methods. Mass spectrometry detector gives a new dimension of  $m/z$  ratio which enables more accurate, precise and sensitive detection of naturally occurring folates. During the optimization of chromatographic conditions, various combinations of eluents and modifiers were tested. Even though acetonitrile is used as a common organic eluent in folate analysis [32,44,48], the sensitivity was significantly lower compared to the use of methanol. The use of acetonitrile resulted in baseline separation of the six different folate forms, but the time of analysis was 6 minutes longer and the instrument response was significantly lower (SOM, Section S2, Figure S2). By using methanol no baseline separation of the four folate compounds ( $H_4$ folate, 5,10- $CH^+=H_4$ folate, 10-HCO-PteGlu and 5-HCO- $H_4$ folate) was obtained. The forms co-eluted, though the retention times were slightly different. The retention time for each of the forms was similar to the internal standard,  $^{13}C_5$ -labeled 5-HCO- $H_4$ folate, which ensured compensation for possible matrix effect, interferences from co-eluted compounds in the system, and variability in detector response. Therefore, the binary gradient mixture of eluent A (2.5 mM ammonium formate in water:methanol (95:5) and eluent B (2.5 mM ammonium formate in methanol) in combination with C18 column were used (Section 2.8.). Figure 2 shows the optimized parameters for LC-MS/MS for a standard and a liver extract.

### 3.3. Enzyme activity

The activity of AtGGH2 in deconjugation of naturally occurring folates such as 5- $CH_3$ - $H_4$ folate tetraglutamate, 5-HCO- $H_4$ folate-triglutamate and PteGlu triglutamate has been shown to be  $100\pm 2\%$ ,  $100\pm 6\%$ , and  $100\pm 4\%$ , respectively [44]. We tested the activity of the enzyme using polyglutamylated PteGlu as a substrate, which was exposed to the activity of different amounts of enzyme and different incubation times (Table 2).

**Table 2** Enzyme activity treatments and release of monoglutamyl folic acid.

De glutamylation step during extraction of folates is a bottle-neck in folate analysis, which is mostly due to the lack of a standard deconjugase enzyme. Commercially available enzymes of animal origin are used to remove polyglutamyl tail in folate analysis, but it was reported that some of them can be inhibited by different food matrices [50]. The choice of enzyme depends widely on the analytical method used for determination of folates. Former studies confirmed the activity of animal origin hydrolases using polyglutamylated folic acid as a substrate. Rychlik et al. [51] investigated deconjugase activity which was 42% when using rat serum, and 96% when using rat serum combined with chicken pancreas. Various use of deconjugases such as: rat plasma [33] or human plasma [21,34], possibility of tri-enzyme treatment or analysis without any enzyme [52], resulted in discrepancies between total folate content results. Czarnowska-Kujawska et al. [53] compared rat plasma and porcine kidney deconjugases on the same samples (strawberries, blackcurrant and green peas), where the use of rat plasma resulted in significantly higher folate content.

The results in Table 2 for 100 µg GGH per g of sample show a satisfactory enzyme activity of >95% already after half an hour in the solution containing green peas extract. This is in agreement with results from Ramos-Parra [44] where the black beans extract was de glutamylated after half an hour using 100 µg of enzyme per g of sample. However, we included a safety margin to ensure that all polyglutamates are transferred to monoglutamate forms, and therefore extended the incubation to 1 h. The fact that monoglutamylated levels of folic acid did not increase after increasing the duration of the enzyme treatment ensured that the total de glutamylation was achieved. Tri-enzyme extraction has been preferred, but it is questionable if it is needed, since former studies showed no difference in folate content between the use of single deconjugation enzyme, and tri-enzyme extraction [46].

### 3.4. Method validation

#### 3.4.1. Limits of detection and quantification

Specificity of the method has been checked by analyzing blank sample. No carryover has been noticed in these runs. For each of the six folate compounds, LOQ was defined as the minimum detectable values with signal-to-noise ratio 10, while limit of detection was regarded as not relevant in this method on quantifying a vitamin. Moreover, as the recommended dietary daily intake is 300  $\mu\text{g}$ , this LOQ provides the possibility to quantify folate forms in foods where 100 g of food contributes to 1% of the recommended daily intake, which would be 3  $\mu\text{g}$  of food folate/100 g of food. By this method, we quantify 6 different folate forms, and therefore, the aimed LOQ for them is 0.5  $\mu\text{g}/100$  g of food.. The lowest S/N for the folates was recorded for the 5,10- $\text{CH}^+=\text{H}_4$ folate and it was approximately 250 for the lowest calibration level. The lowest level of the calibration curve 0.1  $\mu\text{g}/100$  g met the goal set for LOQ of 0.5  $\mu\text{g}/100$  g, and is defined as the LOQ for all folate forms. The quality assurance of the method included injection of a blank sample containing acetate buffer within the sample sequence and no carryover was seen in any of these injections.

#### 3.4.2. Precision and trueness

Results presented in Table 3 show that relative recoveries for PteGlu and 5- $\text{CH}_3$ - $\text{H}_4$ folate are 100-103% and the precision <7% RSD. These folate forms are normally stable and will not interconvert. The recovery of  $\text{H}_4$ folate varied from day to day analysis, which is expected due to its instability and the fact that we did not use an  $\text{H}_4$ folate corresponding internal standard. Concentration of stock solution was corrected for the purity of solid reference substance, but it wasn't quantified spectrophotometrically, which could result in overestimation of the  $\text{H}_4$ folate content and lower precision, due to  $\text{H}_4$ folate instability. In our study, both intra- and inter-day precision varied, and ranged from 7-34% RSD which did not meet the goal set in validation parameters (<10% RSD).

Therefore, these results show that a corresponding internal standard for quantification of H<sub>4</sub>folate should be used in the future. Since this recovery study was done by using a mixture of folate standards, it was not possible to obtain relative recovery around 100% when the formyl forms were quantified separately. As they interconvert, and presumably have the same bioavailability, we decided to quantify them together, which enabled satisfactory recovery of 80%. The recovery of the interconvertible compounds was similar to the recovery of the other folate forms, but showed a higher variation at 11-14%. However, the abundance of the interconvertible compounds in nature is low, the same as their contribution to the folate intake and we evaluate that the lower precision does not have a significant effect on the total folate content.

**Table 3** Precision and recovery of spiked samples

If available, certified reference materials should always be used to check the trueness of a new method. In the method validation, five certified reference materials were analyzed. Table 4 gives the obtained results, including certified values and comparison with results from other studies.

**Table 4** The total folate content ( $\mu\text{g}/100\text{g}$ ) quantified in five certified reference materials (n=2-4). The contents are expressed as total folate content of each of the individual folate forms, and compared to the certified values and values previously published. [54,55]

Values obtained in this study are in agreement with the certified values or results from other studies. However, all CRM analyzed by microbiological assay gave slightly higher results. For pig liver (BCR 487) the three reported results are similar regarding that 5-CH<sub>3</sub>-H<sub>4</sub>folate and H<sub>4</sub>folate are the most abundant compound, besides this minor differences in folate profile appear [20,21]. Vishnumohan et al. [21] performed tri-enzyme treatment which lasted for 20 h when protease and  $\alpha$ -amylase enzymes were applied, and 3 h treatment when human plasma as deconjugase enzyme was used. Furthermore, the pH was adjusted to 4.5, 6.1 and finally to 7.2 for each enzyme treatment, respectively. In our method the deconjugase step is performed at pH 6.0 in 1 h, which resulted in a shorter time of analysis, which could be the reason for the higher content of H<sub>4</sub>folate.

### 3.4.3. Matrix effect

The use of internal standards is necessary for an accurate and reproducible quantification in folate analysis by LC-MS/MS. Internal standards compensate for the losses of analytes during the sample preparations, but also for matrix effects or ion suppression during LC-MS/MS. Freisleben et al. [56] introduced the use of a stable isotope dilution assays in LC-MS/MS analysis which enabled an accurate and sensitive quantification of folates. However, due to quantification of several folate forms and the high costliness of isotopically labeled internal standards; we found it impossible to have corresponding internal standards for each folate form. It is crucial that internal standards possess structural similarity with the analyte of interest and that it undergoes the same changes during the analysis and has similar retention time. Therefore, based on structural similarity and similar chemical behavior, [ $^{13}\text{C}_5$ ]-5-HCO-H<sub>4</sub>folate was used for quantification of H<sub>4</sub>folate, 5,10-CH<sup>+</sup>=H<sub>4</sub>folate, 10-HCO-PteGlu and 5-HCO-H<sub>4</sub>folate, as they eluted at similar retention times; [ $^{13}\text{C}_5$ ]-5-CH<sub>3</sub>-H<sub>4</sub>folate was used for quantification of 5-CH<sub>3</sub>-H<sub>4</sub>folate, and [ $^{13}\text{C}_5$ ]-PteGlu was used for quantification of PteGlu.

Matrix effect (ME) was investigated based on the method of Matuszewski et al. [57] and calculated as follows:  $\%ME = A/B \times 100$ , where A: the area obtained in the matrix; B: the area obtained in phosphate extraction buffer. The results presented in Fig.3 show that the matrix effect was not pronounced for the examined folate forms except on H<sub>4</sub>folate ( $\%ME \sim 40$ ) where ion suppression occurred, and on 5,10-CH<sup>+</sup>=H<sub>4</sub>folate ( $\%ME \sim 140$ ) where ion enhancement occurred. Furthermore, ME of analytes, which were quantified using the corresponding internal standard, were very similar as expected.

### 3.4.4. Interconversion study



In order to examine the behavior of formyl forms in this method, stock solutions (500 ng/mL of 5,10-CH<sup>+</sup>=H<sub>4</sub>folate, 10-HCO-PteGlu and 5-HCO-H<sub>4</sub>folate in extraction buffer) were analyzed individually using our developed method. The final distribution of these formyl forms was studied. The results in Fig. 4 showed that 10-HCO-PteGlu and 5-HCO-H<sub>4</sub>folate did not convert in any significant extent, whereas approximately 50% of 5,10-CH<sup>+</sup>-H<sub>4</sub>folate were recovered in original form, while the other 50% were converted into 5-HCO-H<sub>4</sub>folate during extraction. In spiking experiments (all three forms added together), the recovery of 5-HCO-H<sub>4</sub>folate, for which the corresponding <sup>13</sup>C<sub>5</sub>-labeled internal standard was used, was 138%, which is explained by the interconversion of 5,10-CH<sup>+</sup>=H<sub>4</sub>folate to 5-HCO-H<sub>4</sub>folate during the sample cleanup. When 500 ng/mL standard solution of 5-HCO-H<sub>4</sub>folate was processed as sample during the interconversion study where the interconversion of 5,10-CH<sup>+</sup>=H<sub>4</sub>folate, 10-HCO-PteGlu and 5-HCO-H<sub>4</sub>folate was studied in separated solutions, the recovery was 90%.

Change in the pH in connection with changes in temperature and the presence of antioxidants results in conversion of reduced formyl groups such as 5-HCO-H<sub>4</sub>folate to 10-HCO-PteGlu and 5,10-CH<sup>+</sup>=H<sub>4</sub>folate as intermediate product [47]. Baggott [58] investigated the interconversion of 5,10-CH<sup>+</sup>=H<sub>4</sub>folate between pH 2.5 and 4.5. The concentrations of 5,10-CH<sup>+</sup>=H<sub>4</sub>folate and 5-HCO-H<sub>4</sub>folate were equal at pH 4.0-4.5 in the presence of antioxidants such as ascorbate, which is in accordance to our results. Jastrebova et al. [59] tested the stability of 5-HCO-H<sub>4</sub>folate during heat treatment in different buffers with different antioxidant systems and they recorded that stability of 5-HCO-H<sub>4</sub>folate was strongly pH-dependent, with maximum at pH above 4.8. They showed that the main interconversion product at acidic pH was 5,10-CH<sup>+</sup>=H<sub>4</sub>folate. Furthermore, 5,10-CH<sup>+</sup>=H<sub>4</sub>folate was mainly converted to 5-HCO-H<sub>4</sub>folate in the pH range between 2 and neutral, which was similar to our study. Both stock and working solutions should be prepared separately in order to prevent interconversion. In nature, the most abundant form is 5-CH<sub>3</sub>-H<sub>4</sub>folate followed in

many cases by 5-HCO-H<sub>4</sub>folate, therefore 5-HCO-H<sub>4</sub>folate was included in our method. Chromatographic analysis of formyl forms of folates is very challenging due to the variations in their stability under different pH conditions. To overcome this problem, precaution should be taken against pH conditions used during the analysis. Isotopically labeled internal standards help to overcome problems related to the interconversion. It also need to be stated that data on individual folate forms will reflect the conditions during analysis rather than the real proportion of folate forms in original food sample. We used and propose to express the amount of 5,10-CH<sup>+</sup>=H<sub>4</sub>folate, 10-HCO-PteGlu, and 5-HCO-H<sub>4</sub>folate as their sum calculated as folic acid equivalent.

#### 4. Conclusions

A sensitive, specific, accurate and reproducible method for determination of six folate forms was optimized and single-laboratory validated for the analysis in foodstuffs. The use of a pure plant recombinant AtGGH2 enables an effective folate deglutamylation by using only 100 µg/g of sample. The single and short deconjugation step reduced significantly the duration of folate analysis compared with an over-night incubation using deconjugases of animal origin. Ramos-Parra et al. [44] set a very good starting point using a pure recombinant plant enzyme for deconjugation. Our developed LC-MS/MS method with the application of a stable isotope dilution assay enables fast folate determination and correction of losses during sample purification, which provides more accurate and reproducible results. However, the corresponding internal standard should be used for quantification of unstable H<sub>4</sub>folate in future analysis.

Interconversion study between 10-HCO-PteGlu, 5,10-CH<sup>+</sup>=H<sub>4</sub>folate, and 5-HCO-H<sub>4</sub>folate showed that their content obtained by this method should be expressed as a combination of all of them. This validated method could make the base for establishment of a rapid standardized method for more accurate quantification of folates in food.

**Acknowledgments**

The authors thank to Dr. Andrew Hanson at the University of Florida, for generously sharing the expression AtGGH vectors.

This research was funded by the Technical University of Denmark and by Danish Veterinary and Food Administration.

**Declaration of interest**

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be considered as a potential conflict of interest.

ACCEPTED MANUSCRIPT

**References**

- [1] R.K. Saini, S.H. Nile, Y.S. Keum, Folates: Chemistry, analysis, occurrence, biofortification and bioavailability, *Food Res. Int.* 89 (2016) 1–13. doi:10.1016/j.foodres.2016.07.013.
- [2] A. Imbard, J.-F. Benoist, H.J. Blom, Neural tube defects, folic acid and methylation, *Int. J. Environ. Res. Public Health.* 10 (2013) 4352–4389. doi:10.3390/ijerph10094352.
- [3] MRC Vitamin study research group, Prevention of neural tube defects: Results of the medical research council vitamin study, *Lancet.* 338 (1991) 131–137. doi:10.1016/0140-6736(91)90133-A.
- [4] S. Voutilainen, T. Rissanen, J. Virtanen, T.A. Lakka, T. Salonen, Jukka, Low dietary folate intake is associated with an excess incidence of acute coronary events: The Kuopio ischemic heart disease risk factor study, *Circulation.* (2001) 2674–2681. doi:10.1161/01.CIR.103.22.2674.
- [5] H.J. Blom, Y. Smulders, Overview of homocysteine and folate metabolism. With special references to cardiovascular disease and neural tube defects, *J. Inherit. Metab. Dis.* 34 (2011) 75–81. doi:10.1007/s10545-010-9177-4.
- [6] N. Delchier, A.L. Herbig, M. Rychlik, C.M.G.C. Renard, Folates in Fruits and Vegetables: Contents, Processing, and Stability, *Compr. Rev. Food Sci. Food Saf.* 15 (2016) 506–528. doi:10.1111/1541-4337.12193.
- [7] Nordic Nutrition Recommendations, *Nord. Nutr. Recomm.* 2012. Nord. Counc. Minist. (2014). doi:10.6027/Nord2014-002.
- [8] Institute of Medicine, Dietary Reference Intakes for Thiamin, Riboflavin, Niacin, Vitamin

B6, Folate, Vitamin B12, Pantothenic Acid, Biotin, and Choline, The National Academies Press, Washington, DC, 1998. doi:10.17226/6015.

- [9] J.Y. Park, G. Nicolas, H. Freisling, C. Biessy, A. Scalbert, I. Romieu, V. Chajès, S.C. Chuang, U. Ericson, P. Wallström, M.M. Ros, P.H.M. Peeters, A. Mattiello, D. Palli, J.M. Huerta, P. Amiano, J. Halkjær, C.C. Dahm, A. Trichopoulou, P. Orfanos, B. Teucher, S. Feller, G. Skeie, D. Engeset, M.C. Boutron-Ruault, F. Clavel-Chapelon, F. Crowe, K.T. Khaw, P. Vineis, N. Slimani, Comparison of standardised dietary folate intake across ten countries participating in the European Prospective Investigation into Cancer and Nutrition, *Br. J. Nutr.* 108 (2012) 552–569. doi:10.1017/S0007114511005733.
- [10] K.S. Crider, L.B. Bailey, R.J. Berry, Folic acid food fortification—Its history, effect, concerns, and future directions, *Nutrients*. 3 (2011) 370–384. doi:10.3390/nu3030370.
- [11] European Union, Regulation (EC) No 1925/2006 of the European Parliament and of the Council of 20 December 2006 on the addition of vitamins and minerals and of certain other substances to foods, *Off. J. Eur. Union*. L404 (2006) 26–38.
- [12] European Union, Directive 2002/46/EC of the European Parliament and of the Council of 10 June 2002 on the approximation of the laws of the Member States relating to food supplements, *Off. J. Eur. Communities*. 183 (2002) 51–57.  
doi:10.1016/j.jclepro.2010.02.014.
- [13] J. Selhub, I.H. Rosenberg, Excessive folic acid intake and relation to adverse health outcome, *Biochimie*. 126 (2016) 71–78. doi:10.1016/j.biochi.2016.04.010.
- [14] R.Z. Stolzenberg-Solomon, S.-C. Chang, M.F. Leitzmann, K.A. Johnson, C. Johnson, S.S. Buys, R.N. Hoover, R.G. Ziegler, Folate intake, alcohol use, and postmenopausal breast

cancer risk in the prostate, lung, colorectal, and ovarian cancer screening trial, *Am. J. Clin. Nutr.* 83 (2006) 895–904. doi:10.1093/ajcn/83.4.895.

- [15] J. Bearak, A. Popinchalk, L. Alkema, G. Sedgh, Global, regional, and subregional trends in unintended pregnancy and its outcomes from 1990 to 2014: estimates from a Bayesian hierarchical model, *Lancet Glob. Heal.* 6 (2018) e380–e389. doi:10.1016/S2214-109X(18)30029-9.
- [16] N. Delchier, M. Reich, C.M.G.C. Renard, Impact of cooking methods on folates, ascorbic acid and lutein in green beans (*Phaseolus vulgaris*) and spinach (*Spinacea oleracea*), *LWT - Food Sci. Technol.* 49 (2012) 197–201. doi:10.1016/j.lwt.2012.06.017.
- [17] N. Delchier, C. Ringling, J. Le Grandois, D. Aoudé-Werner, R. Galland, S. Georgé, M. Rychlik, C.M.G.C. Renard, Effects of industrial processing on folate content in green vegetables, *Food Chem.* 139 (2013) 815–824. doi:10.1016/j.foodchem.2013.01.067.
- [18] N. Delchier, C. Ringling, M.E. Cuvelier, F. Courtois, M. Rychlik, C.M.G.C. Renard, Thermal degradation of folates under varying oxygen conditions, *Food Chem.* 165 (2014) 85–91. doi:10.1016/j.foodchem.2014.05.076.
- [19] M. Jägerstad, J. Jastrebova, Occurrence, stability, and determination of formyl folates in foods, *J. Agric. Food Chem.* 61 (2013) 9758–9768. doi:10.1021/jf4028427.
- [20] P.M. Finglas, K. Wigertz, L. Vahteristo, C. Witthoft, S. Southon, I. De Froidmont-Gortz, Standardisation of HPLC techniques for the determination of naturally-occurring folates in food, *Food Chem.* 64 (1999) 245–255. doi:10.1016/S0308-8146(98)00171-X.
- [21] S. Vishnumohan, J. Arcot, R. Pickford, Naturally-occurring folates in foods: Method development and analysis using liquid chromatography–tandem mass spectrometry (LC–

- MS/MS), *Food Chem.* 125 (2011) 736–742. doi:10.1016/j.foodchem.2010.08.032.
- [22] J.W. DeVries, J.I. Rader, P.M. Keagy, C.A. Hudson, G. Angyal, J. Arcot, M. Castelli, N. Doreanu, C. Hudson, P. Lawrence, J. Martin, R. Peace, L. Rosner, H.S. Strandler, J. Szpylka, H. van den Berg, C. Wo, C. Wurz, Microbiological assay tri-enzyme procedure for total folates in cereals and cereal foods: Collaborative study, *J. AOAC Int.* 88 (2005) 5–15.
- [23] C. Ringling, M. Rychlik, Origins of the difference between food folate analysis results obtained by LC – MS/MS and microbiological assays, *Anal. Bioanal. Chem.* 409 (2017) 1815–1825. doi:10.1007/s00216-016-0126-4.
- [24] H. McNulty, K. Pentieva, Folate bioavailability, *Proc. Nutr. Soc.* 63 (2004) 529–536. doi:10.1079/PNS2004383.
- [25] V.E. Ohrvik, C.M. Witthoft, Human folate bioavailability., *Nutrients.* 3 (2011) 475–490. doi:10.3390/nu3040475.
- [26] E. Ginting, J. Arcot, High-performance liquid chromatographic determination of naturally occurring folates during tempe preparation, *J. Agric. Food Chem.* 52 (2004) 7752–7758. doi:10.1021/jf040198x.
- [27] S. Ndaw, M. Bergaentzle, D. Aoude-Werner, S. Lahely, C. Hasselmann, Determination of folates in foods by high-performance liquid chromatography with fluorescence detection after precolumn conversion to 5-methyltetrahydrofolates, *J. Chromatogr. A.* 928 (2001) 77–90. doi:10.1016/S0021-9673(01)01129-3.
- [28] M. Johansson, J. Jastrebova, A. Grahn, M. Jagerstad, Separation of dietary folates by gradient reversed-phase HPLC : Comparison of alternative and conventional silica-based stationary phases, *Chromatographia.* 62 (2005) 33–40. doi:10.1365/s10337-005-0571-2.

- [29] P.J. Bagley, J. Selhub, Analysis of folate form distribution by affinity followed by reversed-phase chromatography with electrochemical detection, *Clin. Chem.* 46 (2000) 404–411. doi:10.1.1.318.7898.
- [30] P.A. Ramos-Parra, C. Garcia-Salinas, C. Hernandez-Brenes, R. Díaz de la Garza, Folate levels and polyglutamylolation profiles of Papaya (*Carica papaya* cv. Maradol) during fruit development and ripening, *J. Agric. Food Chem.* 61 (2013) 3949–3956. doi:10.1021/jf305364x.
- [31] R.J. Pawlosky, V.P. Flanagan, R.F. Doherty, A mass spectrometric validated high-performance liquid chromatography procedure for the determination of folates in foods, *J. Agric. Food Chem.* 51 (2003) 3726–3730. doi:10.1021/jf034017n.
- [32] C. Ringling, M. Rychlik, Analysis of seven folates in food by LC-MS/MS to improve accuracy of total folate data, *Eur. Food Res. Technol.* 236 (2013) 17–28. doi:10.1007/s00217-012-1849-x.
- [33] K. Tyagi, P. Upadhyaya, S. Sarma, V. Tamboli, Y. Sreelakshmi, R. Sharma, High performance liquid chromatography coupled to mass spectrometry for profiling and quantitative analysis of folate monoglutamates in tomato., *Food Chem.* 179 (2015) 76–84. doi:10.1016/j.foodchem.2015.01.110.
- [34] S. Vishnumohan, R. Pickford, J. Arcot, Naturally occurring folates in selected traditionally prepared foods in Southern India, *J. Food Sci. Technol.* 54 (2017) 4173–4180. doi:10.1007/s13197-017-2870-7.
- [35] T. Tamura, Determination of food folate, *J. Nutr. Biochem.* 9 (1998) 285–293. doi:10.1016/S0955-2863(98)00013-8.



- [36] P. Upadhyaya, K. Tyagi, S. Sarma, V. Tamboli, Y. Sreelakshmi, R. Sharma, Natural variation in folate levels among tomato (*Solanum lycopersicum*) accessions, *Food Chem.* 217 (2017) 610–619. doi:10.1016/j.foodchem.2016.09.031.
- [37] S. Luo, H. Duan, Y. Zou, R. Qiu, C. Wang, Quantification of total folate, folate species and polyglutamyl folate distribution in winged beans (*Psophocarus tetragonolobus* (L) DC) from different cultivars and growth stages by ultra-high performance liquid chromatography tandem mass spectrometry, *J. Nutr. Sci. Vitaminol. (Tokyo)*. 63 (2017) 69–80. doi:10.3177/jnsv.63.69.
- [38] J. I Martin, W. O Landen, A.G.M. Soliman, R. R Eitenmiller, Application of a Tri-enzyme Extraction for Total Folate Determination in Foods, 1990.
- [39] P. Puwastien, N. Pinprapai, K. Judprasong, T. Tamura, International inter-laboratory analyses of food folate, *J. Food Compos. Anal.* 18 (2005) 387–397. doi:10.1016/j.jfca.2004.02.011.
- [40] G. Orsomando, R. Díaz de la Garza, B.J. Green, M. Peng, P.A. Rea, T.J. Ryan, J.F. Gregory III, A.D. Hanson, Plant  $\gamma$ -glutamyl hydrolases and folate polyglutamates: Characterization, compartmentation, and co-occurrence in vacuoles, *J. Biol. Chem.* 280 (2005) 28877–28884. doi:10.1074/jbc.M504306200.
- [41] T.A. Akhtar, R.P. McQuinn, V. Naponelli, J.F. Gregory III, J.J. Giovannoni, A.D. Hanson, Tomato gamma-glutamylhydrolases: Expression, characterization, and evidence for heterodimer formation, *Plant Physiol.* 148 (2008) 775–785. doi:10.1104/pp.108.124479.
- [42] C. García-Salinas, P.A. Ramos-Parra, R.I. Díaz de la Garza, Ethylene treatment induces changes in folate profiles in climacteric fruit during postharvest ripening, *Postharvest Biol. Technol.* 118 (2016) 43–50. doi:10.1016/j.postharvbio.2016.03.011.

- [43] S.D. Bhandari, G. Ming, J. Szpylka, Total folate in infant formula and adult nutritionals by trienzyme extraction and LC-MS/MS quantitation: A multilaboratory testing study, *Final Action 2011.06*, *J. AOAC Int.* 101 (2018) 1881–1894. doi:10.5740/jaoacint.18-0114.
- [44] P. A. Ramos-Parra, R. Urrea-López, R.I. Díaz de la Garza, Folate analysis in complex food matrices: Use of a recombinant *Arabidopsis*  $\gamma$ -glutamyl hydrolase for folate deglutamylation, *Food Res. Int.* 54 (2013) 177–185. doi:10.1016/j.foodres.2013.06.026.
- [45] M.M. Bradford, A Rapid and Sensitive Method for the Quantitation Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding, 254 (1976) 248–254.
- [46] NMKL PROCEDURE No. 4 (2009) Validation of chemical analytical methods, 2009. [http://www.nmkl.org/dokumenter/prosedyrer/en/PROC4\\_en\\_may09.pdf](http://www.nmkl.org/dokumenter/prosedyrer/en/PROC4_en_may09.pdf).
- [47] V. de Brouwer, G. Zhang, S. Storozhenko, D. van der Straeten, W.E. Lambert, pH stability of individual folates during critical sample preparation steps in prevision of the analysis of plant folates, *Phytochem. Anal.* 18 (2007) 496–508. doi:10.1002/pca.1006.
- [48] G.-F. Zhang, S. Storozhenko, D. Van Der Straeten, W.E. Lambert, Investigation of the extraction behavior of the main monoglutamate folates from spinach by liquid chromatography–electrospray ionization tandem mass spectrometry, *J. Chromatogr. A.* 1078 (2005) 59–66. doi:10.1016/j.chroma.2005.04.085.
- [49] M.J.I. Shohag, Q. Yang, Y. Wei, J. Zhang, F.Z. Khan, M. Rychlik, Z. He, X. Yang, A rapid method for sensitive profiling of folates from plant leaf by ultra-performance liquid chromatography coupled to tandem quadrupole mass spectrometer, *J. Chromatogr. B.* 1040 (2017) 169–179. doi:10.1016/j.jchromb.2016.11.033.
- [50] M.-M. Wei, J.F. Gregory, Organic acids in selected foods inhibit intestinal brush border

pteroylpolyglutamate hydrolase in vitro: potential mechanism affecting the bioavailability of dietary polyglutamyl folate, *J. Agric. Food Chem.* 46 (1998). doi:10.1021/jf970662g.

- [51] M. Rychlik, K. Englert, S. Kapfer, E. Kirchoff, Folate contents of legumes determined by optimized enzyme treatment and stable isotope dilution assays, *J. Food Compos. Anal.* 20 (2007) 411–419. doi:10.1016/j.jfca.2006.10.006.
- [52] M. V Chandra-Hioe, M.P. Bucknall, J. Arcot, Evaluating folate extraction from infant milk formulae and adult nutritionals: Enzymatic digestion versus enzyme-free heat treatment, *Food Chem.* 234 (2017) 365–371. doi:10.1016/j.foodchem.2017.04.179.
- [53] M. Czarnowska-Kujawska, G. Elzbieta, J. Michalak, Testing of different extraction procedures for folate HPLC determination in fresh fruits and vegetables, *J. Food Compos. Anal.* 57 (2017) 64–72. doi:10.1016/j.jfca.2016.12.019.
- [54] J.E. Camara, M.S. Lowenthal, K.W. Phinney, Determination of fortified and endogenous folates in food-based Standard Reference Materials by liquid chromatography-tandem mass spectrometry., *Anal. Bioanal. Chem.* 405 (2013) 4561–8. doi:10.1007/s00216-013-6733-4.
- [55] L. Chen, R.R. Eitenmiller, Evaluation for the analysis of total food folate by trienzyme extraction and microplate assay, *J. Food Sci.* 72 (2007) 243–247. doi:10.1111/j.1750-3841.2007.00370.x.
- [56] A. Freisleben, P. Schieberle, M. Rychlik, Specific and sensitive quantification of folate vitamers in foods by stable isotope dilution assays using high-performance liquid chromatography-tandem mass spectrometry., *Anal. Bioanal. Chem.* 376 (2003) 149–56. doi:10.1007/s00216-003-1844-y.
- [57] B.K. Matuszewski, M.L. Constanzer, C.M. Chavez-Eng, Strategies for the Assessment of

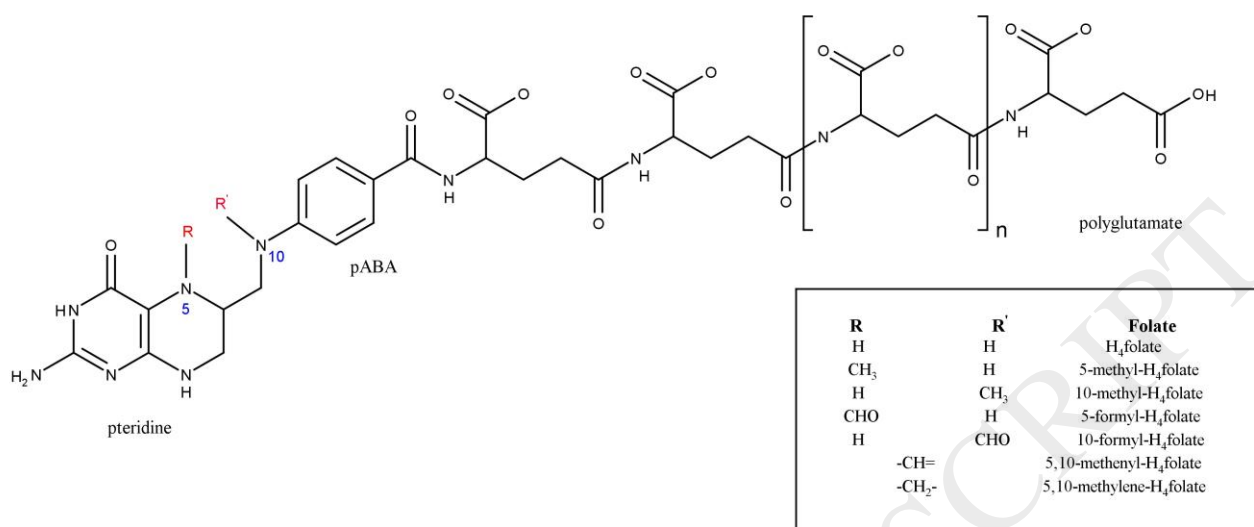
Matrix Effect in Quantitative Bioanalytical Methods Based on HPLC–MS/MS, *Anal. Chem.* 75 (2003) 3019–3030. doi:10.1021/ac020361s.

[58] J.E. Baggott, Hydrolysis of 5,10-Methenyltetrahydrofolate to 5-Formyltetrahydrofolate at pH 2.5 to 4.5, *Biochemistry.* 39 (2000) 14647–14653. doi:10.1021/bi001362m.

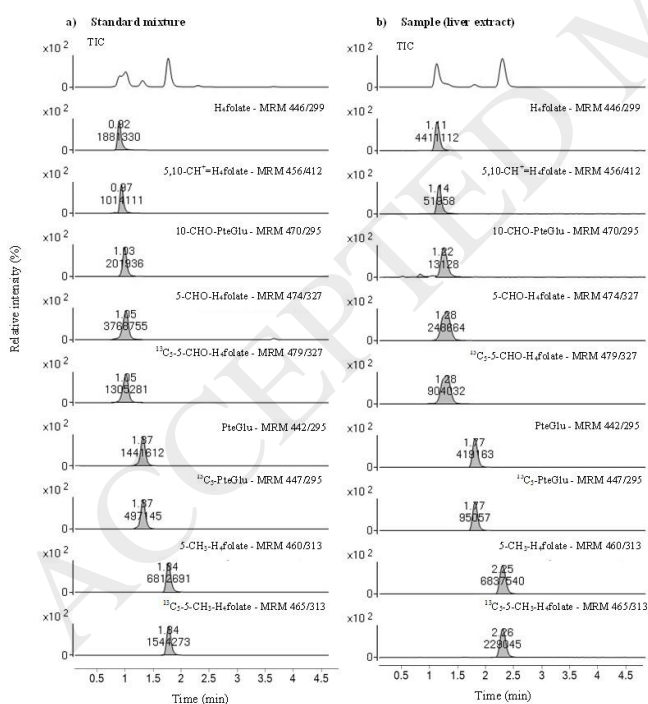
[59] J. Jastrebova, M. Axelsson, H.S. Strandler, M. Jagerstad, Stability of dietary 5-formyl-tetrahydrofolate and its determination by HPLC: a pilot study on impact of pH, temperature and antioxidants on analytical results, *Eur. Food Res. Technol.* 237 (2013) 747–754. doi:10.1007/s00217-013-2046-2.

ACCEPTED MANUSCRIPT

## Figure captions:

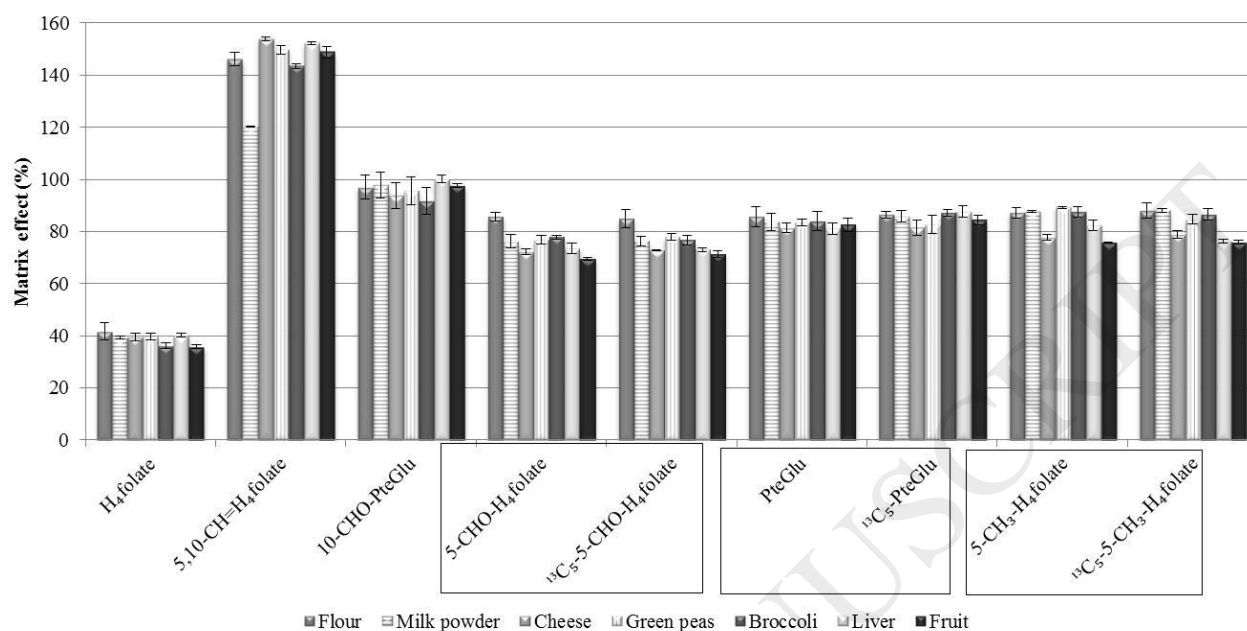


**Figure 1** Chemical structure of polyglutamylated folates. pABA: p-aminobenzoate; H<sub>4</sub>folate: tetrahydrofolate

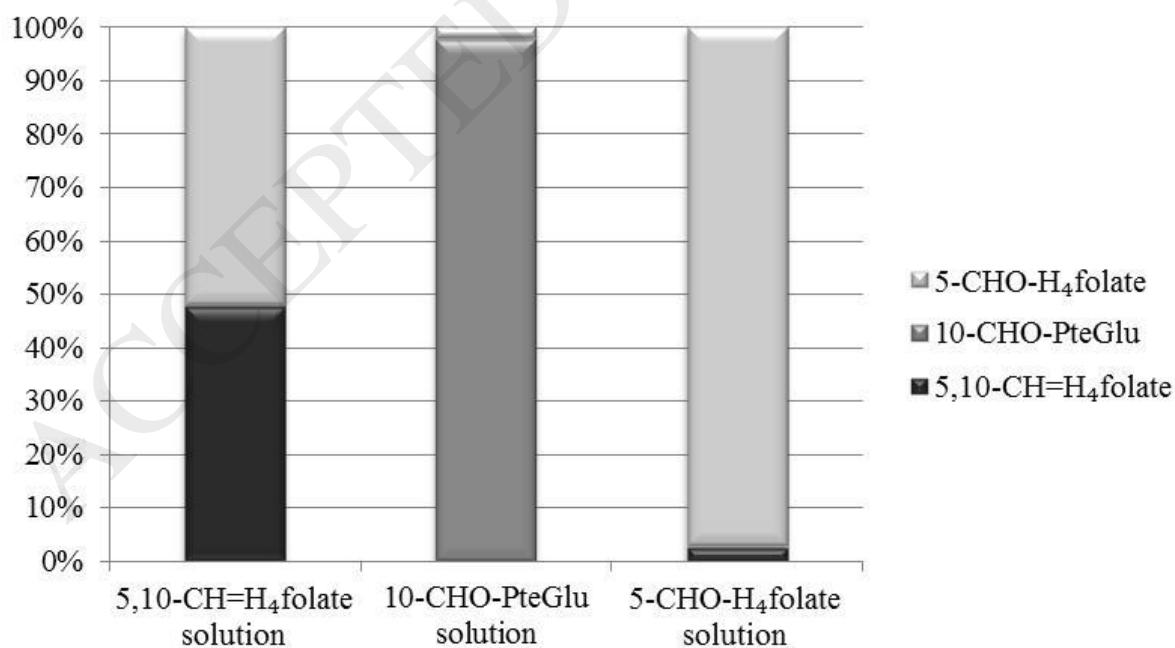


**Figure 2** LC-MS/MS chromatogram of the six naturally occurring monoglutamylated folates and three <sup>13</sup>C<sub>5</sub>-labeled internal standards in a standard mixture (a) and a liver extract from in-

house reference material (b) with their MRM transitions. The figure shows relative intensity of the peaks.



**Figure 3** The matrix effect on each folate and <sup>13</sup>C<sub>5</sub>-labeled internal standards in various food matrices (n=3).



**Figure 4** Distribution of formyl folate forms after the interconversion analysis (n=3).

**Table 1** MRM transitions and corresponding parameters for analyzed folate forms

Compound	Precursor ion (m/z)	Product ion (m/z)	Retention time (min)	Fragmentor (V)	Collision energy (V)
H <sub>4</sub> folate	446.2	299.1	0.92	84	17
		120.0	0.92	84	40
5,10-CH <sup>+</sup> =H <sub>4</sub> folate	456.2	412.2	0.97	156	29
		324.2	0.97	156	55
10-CHO-PteGlu	470.1	295.1	1.03	104	29
		176.1	1.03	104	40
5-CHO-H <sub>4</sub> folate	474.2	327.2	1.05	96	15
		299.1	1.05	96	37
[ <sup>13</sup> C <sub>5</sub> ]-5-CHO-H <sub>4</sub> folate	479.2	327.2	1.05	96	15
		PteGlu	442.1	295.1	1.37
[ <sup>13</sup> C <sub>5</sub> ]-PteGlu	447.1	295.1	1.37	84	9
		5-CH <sub>3</sub> -H <sub>4</sub> folate	460.2	313.2	1.84
[ <sup>13</sup> C <sub>5</sub> ]-5-CH <sub>3</sub> -H <sub>4</sub> folate	465.2	180.2	1.84	100	40
		313.2	1.84	88	17

The order of compounds is based on the use of three internal standards. [<sup>13</sup>C<sub>5</sub>]-5-CHO-H<sub>4</sub>folate is used as internal standard for H<sub>4</sub>folate, 5,10-CH<sup>+</sup>=H<sub>4</sub>folate, 10-CHO-PteGlu, and 5-CHO-H<sub>4</sub>folate, [<sup>13</sup>C<sub>5</sub>]-5-CH<sub>3</sub>-H<sub>4</sub>folate is used as internal standard for 5-CH<sub>3</sub>-H<sub>4</sub>folate, while [<sup>13</sup>C<sub>5</sub>]-PteGlu was used as internal standard for PteGlu. Second product ion in MRM transitions of unlabeled standards was used as a qualifier during the analysis.

**Table 2** Enzyme activity treatments and release of monoglutamyl folic acid.

Substrate	Sample mass (g)	AtGGH2 ( $\mu\text{g}/\text{reaction}$ )	Incubation time (h)	% of folic acid released as monoglutamate form
1 $\mu\text{M}$ of 5-glu-PteGlu	0.0	10	1.0	92 $\pm$ 1
1 $\mu\text{M}$ of 5-glu-PteGlu	0.0	20	1.0	93 $\pm$ 1
1 $\mu\text{M}$ of 5-glu-PteGlu	0.0	30	1.0	95 $\pm$ 1
1 $\mu\text{M}$ of PG PteGlu mixture	0.0	10	1.0	92 $\pm$ 1
1 $\mu\text{M}$ of PG PteGlu mixture	0.0	20	1.0	95 $\pm$ 3
1 $\mu\text{M}$ of PG PteGlu mixture	0.0	30	1.0	94 $\pm$ 2
10 $\mu\text{M}$ of 5-glu-PteGlu + GPE	1.0	100	0.5	96 $\pm$ 5
10 $\mu\text{M}$ of 5-glu-PteGlu + GPE	1.0	100	1.0	98 $\pm$ 2
10 $\mu\text{M}$ of 5-glu-PteGlu + GPE	1.0	100	2.0	99 $\pm$ 2
10 $\mu\text{M}$ of 5-glu-PteGlu + GPE	1.0	100	3.0	98 $\pm$ 2

AtGGH – Arabidopsis  $\gamma$ -glutamyl hydrolase; PG – polyglutamyl; GPE – green peas extract.

Data are means of three replicates  $\pm$  SD. No significant difference was found in any of the treatments (Tukey-Kramer test,  $p \leq 0.05$ ). 5-glu-PteGlu, pteroylpenta- $\gamma$ -L-glutamic acid; GPE, green peas extract.



**Table 3** Precision and recovery of spiked samples

Folate form	Precision (n=3, %RSD)						Recovery (%)		
	Intra-day (n=3)			Inter-day (n=9)			10 ng	500 ng	900 ng
	10 ng	500 ng	900 ng	10 ng	500 ng	900 ng			
H <sub>4</sub> folate	31.4	7.2	26.3	34.2*	14.2*	17.1*	105	100	123
5,10-CH <sup>+</sup> =H <sub>4</sub> folate +10-HCO-PteGlu +5-HCO-H <sub>4</sub> folate	2.6	2.9	3.2	13.9	11.1	10.7	82	83	81
PteGlu	2.1	1.0	1.6	6.8	3.7	4.7	100	103	101
5-CH <sub>3</sub> -H <sub>4</sub> folate	1.1	0.5	0.2	3.9	3.1	2.1	101	101	101

Intra-day precision and recovery results for H<sub>4</sub>folate shown here were obtained by spiking white cabbage (n=3).

\*Inter-day precision is calculated based on the 2 different days (n=6) from rice samples and white cabbage samples. RSD=relative standard deviation

**Table 4** The total folate content ( $\mu\text{g}/100\text{g}$ ) quantified in five certified reference materials ( $n=2-4$ ). The contents are expressed as total folate content of each of the individual folate forms, and compared to the certified values and values otherwise published.

Sample	Certified value	CRM Method	Total folate	H <sub>4</sub> folate	5,10-CH=H <sub>4</sub> folate	10-CHO-PteGlu	5-CHO-H <sub>4</sub> folate	PteGlu	5-CH <sub>3</sub> -H <sub>4</sub> folate	Enzyme <sup>1</sup>	Method <sup>2</sup>	Reference
BCR 487 Pig Liver	1330 ± 130	MA	1291 ± 1	436	16	20	125	130	580	D (AtGGH2)	LC-MS/MS	Present study
			NS	(334-484)	NA	37	146	139	(27-542)	Pancreatic enzyme + D (hog kidney and/or human plasma)	HPLC-UV or F	Finglas et al.[20]
			1443 ± 13	395	NA	NA	291	275	482	P + A + D (human plasma)	LC-MS/MS	Vishnu mohan et al. [21]
NIST 1546a Meat Homogenate	None	None	1.0 ± 0.3	<0	0.1	<0	<0	0.3	0.3	D (AtGGH2)	LC-MS/MS	Present study
NIST 1549a Whole Milk	None	None	19 ± 1	0.6	0.3	<0	<0	0.2	18	D (AtGGH2)	LC-MS/MS	Present study
			21.1 ± 1.4	NA	NA	NA	NA	SF	21.1	P + A + D (rat serum)	LC-MS/MS	Camar a et al. [54]
NIST 1846 Infant Formula	129 ± 28	MA	124 ± 18	<0	<0	<0	<0	116	9	D (AtGGH2)	LC-MS/MS	Present study
			133 ± 10	NR	NR	NR	NR	NR	NR	P + A + D (chicken pancreas)	MA	Chen & Eitenmiller[55]
NIST 1849 Infant Formula	211 ± 13	MA LC-MS/MS	195 ± 16	<0	<0	<0	<0	189	6	D (AtGGH2)	LC-MS/MS	Present study
			225 ± 18	NA	NA	NA	NA	210	16	None	LC-MS/MS	Chandra-Hioe et al. [52]

AtGGH2= $\gamma$ -glutamyl hydrolase from *Arabidopsis thaliana* converts polyglutamates to monoglutamates;  
MA=microbiological assay

NS=not specified; NA=not analyzed; NR=not relevant for a MA; SF=screened for, but not detected.

<sup>1</sup> Enzyme: D=deconjugase; P=protease; A= $\alpha$ -amylase

<sup>2</sup> Detection principle: UV=ultraviolet; F= fluorescence

ACCEPTED MANUSCRIPT