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Title:

Quantification of folate in food using deconjugase of plant origin combined with LC-MS/MS: A method comparison of a large and diverse sample set

Authors:

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Abstract:

A round robin comparison was performed in order to test the performance of a recently developed LC-MS/MS method for quantification of 6 folate forms. Eighty-nine samples representing the food groups of fruits, vegetables, legumes, cereals, dairy products, meat, and offal were analyzed by two LC-MS/MS methods and a microbiological assay (MA). A plant-origin deconjugase enzyme (*Arabidopsis thaliana*) for deconjugation of folates (PE-LC-MS/MS), or animal-origin deconjugase (rat serum and chicken pancreas) (AE-LC-MS/MS) was used in the LC-MS/MS methods, each in a single enzymatic step. In contrast, the MA involved tri-enzyme extraction including human plasma as a deconjugase. A significant bias of 17% lower and 25% higher results was found when PE-LC-MS/MS was compared to MA and AE-LC-MS/MS, respectively. The PE-LC-MS/MS provides fast quantification of various folate vitamers and total folate content, which could be a proper substitute to the currently standardized but imprecise and time-consuming microbiological assay in the future.

Keywords:

Folates; Food; Method comparison; Deconjugase enzymes; LC-MS/MS; Microbiological assay

Chemical compounds studied in this article:

Tetrahydrofolate (PubChem CID: 1129); 5,10-Methenyltetrahydrofolate (PubChem CID: 439237); 10-Formylfolic acid (PubChem CID: 3080544); 5-Formyltetrahydrofolate (PubChem CID: 149436); 5-Methyltetrahydrofolate (PubChem CID: 439234); Folic acid (PubChem CID: 6037)

1. Introduction:

Folate or vitamin B₉ is a generic term for structurally similar compounds that act as coenzymes in numerous reactions in humans and other mammals. Folates are essential compounds, that can't be synthesized by animal organisms, and hence they are ingested from external sources such as food or supplements (Saini, Nile, & Keum, 2016). These compounds play a key role in one-carbon transfer reactions, and consequently

in proliferation and methylation of DNA. Thus, they are indispensable for the prevention of various chronic diseases such as cardiovascular diseases, cancer, and various birth effects (Bailey et al., 2015).

The structure of folates is composed of a pteridine ring that is attached by a methylene bridge to para-aminobenzoic acid (PABA), which is in turn coupled to a polyglutamate chain (Figure 1). The polyglutamate chain is generally composed of 2-8 glutamate residues (Ndaw, Bergaentzle, Aoude-Werner, Lahely, & Hasselmann, 2001), but a length of 17 glutamates has also been recorded in papaya (Ramos-Parra, Garcia-Salinas, Hernandez-Brenes, & Diaz de la Garza, 2013a). In addition, different one-carbon derivatives of tetrahydrofolate (H_4 folate) are present depending on the substituent attached to N^5 or N^{10} , ranging from 5,10-methylenetetrahydrofolate ($5,10-CH_2-H_4$ folate), 5,10-methenyltetrahydrofolate ($5,10-CH^+=H_4$ folate), 10-formyltetrahydrofolate ($10-HCO-H_4$ folate), 5-formyltetrahydrofolate ($5-HCO-H_4$ folate) to 5-methyltetrahydrofolate ($5-CH_3-H_4$ folate), which presumably affects folate bioavailability (McNulty & Pentieva, 2004). The various forms as well as their different number of glutamate residues found in nature alter their affinity for folate-dependent enzymes, and hence, their ability for absorption and physiological functions (Gregory et al., 1992, Gregory 1995).

Naturally occurring folates are sensitive to various parameters such as temperature, light, oxygen, and pH, which makes them vulnerable to losses during food processing (Delchier, Herbig, Rychlik, & Renard, 2016). Folates are present in a large variety of foods, despite often being in minor amounts. The best food sources of folates are green leafy vegetables, legumes, offal, and yeast. However, these sources are mostly not abundant enough in human nutrition to meet the actual daily recommendations in Nordic countries of 300 μ g/day (Nordic Nutrition Recommendations, 2014) and 400 μ g/day, expressed in terms of dietary folate equivalents (DFE) in the USA (Institute of Medicine, 1998). The DFE corrects for a higher bioavailability of synthetic folic acid than that of natural folates; 100 μ g provided as food folate is considered equal to 100 μ g DFE, whereas 100 μ g provided as folic acid in fortified foods is considered equal to 170 μ g DFE (Institute of Medicine, 2003). Accurate determination of folate intake is difficult because food composition databases do not include the essential specific data about folate vitamers that take into consideration the presumable variation of bioavailability of the different folates (Combs, 2012; Ringling & Rychlik, 2017b). In this regard,

Mönch et al. (Mönch et al., 2015, 2016) have shown that bioavailability differs even within varieties of a foodstuff by investigating various sources such as spinach, wheat germ and two different Camembert cheeses, reporting folate bioavailability of 73%, 33% and 9-64%, respectively. In 2004, the European Prospective Investigation into Cancer and Nutrition (EPIC) has initiated the EPIC Nutrient DataBase (ENDB) project as a first attempt to harmonize nutrient databases across European Union (EU) countries. The inclusion of folate was problematic because of the lack of data in the different EU countries (Nicolas et al., 2016).

Folate determination is usually composed of three main steps: extraction, deconjugation and detection (Jägerstad & Jastrebova, 2013). Extraction is normally performed by the combination of heat treatment and enzymes, such as amylase and protease to help folate release from the matrix, and hydrolase enzymes, namely tri-enzyme extraction, which is important for deconjugation of polyglutamates to the respective monoglutamates (Tamura, 1998). Various sources of animal-origin hydrolases such as rat serum/plasma, hog kidney, human plasma, and chicken pancreas have been used for folate deconjugation in combination with different microbiological or chemical techniques for folate determination (Jägerstad & Jastrebova, 2013). However, the data on folate content in national food composition databases are obtained by the use of microbiological assay (MA), which solely quantifies total folate without providing any information on specific folate vitamers. The most recent inter-laboratory study on MA showed the poor precision of this method, besides being laborious and time-consuming (Puwastien, Pinprapai, Judprasong, & Tamura, 2005). Therefore, the need for sensitive and fast chemical methods capable to distinguish between the different folate vitamers is justified. Due to the variability of the hydrolases and detection methods in folate analysis, numerous studies investigating the folate content in food have been reported. The natural variations between different food varieties, and also the differences in the methods used for folate determination, have led to big discrepancies in results (Delchier et al., 2016). Recently, a UPLC-MS/MS method has been approved as an AOAC-method for determination of 5-CH₃-H₄folate, 5-HCO-H₄folate, and PteGlu as the total folate content expressed as PteGlu equivalent in the two food matrices, infant formula and adult nutritionals, by the tri-enzyme treatment including rat plasma as the deconjugase enzyme (Bhandari, Ming, & Szpylka, 2018;

Szpylka, De Vries, Cheney, & House, 2012). Even though tri-enzyme extraction has been proposed as a method of choice, Ringling and Rychlik (2017a) have found no differences between tri-enzyme extraction and single-enzyme extraction, using a combination of chicken pancreas and rat serum as folate hydrolases. In 2013, Ramos-Parra et al. published a study using a hydrolase enzyme of plant origin (*Arabidopsis thaliana*) produced by genetic engineering in *E. coli* (Ramos-Parra, Urrea-López, & Díaz de la Garza, 2013b). The plant enzyme was capable of hydrolyzing polyglutamyl tails in folates within 1 hour of incubation.

The aim of this study was to investigate if a newly developed, LC-MS/MS-method using the enzyme of plant origin (PE-LC-MS/MS) was superior to the currently standardized folate method, the MA, and to an LC-MS/MS-method that uses a commercial combination of deconjugases of animal origin (AE-LC-MS/MS). A round robin study of a large sample set was employed to establish the differences among these methods. The samples included in the round robin comparison were selected to be representative to different food groups and to be ones that contribute significantly to the dietary intake in Denmark. Furthermore, the folate contents and distribution of folate vitamers in the selected foodstuffs was widely distributed and it served as a perfect model to examine the presence of any differences between the methods. In addition, this method comparison provides new information about folate contents and distribution of folate vitamers in various food groups. Finally, to our knowledge, this is the first study that evaluates the differences in performance of a deconjugase enzyme from plant and animal origin in such large and diverse sample set.

2. Materials and methods

A round robin study was performed in three different laboratories. Our recently developed and validated PE-LC-MS/MS was compared to MA as the standard method for folate quantification in food (EN14131, 2003), and also to AE-LC-MS/MS. The analyses were run in duplicates, except for 45 samples analyzed by MA that were performed as a single determination.

2.1. Chemicals and standards

All chemicals were of analytical quality, but for each of the used methods, the following standards and enzyme solutions were used in the round robin study:

2.1.1. PE-LC-MS/MS

The following folate standards were used; (6S)-5,6,7,8-tetrahydrofolic acid (H₄folate), (6R,S)-5,10-methenyl-5,6,7,8-tetrahydrofolic acid chloride (5,10-CH⁺=H₄folate), 10-formylfolic acid (10-HCO-PteGlu), (6S)-5-formyl-5,6,7,8-tetrahydrofolic acid, calcium salt (5-HCO-H₄folate), folic acid (PteGlu) and (6R,S)-5-methyl-5,6,7,8-tetrahydrofolic acid, calcium salt (5-CH₃-H₄folate) from Schircks (Jona, Switzerland). Isotopically labeled internal standards (¹³C₅-5-HCO-H₄folate, ¹³C₅-PteGlu and ¹³C₅-5-CH₃-H₄folate) were from Merck Eprova (Schaffhausen, Switzerland). The folate standards were stored at -80°C until use.

The pure recombinant plant γ -glutamyl hydrolase (GGH) enzyme (EC 3.4.19.9) was produced by genetic engineering at the Institute of Technology and Higher Education, Monterrey, Mexico as previously described (Ložnjak, García-Salinas, Díaz de la Garza, Bysted, & Jakobsen, 2019; Ramos-Parra et al., 2013b). For additional information about the enzyme see Supplementary Online Material (SOM, Section S2, Fig. S1).

2.1.2. MA

The reference for the method is EN14131 (2003), in which PteGlu were used as standard, and creon capsules containing amylase, lipase and protease combined with human plasma as the deconjugase enzyme (Sigma-Aldrich, Steinheim, Germany) were used.

2.1.3. AE-LC-MS/MS

Both the unlabeled reference compounds (H₄folate, 5-CH₃-H₄folate, 5-HCO-H₄folate, and 10-HCO-PteGlu) and isotopically labeled internal standards (¹³C₅-PteGlu, ¹³C₅-H₄folate, ¹³C₅-5-CH₃-H₄folate, and ¹³C₅-5-HCO-H₄folate) were acquired from Schircks Laboratories (Jona, Switzerland), whereas PteGlu was obtained from Fluka (Sigma-Aldrich, Steinheim, Germany).

Rat serum and chicken pancreas containing γ -glutamyl hydrolase (EC 3.4.19.9) were purchased from Biozol (Eching, Germany) and Difco (Sparks, MD, USA), respectively.

2.2. Samples

Two to four different foodstuffs were selected for each of the food groups (fruits, vegetables, legumes, cereals, dairy products, meat, and offal), resulting in a total of 26 foodstuffs. For each foodstuff, 1-4 different sample sets were purchased from local supermarkets in the city of Copenhagen between April and May, 2017. In total, 89 samples were included in the study, as is shown in Table 1. Samples were bought in quantities of 0.5-1 kg and stored at 4°C (fruits, vegetables, dairy products, meat and offal) or at room temperature (legumes and cereals). Representative samples were made within 3 days. Sampling procedure was performed with a purpose to include these data into national food composition database.

2.3. Preparation of test materials

Each of the solid food samples was cleaned from non-eatable parts when necessary and cut into pieces approximately 2.5 cm in size. They were divided into 12 representative samples, ensuring that each subsample contained pieces from different part of the sample (top, middle, and bottom). Each of the 12 subsamples weighed approximately 40 g and was instantly frozen in liquid nitrogen, transferred to a plastic bag from which the oxygen was replaced by nitrogen before closing the bag. An illustration of the process can be found in SOM (Section S2, Fig. S2). The liquid samples (i.e., yoghurt and milk) were divided into 50 mL screw cap tubes and also frozen in liquid nitrogen. All samples were stored and shipped at -80 °C packed with dry ice. Three packages from each test material were sent to each laboratory. The shipped frozen samples were afterward stored at -20 °C for a maximum duration of 3 months before analysis.

Each representative sample of 40 g was homogenized for 30 s in a coffee grinder (EGK 200, Rommelsbacher, Germany) right before the analysis.

2.4. Analytical methods

All analyses were performed under subdued light in order to prevent the degradation of folates.

2.4.1. LC-MS/MS method using a plant enzyme

The method for quantification of six folate vitamers (H_4 folate, 10-HCO-PteGlu, 5,10-CH⁺=H₄folate, 5-HCO-H₄folate, PteGlu and 5-CH₃-H₄folate) by the use of a plant enzyme is described elsewhere (Ložnjak et al.,

2019). In short, 0.5-1 g of a homogenized sample was weighed in a Pyrex glass tube. 10 mL of 0.05 mM phosphate buffer with 1% ascorbic acid and 0.1% β -mercaptoethanol (pH 6.0) and isotopically labeled internal standards ($^{13}\text{C}_5$ -5-HCO- H_4 folate, $^{13}\text{C}_5$ -PteGlu and $^{13}\text{C}_5$ -5- CH_3 - H_4 folate) were added to the sample. The sample was heated in a boiling water bath for 10 min in order to release natural folates from the food matrix. After cooling to room temperature, a single enzymatic step was performed, in which the sample was treated with 100 μg of γ -glutamyl enzyme per 1 g of the sample at 37 °C for 1 hour in a shaking water bath. After the incubation, each sample was heated again, cooled on ice, mixed with 10 mL of acetonitrile and centrifuged, following the sample clean-up by solid-phase extraction (SPE) on strong anion exchange cartridge (SAX, quaternary amine, 55 μm , 70 Å, 500 mg/3 mL, Phenomenex). Each sample was eluted by an elution buffer, composed of methanol containing 1% ascorbic acid and 10% formic acid (v/v), and centrifuged at 10.000 g for 10 min at 4 °C prior the LC-ESI-MS/MS analyses. The results were expressed as a total folate content in terms of PteGlu equivalent ($\mu\text{g}/100\text{ g}$) and the contents of the individual folate vitamers (e.g., H_4 folate, the sum of 10-HCO-PteGlu, 5,10- $\text{CH}^+=\text{H}_4$ folate and 5-HCO- H_4 folate due to their interconversion, PteGlu and 5- CH_3 - H_4 folate) were also evaluated and expressed as PteGlu equivalent ($\mu\text{g}/100\text{ g}$). Limit of quantification was defined as 0.1 $\mu\text{g}/100\text{ g}$ for each folate vitamer.

2.4.2. Microbiological assay using an animal enzyme

The folate extraction was performed using an autoclave, followed by a tri-enzyme extraction. The growth response of *Lactobacillus rhamnosus* (ATCC 7469) related to the amount of the extracted folate was measured turbidimetrically using PteGlu as a calibrant. Due to the costliness, 49% of the samples (n=42) analyzed by MA were analyzed only in a single determination. Precisely, a single determination was used for the group of dairy products representing samples of homogeneous composition, and for some of the samples containing low amount of folates such as apples, tomatoes, pasta, rice, and onions. The uncertainty of the method provided by the laboratory was 15%, whereas the limit of quantification was 5 $\mu\text{g}/100\text{ g}$. The analyses were performed accredited according to ISO17025 at Eurofins Steins Laboratory, Vejle, Denmark.

2.4.3. LC-MS/MS method using an animal enzyme

The method quantifying five different folate vitamers (H_4 folate, 10-HCO-PteGlu, 5-HCO- H_4 folate, PteGlu, and 5- CH_3 - H_4 folate) using a deconjugase of animal origin combined with LC-MS/MS is previously described (Striegel, Chebib, Netzel, & Rychlik, 2018). Briefly, 0.25 – 0.5 g of a homogenized sample was weighed into Pyrex bottles followed by addition of 10 mL buffer for extraction (200 mM MES-hydrate, containing 0.2% ascorbic acid and 0.01% DTT (pH 5.0), and internal standards ($^{13}C_5$ - H_4 folate, $^{13}C_5$ -5-HCO- H_4 folate, $^{13}C_5$ -PteGlu and $^{13}C_5$ -5- CH_3 - H_4 folate) in amounts adjusted to the expected contents of analytes to fall in the given calibration line. Each sample was boiled for 10 min, cooled on ice, and treated with enzymes for deconjugation, for which 2 mL chicken pancreas suspension and 0.8 mL rat serum were added. Samples were incubated for a minimum duration of 12 h in a shaking water bath at 37 °C, following a boiling step in order to stop the enzyme activity. They were cooled on ice and mixed with acetonitrile, following centrifugation and a sample clean-up with the SPE (SAX, quaternary amine, 55 μ m, 70 Å, 500 mg/3mL, Phenomenex). Folates were eluted using an elution buffer, which was prepared by mixing 5% aqueous sodium chloride, 100 mM aqueous sodium acetate, 0.1% ascorbic acid, and 0.01% DTT. The final eluate was membrane filtered (PVDF, 0.22 μ m) and measured by LC-ESI-MS/MS. Pteroylpolyglutamates were included in the LC-MS/MS method to check for complete deconjugation. Limit of quantification was defined as 0.76 μ g/100g, 0.93 μ g/100g, 0.96 μ g/100g and 0.51 μ g/100g for H_4 folate, 5-HCO- H_4 folate, PteGlu and 5- CH_3 - H_4 folate, respectively. The method was run at the Technical University of Munich (TUM).

2.5. Calculation of the total folate content

The total folate content is expressed as PteGlu equivalent, which is the sum of the concentrations of each folate vitamer quantified by LC-MS/MS analysis. To convert each of the folate vitamer to PteGlu equivalent, the content was multiplied by 0.99, 0.97, 0.94, 0.93, 1.00, and 0.96 for H_4 folate, 5,10- $CH^+=H_4$ folate, 10-HCO-PteGlu, 5-HCO- H_4 folate, PteGlu, and 5- CH_3 - H_4 folate, respectively. These factors represent ratios of molecular weights of PteGlu and the folate vitamers of interest.

2.6. Statistical evaluation

The data for method comparison were statistically evaluated using JMP® Statistical Discovery software version 13.0 (SAS Institute Inc. Cary, NC, USA). The newly developed PE-LC-MS/MS method was used as a basis for comparison of the MA and AE-LC-MS/MS methods. The Bland-Altman diagram (Bland & Altman, 1986; Bland & Altman, 1999) was used to investigate the differences between the two methods. A paired t-test was used to evaluate if there was a difference between the folate distributions within the food groups. A p-value ≤ 0.05 was classified as a significant difference. The results are given as mean $\pm$ standard deviation (SD). The precision of each method was assessed from the duplicate within-day analyses. The mean of the range (R) for the duplicate analyses divided by 1.128 is the estimate for the standard deviation (NMKL, 2016).

3. Results and discussion:

The within-day precision of PE-LC-MS/MS, MA and AE-LC-MS/MS was estimated to be similar being 5.9%, 6.2% and 5.8%, respectively. The two LC-MS/MS methods were single-laboratory validated and MA performed in an accredited laboratory, thereby the trueness of the methods were verified. During the analyses by the PE-LC-MS/MS method, the consistency of the folate level was checked by analyzing a certified reference material NIST 1869 Infant/Adult Nutritional Formula. The total folate content expressed as folic acid equivalent was $232 \pm 12 \mu\text{g}/100 \text{ g}$ (n=5), which was in accordance with previous study (Bhandari et al., 2018). Moreover, in-house reference material (freeze-dried white cabbage) was analyzed on each day of analyses. The total folate content expressed as a folic acid equivalent in white cabbage was $238 \pm 32 \mu\text{g}/100 \text{ g}$ (n=17). Normal distribution of residuals was observed during the analyses in duration of 6 months.

Table 1 presents the distribution of foodstuffs within different food groups, the number of analyzed samples, and the folate content quantified by PE-LC-MS/MS.

The results are presented as the mean $\pm$ SD of the analyzed foodstuffs, which was composed of 1-4 different samples. High SD indicates a variation of folate content within the foodstuff. The folate content in foodstuffs can vary according to the variety, state of harvest or storage of fruits and vegetables, and the processing conditions of legumes, cereals, and dairy products (Delchier et al., 2016; Upadhyaya et al., 2017). To

compare the new data, folate contents measured by MA provided in two food composition databases is also shown in the Table 1. It presents discrepancies in the data, indicating inconsistency of the reference data between different countries.

Figure 2 displays a graphical presentation of the folate contents analyzed using the three methods for all the foodstuffs divided into 7 food groups. The detailed results are also provided in SOM (Section S3, Table S3).

Plotting the results by the food group shows that the two LC-MS/MS methods using GGH enzymes of different origin gave very similar results for fruits, legumes, cereals, dairy products, and meat. On the other hand, the differences in the results obtained by these two methods can be seen in food groups of vegetables and offal. In addition, it seems that the MA provides slightly higher folate content in legumes, cereals, and dairy products.

3.1. Comparison of PE-LC-MS/MS vs. MA

In 1986, Bland and Altman proposed a statistical analysis for testing of method agreement, based on the differences and constructing limits of agreement. They presented the differences between the pairs of measurements plotted against their means that can offer insight into the pattern of agreement. Bland-Altman plot evaluates bias between the mean differences, and it estimates an agreement interval, which presents 95% of the differences when the second method is compared to the first one (Watson & Petrie, 2010). A scatter of the differences along the line of equality, and the line of equality that is within the 95% of the confidence interval of the mean, are an indication of agreement between two methods (Bland & Altman, 1999). Furthermore, expressing the differences as percentages of the values on the y-axis is useful when there is an increase in variability of the differences with the increase of the magnitude of the measurement (Giavarina, 2015). More specifically, large variability in the results can be clearly seen when comparing the PE-LC-MS/MS and MA and when measuring a wide range of concentration. The Bland-Altman plots shown in Figure 3 for the PE-LC-MS/MS vs. MA do not present results of samples with $<5 \mu\text{g}/100 \text{ g}$, because this is the limit of quantification of MA. The folate contents in food ranged from very low values (e.g., in meat) to high values (e.g., in green leafy vegetables, legumes, and offal). Our results are in agreement with the

literature, which reports that meat is not a good source of folates, while green leafy vegetables, legumes, and offal are considered to be among the best natural folate sources (Saini et al., 2016).

The Bland-Altman plot presented in Figure 3a has shown a relative difference of -17% between PE-LC-MS/MS and MA, which is a significant bias ($p \leq 0.05$) as the line of equality is not in the confidence interval of the mean. Figure 3b shows that the differences between the two methods in food groups of cereals ($n=16$) and dairy products ($n=14$) were significantly lower ($p \leq 0.05$) by the use of PE-LC-MS/MS method. The results of the PE-LC-MS/MS were significantly higher ($p \leq 0.05$) when compared to the MA for offal.

It may be supposed that the higher level obtained by the MA analysis is due to the use of tri-enzyme extraction. Several studies have suggested this extraction as a standard procedure in folate analysis, in order to release folates from food matrices rich in carbohydrates and proteins (Hyun & Tamura, 2005). However, our trial studies indicated that the use of protease and amylase did not increase the amount of folate monoglutamates, corroborating the previous findings (Ndaw et al., 2001; Ringling & Rychlik, 2017a). Furthermore, no significant difference was shown between these two methods in the food group of legumes, which is considered as a complex food matrix, where the tri-enzyme treatment could be needed. Moreover, previous study indicated that exclusion of some folate vitamers such as 10-formyldihydrofolate from the LC-MS/MS analysis could contribute to the significant differences ($p \leq 0.05$) between LC-MS/MS methods and MA in the food group of cereals, as this folate form is contributing by 20% to the total folate content in this group (Pfeiffer, Rogers, & Gregory, 1997).

In addition, the microbiological assay is known to be dependent on the calibrant used for the quantification of total folate content (Ringling & Rychlik, 2017a). The calibrant in the CEN-method, and thereby in our study, is PteGlu, which is not naturally found in food, and therefore could give inaccurate results (Martin, O'Mahony, & Sheehy, 1996; Reingold & Picciano, 1982). *L. rhamnosus* is the preferred microorganism for this method since it exhibits a growth response to the majority of folate vitamers found in biological systems (Rader, Weaver, & Angyal, 1998). However, the ability of *L. rhamnosus* to respond equally, on a molar basis, to naturally occurring monoglutamate forms of folates is questionable (Koontz et al., 2005). Other

non-folate compounds could also be potential sources of the variability between the methods, being responsible for the bacterial growth. Thymidine, amino acids, purines, and pyrimidines have been reported to positively affect the growth of *L. rhamnosus*, even when no PteGlu is present (Koontz et al., 2005). In 2001, Konings et al. analyzed 35 food samples such as milk, vegetables, fruit, potatoes, and bread using an HPLC method with fluorescence and diode array detection and compared the results with reports from three food databases based on MA. In their study, the total folate quantities, analyzed by chemical assay including extraction at pH 7.8 and tri-enzyme treatment by rat plasma as a deconjugase, resulted in 25% lower results than the amounts listed in the food composition databases, which is also well in line with our findings of higher MA values.

3.2. Comparison of the two LC-MS/MS methods

Figure 4 presents the comparison between the two LC-MS/MS methods that used GGH of plant or animal origin.

Plotting the relative differences between the results of the two LC-MS/MS methods, a constant bias (mean difference) of 25% was observed, in a confidence interval of the mean difference from 14% to 37%. The bias was significant ($p \leq 0.05$) since the line of equality is not in the confidence interval of the mean. The use of absolute values would be appropriate when comparing low contents by Bland-Altman system since the relative differences in these samples were large. When the samples containing $<5 \mu\text{g}/100 \text{ g}$ were excluded from the comparison between the two LC-MS/MS methods, the bias decreased to 20%. By comparing the two LC-MS/MS methods, it can be seen that the relative differences observed in the samples containing $<20 \mu\text{g}/100 \text{ g}$ of total folate contribute to the observed bias, as shown in Figure 4a. EU regulations apply health claims “source of” to foodstuffs that contain at least 15% of the folate reference intake of $200 \mu\text{g}/100 \text{ g}$, or $30 \mu\text{g}/100 \text{ g}$ of food (European Union, 2012). Even though those samples are not considered as a “source of folate”, foodstuffs containing $<20 \mu\text{g}/100 \text{ g}$, such as milk, yoghurt, cereals, meat etc., are widely consumed; and therefore, they contribute to the dietary folate intake. Figure 4b indicates that the results obtained in food groups of fruits, vegetables and cereals are significantly higher when being analyzed by PE-LC-MS/MS

method, whereas no significant difference was found in the food group of legumes, dairy products, meat and offal. In addition, a qualitative control of the presence of polyglutamylated forms was done during the AE-LC-MS/MS analysis. Peaks of 5-CH₃-H₄folate diglutamate were detected in a variety of foods i.e. in broccoli, romaine salad, leek, and offal samples, indicating incomplete deconjugation of pteroylpolyglutamates (SOM, Section S3, Fig. S4). Moreover, former studies have shown that the activity of GGH enzyme of animal origin can be inhibited in plant matrices (Bhandari & Gregory, 1990; Gregory & Toth, 1988), which supports the significant difference in our results obtained in fruits, vegetables, and cereals. In addition, liver is one of the best sources of folates, and the amount of 2 mL chicken pancreas solution and 0.8 mL rat serum solution has been shown to be insufficient to deglutamylate all polyglutamates to the respective monoglutamates using the AE-LC-MS/MS method. Thus, the amount of enzymes needs to be adjusted for each food upon using the enzyme of animal origin. However, the amount of 100 µg of plant-origin GGH per gram of sample, which corresponds to approximately 50 µL of the enzyme solution (2 mg/mL), has been shown to be more efficient in the analyses of folates in a whole range of different foods.

Figure 5 presents a comparison between vitamer distributions in different food groups obtained by the PE-LC-MS/MS and AE-LC-MS/MS methods. No significant differences in the distribution of folate monoglutamates within the food groups of fruits, vegetables, and dairy products were found. However, some significant differences ($p \leq 0.05$) in the distribution of formyl vitamers within legumes and cereals, of H₄folate in meat and offal food groups were found, in addition to a significant difference ($p \leq 0.05$) in the distribution of PteGlu and 5-CH₃-H₄folate in the offal group (for numeric data and p-values see SOM, Section S3, Table S5 and S6, respectively). The 5-CH₃-H₄folate is the most abundant folate form in fruits and vegetables, and it is the second most abundant form in legumes, dairy products, and offal. In offal, 5-CH₃-H₄folate represents 50% of the present folate. 5-HCO-H₄folate is the second most abundant folate form representing the most abundant folate form in legumes and cereals. In meat and offal, H₄folate is the most abundant form, which is in agreement with the results reported by Konings et al. (2001), who found >40% of H₄folate in the samples from this groups. The difference in the distribution of folate vitamers in offal between the PE-LC-MS/MS and AE-LC-MS/MS methods might be due to the incomplete deconjugation that

was observed in liver samples analyzed by the AE-LC-MS/MS method (SOM, Section S3, Fig. S4). Furthermore, the differences in the corresponding labeled internal standard for H₄folate within the two methods would have probably introduced a bias, especially for samples with high content of this folate form (e.g., in meat and offal). In addition, deconjugases of animal origin show preference differences in deconjugation of various folate vitamers as reported by Ramos-Parra et al. (2013b). In their study, the deconjugase from rat plasma completely deconjugated PteGlu triglutamate in 30 min, whereas only 85% of 5-HCO-H₄folate triglutamate was deconjugated within 1 h. A significant difference in a distribution of formyl forms in legumes and cereals can also derive from the quantification of 5,10-CH⁺=H₄folate by PE-LC-MS/MS method, whereas the analysis of this folate vitamer was not included in the AE-LC-MS/MS method. Previous study showed that 50% of 5,10-CH⁺=H₄folate is converted to 5-HCO-H₄folate, indicating that the rest could contribute to the total folate content of the foodstuff (Ložnjak et al., 2019).

3.3. Overall comparison

In our study, all three methods were performed with repeatability between 5.8%-6.2%, and internal reproducibility of the PE-LC-MS/MS method and MA of 13%, and 15%, respectively. LC-MS/MS analysis enables the differentiation between folate vitamers, which can give insights about bioaccessibility, bioavailability, as well as folate intake. This method comparison showed that there are some differences between the use of deconjugase enzyme of plant or animal origin. The enzyme of plant origin has shown to be superior in folate analysis, providing fast deglutamylation (1 h) by the use of low amount of the enzyme (ca 50 µL per gram of sample) as when compared to the use of animal enzyme (min 12 h). Furthermore, two deconjugase enzyme solutions, such as chicken pancreas and rat serum, were used in higher volumes of 2 ml and 0.8 mL, respectively in AE-LC-MS/MS. The use of a pure and recombinant enzyme enables a fast and reproducible method, which could provide reproducible results all over the world, as it is proved to be efficient in various food matrices. Therefore, the combination of deconjugase of plant origin and LC-MS/MS represents a candidate for a future standard method for food that is fast and can provide precise results. The next steps for the new method are an inter-laboratory study including large number of laboratories, as well as enabling the commercial availability of the plant-origin deconjugase.

The strength of the study is the large number of samples from the food groups of fruit, vegetables, legumes, cereals, dairy product, meat, and offal combined with the inclusion of three different analytical methods. However, we also see the limitation in the lack of a corresponding isotopically labeled internal standard for quantification of H₄folate in the PE-LC-MS/MS. It contributed to lower trueness for foods with high content of this folate vitamer. Furthermore, the lack of labeled internal standard for 5,10-CH⁺=H₄folate and 10-HCO-PteGlu also contributed to discrepancies in results. Furthermore, it would have been optimal to confirm complete deglutamylation in all samples. However, that was not possible within the budget for the project, but it shall be mentioned that the PE was tested for efficacy. Moreover, it would have been an advantage to check the consistency of the folate level for the AE-LC-MS/MS method by running the in-house reference materials, which was included during the analyses by the PE-LC-MS/MS, or the certified reference materials, while the MA was performed accredited according to ISO17025 which included check of trueness.

4. Conclusion:

Extraction by the use of a single, pure deconjugase of plant-origin combined with quantification by LC-MS/MS provided folate profiles and contents for 89 foodstuffs. The content of folate were 17% lower ($p \leq 0.05$) than those obtained by the standardized microbiological assay using tri-enzyme treatment, but 25% higher ($p \leq 0.05$) compared to results achieved by using an LC-MS/MS method using two deconjugases of animal origin in the extraction. Due to incomplete deconjugation, the AE-LC-MS/MS method is underestimating folates and MA assay might overestimate folate levels due to the microbial growth sustained by other metabolites present in the food matrices. However, for legumes, which are usually considered as a problematic matrix to extract folate from, no significant differences were observed between either of the methods. The use of a pure enzyme of plant origin enables enzyme treatment in 1 hour, and preparation of 20 extracts within one working day, resulting in reduction of costs in terms of especially manpower. Additionally, it seems to be more sustainable and ethical preferable to use an enzyme of plant origin compared to enzymes derived from animal tissues and fluids. Beyond the comparison, the sampling was done that the results presented in this work may be included in food databases. In the future, the proposed

method can be included in projects aimed to elucidate the faith of folate vitamers from different food sources and supplements in the human body.

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Figure captions:

Figure 1 The structure of naturally-occurring folates and the role of γ -glutamyl hydrolase (GGH) in analysis of folate. PABA: *para*-aminobenzoic acid

Figure 2 The results for the 89 samples analyzed by the three different analytical methods. Values are presented as total folate content ($\mu\text{g}/100\text{ g}$) expressed as PteGlu equivalent. PteGlu: folic acid; MA: microbiological assay; PE-LC-MS/MS: using deconjugase enzyme of plant origin; AE-LC-MS/MS: using deconjugase enzyme of animal origin.

Figure 3 Bland-Altman plots for method comparison between the PE-LC-MS/MS and the MA. a) All samples together and total bias, b) Samples separated by the food groups. Blue lines: UCL – upper confidence limit, LCL – lower confidence limit; Red line: Bias – mean difference between two methods; n – number of the analyzed samples. PE-LC-MS/MS: using deconjugase enzyme of plant;

MA: microbiological assay. Axis are the same on a) and b) part of the figure, however, the scale depends on the folate content in specific food group. If 0 (black line) is not within 95% confidence interval (dotted red lines) of the bias (red line), there is a significant difference between two methods.

Figure 4 Bland-Altman plots for the two LC-MS/MS methods using the GGH enzymes of different origin. a) All samples together and total bias, b) Samples separated by the food group. Blue lines: UCL – upper confidence limit, LCL – lower confidence limit; Red line: Bias – mean difference between two methods; n – number of analyzed samples. PE-LC-MS/MS: using deconjugase enzyme of plant origin; AE-LC-MS/MS: using deconjugase enzyme of animal origin. Axis are the same on a) and b) part of the figure, however, the scale depends on the folate content in specific food group. If 0 (black line) is not within 95% confidence interval (dotted red lines) of the bias (red line), there is a significant difference between two methods.

Figure 5 Distribution of different folate monoglutamate forms (%) from the two LC-MS/MS methods using GGH enzymes of either animal or plant origin. The percentage values can be found in SOM Table S5.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Table 1. The content of total folate in 26 foodstuffs included in the study obtained by the analyses of the six folate vitamers by PE-LC-MS/MS and the values provided in the national food composition databases from Denmark and the US.

Food group	Foodstuff	No. of samples	H ₄ folate (µg/100g) ^{ac}	HCO-folate vitamers ^b (µg/100g) ^{ac}	PteGlu (µg/100g) ^{ac}	5-CH ₃ -H ₄ folate (µg/100g) ^{ac}	Total folate (µg/100g) ^{ac}	Reference data (µg/100g)	
								FRIDA ^{cd}	USDA ^{ce}
Fruit	Apple	4	<0.1	<0.1	<0.1	3 ± 2	3 ± 2	9	3
	Banana	2	<0.1	3	<0.1	18	21	38	20
	Strawberry	4	2 ± 1	7 ± 5	<0.1	80 ± 9	89 ± 6	117	24
	Tomato	4	<0.1	2 ± 1	<0.1	14 ± 4	16 ± 5	29	15
Vegetables	Broccoli	4	13 ± 9	9 ± 4	<0.1	108 ± 18	130 ± 22	239	63
	Leek	4	1 ± 2	2 ± 1	<0.1	110 ± 13	109 ± 13	82	64
	Onion	4	<0.1	1 ± 0.4	<0.1	19 ± 4	20 ± 4	36	19
	Romaine salad	4	1 ± 1	9 ± 8	<0.1	101 ± 31	111 ± 40	136	136
Legumes	Green peas, frozen	4	6 ± 2	6 ± 2	1 ± 0.3	75 ± 7	87 ± 7	121	53
	Kidney beans, canned	4	<0.1	10 ± 9	2 ± 0.5	23 ± 2	34 ± 12	13	28
	Kidney beans, dry	1	19	60	9	27	115	/	/
	Lentils	4	22 ± 7	21 ± 3	2 ± 0.4	36 ± 6	81 ± 12	35	204
	Peanut	4	2 ± 1	65 ± 9	7 ± 3	7 ± 1	81 ± 6	53	120
Cereals	Oat, dry	4	<0.1	20 ± 2	2 ± 0.5	6 ± 2	28 ± 3	47	32
	Pasta, dry, uncooked	4	<0.1	10 ± 2	1 ± 0.2	1 ± 0.6	12 ± 2	29	18
	Parboiled rice, uncooked	4	<0.1	3 ± 2	2 ± 0.4	2 ± 2	7 ± 4	18	8
	Wheat flour	2	2	7	<0.1	3	12	18	33
	Wheat flour, wholemeal	2	5	24	1	7	37	18	44
Dairy products	Hard cheese, +45 FDM	4	1 ± 1	22 ± 7	<0.1	5 ± 1	28 ± 7	60	21
	Soft cheese, +60 FDM	4	5 ± 3	13 ± 5	1 ± 2	17 ± 6	37 ± 6	90	65
	Milk, 1.5% fat	4	<0.1	1 ± 1	<0.1	5	6 ± 1	12	6
	Yoghurt 1.5 % fat	2	<0.1	6	<0.1	6	12	12	12
Meat	Chicken breast, raw	4	1 ± 0.3	2 ± 1	<0.1	2 ± 1	5 ± 2	4	4
	Pork tenderloin, raw	4	<0.1	1 ± 1	<0.1	<0.1	1 ± 1	2	0

Offal	Liver, calf	2	860	37	1	859	1757	2300	125
	Liver, pork	2	514	53	1	554	1121	1000	212

^aResults are expressed as mean $\pm$ standard deviation (SD) from duplicates from 2-4 random selected brands of the same product.

^b10-HCO-PteGlu, 5,10-CH⁺=H₄folate and 5-HCO-H₄folate

^cExpressed as folic acid equivalent

^dFRIDA Danish Food Composition Databank (2015)

^eUSDA Nutrient Database (2015)

FDM: fat in dry matter; PE-LC-MS/MS: liquid chromatography tandem mass spectrometry method using the plant-origin enzyme

Highlights:

- Plant enzyme shortened deglutamylation time when compared to animal enzyme
- Plant enzyme is superior to animal enzyme in plant-origin food groups
- Repeatability of all used methods was ~6%
- Reported results for microbiological assay are generally higher than for LC-MS/MS
- No difference is seen between the three methods in complex matrix such as legumes

Figure 1

Figure 2

a)

b)

Figure 3

a)

b)

Figure 4

Figure 5