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Title

Collaborative study: Quantification of total folate in food using an efficient single-enzyme extraction combined with LC-MS/MS

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Abstract

Quantification of the specific folate vitamers to estimate total folate in foods is not standardized. A collaborative study, including eight European laboratories, was conducted in order to determine the repeatability and reproducibility of the method for folate quantification in foods using the plant-origin γ -glutamyl hydrolase as part of the extraction procedure. The seven food samples analyzed represent the food groups; fruits, vegetables, dairy products, legumes, offal, fish, and fortified infant formula. The homogenization step was included, and six folate vitamers were analyzed using LC-MS/MS. Total folate content, expressed as folic acid equivalent, was 17 – 490 $\mu\text{g}/100\text{ g}$ in all samples. Horwitz ratio values were within the acceptable range (0.60 – 1.94), except for fish. The results for fortified infant formula, a certified reference material (NIST 1869), confirmed the trueness of the method. The collaborative study is part of a standardization project within the Nordic Committee on Food Analysis (NMKL).

Keywords

Tetrahydrofolates; Inter-laboratory study; Deconjugase; Plant enzyme; Homogenization

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 group of water-soluble vitamers synthesized by plants and microorganisms. Together with vitamins B₆, B₁₂, and choline, folate plays a crucial role in the synthesis, reparation, and methylation of DNA, being involved in two important metabolic cycles, namely methylation cycle and DNA biosynthesis cycle (Crider, Yang, Berry, & Bailey, 2012). Inadequate folate intake affects cellular function and metabolism and leads to various diseases. Folate plays a significant role in the prevention of neural tube defects (NTDs) and other birth disorders, cardiovascular diseases, and cancer (Quinlivan, Hanson, & Gregory, 2006). In 1996, the USA and Canada introduced mandatory fortification of grain products by folic acid, a synthetic form of folate, which significantly reduced the prevalence of NTDs (FDA, 1996).

The European Prospective Investigation into Cancer and Nutrition (EPIC) studied dietary folate intake from foods across EPIC cohorts in ten European countries using a standardized nutrient database. The dietary folate intake ranged from 250 to 350 µg/day in men and 200 to 300 µg/day in women (Park et al., 2012). The recommended daily intake is increased from 300 µg/day to 400 µg/day and 500 µg/day for women of reproductive age and women in pregnancy and lactation, respectively (NNR, 2014). Thus, a supplementation by folic acid is recommended for the two groups (EFSA NDA Panel, 2014). The primary food sources of folate are green vegetables and legumes, but it is also found in other food groups such as yeast, offal, certain fruits and cereals (Delchier, Herbig, Rychlik, & Renard, 2016; Ložnjak, Striegel, Díaz de la Garza, Rychlik, & Jakobsen, 2020). Even though naturally occurring folate vitamers are widely and unevenly distributed in different food types, they are sensitive compounds that can easily degrade if exposed to oxygen or heat (Delchier et al., 2016).

By the chemical structure, folate is composed of a pteridine ring connected by a methylene bridge to para-aminobenzoic acid (PABA) that is linked to one or more glutamyl residues. The folate vitamers differ in the oxidative state of the pteridine ring, ranging from reduced form, tetrahydrofolate, to the oxidized form, folic acid (Jägerstad & Jastrebova, 2013). Different one-carbon substituents can be present on the N₅ and N₁₀ positions of the folate molecule, which in combination with a different oxidation state of pteridine ring, and various number of glutamyl residues may result in numerous folate derivatives. The most common naturally occurring folates are 5-methyltetrahydrofolate (5-CH₃-H₄folate), tetrahydrofolate (H₄folate), 5-formyltetrahydrofolate (5-HCO-H₄folate), 5,10-methenyltetrahydrofolate (5,10-CH⁺=H₄folate) and 5,10-methylenetetrahydrofolate (5,10-CH₂-H₄folate) as shown in Fig. S1 in Supplementary Online Material (SOM) (Jägerstad & Jastrebova, 2013; Strandler, Patring, Jägerstad, & Jastrebova, 2015). Folic acid and 10-formylfolic acid have also been studied as oxidation products from naturally occurring folates. The bioavailability of natural folate vitamers is approximately 60% compared to its synthetic form folic acid (Institute of Medicine, 1998). Therefore, the term *dietary folate equivalent* (DFE) takes into account these differences, and it is defined as 1 µg DFE = 1 µg food folate = 0.6 folic acid from fortified food or as a supplement consumed with food (Institute of Medicine, 1998; EFSA NDA Panel, 2014).

The number of glutamyl residues found in nature can span up to 17 glutamates (Ramos-Parra, Garcia-Salinas, Hernandez-Brenes, & Diaz de la Garza, 2013). In order to analyze folate monoglutamates, the polyglutamate tail is removed during extraction by the use of γ -glutamyl-hydrolases (GGHs), enzymes that are commonly called deconjugases. Deconjugases from various animal sources are generally used in folate extraction, such as chicken pancreas, rat serum/plasma, hog kidney or human plasma (Hyun & Tamura, 2005; Kariluoto et al., 2010; Pedersen, 1988; Vishnumohan, Pickford, & Arcot, 2017). The only standardized method for determination of total folate content in food is a microbiological assay recommending either of these deconjugases combined with amylase and protease in a tri-enzyme extraction (EN14131, 2003). During the years, chromatographic methods using UV, fluorescence or mass spectrometry detection have been introduced that enabled quantification of the individual folate vitamers, which is not possible when using non-specific microbiological assay (Patring, Jastrebova,

Hjortmo, Andlid, & Jagerstad, 2005; Pawlosky, Flanagan, & Doherty, 2003). The use of internal standards that compensate for folate loss during sample preparation and LC-MS/MS analysis enabled specific and accurate quantification of different folate vitamers (Freisleben, Schieberle, & Rychlik, 2003). An alternative to deconjugase of animal origin is pure GGHs of plant or human origin, that have been combined with specific electrochemical or MS/MS detection (Ramos-Parra, Urrea-López, & Díaz de la Garza, 2013; Zou, Duan, Li, Chen, & Wang, 2019). Ringling & Rychlik (2017) reported effective extraction of folate from wheat germs, as a food matrix rich in starch and protein, using a single-enzyme extraction (deconjugase) when compared to a commonly used tri-enzyme extraction. Recently, a single-laboratory validated LC-MS/MS method using a highly efficient pure plant-origin GGH was developed for quantification of six folate vitamers in various food matrices (Ložnjak, García-Salinas, Díaz de la Garza, Bysted, & Jakobsen, 2019). Satisfactory trueness (85-115%) and inter-day precision (<7% RSD) were reported for analysis of folic acid and 5-CH₃-H₄folate, which served as a basis for our study (Ložnjak et al., 2019). Furthermore, the method was satisfactory when evaluated against the current standardized microbiological method in a sample set of 25 different food types (Ložnjak et al., 2020).

The purpose of this collaborative study was to document the quantification of the individual folate vitamers, and total folate analyzed in foods using a single-enzyme extraction by pure GGH of plant-origin followed by LC-MS/MS. The study was designed and organized following the guidance for collaborative studies in the Nordic Committee on Food Analysis (NMKL) to estimate repeatability, internal reproducibility, and reproducibility of the method. It included analysis of six folate vitamers in seven food matrices representing different food groups such as cereals rich in starch, containing low levels of folate, or offal rich in proteins containing high levels of folate. Eight European laboratories implemented the method, including the sample homogenization step, and successfully analyzed the seven test samples.

2. Materials and methods

2.1. The collaborative study

2.1.1. Study design

A study plan for quantification of the six folates; 5-CH₃-H₄folate, H₄folate, folic acid (PteGlu), 5-HCO-H₄folate, 5,10-CH⁺=H₄folate and 10-formylfolic acid (10-HCO-PteGlu), in foods by a method using pure GGH of plant origin combined with detection by LC-MS/MS (Ložnjak et al., 2019) was designed by the description for collaborative studies in NMKL (NMKL, 2005). Seven test samples were analyzed in a uniform model. Apart from duplicate analyses on the same days, a third determination was performed on another day within one week in order to obtain data for internal reproducibility. Before the analyses of the test samples, the participating laboratories analyzed a standard solution and five practice samples in order to get familiar with the method. Practice samples included non-homogenized samples of strawberry, high-fat cheese and green peas, and homogenized samples of other batches of strawberry and high-fat cheese than the ones used in the main study. In this way, all participants got familiar with homogenization procedure (data not presented).

2.1.2. *Participating laboratories*

Eight laboratories competent in the analyses of folate and LC-MS/MS accepted to participate in the study. They were located in seven different European countries at universities, research institutes, contract analytical services, and food manufacturers.

2.1.3. *Instruction and delivered materials to the participating laboratories*

Each participating laboratory received a registration form, a study protocol, a detailed method description, a questionnaire, electronic templates for the report of the results, a package containing the above mentioned standard solution, the five practice samples, and seven test samples (Section 2.2). In order to ensure the optimal performance of the method and to avoid an economic burden, participating laboratories were provided with the GGH plant enzyme, the SPE cartridges, the standards, and the internal standards (Section 2.3). All participants used the same coffee grinder (Rommelsbacher EGK 200) for sample homogenization.

2.1.4. *Reports from the participating laboratories*

The detailed method description was supposed to be strictly followed, and any deviation from the method had to be reported together with the information on equipment in the questionnaire. The reported results from the standard solution and the practice samples were evaluated and approved by the study coordinator (DTU Food) before the laboratory could start the analyses of the test samples. The reports on the test samples included the content of each of the six folate vitamers in the test samples, information on the weight of the test sample taken for analysis, calibration curves of each folate form, and additional comments where needed.

2.1.5. Conduct of the study

The invitation letters were sent in April 2019, the package of information and material was shipped on 22nd of May 2019 and received by the laboratories within two days. The last results from the laboratories were received on the 13th of September 2019.

2.2 Samples

2.2.1 Rationale for the form and type of samples

The critical step in collaborative studies is to ensure that samples are homogeneous in order to decrease the variance in the analytical results that are not related to the intrinsic method variability caused by e.g. the interaction of the analyte with reagents from various suppliers (AOAC, 2005). The homogenization step of fresh foods is part of the method description and was thus included. Two practice samples and four test samples were delivered as representative subsamples, which each laboratory would homogenize prior to analysis. Three practice and three test samples were delivered in homogenized frozen form, ready for analysis without further homogenization. The seven test samples were representative of fruits, vegetables, dairy products, legumes, offal, meat/fish, and fortified infant formula. These foods were chosen based on our previous round robin study performed on 25 different foods, except for salmon which replaced meat (Ložnjak et al., 2020). Information about each of the seven samples is provided in Table 1. The fortified infant formula was a certified reference material, NIST 1869, which was included in order to ensure documentation for trueness in the collaborative study. The LOQ of the method for each

of the folate vitamers was previously assessed in the single-laboratory validation to 0.1 µg/100 g when 1 g of sample was taken for analysis (Ložnjak et al., 2019).

Table 1 Information about the test samples included in the collaborative study

2.2.2 *Non-homogenized test samples*

Fresh strawberries, fresh spinach, yogurt, and dry lentils were each bought from the same batch in the amount specified in Table 1. Strawberries were each cut into eight parts separated in the middle of the strawberry followed by division into subsamples of 40 g, each containing a maximum of 1 slice from the same strawberry. The whole spinach leaves taken from the six packages were divided into the subsamples of 40 g. The two litres of yogurt were mixed, followed by sampling into each subsample of 40 mL. The three bags of lentils were treated similar to yogurt and weighed into 40 g subsamples. The procedure to ensure each subsample to be representative is described in detail elsewhere (Ložnjak et al., 2020). Each subsample was frozen in liquid nitrogen and packed in vacuum-sealed silver aluminum foil bags, followed by replacing atmospheric air by pure nitrogen gas, in order to protect the sample from oxygen and light during transport and storage.

2.2.3 *Homogenized test samples*

Pork liver and farmed Atlantic salmon were bought in an amount of approximately 0.5 kg each, and treated similarly to produce representative subsamples. The fresh samples were cut into smaller pieces and instantly frozen by liquid nitrogen. Frozen pieces were transferred into a coffee grinder in batches of 40 g, where they were homogenized in 2 intervals using 20 s cycles. The 4-bladed cup was used, and it was important to stop grinding after 20 s in order to keep the temperature low and prevent the homogenized sample from thawing. The homogenized sample was collected in a box surrounded by liquid nitrogen (for salmon illustrated in SOM, Fig. S2). Prepared homogenized samples were divided into subsamples of 5 g. Six packages of infant formula (NIST 1869) were mixed and divided into bags containing 2 g each.

2.2.4. *Storage and shipment*

All produced bags were stored at -80 °C for a maximum of three weeks until shipped with dry ice. The bags were received at the participant's laboratories within two days and stored at -80 °C, but at one laboratory at -18 °C until analyses.

2.3. Analytical method

The analytical method has previously been described in detail elsewhere (Ložnjak et al., 2019). All work was performed in fume hoods under yellow light, using brown glass flasks or wrapping the transparent glass with aluminum foil in order to protect folates from light. Apart from general analytical or MS-grade chemicals present in an analytical lab, specifications of the GGH deconjugase and standards are provided below.

2.3.1 Deconjugase

Expression construct harboring a short version of *Arabidopsis* γ -glutamyl-hydrolase was produced by genetic engineering from *Arabidopsis thaliana* at University of Florida (Orsomando et al., 2005). The recombinant plant-origin GGH enzyme (EC 3.4.19.9) was produced and recovered from the *Escherichia coli* BL21 (DE3) pLysS supernatant and purified by Ni-NTA Agarose column. The procedure of enzyme production was previously described in detail (Ložnjak et al., 2019; Orsomando et al., 2005; Ramos-Parra et al., 2013). Recovered protein was stored in a buffer (100 mM potassium phosphate, pH 6.0, 10% glycerol, and 10 mM β -mercaptoethanol) and its purity was checked by SDS-electrophoresis at DTU Food by a procedure described in details elsewhere (Ložnjak et al., 2019; Orsomando et al., 2005). The activity of enzyme solution (100 μ g of enzyme/g of sample) was tested in the green peas extract spiked by 5-glutamyl-folic acid, and it was determined to be $119 \pm 7\%$ during 1-hour incubation.

2.3.2 Standards and calibration curve

The standards 5-CH₃-H₄folate as calcium salt, H₄folate, PteGlu, 5-HCO-H₄folate as calcium salt, 5,10-CH⁺=H₄folate as chloride, and 10-HCO-PteGlu were bought from Schircks (Jona, Switzerland). The labeled

internal standards $^{13}\text{C}_5\text{-5-CH}_3\text{-H}_4\text{folate}$, $^{13}\text{C}_5\text{-H}_4\text{folate}$, $^{13}\text{C}_5\text{-PteGlu}$, and $^{13}\text{C}_5\text{-HCO-H}_4\text{folate}$ were generously provided by Merck Eprova (Schaffhausen, Switzerland).

Each participating laboratory received weighed standard unlabeled powder (approx. 10 mg) from which stock solutions ($\sim 400\text{ }\mu\text{g/mL}$) in Buffer I (0.05 M acetate buffer, 2% ascorbic acid, 0.1% β -mercaptoethanol, pH=7.0) were prepared. The calculation of the concentration of each standard included correction for the moisture content, the purity (provided by the supplier), and salt content in the standard powder. Moisture content for PteGlu was 7.9% and for 5-HCO-H₄folate 14.9%. The purity of the standards was in the range from 96.4 to 99.6%. The stock solutions were divided into 0.2 mL aliquots in microcentrifuge tubes in order to avoid freeze and thaw cycles. The preferable storage temperature was $-80\text{ }^\circ\text{C}$; however, one laboratory stored at $-18\text{ }^\circ\text{C}$.

Standard solutions of 1, 5, 10, 50, 100, 500, and 1000 ng/mL containing a mixture of unlabeled standards (5-CH₃-H₄folate, H₄folate, PteGlu, 5-HCO-H₄folate, 5,10-CH⁺=H₄folate and 10-HCO-PteGlu) were prepared fresh in Buffer II (0.05 M acetate buffer, 1% ascorbic acid, 0.1% β -mercaptoethanol, pH= 6.0). Each of them contained 20 ng/mL of $^{13}\text{C}_5\text{-5-CH}_3\text{-H}_4\text{folate}$, and 40 ng/mL of $^{13}\text{C}_5\text{-H}_4\text{folate}$, $^{13}\text{C}_5\text{-PteGlu}$, and $^{13}\text{C}_5\text{-5-HCO-H}_4\text{folate}$. The calibration curve was the ratio of the concentration of each calibration standard (ng/mL) of a specific folate vitamer and its internal standard (ng/mL) against the corresponding measured peak areas of that particular folate vitamer and its internal standard. $^{13}\text{C}_5\text{-5-HCO-H}_4\text{folate}$ was furthermore used as the internal standard for quantification of both 5,10-CH⁺=H₄folate and 10-HCO-PteGlu, as they were co-eluting with 5-HCO-H₄folate (Fig. 1), whereas for the other folate vitamers respective internal standards were used. The recommended calibration curves for all vitamers were second-order or quadratic, $1/x^2$ weighed, and not forced through curve origin (0,0). However, one laboratory used a linear calibration curve for quantification of 5-CH₃-H₄folate, H₄folate, PteGlu, and 5-HCO-H₄folate, as it had satisfactory $R^2 > 0.999$. All laboratories received aliquots of two separate standard solutions (approx. 500 ng/mL) of 5-CH₃-H₄folate and PteGlu, which served to compare calibration between laboratories.

Figure 1 Extracted ion chromatogram of folate analysis showing the elution order and corresponding retention time of folate vitamers.

2.3.3 Procedure

An overview of the method procedure is shown in Fig. 2.

Figure 2 Overview of the analytical method for the analysis of folate vitamers in food. Participating laboratories performed homogenization step for strawberry, spinach, yogurt and lentils, and DTU for pork liver and salmon. No homogenization was done for infant formula. The LC-MS/MS analysis was performed using multiple reaction monitoring (MRM).

Non-homogenized samples, i.e., strawberry, spinach, yogurt, and lentils, had to be homogenized by each laboratory. The 40 g was taken directly from the freezer into the coffee grinder, where it was homogenized for a maximum of 20 s or until the sample became a powder. The weight of test samples used for the analysis was 0.5 ± 0.1 g for strawberries, spinach, yogurt, lentils, and salmon, while 0.25 ± 0.025 g of pork liver and infant formula was weighed for analysis.

The system of choice was LC-MS/MS equipped with electrospray ionization (ESI) source. Equipment and conditions used in the collaborative study are provided in SOM, Table S3. All laboratories used the mobile phase A (2.5 mM ammonium formate in water: methanol (95:5)) and mobile phase B (2.5 mM ammonium formate in methanol). The primary gradient used: 0.0 - 0.1 min 5% B; 2.0 - 2.10 min 20% B; 4.10 - 9.0 min 5% B. One laboratory changed the gradient in order to have the separation required for their system. The column temperature was 30 °C, the flow rate of 0.4 mL/min, and the injection volume was 5 µL. The temperature in the autosampler was kept at 5 °C.

The method by Ložnjak et al. (2019) was used and slightly modified, as $^{13}\text{C}_5\text{-H}_4\text{folate}$ was included as the internal standard for H_4folate in this study. The multiple reaction monitoring (MRM) transitions are shown in Table 2.

Table 2 MRM transitions and corresponding MS parameters for Agilent 6470 Triple Quadrupole for different folates used for their detection. Underlined $^{13}\text{C}_5$ -labeled internal standards were used for the quantification of following folate vitamers.

2.4 Calculations

2.4.1 Calculation of the total folate content

In order to express folate content as PteGlu equivalents, the content of the individual folate vitamer was multiplied by the ratio of the molecular weight of PteGlu and the corresponding folate vitamers, as shown in SOM, Table S4. The sum of obtained values represented total folate content as PteGlu equivalent in $\mu\text{g}/100\text{ g}$.

2.4.2 Statistical evaluation

All data were statistically analyzed using the NMKL guidelines for the performance of collaborative studies (NMKL, 2005). The overall mean, intra-laboratory repeatability, repeatability relative standard deviation (RSD_r), internal reproducibility, internal reproducibility relative standard deviation (RSD_{IR}), inter-laboratory reproducibility, reproducibility relative standard deviation (RSD_R), and Horwitz ratio (HorRat value) were calculated. One-tail Cochran ($p=0.025$) and single and double two-tail Grubbs tests were used to determine outliers (AOAC, 2005; Horwitz, 1995; NMKL, 2005). Data below LOQ were not taken into calculation. Furthermore, if a particular laboratory reported only one determination or if the number of reported double determinations was $n < 7$, that laboratory was not statistically evaluated. HorRat values of 0.5 – 2.0 were considered satisfactory (Horwitz, 1995; Wehling & DeVries, 2012). All mean values are given with three significant digits, but two if the concentration is lower than $1\text{ }\mu\text{g}/100\text{ g}$.

3 Results

Each laboratory received a mixed standard solution of 5- CH_3 - H_4 folate and PteGlu, measured to $489 \pm 36\text{ ng/mL}$, and $589 \pm 77\text{ ng/mL}$, respectively. One laboratory did not report these results. The internal reproducibility was

4.3% for 5-CH₃-H₄folate and 2.9% for PteGlu, whereas inter-laboratory reproducibility was 7.9% and 13.3%, respectively.

The individual results received from 8 laboratories are summarized in SOM, Table S5, and S6. Eleven Cochran outliers and three Grubbs outliers were determined in a within-day analysis of six folate vitamers and total folate content in seven analyzed samples. Three Cochran and three Grubbs outliers were determined in between-day analysis. The results were statistically analyzed, as described in section 2.4.2.

In Table 3, the precision, including RSD_r, RSD_R, and HorRat values for the content of each of the six vitamers, are provided. In Table 4, similar information for the content of total folate in the samples is shown. The data for internal reproducibility RSD_{IR} are shown in SOM, Table S7. These values were, as expected, between RSD_r and RSD_R values and for total folate ranged from 7.2% for RSD_{IR} in pork liver to 49.0% for RSD_{IR} in salmon.

Table 3 Summary of precision of the method for the content of the six folate vitamers in the seven test samples.

Table 4 The method precision for content of total folate in the seven test samples

NIST 1869 was used as reference material in order to evaluate the trueness of the method. The certified mean (reference interval) for PteGlu in NIST 1869 is 224 µg/100 g (215-233 µg/100 g) (NIST, 2018). The vitamer distributions of the test samples are shown in SOM, Table S8. 5-CH₃-H₄folate was the most abundant folate vitamer in all tested samples except in salmon and NIST 1869 Infant/Adult Nutritional Formula, in which the major part was represented by H₄folate and PteGlu, respectively. H₄folate was the second most abundant vitamer in pork liver and lentils, and 5-HCO-H₄folate in spinach and yogurt.

4 Discussion

Eight laboratories reported results for all the seven test samples in this collaborative study in order to validate further the previous single laboratory study using LC-MS/MS for quantification of six folate vitamers in foods (Ložnjak et al., 2019). The seven food groups included were fruits, vegetables, dairy products, legumes, offal,

meat/fish, and fortified foods, represented by strawberries, spinach, yogurt, lentils, pork liver, salmon, and infant formula. Salmon was the only sample that did not result in acceptable HorRat values neither for the individual folate vitamers nor for total folate. Therefore, the results for folate in salmon are discussed in a separate paragraph following the discussion of the results for the other sample types.

5-CH₃-H₄folate is the most abundant folate form found in nature. Acceptable HorRat values (<2.0) for 5-CH₃-H₄folate in the range of 0.53 – 1.94 were found in strawberries, spinach, yogurt, lentils, pork liver, and NIST 1869. The content varied from 8.7 to 248 µg/100 g, with a repeatability of 4.4% - 10%, and reproducibility of 9.0% - 28% (Table 3). The repeatability was similar to that found in the single-laboratory validation study for foods and obtained for NIST 1869 in a multi-laboratory study on infant formula and adult/pediatric nutritionals (Bhandari, Ming, & Szpylka, 2018; Ložnjak et al., 2019).

The most sensitive folate vitamer, H₄folate, was present at the lowest level of 1.5 µg/100 g in yogurt up to 178 µg/100 g in liver. The HorRat values were in the range of 1.09 – 1.94 and considered acceptable, with repeatability and reproducibility between 8.4% - 11% and 21% - 31%, respectively. In contrast, the HorRat values for strawberry (2.36) and spinach (3.26) were not acceptable (Table 3). Even though the H₄folate content in strawberry was low (2.48 µg/100 g), the repeatability was 4.7%, while the content of 28.5 µg/100 g in spinach showed repeatability of 19%. Together with yogurt and lentils, both strawberry and spinach were distributed into representative subsamples at DTU Food, followed by homogenization at the individual laboratories prior to weighing for the analysis. The repeatability for strawberry indicates that the homogenization was successfully conducted for this matrix, which was not the case for spinach. The reproducibility values, ranging from 54% to 63%, indicate that even though the process of distributing the strawberry and spinach into representative 40 g subsamples was made as fast as possible, this process should be further optimized. The sample distribution should have been performed at a lower temperature under yellow light and/or under nitrogen. Yogurt and lentils were also distributed into representative subsamples, but for these matrices, the distribution process into subsamples was considerably shorter. The reason for the unacceptable HorRat value, i.e., high RSD_R, could also indicate that

degradation of this sensitive folate form occurred during different handling and storage conditions during sample transport and at laboratories, as within-day precision %RSD_r was shown to be acceptable. The use of internal standard compensates for the losses during extraction and chemical analysis due to the same properties of H₄folate and corresponding internal standard. Therefore, the difference in the repeatability and reproducibility in analysis of H₄folate was a result of conditions applied during sampling, storage, and homogenization.

The level of PteGlu found in tested samples was generally low and inconsistently identified and quantified by the laboratories. PteGlu was found in minor concentrations less than 1 µg/100 g in spinach, lentils, and pork liver, though in pork liver, one lab reported content of 37 µg/100 g (Table 3, SOM, Table S5 and S6). PteGlu is the most stable folate vitamer used as a food fortifier, that also occurs in unfortified foods as an oxidation product of H₄folate in minor amounts (Forssén, Jägerstad, Wigertz, & Witthöft, 2000). HorRat value for lentils was acceptable, but not acceptable for spinach and pork liver. In these three samples, the content of H₄folate was relatively higher than in strawberry and yogurt (Table 3), and the oxidation to PteGlu occurred at a quantifiable level. NIST 1869 Infant/Adult Nutritional Formula is fortified with PteGlu at a level 225 µg/100 g (certified range 215 – 233 µg/100 g), showing a HorRat value of 0.70. One laboratory identified as an outlier reported an issue with the gelatinization of the NIST 1869 sample, which might be the reason for their exclusion.

5-HCO-H₄folate and 5,10-CH⁺=H₄folate were identified in all samples. 5-HCO-H₄folate was present in the range of 1.4 µg/100 g in strawberries to 32 µg/100 g in spinach, whereas the content of 5,10-CH⁺=H₄folate varied from 1.5 µg/100 g in strawberries to 37 µg/100 g in pork liver. HorRat values for 5-HCO-H₄folate were acceptable (0.92 – 1.85), except for pork liver (2.40), while for 5,10-CH⁺=H₄folate, none of the HorRat values was acceptable. 10-HCO-PteGlu was present in spinach, lentils, and NIST 1869 at levels from 1.7 µg/100 g to 4.9 µg/100 g, showing unsatisfactory HorRat values (Table 3). Ložnjak et al. (2019) studied interconversion between 5-HCO-H₄folate, 5,10-CH⁺=H₄folate, and 10-HCO-PteGlu, and found that ~50% of 5,10-CH⁺=H₄folate and <3% of 10-HCO-PteGlu was converted to 5-HCO-H₄folate during the analytical procedure. Jägerstad & Jastrebova (2013) reported that 5,10-CH₂=H₄folate is less stable than 5-HCO-H₄folate and 10-HCO-PteGlu at pH values around neutral,

which might be the reason for its interconversion. Pfeiffer, Rogers, & Gregory (1997) reported complete oxidation of naturally occurring 10-HCO-H₄folate to 10-formyldihydrofolate (10-HCO-H₂folate) or 10-HCO-PteGlu in cereals. These results indicate the occurrence of interconversion between sensitive formyl folate vitamers and that inconsistency of interconversion affects the reproducibility of the results for the content of formyl folates. Konings et al. (2001) reported the presence of 10-HCO-PteGlu in spinach and legumes, which is in accordance with our results (Table 3). In the same study, contents of 10-HCO-H₂folate in raw spinach, and especially in cooked vegetables, were also reported. It is very likely that the food matrix and the content and distribution of folate vitamers affect their stability related to interconversion. Due to the interconversion of formyl folates in food, it is recommended to report their content as the sum of 5-HCO-H₄folate, 5,10-CH⁺=H₄folate and 10-HCO-PteGlu calculated as folic acid equivalents (Jägerstad & Jastrebova, 2013; Ložnjak et al., 2019). The sum of formyl forms did not result in acceptable HorRat values in strawberries, spinach, pork liver, and NIST 1869, although the HorRat values for 5-HCO-H₄folate in tested food matrices are acceptable, except in pork liver.

The sum of identified folate vitamers, or total folate, ranging from 17 µg/100 g in yogurt to 490 µg/100 g in pork liver, showed acceptable HorRat values (0.6 – 1.94), as well as repeatability and reproducibility between 2.3% - 15% and 8.7% - 28%, respectively (Table 4). In the multi-laboratory study using LC-MS/MS system for the AOAC-method, NIST 1869 was reported to 6.8% and 11.1% for repeatability and reproducibility (Bhandari et al., 2018). In our study, a better precision was indicated, by repeatability and reproducibility being 4.5% and 8.7%, respectively. Using microbiological assay for total folate content ranging from 50 to 1330 µg total folate/100 g in whole-meal flour, milk powder, mixed vegetables, and pig's liver, the intra- and inter-laboratory precision were estimated to 4.9% - 14.8% and 13.8 - 22.3%, respectively (Finglas et al., 1999). Homogeneous and dry reference materials intended to become certified reference materials were used, whereas, in our study, fresh samples were handled. The reason for the differences in repeatability and reproducibility between spinach and matrices in our study could be the combination of the thin spinach leaf, which might break and increase the loss of H₄folate, which contributes 15% to the total folate content.

In salmon, the results for the individual folate vitamers (1.6 – 13.1 $\mu\text{g}/100\text{ g}$) and total folate (23.2 $\mu\text{g}/100\text{ g}$) were not acceptable, due to the HorRat values being above 2. The repeatability and reproducibility were between 8.6% - 18%, and 58% - 69%, respectively (Table 3 and 4). Thus, the repeatability was similar to the other sample types, while the reproducibility values were higher. Salmon was the representative for a fat containing matrix in this trial. The fat-containing samples of peanuts and cheese were included in a round robin study that compared the method used in this study with a LC-MS/MS method using deconjugase of animal origin and microbiological assay using tri-enzyme extraction. The results from the round robin study indicated that the presence of fat did not interfere in the quantification of folate (Ložnjak et al., 2020). Our results for H₄folate and 5-CH₃-H₄folate as the most abundant folate vitamers in salmon, are in accordance with a previous study that analyzed folate distribution in fish (Vahteristo, Ollilainen, & Varo, 1997). The reason for the unsatisfactory results for the reproducibility might be due to the level and type of folate vitamer combined with the procedure used for salmon. The content of 5-CH₃-H₄folate in salmon is at the same level as in yogurt, while the internal reproducibility is 38% in salmon, but 13% in yoghurt. Lentils were the matrix which contained a comparable amount of H₄folate as in salmon, however the internal reproducibility was 52% for salmon and 13% for lentils (SOM, Table S7). Yogurt and lentils were homogenized by the participants, whereas salmon was provided to participants in homogenized form in small amounts (5 g). This sample condition could be prone to freeze-thaw cycles during the transport and storage of the sample in the participating laboratories. The pork liver was delivered in the same way as salmon, but the content of folate vitamers in pork liver is at a minimum, 10 times higher, and therefore showed acceptable HorRat values for 5-CH₃-H₄folate and H₄folate.

Infant formula (NIST 1869), with a certified value at 224 $\mu\text{g PteGlu}/100\text{ g}$ (NIST, 2018), was used to evaluate the trueness for PteGlu obtained by 8 different laboratories. One laboratory experienced problems, gelatinization after the second boiling step, and was statistically identified as an outlier and excluded from the calculation of the trueness. The overall mean of 7 laboratories was 225 $\mu\text{g PteGlu}/100\text{ g}$, which was in agreement with the certified value. Besides, the trueness of the method has been evaluated satisfactory as a part of single-laboratory validation

by the analyses of the certified reference material of the foods as pig liver (BCR 487), meat homogenate (NIST 1546a), and infant formula (NIST 1846 and NIST 1849) (Ložnjak et al., 2019).

Due to the cost of standards, we chose to supply the participating laboratories with the standards and include a standard solution of 5-CH₃-H₄folate and PteGlu to compare quantification in different laboratories. The reported results obtained at two different days were acceptable as RSD_{IR} and RSD_R were generally lower than expected for food samples i.e. <5% and 15%, respectively. It should be mentioned that quality control of standards by a spectrophotometric method, as introduced by van der Berg et al. (1994), should be part of a future standardized NMKL-method, and if implemented in laboratories in the future.

Recommended storage temperature for standards was -80 °C. One laboratory stored standards and samples at -18 °C, which did not result in statistical outlier results for any of the samples stored at -18 °C. The chromatographic system used in this study was chosen to have a generic LC-system, which could also be used for analyses of vitamin D (Barnkob, Petersen, Nielsen, & Jakobsen, 2019). It was known to provide a shift in retention time between standard solution and sample extract but resulted in a prolonged duration of analysis for some laboratories. The alternative chromatographic system could consist of an alternative column and eluents containing acetic acid. Comments to the method procedure were also to use a larger volume to reconstitute after SPE step e.g., 500 µL instead of 250 µL in order to enable easier use of the method, which could improve the precision. The recommended calibration curve was of second-order and 1/x² weighed, but due to the ability at each laboratory, 1/x² weighed quadratic and linear calibration curves were also applied. This change did not result in outliers, even though the risk in using a linear calibration curve is a decrease in accuracy obtained at lower levels of the curve, even when the variation coefficient is >0.99.

The strength of the study is that we met the requirement for standardization of an NMKL method on the number of accepted results from a minimum of five laboratories if the method involves very special equipment or very specialized technical skills. For this study, the participants were selected due to their equipment and skills. Eight

European laboratories from seven countries successfully implemented the method. The homogenization step, including the freezing of the sample by liquid nitrogen, enabled a fast sample preparation and resulted in satisfactory homogeneity. Despite different storage temperatures of the samples and standards, instruments, chromatographic conditions, and calibration curves were applied, the results were still acceptable, which confirmed the robustness of the method.

The limitation of the study is that due to restriction of cost, no spectrophotometric control of standard solutions was performed at each laboratory. DTU Food provided the weighted standards to each laboratory, which might have resulted in an improvement of the precision. The method includes quantification of six folate vitamers, but especially for cereals, the inclusion of 10-HCO-H₂folate is essential. Sample amount for pork liver was half of the amount for the other fresh samples, which might have resulted in a lower precision. Preferably, the number of the participating laboratories should have been higher to allow the evaluation of all combinations of folate vitamers and test samples with a minimum of eight laboratories, as preferred, e.g., by the European Committee for Standardization.

The reported results from the collaborative study for a fast and efficient single-enzyme extraction of folate vitamers obtained by the use of plant-origin GGH combined with specific LC-MS/MS provided acceptable documentation for an NMKL-method. Previous studies have reported discrepancies of ~20% between microbiological assay and chromatographic methods for folate analysis in food (Finglas et al., 1999; Konings et al., 2001; Ložnjak et al., 2020). This difference indicates that there is an uncertainty in folate content assessment. Microbiological assay might overestimate folate content due to other non-folate compounds responsible for microbial growth (Koontz et al., 2005). In contrast, chemical methods combined with the use of internal standards enable specific folate quantification. In the future, this new method may be used for studies on data for food composition databases, retention of specific folate vitamers, bioaccessibility, and bioavailability, as it distinguishes naturally occurring folates from folic acid. To make the enzyme of plant origin commercially available, the possibilities of production are being investigated. Costs are expected to be at the same level as for the currently used enzymes.

5 Conclusion

A collaborative study was conducted with eight European laboratories in order to establish documentation for a future standard method capable of quantifying folate vitamers, including folic acid, and total folate in foods. The method included plant-origin GGH in the extraction and LC-MS/MS for the quantification. The method proved satisfactory repeatability and reproducibility to quantify total folate content as the sum of 5-CH₃-H₄folate, H₄folate, PteGlu, 5-HCO-H₄folate, 5,10-CH⁺=H₄folate, and 10-HCO-PteGlu expressed as PteGlu equivalents in strawberry, spinach, yogurt, lentils, pork liver, and infant formula, but not in salmon. Similar results were obtained for the most abundant folate vitamer, 5-CH₃-H₄folate, while for 5-HCO-H₄folate, PteGlu, and H₄folate, the results depended on the food type. Lower precision was observed for vitamers present at low levels in the sample, whereas quantification of 5,10-CH⁺=H₄folate and 10-HCO-PteGlu was unacceptable. The method met NMKL criteria for most vitamers and total folate content in the successfully analyzed matrices mentioned above.

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Table 1: Information about the test samples included in the collaborative study.

Sample ID	Sample type	Foodstuff	Sample condition	Homogenization in each laboratory*	Sample purchased	Representative sample per bag
IC-S6	Fruit	Strawberry	Fresh	Yes	2.0 kg	40 g
IC-S7	Vegetable	Spinach	Fresh	Yes	1.2 kg	40 g
IC-S8	Dairy product	Yogurt	Fresh	Yes	2.0 L	40 mL
IC-S9	Legumes	Red lentils	Dry	Yes	1.2 kg	40 g
IC-S10	Offal	Pork liver	Fresh	No	0.5 kg	5 g
IC-S11	Fish	Salmon	Fresh	No	0.5 kg	5 g
IC-S12	Infant formula	NIST 1869 Infant/Adult Nutritional Formula	Dry	No	0.06 kg	5 g

* No: the sample was homogenized at DTU Food.

^aLožnjak et al., 2020

^bDelchier et al., 2016

^cVahteristo et al., 1997

^dNIST, 2018

Table 2 MRM transitions and corresponding MS parameters for Agilent 6470 Triple Quadrupole for different folates used for their detection. Underlined $^{13}\text{C}_5$ -labeled internal standards were used for quantification of following folate vitamers.

Analyte	Precursor ion (m/z)	Quantifier	Qualifier	Fragmentor	Collision energy (eV)
$^{13}\text{C}_5$ -5- CH_3 - H_4 folate	465.2	313.1		88	17
5- CH_3 - H_4 folate	460.2	313.2		100	17
	460.2		180.2	100	40
$^{13}\text{C}_5$ - H_4 folate	451.2	299.1		84	17
H_4 folate	446.2	299.1		84	17
	446.2		120.0	84	40
$^{13}\text{C}_5$ -PteGlu	447.1	295.1		84	9
PteGlu	442.1	295.1		84	8
	442.1		176.0	84	40
$^{13}\text{C}_5$ -5-HCO- H_4 folate	479.2	327.2		96	15
5-HCO- H_4 folate	474.2	327.2		96	15
	474.2		299.1	96	37
5,10- $\text{CH}+=\text{H}_4$ folate	456.2	412.2		156	29
	456.2		324.2	156	37
10-HCO-PteGlu	470.1	295.1		104	29
	470.1		176.1	104	40

Table 3: Summary of precision of the method for the content of the six folate vitamers in the seven test samples.

Folate vitamer	Sample No.	Sample	Total No. of labs	Total No. of replicates	Overall mean of all data	RSD _T	RSD _R	HorRat
					µg/100 g	%	%	
5-CH ₃ -H ₄ folate	1	Strawberry	7	14	68.5	4.4	9.0	0.53
	2	Spinach	8	16	117	12	28	1.79
	3	Yogurt	8	16	8.70	7.2	21	0.92
	4	Lentils	7	14	62.7	2.3	25	1.45
	5	Liver, pork	8	16	248	10	27	1.94
	6	Salmon	8	16	5.39	8.6	67	2.91*
	7	NIST 1869	8	16	26.6	8.3	13	0.67
H ₄ folate	1	Strawberry	7	14	2.48	4.7	54	2.36*
	2	Spinach	8	16	28.5	19	63	3.26*
	3	Yogurt	7	14	1.49	10	31	1.37
	4	Lentils	8	16	26.0	11	21	1.09
	5	Liver, pork	8	16	178	8.4	28	1.94
	6	Salmon	8	16	13.1	14	69	3.17*
	7	NIST 1869	5	10	<0.1 – 0.50	n.e.	n.e.	n.e.
PteGlu	1	Strawberry	8	16	<0.1 – 0.36	n.e.	n.e.	n.e.
	2	Spinach	7	14	0.69	20	69	3.01*
	3	Yogurt	8	16	<0.1 – 0.56	n.e.	n.e.	n.e.
	4	Lentils	6	12	1.01	8.0	43	1.87
	5	Liver, pork	8	16	<0.1 – 2.88	n.e.	n.e.	n.e.
	6	Salmon	7	14	1.25	7.4	52	2.26*
	7	NIST 1869	7	14	225	4.9	10	0.70
5-HCO-H ₄ folate	1	Strawberry	8	16	1.40	18	25	1.06
	2	Spinach	8	16	31.5	14	35	1.85
	3	Yogurt	7	14	4.92	8.3	23	1.00
	4	Lentils	7	14	14.2	2.8	23	1.07
	5	Liver, pork	8	16	20.8	17	49	2.40*
	6	Salmon	8	16	1.55	18	66	2.89*
	7	NIST 1869	7	14	1.60	15	21	0.92
5,10- CH ⁺ =H ₄ folate	1	Strawberry	6	12	1.49	18	68	2.95*
	2	Spinach	7	14	11.6	15	122	5.53*
	3	Yogurt	6	12	1.94	12	94	4.09*
	4	Lentils	8	16	6.54	27	79	3.46*
	5	Liver, pork	8	16	37.1	37	105	5.69*
	6	Salmon	6	12	2.48	19	58	2.53*
	7	NIST 1869	5	10	<0.1 – 2.2	n.e.	n.e.	n.e.

Table 3: Summary of the precision of the method for the content of the six folate vitamers in the seven test samples. (*continued*)

Folate vitamer	Sample No.	Sample	Total No. of labs	Total No. of replicates	Overall mean of all data	RSD _r	RSD _R	HorRat
					µg/100 g	%	%	
10-HCO-PteGlu	1	Strawberry	8	16	<0.1 – 0.24	n.e.	n.e.	n.e.
	2	Spinach	7	14	4.81	42	103	4.50*
	3	Yogurt	8	16	<0.1 – 0.88	11	57	2.51*
	4	Lentils	6	12	1.73	15	64	2.78*
	5	Liver, pork	8	16	<0.1 – 0.72	n.e.	n.e.	n.e.
	6	Salmon	8	16	<0.1 – 0.19	n.e.	n.e.	n.e.
	7	NIST 1869	7	14	4.87	49	105	4.55*
Formyl forms	1	Strawberry	8	16	2.59	11	51	2.24*
	2	Spinach	8	16	52.9	13	53	3.00*
	3	Yogurt	7	14	6.92	7.2	34	1.50
	4	Lentils	8	16	5.83	11	35	1.74
	5	Liver, pork	8	16	276	29	84	4.81*
	6	Salmon	8	16	3.44	18	75	3.26*
	7	NIST 1869	8	16	6.48	43	85	3.70*

Formyl forms – sum of 5-HCO-H₄folate, 5,10-CH₂=H₄folate, 10-HCO-PteGlu expressed as PteGlu equivalent

n.e. – not statistically evaluated due to the lack of reported data

n.q. – not quantified

* – HorRat values were considered unacceptable

NIST 1869 – Infant/Adult Nutritional Formula

Table 4: The method precision for content of total folate in the seven test samples.

Sample No.	Sample	Total No. of labs	Total No. of replicates	Overall mean of all data, $\mu\text{g}/100\text{ g}$	RSD _r	RSD _R	HorRat
					%	%	
1	Strawberry	7	14	73.8	4.2	10	0.60
2	Spinach	8	16	200	9.9	28	1.94
3	Yogurt	8	16	17.2	15	22	1.04
4	Lentils	7	14	112	2.3	19	1.22
5	Liver, pork	8	16	490	6.0	22	1.77
6	Salmon	8	16	23.2	11	58	2.93*
7	NIST 1869	7	14	259	4.6	8.7	0.63

* – HorRat values were considered unacceptable

NIST 1869 – Infant/Adult Nutritional Formula

Highlights:

- Specific LC-MS/MS method for folate analysis in food is inter-laboratory validated
- Total folate results acceptable in six tested food samples; for salmon unacceptable
- HorRat values for total folate and 5-CH₃-H₄folate were within the acceptable range
- Acceptability of HorRat values for other studied vitamers depends on food matrix
- For total folate: repeatability at 2.3% to 15% and reproducibility at 8.7% to 58%

CRedit authorship contribution statement:

Petra Ložnjak Švarc: conceptualization, methodology, validation, formal analysis, investigation, visualization, writing – original draft, writing – reviewing and editing. **Eystein Oveland:** conceptualization, investigation, writing – reviewing and editing. **Hanna Sara Strandler:** conceptualization, investigation, writing – reviewing and editing. **Susanna Kariluoto:** conceptualization, investigation, writing – reviewing and editing. **Esther Campos Giménez:** investigation, writing – reviewing and editing. **Elise Ivarsen:** investigation, writing – reviewing and editing. **Isabelle Malaviole:** investigation, writing – reviewing and editing. **Carla Motta:** investigation, writing – reviewing and editing. **Michael Rychlik:** writing – reviewing and editing. **Lisa Striegel:** investigation. **Jette Jakobsen:** conceptualization, methodology, validation, writing-original draft, writing – reviewing and editing, supervision, project administration, funding acquisition.