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Origins of the difference between food folate analysis by LC-MS/MS and microbiological assay and quantitation of an oxidation product of 5-CH_3-H_4folate

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***Running head*: LC-MS/MS vs. Microbiolocial assay**

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ABSTRACT

To investigate the often reported disagreement in food folate quantitation between the microbiological assay and HPLC methods, different foods were analyzed both by the microbiological assay and an LC-MS/MS method in the present study.

For the LC-MS/MS analysis we emphasize the need for a complete deconjugation of polyglutamic folate forms. Moreover, our results revealed no necessity for an additional enzyme treatment except from the deconjugation step. To check the efficiency of deconjugation without additional sample preparations a quantification of the amount of diglutamates and a screening for additional polyglutamates was applied. An intense investigation of a substance with a polyglutamate chain being deconjugated like the folates revealed that it was an oxidation product of 5-CH_3-H_4folate, a pyrazino-s-triazine called MeFox in previous reports. The latter is not microbiologically active and, therefore, does not contribute to the amount of total folates. But we found it being commonly present in foods, especially in those being low in ascorbic acid.

The microbiological assay showed different responses to the single vitamers. Therefore, it was necessary to calibrate with the folate, which had the highest portion of the folate distribution.

The investigations showed that both methods can provide similar results, when including for both a deconjugation step. This is particularly important for LC-MS/MS but probably also for the microbiological assay. Additionally, considering the folate distribution was found to be crucial for the accurate calibration of the microbiological assay.

1. Introduction

Folates are a group of water-soluble vitamins playing a key role in C_1-metabolism. For their analysis, a microbiological assay and HPLC-methods with various detectors (i.a. UV, Fluorescence and MS) are commonly applied. Whereas the microbiological assay is very sensitive but reveals only the total amount of folates the HPLC allows to differentiate and quantitate the single vitamers. However, in particular for HPLC attention must be paid to a good sample clean up and to a complete deconjugation to the respective folate monoglutamates. But even for the microbiological assay a certain degree of deconjugation is necessary, because the mostly applied microorganism *Lactobacillus rhamnosus* has a similar response to mono-, di- and triglutamates, but a decreasing activity with further increasing chain length [1].

For both methodologies, an extraction with three different kinds of enzymes is recommended [2], the so called “trienzyme treatment” introduced by Martin et al. [3]. Here, in addition to the enzymes for deconjugation, additional treatment with α-amylase and protease were applied.

There are several reports on the results from the microbiological assay being higher than those from HPLC-methods [4–7]. However, not always a direct comparison with the same samples was executed. As possible reasons for the differences observed in direct comparisons either unidentified folates or substances being active in the microbiological assay were hypothesized. [5,6]. In contrast to this, other studies did not find a difference between these two methods [2]. To answer the question about a difference between the microbiological assay and the LC-MS/MS, we performed a direct comparison. A further objective was to investigate the need for the “trienzyme treatment” by surveying and if necessary improving the efficiency of deconjugation. Moreover, we had a look on additional peaks in the chromatograms of the MRM traces of 5-HCO-H_4folate and its

polyglutamates and investigated the reportedly different response of the microbiological assay to the different vitamers[9–11].

2. Experimental

2.1. Synthesis of pyrazino-s-triazine (MeFox)

MeFox was synthesized according to Gapski et al. [12] with slight modifications. 1 mg 5-CH_3-H_4folate was dissolved in 1 ml phosphate buffer (0.1 mol/l, pH 6 instead of 5), 50 µl aqueous H_2O_2 (30%) was added and the solution was stirred for 2 h (remaining amount of 5-CH_3-H_4folate thereafter was 2 %). The residual H_2O_2 was removed by $Na_2S_2O_3$ (1 mol/l) and a spatula of NaI. The absence of H_2O_2 was verified with a test strip. After purification by semi-preparative HPLC (Pro C18 EC, 150 x 10 mm, 5 µm, 120 Å; gradient with 0.1 % (v/v) formic acid and acetonitrile) the solution was lyophilized and the product dried over concentrated sulphuric acid in a desiccator.

2.2. Folate analysis by LC-MS/MS

Distribution and amount of total folates were determined by LC-MS/MS according to a previous publication [8]. Briefly, after addition of [$^2H_4$]-labeled standards, the folates were extracted in 10 ml MES-buffer (with 0.1 % (m/v) DTT and 0.1 % (v/v) mercaptoethanol as a mixture or 1.3 % (v/v) mercaptoethanol or 0.1 % (m/v) DTT) and deconjugated by addition of 150 µl rat serum and 2 ml chicken pancreas suspension (0.33 mg/2 ml) over night. Afterwards 10 ml acetonitrile was added for precipitation of the proteins (mostly for column care), then the sample was centrifuged and purified by SAX-SPE. The cleaned extracts were measured with LC-MS/MS. To food samples, for which a higher amount of enzymes for deconjugation was necessary, 300 µl rat serum and 2 ml of a more concentrated chicken pancreas solution (2 mg/2 ml) were added, respectively 450 µl rat

serum and 4 ml of the chicken pancreas solution (2 mg/2ml). For investigating the necessity of a di- or trienzyme treatment, 10 mg α-amylase per g food sample were added and incubated for 2 h at 37°C. Thereafter, or alternatively, 3 mg protease per g food sample were added and incubated for 4 h at 37°C. After the protease treatment the enzymes were inactivated in a boiling water bath for 10 min, the sample extract was cooled in an ice bath and the enzymes for deconjugation were added (see above). When only the amylase treatment was applied no additional heat treatment was necessary.

In contrast to our publication mentioned above [8], a different HPLC column (Pro-C18, 250 x 3.2 mm, 3 µm, 130 Å, YMC, Japan; precolumn: C18 4 x 2 mm, Phenomenex, Torrance, USA), other mobile phases (A: 0.1% (v/v) formic acid, B: acetonitrile with 0.1 % formic acid (v/v)) and an adapted gradient was used. The gradient elution started at 5 % B, raising up to 10 % B in 5 minutes and staying at this composition for another 5 minutes, followed by raising to 15 % B during 10 minutes and to 50 % in 2 minutes. This composition was held for 4 minutes before being brought back to 5 % B in 2 minutes and equilibrating the column for 9 minutes.

Additionally, MeFox, folate diglutamates and higher folate polyglutamates were included into the LC-MS/MS-method. The specific MS parameters for the monoglutamates are the same as described in [8], those for the diglutamates and higher polyglutamates are given in table 1.

The response factors for quantifying the folate monoglutamates were the same as described in [8], except for 10-HCO-H_2folate (1.149 ± 0.056, internal standard: [$^2H_4$]-10-HCO-PteGlu) and 5,10-CH^+-H_4folate (1.056 ± 0.057, internal standard: [$^2H_4$]-5-CH_3-H_4folate). For the response factor of MeFox using [$^2H_4$]-10-HCO-PteGlu as the internal standard, seven concentration levels of MeFox along with a constant concentration of [$^2H_4$]-10-HCO-PteGlu were prepared in the elution buffer (5 % NaCl, 1 % ascorbic acid, 0.1 % DTT) with concentration ratios between 0.02:1 and 200:1.

For determining the response factors for the diglutamates, three different food stuffs (broccoli, baguette and pea soup) were processed in two different ways. One approach included the usual sample preparation with rat serum and chicken pancreas to generate the monglutamates and the second approach used only chicken pancreas to stop deconjugation on the diglutamate level. The difference in the amount of both approaches ($\Delta n_{Monoglutamate}$) represented the amount of the diglutamates in the second approach. The response factors RF of the diglutamates to the corresponding [$^2H_4$]-labeled monoglutamates (ISTD) were calculated as follows:

$$RF = \frac{Area(Diglutamate)}{Area(ISTD)} \cdot \frac{\Delta n(Monoglutamate)}{n(ISTD)}$$

RFs are summarized in table 1.

2.3. Microbiological assay

In lab A the sample preparation was adapted to the LC-MS/MS method: 0.2 to 2 g food sample were weighed into 50 ml PET centrifugal tubes and10 ml MES-buffer was added. The tubes were placed into a boiling water bath for 10 min and cooled in an ice bath thereafter. Then, 150 µl rat serum and 2 ml chicken pancreas solution (0.33 mg/2 ml) were added and the samples were incubated over night in a shaking water bath at 37°C. The next day the samples were cooked in a boiling water bath for 10 min, the volume of the samples was added to 20 ml and the tubes were centrifuged. The extract was diluted with a phosphate buffer (0.05 mol/l, pH 7.2, with 0.1 g/l ascorbic acid and 1.3 % v/v mercaptoethanol) and filtered with aseptic membrane filters (regenerated cellulose, 0.2 µm). For calibration, (6*S*)-5-CHO-H_4folate or (6*S*)-5-CH_3-H_4folate was used. The calibrants were dissolved in MES-buffer and diluted with the same phosphate buffer, which was used for the food samples. 150 µl of the sample respectively 150 µl of the standard solutions were pipetted into the cavities of the microbiological assay kit from R-

Biopharm (using *Lactobacillus rhamnosus*) and subsequently 150 µl of the assay medium was added. The assay tray was sealed with a self-adhesive foil and the cavities were incubated at 37°C in a small incubator. After 46 h the cavities were shaken overhead, the foil was removed and the turbidity of the sample solutions was measured at 540 nm.

In lab B the same kit was used. Briefly, according to the description of the kit, a phosphate buffer (0.05 mol/l, pH 7.2, 0.1 % ascorbate) was used for extraction and pig or chicken pancreatin for deconjugation (2 h). Afterwards, the extracts were heated at 95°C for 30 minutes, centrifuged and diluted with sterile water. For calibration, (6*S*)-5-HCO-H_4folate or PteGlu were used.

Lab C used a more classic version of the microbiological assay in test tubes, but with the same microorganism (*Lactobacillus rhamnosus*). Briefly, chicken pancreas and phosphate buffer A at pH 7.8 (0.1 mol/l, with 1% (m/v) sodium ascorbate: 13.8 g sodium dihydrogen phosphate monohydrate, 10.0 g sodium ascorbate were diluted in 900 ml distilled water, the pH was adjusted to 7.8 ± 0.1 with sodium hydroxide solution (5 % and 50 %) and the volume was added to 1 L with distilled water) were added to the samples and incubated approximately for 15 h at 37°C. The samples were centrifuged and diluted with another phosphate buffer B (0.1 mol/l, with 0.1% (m/v) ascorbic acid: 6.96 g sodium hydrogen phosphate dihydrate, 21.76 g sodium dihydrogen phosphate monohydrate and 2 g ascorbic acid were diluted in distilled water and the volume was added to 2 L). Then the medium of pH 6.1 (9.4 g Folic Acid Casei Medium from difco + 100 g phosphate buffer B) was added in equal parts and the solution was heated for 7 min at 121°C. Afterwards, the suspension of the microorganisms was added and the samples were incubated for approximately 16 to 24h at 37°C. For calibration, (6*S*)-5-HCO-H_4folate and PteGlu were used. Optical density was assessed at 546 nm.

3. Results

3.1. Optimization for the LC-MS/MS-method

3.1.1. Trienzyme treatment

The di- and trienzyme treatment is recommended for the extraction of folates from food matrices. To verify the need for these additional treatments we added α-amalyse and/or protease to the food extract of wheat germs, which are rich in starch and protein, before adding the deconjugases (chicken pancreas plus rat serum). The results are presented in figure 1.

The approach without an additional treatment, thus adding only the two deconjugases, yielded the highest amount of total folate. This amount was not significantly different ($p > 0.05$) from the result of the second approach, where α-amylase was added. Besides, adding protease gave a significant lower amount of total folates ($p < 0.01$) and adding both enzymes (trienzyme treatment) an even lower amount ($p < 0.01$). This result has not yet been optimized for deconjugation, which is described in the following paragraph.

3.1.2. Polyglutamates

To control the efficiency of deconjugation, the remaining amount of diglutamates was quantified and additionally the sample solution was screened for the presence of higher polyglutamates.

In various samples a complete deconjugation could be achieved, for example in corn meal, orange juice and baguette bread (14 µg/100g, 25 µg/100g and 30 µg/100g, respectively) or at least a nearly complete deconjugation, for example in broccoli, spinach and soybeans (176 µg/100g, 113 µg/100g and 245 µg/100g). In contrast to this, in many sample solutions of different foods di- and often even higher polyglutamates were still present after deconjugation, for example in wheat germs and peas (figure 3).

Chromatograms of the polyglutamates of 5-CH_3-H_4folate in peas are shown in figure 2 (left side).

In some cases the amount of diglutamates in relation to the sum of mono- and diglutamates (∑) was negligible, for example in broccoli with 0.3 % (∑ = 176 µg/100g), basmati rice with 0.1 % (∑ = 10 µg/100g) and tomato juice with 0.4 % (∑ = 19 µg/100g), in other cases the amount was within the range of analytical variation, e.g. cauliflower with 2 % (∑ = 88 µg/100g), lentils with 5 % (∑ = 143 µg/100g), potatoes with 4 % (∑ = 18 µg/100g), couscous with 2 % (∑ = 17 µg/100g), carrots with 3 % (∑ = 55 µg/100g) and calf's liver with 2 % (∑ = 663 µg/100g). But in pistachios this quantification revealed up to 40 % (∑ = 40 µg/100g). In contrast to this, other foods revealed high amounts of diglutamates such as mung beans with 29 % (∑ = 422 µg/100g), peanuts with 20 % (∑= 99 µg/100g), camembert with 24 % (∑ = 108 µg/100g), wheat germs with 24 % (∑ = 321 µg/100g) and peas with 33 % (∑ = 101 µg/100g).

The screening for higher polyglutamates indicated that in the samples with a higher amount of diglutamates often distinct amounts of tri- and higher polyglutamates were present. Therefore, in these cases the total amount of folates was still higher than the sum of the mono- and diglutamates. In the case of lentils, couscous and basmati rice there were no other polyglutamates than diglutamates and, therefore, the sum of mono- and diglutamates represented the total amount of folates. In the case of cauliflower, carrots and calf's liver there was only a little residue of triglutamates, which can be neglected. But there was a food sample (adzuki beans) which had only an amount of diglutamates of 4 %, which seemed not really high, but the screening of higher polyglutamates revealed a noticeable amount of these.

3.1.3 Buffer optimization for deconjugation

Due to toxicity and unpleasant odor we wanted to replace mercaptoethanol (1.3 % v/v) by DTT (0.1 % m/v), but the efficiency of deconjugation seemed worse (fig. 4). In fact, the amount of diglutamates was not significantly different, but the amount of monoglutamates appeared to be different ($p = 0.04$). When using a combined buffer of DTT and mercaptoethanol (0.1 % each) a significant rise in the amount of monoglutamates ($p= 0.002$ based on DTT buffer and $p = 0,003$ based on MCE-buffer) and a significant decrease in the amount of diglutamates ($p = 0.0014$ based on DTT-buffer and $p = 0.004$ based on MCE-buffer) could be detected. Nevertheless, the sum of mono- and diglutamates was still the same.

3.1.4. Optimization of deconjugation

The effect of a smaller sample weight as well as a higher amount of deconjugases was investigated to obtain a complete deconjugation. As can be seen in figure 3 a smaller sample weight in combination with an additional amount of enzymes was effective. In most cases 300 µl of rat serum instead of 150 µl plus a sixfold higher amount of chicken pancreas was satisfactory. But in the case of the pistachios a very low sample weight of 0.12 g, 450 µl rat serum and twice the amount of the sixfold chicken pancreas solution was necessary to reach an acceptable deconjugation (only 0.4% diglutamates were left, but no polyglutamates). In conclusion, by adapting the sample weight and the amount of enzymes for deconjugation, a successful deconjugation could be achieved.

Another possibility instead of rising the amount of enzymes is a longer incubation time. For wheat germs a low sample weight and incubation over two nights gave the same result as the addition of the higher amount of enzymes.

3.1.4 Pyrazino-s-triazine (MeFox)

In several food stuffs an additional peak was visible in the MRM chromatogram of the 5-CHO-H_4folate trace (MRM 474 ➔ 327). Interestingly, in each MRM chromatogram of the respective polyglutamates of 5-CHO-H_4folate (fragments always after splitting the whole glutamate chain) an additional peak was visible, too. The same peak in the 5-CHO-H_4folate trace appeared after incubating 5-CH_3-H_4folate in the context of investigating the stability of 5-CH_3-folate during digestion in a simulation model [13], i.e. at pH 2 in diluted hydrochloric acid for 2 h followed by adding sodium hydrogen carbonate, at pH 5.5 (for 3 h) and 6.5 (an additional hour). When adding ascorbic acid, the amount of this substance decreased. Therefore, it was assumed, that this substance, with the MRM transition of 474 ➔ 327 is an oxidation product of 5-CH_3-H_4folate. An oxidation product of 5-CH_3-H_4folate has already been reported, i. e. a substance with a pyrazino-s-triazine structure [14], which was formerly misidentified as 4a-hydroxy-5-methyl tetrahydrofolate (hmTHF) [12,15]. According to Hannisdal et al. [16] and Fazili et al. [17] this oxidation product, which has also been abbreviated MeFox, shows the MRM-transition of 474 ➔ 327. When looking at the fragments of the MS/MS-spectrum of our peak, we found it in agreement with the structure of this pyrazino-s-triazine. Therefore, we synthesized MeFox and compared it with the substance found in several food stuffs by its retention time, UV-spectrum and MS/MS-spectrum. All were in good agreement. Additionally, MeFox is known to show no activity in the microbiological assay [18], which we could also confirm.

To get an impression about its amount in foods, we quantified MeFox using [$^2H_4$]-10-CHO-PteGlu as the internal standard. We chose this standard due to the fact that it shows no interconversions during analysis and already showed good validation results in the analysis of 10-CHO-H_2folate [8], which has a similar retention time to MeFox. The results are shown in table 2. MeFox can be found in different kinds of food stuffs: in fresh vegetables like cauliflower, carrots and peas; in dried legumes like adzuki beans and soy beans; in grain respectively grain products like wheat germs, couscous, oat flakes and

spaghetti and in nuts and similar food stuffs like hazelnut , almonds, walnuts and peanuts. In some samples the amount of MeFox was quite high, for example in wheat germs, oat flakes and walnuts and the ratio to the amount of total folate was highest in couscous, rice and spaghetti. This was not surprising as these products are low in ascorbic acid and were dried or further processed.

3.2. Microbiological assay

As mentioned in the introduction the response of the microbiological assay to food folate vitamers is different [9–11]. To investigate this effect in more detail, we compared the results of folate quantitations in three different labs using different folates for calibration. As can be seen in figure 5, in all three labs there were distinct differences with respect to the folate utilized for calibration. The results for the total amount of folates differed depending on the folate and the lab between 7 and 37 %.

3.3. Comparison between LC-MS/MS and microbiological assay

After optimizing the LC-MS/MS method particularly with regard to a complete deconjugation, several food stuffs were analyzed both with LC-MS/MS and with the microbiological assay. For calibrating the microbiological assay both PteGlu and 5-CHO-H_4folate were used (lab C), except from broccoli, for which we used 5-CH_3-H_4folate as a calibrant instead of PteGlu (lab A). The comparison is shown in table 3 and the folate composition of the corresponding food in table 4.

For broccoli, the vegetable mix (BCR 485), wheat germs, chick peas and pistachios there was no significant difference between the two methods when using the folate with the highest proportion for calibration. Also for spaghetti and mung beans there was no significant difference between the results of the LC-MS/MS measurement and the

microbiological assay when using a folate as a calibrant, which was not the one with the highest proportion.

4. Discussion

As many publications report a smaller amount of folates analyzed by HPLC-methods compared to the microbiological assay we optimized our sample preparation for LC-MS/MS for completeness of extraction and deconjugation before executing the comparison.

An often recommended method is the di- or trienzyme treatment. When we compared our usual treatment before LC-MS/MS including only deconjugases (“single treatment”) with the additional treatments with α-amylase and / or protease (“dienzyme” and “trienzyme treatment”) the latter two showed no benefits compared to the “single treatment”. We assume that the application of chicken pancreas for deconjugation made an additional enzyme treatment with α-amylase and / or protease obsolete due to the fact that this source of deconjugase also contains further enzymes like amylases [19].

Then we had a look on the completeness of folates’ deconjugation by analyzing the amount of diglutamates and screening for the presence of higher polyglutamates by LC-MS/MS. In several samples the deconjugation was complete, whereas in other samples the deconjugation activity was not sufficient (diglutamates were present and in some samples tri- and higher polyglutamates, too). However, the amount was not expected to be too high, for example in pistachios, buck wheat and camembert. One reason could be the presence of other polyglutamates, which were deconjugated by the same enzymes, for example MeFox, the oxidation product of 5-CH_3-H_4folate. However, in camembert neither the amount of folates nor the amount of MeFox should be too high. Therefore, deconjugation inhibitors may be present, as was already mentioned in earlier publications

[7,20]. As such, glutamic acid [21], citric acid [22] or even fatty acids could be conceivable. The latter can inhibit the activity of enzymes [23], so that an inhibition of the deconjugation enzymes in rat serum and chicken pancreatin is likely. Even the components of the buffer can influence the deconjugation, as revealed by our experiment with different amounts of DTT and mercaptoethanol. It seemed, that mercaptoethanol can both promote and in higher amounts inhibit the deconjugation. The promoting effect of mercaptoethanol on the deconjugases of rat serum was already mentioned in [24].

In most cases, analyzing the amount of diglutamates gave a good hint about the efficiency of deconjugation. Sometimes already the sum of mono- and diglutamates was sufficient for calculating the amount of total folates, especially when the amount of diglutamates was low and no higher polyglutamates were present. But there are a few exceptions, for example adzuki beans. Here, the amount of diglutamates was low, but the screening for higher polyglutamates and an analysis with a higher amount of deconjugases revealed that the efficiency of deconjugation was lower than expected by the amount of diglutamates. Therefore, this food stuff showed that a screening for higher polyglutamates can sometimes be essential. Probably, in these cases the deconjugase of the chicken pancreas is more inhibited than the deconjugase of the rat serum.

In those foods with higher amounts of diglutamates, the sum of mono- and diglutamates was not enough to represent the total amount of folates. Therefore, the method needed to be optimized. The reduction of the samples size can improve the efficiency of deconjugation. But to keep the sample amount in several samples feasible, a higher addition of deconjugation enzymes was necessary. Prolonging the incubation time is another possibility to improve deconjugation. With respect to the stability of folates and the total analysis time, this alternative is recommended for food stuffs with low amounts, where reducing the sample amount and subtraction of the even higher amount of the

intrinsic folates of the added enzymes would result in a higher uncertainty of measurement.

We could confirm the additional peaks both in the chromatograms of the 5-CHO-H_4folate MRM trace and the MRM traces of the corresponding polyglutamates to be the oxidation product of the folate 5-CH_3-H_4folate, namely MeFox. But MeFox has no microbiological activity and, therefore, cannot be a reason for the differences between HPLC-methods and the microbiological assay. Nevertheless, this substance seemed to be related to the stability of 5-CH_3-H_4folate in blood during storage [25, 26] and also to the stability of the latter folate in food. In many samples the amount of MeFox is considerably higher than the amount of folates, especially when they have been processed and are low in ascorbic acid. Nevertheless, even in fresh carrots, the amount respectively the ratio to folates is not negligible. It could be interesting from the technological and nutritive point of view to have a closer look on the formation of MeFox with respect to its reduction or the question of its formation during plant growth.

When regarding the microbiological assay, we confirmed in three labs that there were differences in the response to the single folate vitamers. One reason could be the pH during incubation. Lab A and B used a commercial kit from the same manufacturer. Here the buffer capacity was probably not high enough, due to the dropping of pH from 6.5 to 5.2 with rising amount of folates. In contrast to this, in lab C a buffered pH of 6.1 ± 0.1 was used. That pH is similar to the recommended pH of 6.2 by Phillips and Wright [9], at which the difference in response shall be virtually minimal. Nevertheless, distinct differences between the results based on calibration with 5-CHO-H_4folate and PteGlu were observed. The stability of the used folates cannot be the reason, because 5-CHO-H_4folate and PteGlu are relatively stable folates. Moreover, in lab A using 5-CH_3-H_4folate for calibration, a folate being more prone to oxidative degradation, mercaptoethanol was used to prevent oxidation thus excluding lack of stability as a reason for the differences in the response.

Due to the fact that even when holding the pH range very close to the recommended value, the response of the microbiological assay differs between the folate vitamers, it was advantageous to use the folate with the highest proportion for calibration (“main” folate). The comparison showed that the results of LC-MS/MS are even higher on average than the results from the microbiological assay calibrated with PteGlu, which is the calibrant with the highest utilization. This is in contrast to the presumption that the microbiological assay often results in significantly higher amounts. For broccoli a really good correlation between the microbiological assay and the SIDA was achieved when using 5-CH_3H_4folate for calibration. With an amount of 81%, 5-CH_3-H_4folate can be considered as the “main” folate. Calibrating with 5-CHO-H_4folate would result in an overestimation. As well as in broccoli, the “main” folate in the vegetable mix is 5-CH_3-H_4folate. In this case PteGlu was used for calibration as this is also the usual calibrant in the reference microbiological assay. This vegetable mix is a certified reference material (BCR 485) with a certified value for total folate of 315 ± 28 µg/100g. Due to the fact that both, the result from LC-MS/MS and the microbiological assay via PteGlu, showed no significant difference, we assume that the responses of the used microbiological assay for PteGlu and 5-CH_3-H_4folate were similar. Therefore, PteGlu could be used instead of 5-CH_3-H_4folate. In these two cases, an explicit “main”-folate existed, which yielded results showing no significant difference from the results by LC-MS/MS.

For wheat germs, chick peas and pistachios the folate with the highest proportion was 5-CHO-H_4folate. Using this folate as a calibrant gave results also being not significantly different from the results by LC-MS/MS. A calibration with the most frequently used PteGlu resulted in an underestimation. Therefore, all the mentioned food stuffs confirm that it is necessary to calibrate the microbiological assay with 5-HCO-H_4folate, if it is the vitamer with the highest proportion, otherwise with PteGlu or 5-CH_3-H_4folate. Nevertheless, earlier publications already used 5-HCO-H_4folate as a calibrant [27]. In the

respective samples, 5-HCO-H_4folate had the highest proportion, but only with an average of 35 %, so that the other folates with an amount of 65 % could have influenced the overall response in the microbiological assay. Therefore, we assume that having no explicit “main” folate could over- or underestimate the results. This most likely happened to our result for spaghetti. Here the microbiological assay using 5-HCO-H_4folate as a calibrant produced an amount for total folate distinctively higher than the LC-MS/MS, whereas using PteGlu showed no significant difference. The distribution in spaghetti revealed a similar amount of 5-HCO-H_4folate and 10-HCO-PteGlu. The latter shows a microbiological activity of 95 % compared to PteGlu, which is not significantly different. PteGlu and 10-HCO-PteGlu represent approximately half of the folate vitamers. Considering that 5-CH_3-H_4folate has a similar response as PteGlu, as evident from the results of the vegetable mix mentioned above, more than 50 % of the vitamers would be better represented by the calibration using PteGlu.

For mung beans the calibration with 5-CHO-H_4folate and with PteGlu gave lower results compared to the LC-MS/MS, although the result via 5-CHO-H_4folate was not significantly different. But actually 5-CHO-H_4folate was not the “main” folate. 5-CH_3-H_4folate was the largest fraction amounting to 45 %. Therefore, a calibration with PteGlu should be sufficient. But the proportion of 5-CHO-H_4folate is 30 % and additionally H_4folate is accounting for 14 %. We assume that the latter folate is degraded to a certain amount during incubation, at least in lab B and C (in lab A mercaptoethanol was used for stabilization). Besides, the results for the mung beans can be explained when taking into account that the deconjugation to the diglutamates was not complete in combination with the knowledge, that according to Tamura et al. [1] the response of the microbiological assay to higher polyglutamates is lower than for mono-, di- and triglutamates. Even for wheat germs, chick peas and pistachios an incomplete deconjugation as well as a certain loss of H_4folate could have lowered the results of the microbiological assay. Nevertheless,

this does not disagree with the former observation that LC-MS/MS and the microbiological assay achieve results being not significantly different, because using 5-HCO-H_4folate solely as a calibrant could have very probably overestimated the amount and compensated for the converse effects. Therefore, in some cases it may be helpful to use a mixture of calibrants with a folate composition representing the distribution of the food. Nevertheless, we recommend to use a calibrant representing the main vitamer for the microbiological assay and a sufficient deconjugation for both methods. Then this will give similar results of the microbiological assay and the LC-MS/MS coming close to the “true” value. However, as the microbiological assay is not able to reveal the vitamer distribution and the correct calibrant, we see significant drawbacks for the method in those samples.

5. Conclusion

From our results, several reasons for the differences between the microbiological assay and LC-MS/MS can be concluded.

First, insufficient deconjugation can lead to lower results in HPLC methods. As an incomplete deconjugation hardly can be predicted, a screening for polyglutamates or their respective quantitation is recommended.

Second, the microbiological assay may give inaccurate results due to different responses and stabilities of the single vitamers. This problem hardly can be solved with the microbiological assay alone, information about vitamer distribution from other methods are necessary.

Third, and what we did not mentioned up to now, HPLC methods can be inaccurate when using inequivocal UV extinction coefficients for calibration of the single vitamers. Significant disagreement of this values appears from several reports [28,29] and we recommend to use the values from the most recent study [29].

6. Acknowledgments

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Tables

Table 1: MRM transitions and corresponding parameters for MeFox, folate diglutamates and higher polyglutamates. As internal standards, the corresponding [$^2H_4$]-labeled folate monoglutamates were used. However, for MeFox and 10-HCO-H_2PteGlu$_2$ [$^2H_4$]-labeled 10-HCO-PteGlu served as internal standard.

Compound	Precursor ion (m/z)	Product ion [1] (m/z)	DP [2]	EP [3]	CE [4]	CXP [5]	RF ± s [6]
MeFox (pyrazino-s-triazine)	474	327	66	10	29	14	1.496 ± 0.063
5-CH_3-H_4PteGlu$_2$	589	313	60	10	40	8	0.721 ±
		180	60	10	65	10	0.071
5-CH_3-H_4PteGlu$_3$	718	313	70	10	50	8	
		180	70	10	75	10	
5-CH_3-H_4PteGlu$_4$	847	313	80	10	60	8	
		180	80	10	85	10	
5-CH_3-H_4PteGlu$_5$	976	313	90	10	70	8	
		180		10	95	10	
5-CH_3-H_4PteGlu$_6$	1105	313	90	10	80	8	
5-CH_3-H_4PteGlu$_7$	1234	313	90	10	90	8	
5-HCO-H_4PteGlu$_2$	603	327	61	10	35	14	0.583 ±
		299	61	10	57	16	0.052
5-HCO-H_4PteGlu$_3$	732	327	80	10	50	14	
		299	80	10	60	16	
5-HCO-H_4PteGlu$_4$	861	327	80	10	60	14	
		299	80	10	70	16	
5-HCO-H_4PteGlu$_5$	990	327	70	10	70	14	
		299	70	10	80	16	
5-HCO-H_4PteGlu$_6$	1119	327	70	10	80	14	
5-HCO-H_4PteGlu$_7$	1248	327	70	10	90	14	
10-HCO-PteGlu$_2$	599	295	70	15	50	16	0.774 ±
	599	176	70	15	70	8	0.131
10-HCO-PteGlu$_3$	728	295	70	15	55	16	
	728	176	70	15	80	8	
10-HCO-PteGlu$_4$	857	295	70	15	65	16	
	857	176	70	15	90	8	
10-HCO-PteGlu$_5$	986	295	70	15	75	16	
	986	176	70	15	100	8	
H_4PteGlu$_2$	575	299	60	10	35	16	0.772 ±
	575	166	60	10	65	8	0.026
H_4PteGlu$_3$	704	299	60	10	50	16	
	704	166	60	10	80	8	

$H_4PteGlu_4$	833	299	70	10	60	16		
	833	166	70	10	90	8		
$H_4PteGlu_5$	962	299	80	10	70	16		
	962	166	80	10	100	8		
$PteGlu_2$	571	295	60	8	30	14	0.480	±
		176	60	8	65	8	0.068	
$PteGlu_3$	700	295	60	8	40	14		
		176	60	8	70	8		
$PteGlu_4$	829	295	70	8	50	14		
		176	70	8	80	8		
$PteGlu_5$	958	295	80	8	60	14		
		176	80	8	90	8		
10-HCO-$H_2PteGlu_2$	601	178	75	10	45	8	0.183	±
		297	75	10	40	16	0.056	

[1] Dwell time: 50 s, [2] DP: Declustering potential (in V), [3] EP: Entrance Potential (in V), [4] CE: Collision Energy (in V), [5] CXP: Cell Exit Potential (in V), [6] RF: response factor ± standard deviation

Table 2: Amount of MeFox and ascorbic acid in different foods. Additionally, the ratio of MeFox to total folate is presented.

foodstuff	MeFox (in µg/100 g)	Ratio of MeFox to total folate	ascorbic acid (in mg/100 g)
carrot juice	159	8.6	3.8 [1]
carrots	364	6.2	7 [1]
cauliflower	19	0.19	64 [1]
peas	113	0.96	25 [1]
soy beans	187	0,57	34 [2]
adzuki beans	551	2.5	
whet germs	2581	5.4	0 [2]
buckwheat	140	2.4	0 [1]
couscous	591	34	0 [2]
corn semolina	139	9.0	0 [1]

pearl barley	54	1.7	0 [1]
oatmeal	1182	30	0 [1]
millet	216	5.1	0 [2]
basmati rice	759	59	0 [1]
spaghetti	767	41	0 [1]
potatoes	13	0.61	17 [1]
walnuts	2425	17	2.6 [1]
hazelnuts	597	8.4	3 [1]
peanuts	1394	8.6	0 [1]
almonds	95	3.1	3.7 [1]
pistachios	943	7.3	7 [1]

[1] Souci, Fachmann, Kraut [30], [2] Bundeslebensmittelschlüssel [31]

Table 3: Amount of total folate (calculated as PteGlu in µg/100g) in different kinds of food analyzed by two methods: LC-MS/MS and microbiological assay (with three different folates for calibration; results from lab C, except the result for broccoli with 5-CH_3-H_4folate as calibrant, which was from lab A). The results from the microbiological assay in bold characters are not significantly different ($p>0.05$) from the results of LC-MS/MS.

food stuff	LC-MS/MS	Microbiological assay		
		5-CH_3-H_4folate	5-CHO-H_4folate	PteGlu
broccoli	183	**188**	250	-
vegetable mix	336	-	459	**311**
wheat germs	392	-	**438**	277
chick peas	393	-	**362**	254
pistachios	112	-	**124**	93
spaghetti	17	-	28	**20**
mung beans	433	-	**367**	238

Table 4: Folate composition in different kinds of food (in %).

food stuff	5-CH_3-H_4folate	5-CHO-H_4folate	10-CHO-PteGlu	H_4folate	PteGlu	10-HCO-H_2folate	5,10-CH^+-H_4folate
broccoli	81	13	3	2	0	1	1
vegetable mix	96	1	0	2	1	0	0
wheat germs	9	68	9	1	12	1	1
chick peas	28	49	6	11	4	1	2
pistachios	22	56	5	10	4	2	1
spaghetti	5	42	40	1	9	1	1
Mung beans	45	30	5	14	2	3	2

Legends to the figures

Figure 1: Amount of folates (Sum of the single vitamers, monoglutamates; calculated as PteGlu in µg/100g) after different sample preparations or after enzyme treatments in addition to the deconjugation step. This treatment has not yet been optimized for deconjugation.

Figure 2: Chromatograms of 5-CH_3-H_4PteGlu$_{1-5}$ in a sample solution of peas before (left side) and after optimization of deconjugation (right side).

Figure 3: Amount of mono- and diglutamates (calculated as PteGlu) of different types of food after original sample preparation and with varying sample weights and an extra addition of deconjugases (+ E).

Figure 4: Comparison of the amount of folates in wheat germs calculated from mono- and diglutamates (calculated as PteGlu) after the deconjugation step using different thiols or a combination of these in the extraction buffer.

Figure 5: Results from the microbiological assay via calibration with different vitamers in three different labs (A, B and C).

Figures

Figure 1

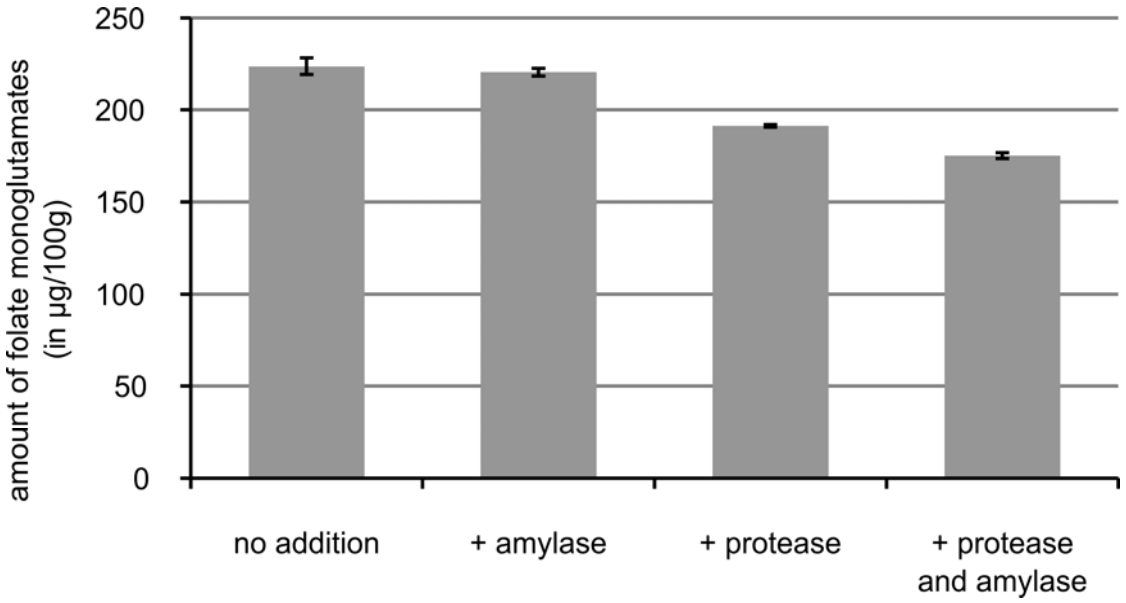


Figure 2

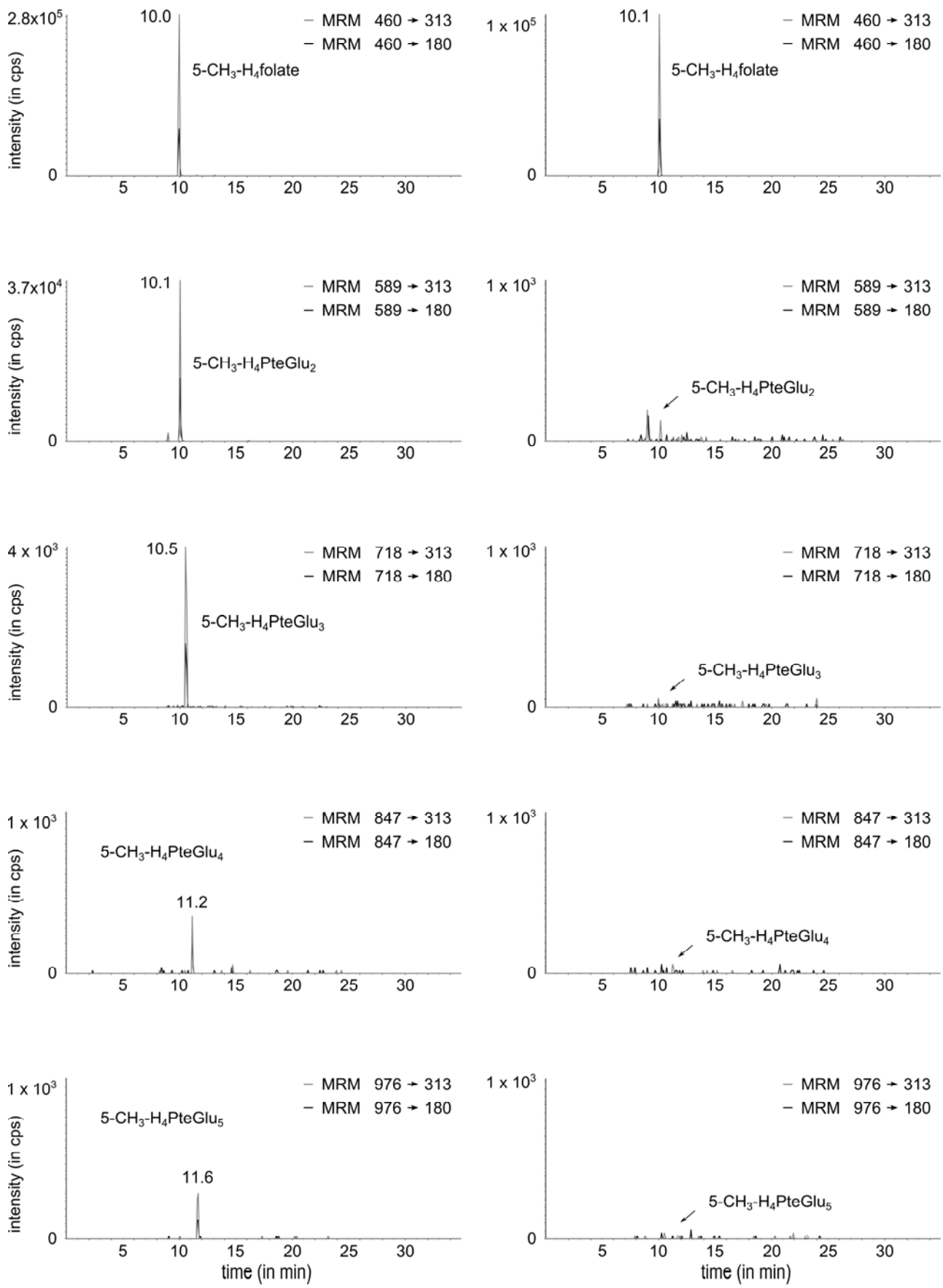

2.8x10^5
10.0
MRM 460 → 313
MRM 460 → 180
5-CH3-H4folate
intensity (in cps)
1 x 10^5
10.1
3.7x10^4
5-CH3-H4PteGlu2
1 x 10^3
4 x 10^3
10.5
MRM 718 → 313
MRM 718 → 180
5-CH3-H4PteGlu3
MRM 847 → 313
MRM 847 → 180
5-CH3-H4PteGlu4
11.2
MRM 976 → 313
MRM 976 → 180
5-CH3-H4PteGlu5
11.6
0
5
10
15
20
25
30
time (in min)

Figure 3

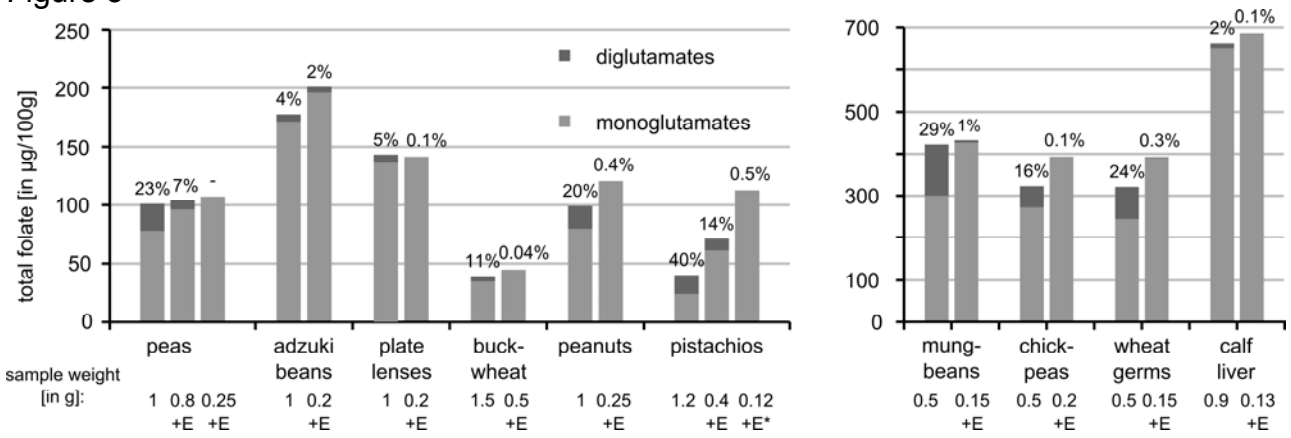


Figure 4

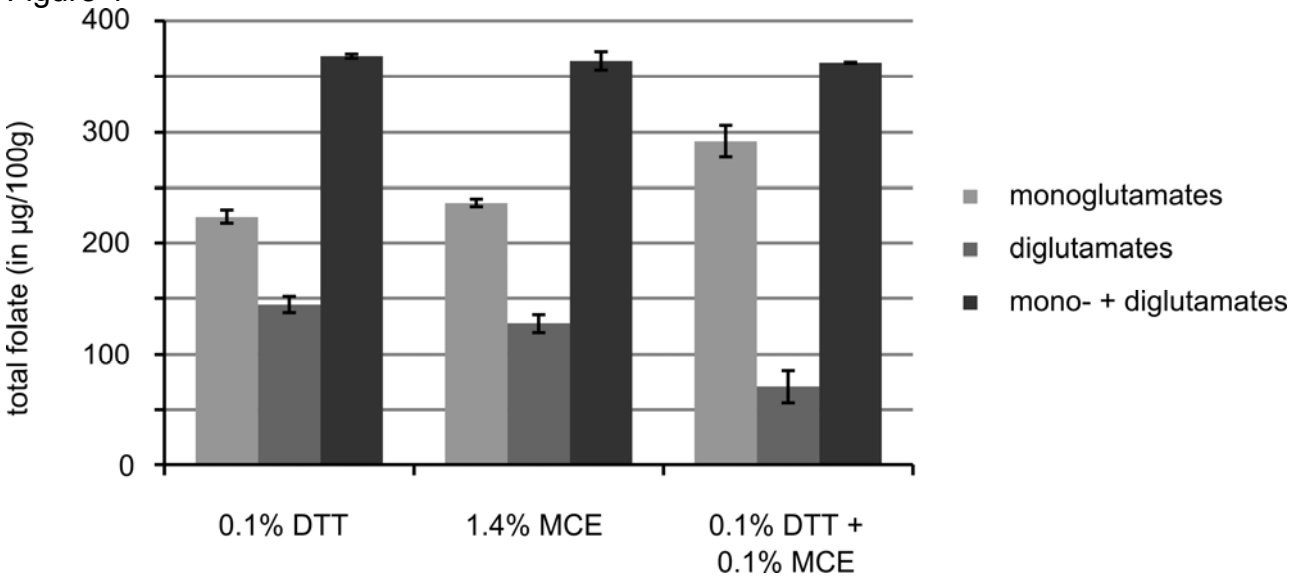
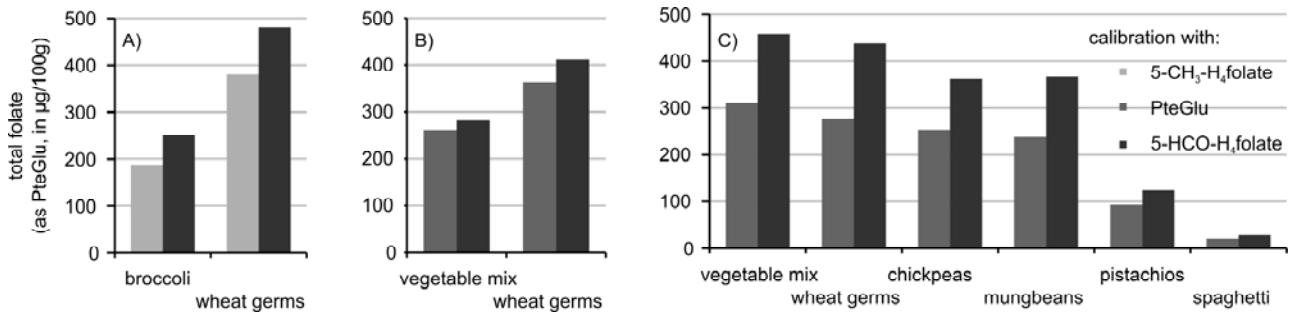


Figure 5


A)
B)
C)
total folate
(as PteGlu, in µg/100g)
500
400
300
200
100
0
broccoli
wheat germs
vegetable mix
wheat germs
calibration with:
5-CH3-H4folate
PteGlu
5-HCO-H4folate
vegetable mix
wheat germs
chickpeas
mungbeans
pistachios
spaghetti