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Specific and Sensitive Quantitation of Folate Vitamers in Foods by Stable Isotope Dilution Assays Using High-Performance Liquid Chromatography-Tandem Mass Spectrometry

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ABSTRACT

Stable isotope dilution assays were developed for the quantitation of the folate vitamers 5-methyltetrahydrofolate, 5-formyltetrahydrofolate, tetrahydrofolate, 10-formylfolate and pteroylglutamic acid in food samples by using deuterated isotopomers as internal standards. Vitamers and their labeled analogues were analyzed simultaneously by HPLC/MS/MS using selected reaction monitoring (SRM), which allowed a higher specifity than other methods published previously. Sample preparation involved treatment by protease in sequence with α-amylase and rat serum deconjugase followed by anion exchange chromatography. The detection limits for 5-methyltetrahydrofolate, 5-formyltetrahydrofolate, tetrahydrofolate, 10-formylfolate and pteroylglutamic acid were found to be 0.5, 1.2, 1.5, 0.6 and 2.6 µg/100g fresh weight, respectively. Using the new method, folate contents were determined in meat, cereals, and vegetables. Data were in good agreement with literature data, except results for broccoli, which were much lower than reported in previous studies.

INTRODUCTION

There is increasing evidence based on physiological studies that folate deficiences may be associated with the prevalence of neural tube defects [1], coronal heart desease [2] and cancer [3]. Based on these proposals, fortification of grain products with folic acid is mandatory in the USA since 1998 and seems to lower the occurrence of neural tube defects since then [4].

Nevertheless, there is an increased need for accurate folate data in order to recommend daily intakes for non-fortified foods.

Since decades, the standard method for quantifying folates is a microbiological assay measuring the growth of *Lactobacillus rhamnosus* turbidimetrically in a folate-deficient medium. This method, however, has increasingly been questioned as it cannot distinguish between the different folates. However, differentiation is crucial in folate analysis, since the vitamers show different bioavailability [5] and stability [6]. Moreover, the microorganism show different responses to the single folates, thus providing invalid results for samples containing mixtures of different vitamers.

Another type of methodology is based on the reaction of folates with folate binding protein [7] in protein-binding assays (PBA) or with monoclonal antibodies in biosensor assays [8]. In analogy to the microbiological assay, these methods also (i) cannot distinguish between the single vitamers and (ii) show different responses to them. In case of PBAs, the values obtained have reported to be too high [9].

Currently, the only method to differentiate between folates is HPLC coupled either (i) to fluorescence detection (FD) or (ii) to electrochemical detection (ED) or (iii) to mass spectrometry (MS). As HPLC/FD and HPLC/ED are subject to matrix interferences, sophisticated clean-up procedures such as affinity chromatography using folate binding protein [10] are applied. In order to overcome these obstacles, Stokes and Webb [11] recently developed an LC/MS method, which enabled quantitation of folic acid and 5-/10-formyltetrahydrofolic acid. Because this method did not use an internal standard (IS) to correct for losses during sample clean-up and to address variations in ionization efficiency, Garbis et al. [12] recently reported on the quantitation of folate vitamers employing methothrexat as IS. Further improvement of the methodology was achieved by Pawlosky

and coworkers who used [^{13}C]-labeled folates as IS for quantitation of folic acid in fortified foods [13] and 5-methyltetrahydrofolate in blood serum [14]. However, due to the low specifity of single stage MS, the mass chromatograms published by latter authors revealed significant matrix interferences. These drawbacks were circumvented by Nelson et al. [15] and Hart et al. [16] using RP-18 cartridges or affinity chromatography, respectively, for sample clean-up. Both groups applied [$^{13}C_5$]-5-methyltetrahydrofolate as IS for quantifying blood folates.

As analogues labeled with stable isotopes behave almost identically to the analytes, these isotopomers are considered the most suitable IS. Recently we reported on the synthesis of a couple of deuterated folates [17]. The purpose of the present study was, first, to develop a stable isotope dilution assay in order to quantify the most important naturally occurring folates in foods by LC/MS and, second, to improve the method's specifity by employing LC/ tandem MS.

EXPERIMENTAL

Reagents

The following chemicals were obtained commercially from the sources given in parentheses: ascorbic acid, formic acid, H_2O_2, hydrochloric acid, 2-mercapto ethanol, potassium dihydrogenphosphate, sodium acetate, sodium hydroxide, sodium chloride, *di*-sodium hydrogenphosphate (Merck, Darmstadt, Germany), 2-[N-cyclohexylamino]ethanesulfonic acid (CHES), N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES), sodium ascorbate (Sigma, Deisenhofen, Germany), acetonitrile (Baker, Gross-Gerau, Germany). Unlabeled folate vitamers tetrahydrofolic acid (H_4folate), 5-methyltetrahydrofolic acid (5-CH_3-H_4folate), 10-formylfolic acid (10-CHO-PteGlu) and pteroyltriglutamate ($PteGlu_3$) were obtained from Dr. Schircks Laboratories (Jona, Switzerland), 5-formyltetrahydrofolic acid (5-CHO-H_4folate) was purchased from Sigma (Deisenhofen, Germany) and folic acid (PteGlu) was from Fluka (Neu-Ulm, Germany).

Extraction and Incubation Buffer

For storage, enzymatic incubations and extractions of folates, the following buffer system according to Wilson and Horne [18] was used, henceforth referred to as extraction buffer: aqueous HEPES (50 mmol/L) and aqueous CHES (50 mmol/L) at pH 7.85 containing sodium ascorbate (2 %) and 2-mercapto ethanol (20 mM).

Isotopically Labeled Standards

[$^2H_4$]-labeled folate standards were synthesized as reported recently [17]. Crystalline [$^2H_4$]-5-CH_3-H_4folate and [$^2H_4$]-H_4folate were stored under argon at -30°C, whereas [$^2H_4$]-folic acid, [$^2H_4$]-5-CHO-H_4folate and [$^2H_4$]-10-CHO-folic acid were kept at room temperature in amber vessels to exclude light. No measurable degradation was observed over a period of several months. Ready-to-use working solutions of the internal standards (approx. 1.5 µg/mL) in extraction buffer were stored under argon at -60°C and also showed no measurable degradation.

Preparation and Determination of the Concentration of Standard Solutions

The crystalline compounds (labeled as well as unlabeled) were suspended in re-destilled water, dissolved by additon of an aqueous solution of NaOH (0.1 mol/L) and checked for purity by HPLC/UV (see below). Concentrations were determined by UV spectrometry using the molar extinction coefficients at pH 7 and pH 1 reported by Blakley [19]. The UV spectrometer U-2000 (Hitachi, Berks, GB) was calibrated using potassium dichromate [20]. Immediately after UV spectrometry, the standard solutions were diluted with extraction buffer and stored in small portions under argon at –60°C until use.

Enzymes

Rat serum containing folate conjugase was obtained from Biozol (Eching, Germany) and stored in small aliquots at –30°C until use.

Bacterial protease from *Streptomyces griseus* (P-5147; 10 mg/mL), α-amlyase from *Bacillus species* (A-6380; 20 mg/mL), and catalase from bovine liver (0.3 mg/mL) were purchased from Sigma (Deisenhofen, Germany) and diluted in extraction buffer to the concentrations given in parentheses.

Protease and catalase contained no measurable amounts of folates, whereas the fungal α-amylase generally used for folate assays [21] (Sigma A-6211) contained 0.07 ng 5-CH_3-H_4folate per mg solid. Therefore, bacterial amylase was chosen, which was devoid of endogenous folate. Rat serum contained 7.5 ng 5-CH_3-H_4folate per 100 µL. The corresponding values were subtracted when calculating the contents in the sample.

Sample Preparation

Samples of vegetables, cereals and meat were purchased at local retail stores, bakeries and markets in the city of Munich, Germany. Orange juices (two juices not from concentrate and one juice from concentrate) as well as orange fruits used for hand squeezing were also from local stores in Munich.

In order to achieve a homogenous material for representative sampling, large quantities of each sample (100-150 g) were comminuted and frozen with liquid nitrogen before mincing them with a blender (Privileg, Quelle, Fürth). Aliquots (1-2 g, triplicate analysis per sample) were taken from the resulting powder-like homogenate and overlaid with 10 ml of extraction buffer. [$^2H_4$]-labeled internal standards were added to the suspension in an amount to adjust a mass ratio of standard to analyte ranging from 1 to 5.

Sample suspensions were then purged with argon, capped tightly and placed in a boiling water bath for 10 min. Subsequently the extracts were rapidly cooled in an ice-bath and subjected to two types of enzyme treatments: (a) Samples rich in protein were digested with protease (3 mg/g sample) for 6 h at 37°C and (b) samples rich in starch were incubated first with amylase (20 mg/g sample) for 4 h at 37°C and then with protease (3 mg/g sample) for 2 h at 37°C. After the enzyme digestions, the samples were heated at 100°C for 10 min, cooled on ice and spiked with 100 µL of rat serum. The deconjugation was performed at 37°C overnight.

At the end of the conjugase treatment, the samples were again heated at 100°C for 10 min, then cooled on ice and centrifuged at 6000 g for 20 min. If necessary, the residue was adjusted to pH 7.5, passed through a syringe filter (0.4 µm, Millipore, Bedford, MA, USA) and subjected to clean-up by solid phase exctraction.

Orange juices (5 mL) were diluted with extraction buffer (10 mL), the standards were added and the extracts were heated for 10 min at 100°C. After cooling on ice, the extracts were incubated with 100 µL of rat serum for 4 h at 37°C and subjected to solid phase extraction.

In order to assure the completeness of deconjugation, pteroyltriglutamate ($PteGlu_3$) was added to a sample of spinach which was quantified by SIDA after treatment with proteinase and rat serum as detailed above. As $PteGlu_3$ was converted to folic acid at a degree of 100 ± 10 %, the enzyme treatment was deemed satisfactory.

Sample Clean-up by Solid Phase Extraction (SPE)

Extracts were purified by SPE according to the method described by Gounelle et al. [22], using an 12-port vacuum manifold (Alltech, Bad Segeberg, Germany) equipped with Bakerbond SAX cartridges (quaternary amine, 500 mg, No. 7091-3, Baker, Gross-Gerau, Germany). The cartridges were successively activated with 2 volumes of hexane, methanol and water, and then conditioned with 7 to 8 volumes of phosphate buffer (pH 7.5, 0.01 mol/L, containing 0.2 % mercapto ethanol).

After applying the sample extracts, the columns were washed with 6 volumes of conditioning buffer, and the folates were eluted with 3 mL of aqueous sodium chloride (5 %, containing 1 % sodium ascorbate and 0.1 mol/L sodium acetate; pH 5.4). 100 µL mercapto ethanol was added to each eluate and the purified extracts were stored under argon at –30°C until analysis (typically within two days).

LC/MS/MS and LC/UV Analysis

LC was performed on an Aqua C-18 reversed phase column (250 x 4.6 mm; 5 µm, Phenomenex, Aschaffenburg, Germany) by eluting the samples (100 µL) with variable mixtures of aqueous formic acid (0.1 %) and acetonitrile, at a flow of 0.8 mL/min. An initial

9-min isocratic hold of 7% acetonitrile was followed by a 13-min linear gradient to 13 % and a further 4-min linear gradient to 25 % acetonitrile. Then, the mobile phase was programmed to 100 % acetonitrile over 4 min before being brought back to the initial mixture for another 5 min to allow for column equilibration. The HPLC system consisted of a spectra series chromatograph (Thermo Separation Products, San Jose, CA, USA) coupled to an UV-Detector and an LCQ ion-trap mass spectrometer (Finnigan MAT, Bremen, Germany). During the first 8 min of the gradient programme, the column effluent was diverted to waste to ensure an adequate spray stability. The spectrometer was operated in the positive electrospray mode using selected-reaction monitoring (SRM). The spray voltage was set to 5.5 kV, the capillary temperature to 200°C and the capillary voltage to 24.3 V. The maximum ionization time was set to 200 ms and the MS/MS transition was measured using 3 microscans in order to obtain reproducible peak areas. The following mass transitions for the respective folates were chosen (*m/z* precursor ion/ *m/z* product ion): [$^2H_4$]-H_4folate 450/303, H_4folate 446/299, [$^2H_4$]-5-CH_3-H_4folate 464/317, 5-CH_3-H_4folate 460/313, [$^2H_4$]-10-CHO-PteGlu 474/456, 10-CHO-PteGlu 470/452, [$^2H_4$]-5-CHO-H_4folate 478/331, 5-CHO-H_4folate 474/327, [$^2H_4$]-PteGlu 446/299, PteGlu 442/295. For maximum sensitivity the isolation width of the precursor ion was adjusted to 3 Da and the isolation width of the product ion was set to 1 Da in order to detect the product ion most selectively. The sheath and auxiliary gas flow rates were set to 68 % and 19 % of their maximum flow rates, respectively.

Calibration and Quantitation

For each folate vitamer, solutions of unlabeled and labeled compound were mixed in nine mass ratios ranging from 0.06 to 16. To ensure suitable peak shapes and areas, the concentration of each compound was set to a range between 0.09 µg/mL and 1.5 µg/mL. LC/MS/MS analysis of each mixture was performed in triplicate as outlined before. Calibration curves and regression lines were calculated for each mixture as exemplarily illustrated in Figure 1 for 5-methyltetrahydrofolate. The curve reveals a linear response of the peak area ratios to the mass ratios of unlabeled to labeled compound.The resulting regression equation is the basis for calculating the concentration of each analyte in the sample extracts.

Detection and Quantitation Limits

Detection (DL) and quantitation limits (QL) were determined using a commercially available carrot pulp, which contained low amounts of 5-CH_3-H_4folate and no detectable concentrations of other vitamers. In order to degrade remaining 5-CH_3-H_4folate, the following procedure was applied: aliquots of the pulp (approx. 1.5 g) were diluted with water (4 mL), heated for 10 min in a boiling water bath and cooled on ice. Subsequently the aliquots were subjected to protease (4.5 mg) treatment for 2 h at 37°C. The folates released by this procedure were then oxidated by adding 750 µL H_2O_2 (30 %) to each aliquot and stirring it at room temperature for 60 min. The peroxide was then destroyed by addition of 0.3 mg catalase per tube and stirring for 15 min at room temperature. To the folate-free matrix the following amounts of vitamers (unlabeled as well as labeled compounds) were added: 0.5, 1.0, 2.5 and 5 µg/100 g (for 5-methyl-H_4folate) and 1.0, 2.5, 5.0 and 10 µg/100 g (for H_4folate, 5-formyl-H_4folate, folic acid and 10-formylfolic acid). Extraction and sample clean-up was continued as described before, except that the conjugase treatment was omitted. Then, LC/MS/MS analysis was conducted as outlined above. Each addition assay was performed in triplicates and DLs as well as QLs were calculated according to Hädrich and Vogelgesang [23]. In short, a calibration graph of measured versus added folate amounts was plotted. Considering this graph, DL is the concentration calculated from the maximum height of the 95 % confidence interval at the zero addition level. QL is the addition level for which the lower 95 % confidence limit equals the upper 95 % confidence limit of the addition level at the DL.

Stability of Deuterium Labeled Standards to Protium-Deuterium Exchange

A carrot pulp sample (1.5 g) was spiked with [$^2H_4$]-folic acid (19 µg). Analysis and extraction was performed as outlined before, except that the mass spectrometer was operated in the fullscan electrospray mode ranging from m/z 100 to 500.

RESULTS AND DISCUSSION

Choice and suitability of stable isotopomeric standards

In a first series of experiments, the occurrence of the different folate vitamers was surveyed in the foods to be analyzed. Consistent with several prior reports [24-26], in spinach, carrots and broccoli as well as in cereals and orange juices only folic acid, H_4folate, 5-CH_3-H_4folate, 5-CHO-H_4folate and 10-CHO-folic acid could be detected in the sample extracts. In some other publications the occurrence of 10-CHO-H_2folate has been reported [28, 29]. However, as this compound appears to be an oxidation product of 10-CHO-H_4folate and is readily oxidized to 10-CHO-folic acid, we consider it to be an artefact from sample preparation. In our study, no 10-CHO-H_4folate or 10-CHO-H_2folate could be detected, which prompted us to quantify only the final oxidation product 10-CHO-folate.

In view of these results, we chose [$^2H_4$]-folic acid, [$^2H_4$]-H_4folate, [$^2H_4$]-5-CH_3-H_4folate, [$^2H_4$]-5-CHO-H_4folate and [$^2H_4$]-10-CHO-folic acid (structures shown in fig. 2) as internal standards.

In several respects, the deuterated folates used in this study reveal ideal properties for their use as internal standards in SIDAs. First, the labeled material did not contain significant amounts of residual unlabeled folates, the four-fold labeled isotopomers accounted for 95%. The low 1% content of unlabeled folates may be attributed to a protium deuterium exchange during the cleavage of the N-2 acetyl group in the presence of sodium hydroxide [17]. Second, the mass increment of 4 u ensures that less than 0.1 % intensity of the unlabeled folates fall on the ion monitored for the standard due to naturally occurring heavier isotopes. Third, it is advantageous that the labelling is maintained in MS/MS mode, as the unlabeled glutamic acid moiety is split off in course of collision-induced dissociation (CID). Consequently, the product ions of standard and analyte still bear a mass difference of 4 u, which enhances the specifity of detection. These three properties avoid a spectral overlap between standard and analytes, resulting in a linear calibration function as shown in Fig. 1 and, in improved accuracy.

Another advantage of the standards used is that a deuterium-protium exchange during the extraction steps is excluded. This was proved by spiking a carrot pulp sample devoid of

folates with [$^{2}H_4$]-folic acid and analyzing it in duplicate. The fullscan mass spectra recorded (a) before and (b) after extraction and sample clean-up were not distinguishable ($P < 0.001$), thus indicating that no further protium-deuterium exchange had occurred.

LC/MS/MS

When optimizing an LC/MS method, it is of prime importance to resolve all analytes from each other and from matrix interferences, while maintaining a maximum of detection sensitivity. As eluents containing phosphate buffers or containing ion pair reagents are not compatible to atmospheric pressure ionization, a gradient consisting of acetonitrile and diluted formic acid was chosen as the mobile phase and a polar endcapped RP-18 column as the stationary phase. Contrary to several alternatively tested RP-18 columns, in this system all important folates were base-line separated within a run time of 13 min.

In contrast to Garbis et al. [12], who used negative electrospray ionisation (ESI), we found the positive ESI mode to show better sensitivity. Addition of ammonium salts or formic acid did not enhance signal intensities.

Chromatographic base-line separation of all folates, however, did not prove to be fully adequate for differentiating them from matrix interferences. This observation is in accordance with the findings of Pawlosky et al. [13, 14], indicating that the use of [^{13}C]-labeled folic acid and 5-CH_3-H_4folate as internal standards is not sufficient for unequivocal identification. Consequently, the latter authors had to confirm the presence of folates by MS/MS. For this reason we developed a tandem MS method by applying a suitable activation energy to each compound in order to induce collision-induced dissociation. This procedure, denoted **S**elected **R**eaction **M**onitoring (SRM), enabled us to monitor simultaneously all vitamers and the respective standards in their specific mass transition traces. Fig. 3 shows a typical LC/MS/MS chromatogram for the 5 analytes and their isotopically labeled standards. The analytes and internal standards coeluted in a single peak, respectively, although the SRM traces reveal the standards to elute slightly earlier. Likewise apparent from the SRM traces is the mass shift of 4 u between standards and analytes, which is present in both, the precursor and the product ion. Hence, an unambiguous detection of standard and analyte was possible.

In wholemeal bread, however, we observed matrix peaks in the SRM traces of 5-CHO-H_4folate, which forced us to modify the elution gradient to achieve a better and prolonged

resolution of the vitamers. This resulted in an improved separation from matrix peaks, but led, however, to analysis times of 35 min instead of 13 min.

Limits of Detection and Quantifiction

Determination of detection (DL) and quantitation limits (QL) is a matter of conflicting approaches. The most simple method is to analyze standard solutions and set the DL to a signal to noise ratio of 3. However, (i) losses during extraction and clean-up, (ii) background noise due to matrix interferences and (iii) data scattering in low concentration ranges are not taken into account and, therefore, this approach may render quantitation inaccurate.

Therefore, we used a method proposed by Hädrich and Vogelgesang [23] for calculating the DL from the confidence interval of a calibration line prepared by spiking a suitable matrix with variable amounts of the analyte.

However, determination of DL is often impaired by endogeneous contents of analytes in the matrix. As carrots contain only minute amounts of 5-CH_3-H_4folate, we chose this vegetable as representative matrix and destroyed endogenous folates by treatment with hydrogen peroxide. This method was first reported by Pawlosky et al. [14] in blood plasma, but their procedure was not sufficient to destroy all amounts of 5-CH_3-H_4folate in carrots. Therefore, we increased the amount of added H_2O_2, thereby eliminating all detectable traces of folates and subsequently destroyed the peroxide excess by addition of catalase. Subsequent addition experiments revealed DLs of 1.5, 0.5, 1.2, 0.6, 2.6 and QLs of 4.4, 1.5, 3.5, 1.9, 7.7 µg/100 g fresh weight for H_4folate, 5-CH_3-H_4folate, 5-formyl-H_4folate, 10-formylfolate and pteroylglutamic acid, respectively. These data proved the SIDAs to be sensitive enough to quantify the folate contents in all food samples analyzed. Compared to HPLC/FD methods the new SIDA revealed DL in a similar or lower range as reported by Gregory et al. [30] who found DLs of 2, 0.4, 50, and 30 µg/100g for H_4folate, 5-CH_3-H_4folate, 5-formyl-H_4folate, and pteroylglutamic acid, respectively. However, in comparison to the LC/MS method developed by Stokes and Webb [11] showing an DL of 12 µg/100 g pteroylglutamic acid, the SIDA approach reported here was twice as sensitive. In contrast to this, some modern applications of affinity chromatography (AC) reach DL below 0.1 µg/100g [27, 31]. However, as sensitivity of SIDA was sufficient to detect the folates

occurring in foods, we decided in this study not to go to the trouble of the elaborate and more expensive AC procedure.

Precision and Recovery

In order to evaluate intra-sample precision, a sample of frozen spinach was extracted and 4 repetetive analyses were performed within a time frame of 8 weeks. The sample extract was stored at –30°C under argon during the analyses. The mean value was 45.8 µg/100 g with a CV of 5.3 %.

Inter-assay precision was determined by repeatedly extracting aliquots of another frozen spinach over one week and analyzing the extracts as detailed before. For N=3 determinations, the mean value was 34.0 µg/100 g with a CV of 6.2 %.

The recoveries obtained during the determination of the QLs ranged from 80 to 110 % depending on the individual vitamers.

Sample Extraction and Purification

The conditions during sample extraction and purification were adjusted to ensure solubility, stability and equilibration of folate isotopomers. A minimum of degradation was observed in the buffer system reported by Wilson and Horne [18]. This buffer contained ascorbic acid as antioxidant and 2-mercapto ethanol to suppress interconversion caused by release of formaldehyde from ascorbic acid.

In order to prevent folate degradation by endogenous enzymes, these proteins were inactivated by heating the samples at 100°C immediately after adding the extraction buffer and the internal standards. During this treatment and the subsequent cooling the added standards were allowed to equilibrate with endogenous folates. To release matrix-bound folates, incubation with protease and amylase followed, depending on the composition of the samples. As the majority of folates occur as polyglutamates in foods, their cleavage into monoglutamatyl derivatives was accomplished by addition of rat serum deconjugase. In addition experiments of pteroyltriglutamate to spinach SIDAs proved the deconjugation process to be complete.

According to the literature, further sample clean-up can be achieved by the following methods: (i) chromatography on either anion exchange [22] or on reversed-phase cartridges [13], or (ii) by affinity chromatography using folate binding protein (FBP) [32]. When comparing the first alternatives, anion exchange chromatography provided extracts with less interfering substances during LC/MS/MS. Affinity chromatography on FBP, however, offers the perspective to remove even more matrix interferences and will be tested in future studies.

Folate Content in Vegetables

The folate content in carrots, spinach and broccoli is presented and compared to literature data in Table 1. 5-CH_3-H_4folate is the predominating vitamer in all vegetables analyzed. The values obtained for H_4folate by SIDA exceeded those analyzed by HPLC/FD, which may be attributed to its low stability and the higher recovery yielded by using the labeled IS. On the other hand, in some samples even SIDA could not detect H_4folate thus indicating that this compound had already been degraded during storage. In carrots, the values for 5-methyl- and 5-formyltetrahydrofolic acid were found to be significantly lower than previously published data. Although fresh as well as stored carrots were analyzed, even the fresh samples did not give amounts comparable to the values reported.

In contrast to our results for carrots, the amounts of folate vitamers quantitated in spinach were in good agreement with those obtained by HPLC/FD analysis.

Very puzzling, however, was the unexpectedly low folate content in broccoli, for this vegetable is generally known as a good source for folate. At first, we thought that an insufficient homogenity of the samples had caused the low values, as we suspected the stems of the broccoli to contain fewer folates as the buds. Subsequently, the stems were removed and a complete head (approx. 400 g) was homogenized for each batch. This procedure prevented errors due to incomplete homogenization and was maintained for all subsequent analyses. Alas, the low folate values in broccoli did not mount to the literature data, but the improved sample preparation reduced the standard deviation between multiple extractions of a defined batch.

As indicated before, interfering matrix signals can cause doubtful peak identification and quantitation. Especially Vahteristo et al. [24] stated the need for careful evaluation of the

chromatograms. Because of the high selectivity of the MS/MS mode and the complete correction for losses during extraction by using isotopically labeled standards, our data for broccoli should be more accurate than the results published previously.

In contrast to Vahteristo et al. [24] we could not detect any traces of 10-CHO-folate.

Similarly to the literature data based on HPLC/FD we found significantly lower values for total folates in vegetables compared to those microbiologically analyzed. A possible explanation for the low data might be the occurrence of conjugase inhibitors or of compounds enhancing microbial growth without being folate vitamers. Therefore, we plan to solve these discrepancies in future studies.

Folate Contents in Other Foods

The method developed was applied exemplarily to foods rich in protein, such as meat, to those containing starch, such as cereal products, and to fruit products, such as orange juice.

Regarding bovine meat, we could not detect any folates, which is in agreement with the reports of Müller [26], who found contents as low as 4 µg/100 g.

Among cereal products wheat bread and fortified wheat flour were analyzed. Of these, wheat bread was found to contain a total of 36.9 µg/100g folates, which consisted mainly of 5-CH_3-H_4folate. This result was in compliance with the amount of 29.8 µg/100g found by Pfeiffer et al. [10]. In wheat flour, which had been fortified by the producer, we found 299.4 µg/100g folic acid and only negligible amounts of 5-CH_3-H_4folate.

The analysis of several orange juices resulted in a very homogeneous pattern (Table 2). The only vitamer that could be detected was 5-CH_3-H_4folate. The content of the different types of juices did not differ significantly and confirmed this beverage to be an important source for dietary folate. The values were in good accordance with the data of Vahteristo et al. [24] who reported a folate content of 15 µg/100g.

CONCLUSION

Since the decovery of folates as vitamins in the fourties of the last century, analysis of these substances in foods has been the subject of numerous studies. However, the structural variety, instability, and occurrence of folates in only trace amounts were substantial obstacles for accurate quantitation during the last decades. As SIDAs have been shown to be the method of choice for the determination of unstable analytes occurring in trace amounts, we provide here a method that offers discernable advantages over reported SIDAs of folates. For one, different folate vitamers can be quantified simultaneously without derivatization. What is more, three dimensional specifity is obtained by combining HPLC with double-stage mass spectrometry and, therefore, also allows unequivocal identification.

Applying the new method to different foods, we could confirm the majority of existing literature data, whereas our results were significantly lower in the case of broccoli. In order to resolve this contradiction, a comparison of our SIDA with the alternative methods such as HPLC/FD or microbiological assays is currently underway. Furthermore, the new SIDA is planned to be exploited to blood folate analysis. Given the new methods potential to provide accurate folate data in foods as well as in blood, this technique opens new avenues on the accurate assessment of folate bioavailability.

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Table 1. Folate contents (µg/100 g fresh weight) in carrot, spinach and broccoli determined by SIDA. Comparison with literature data

Food	Vitamer	SIDA	Literatu
			HPLC-FD [18]
Carrot (N=2)	5-CH_3-H_4folate	8.1-9.4	16
	H_4folate	4.9-6.7	1
	5-CHO-H_4folate	n.d.	msk
	Total folate[a]	12.6-15.7 (14.2 ± 2.2)[b]	16
Spinach (N=6)	5-CH_3-H_4folate	72.8-140.0	46
	H_4folate	n.d.-18.7	n.d.
	5-CHO-H_4folate	4.8 – 54.7	n.d.
	10-CHO-PteGlu	n.d.	11
	Total folate[a]	96.1-159.2 (127.9 ± 24.5)[b]	100 [c]
Broccoli (N=4)	5-CH_3-H_4folate	24.6 – 35.7	98
	H_4folate	n.d.	18
	5-CHO-H_4folate	n.d. – 8.1	n.d.
	10-CHO-PteGlu	n.d.	1
	Total folate[a]	27.2 – 41.9 (33.8 ± 6.1)[b]	114

n.d.: not detected, n.a.: not analyzed, msk: masked.
[a] calculated as folic acid.
[b] range (mean value ± standard deviation).
[c] results include 48 µg/100g 10-formyl-H_2folate; sum calculated as folic acid.

Table 2. Folate contents (in µg/100 g fresh weight) in foods analyzed by SIDA

Food	Total (mean) [a]	5-CH_3-H_4folate	5-CHO-H_4folate	H_4fo
Meat				
Bovine	n.d.	n.d.	n.d.	n.
Cereals				
Wheat bread	36.9	29.8	7.1	n.
Fortified wheat flour	299.4	2.0	n.d.	n.
Orange juices				
Juice A not from concentrate (NFC)	16.2	16.9	n.d.	n.

Juice B not from concentrate (NFC)	17.2	17.9	n.d.	n.d.
Juice from concentrate	15.8	16.5	n.d.	n.d.
Fresh hand squeezed juice	15.1	15.7	n.d.	n.d.

n.d.: not detected; n.q.: not quantified; n.a.: not analyzed.
[a] calculated as folic acid.

LEGENDS TO THE FIGURES

Figure 1. Calibration curve for 5-CH_3-H_4folate obtained by plotting area ratios vs. mass ratios of isotopomeric acids

Figure 2. Structures of the [$^2H_4$]-labeled internal standards used

Figure 3. LC/MS/MS chromatograms of (A) a standard mixture of [$^2H_4$]-H_4folate(1) H_4folate (2), [$^2H_4$]-5-CH_3-H_4folate (3) 5-CH_3-H_4folate (4), [$^2H_4$]-10-CHO-PteGlu (5), 10-CHO-PteGlu (6), [$^2H_4$]-5-CHO-H_4folate (7), 5-CHO-H_4folate (8), [$^2H_4$]-PteGlu (9), PteGlu (10) and (B) an extract from spinach leaves showing the mass ranges of folate vitamers as well as of the corresponding internal standards

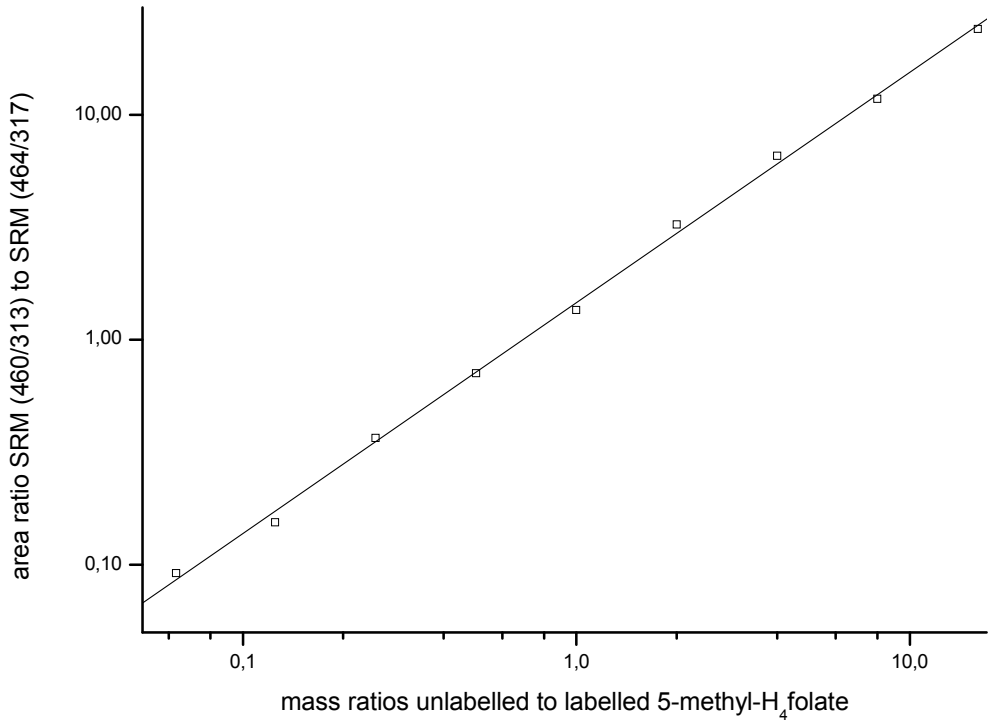
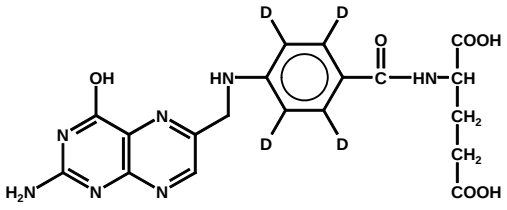
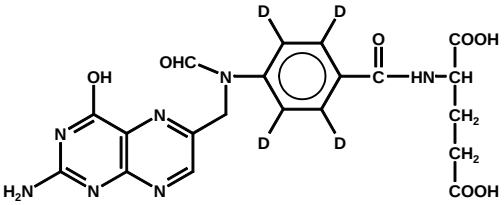

area ratio SRM (460/313) to SRM (464/317)
10,00
1,00
0,10
0,1
1,0
10,0
mass ratios unlabelled to labelled 5-methyl-H4folate

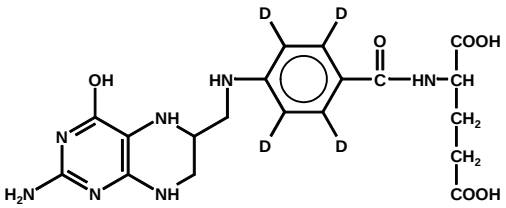
Figure 1:



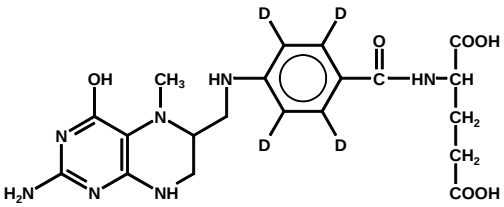
[$^2H_4$]-Folic acid



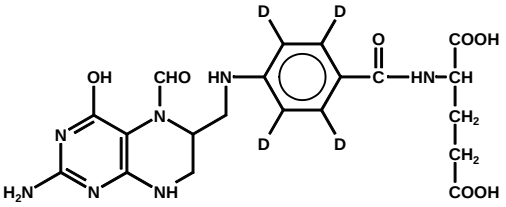
[$^2H_4$]-10-CHO-folate



[$^2H_4$]-H_4 folate

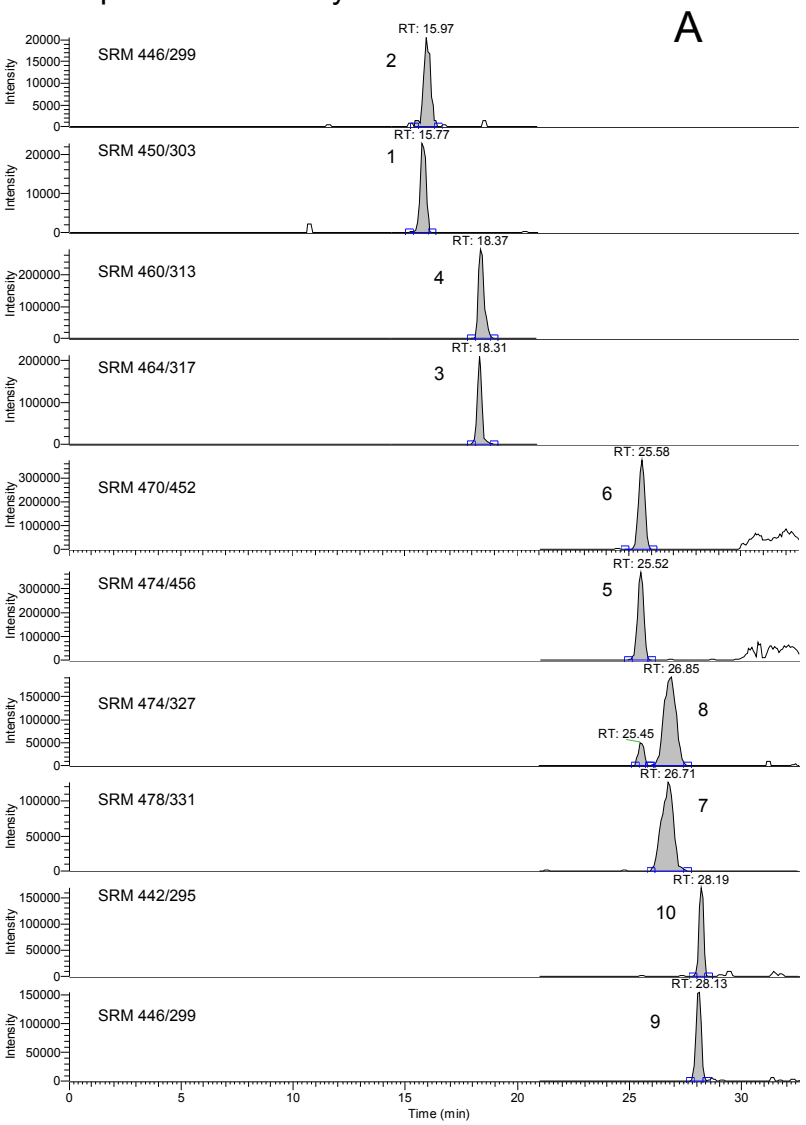


[$^2H_4$]-5-CH_3-H_4 folate



[$^2H_4$]-5-CHO-H_4 folate

Figure 2: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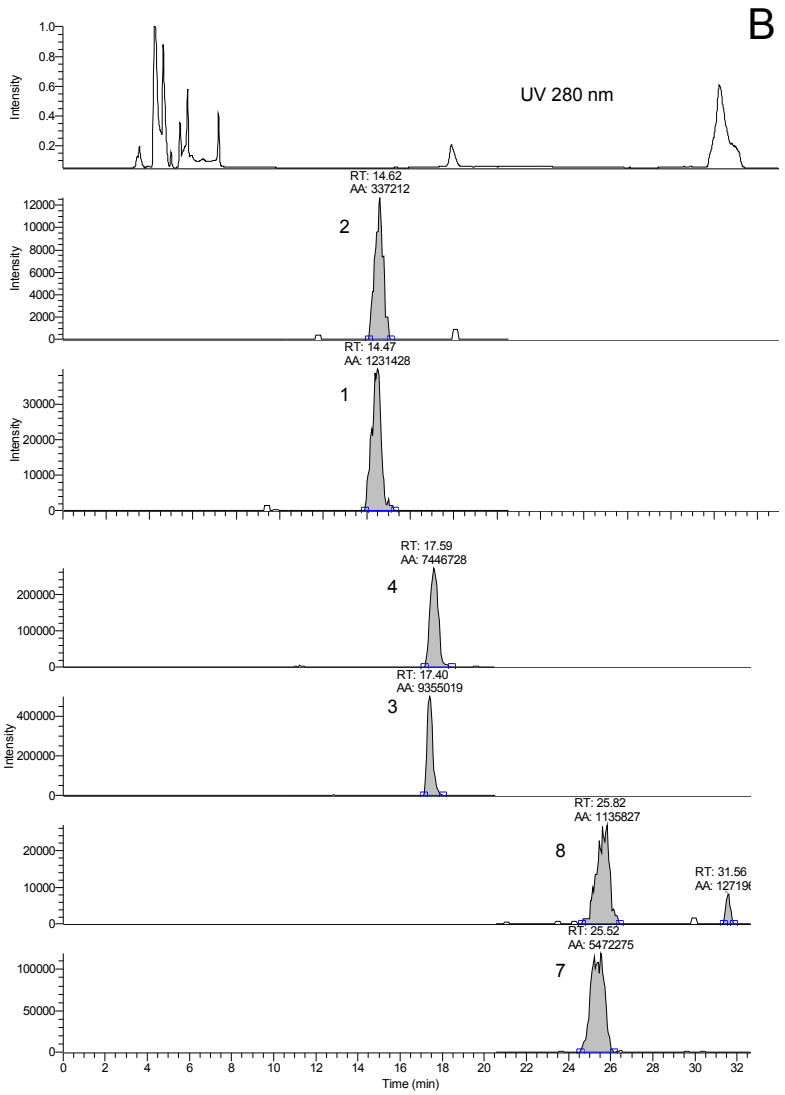
RT: 15.97
2
SRM 450/303
RT: 15.77
1
SRM 460/313
RT: 18.37
4
SRM 464/317
RT: 18.31
3
SRM 470/452
RT: 25.58
6
SRM 474/456
RT: 25.52
5
SRM 474/327
RT: 26.85
RT: 25.45
8
SRM 478/331
RT: 26.71
7
SRM 442/295
RT: 28.19
10
SRM 446/299
RT: 28.13
9
Intensity
Time (min)


B
UV 280 nm
Intensity
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2
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AA: 1231428
1
RT: 17.59
AA: 7446728
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RT: 17.40
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3
RT: 25.82
AA: 1135827
8
RT: 31.56
RT: 25.52
AA: 5472275
7
Time (min)