

Published in:

Analytical Biochemistry 315 (2003) 247–255

The final publication is available at Elsevier via [https://doi.org/10.1016/S0003-2697\(03\)00029-0](https://doi.org/10.1016/S0003-2697(03)00029-0)

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Comparison of Folate Quantification in Foods by High-

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Performance Liquid Chromatography-Fluorescence

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Detection To Stable Isotope Dilution Assays Using High-

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Performance Liquid Chromatography-Tandem Mass

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Spectrometry

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1 ABSTRACT

2 A comparison study on folate quantitation was carried out between the recently
3 developed stable isotope dilution assay using liquid chromatography-tandem mass
4 spectrometry (LC-MS-MS) and the frequently used HPLC with fluorimetric detection
5 (LC-FD). By applying LC-MS-MS, spinach, wheat bread, beef and blood plasma
6 were found to contain 159.2, 19.8, 1.2 and 5.6 $\mu\text{g} / 100 \text{ g}$ total folates, repectively,
7 whereas the repective quantitative data obtained by LC-FD were 95.5, 16.2, 0.7
8 and 6.8 $\mu\text{g} / 100 \text{ g}$. In all samples, LC-MS-MS revealed superiour selectivity and
9 precision and circumvented the shortcomings of conventional LC techniques, i. e.
10 ambiguous peak assignment as well as high detection limits for 5-
11 formyltetrahydrofolate, 10-formylfolic acid and folic acid. The affinity
12 chromatography columns used in this study showed excellent clean-up
13 performance and permitted detection limits as low as 0.1, 0.5, 0.1, 0.08 and 0.1 $\mu\text{g} /$
14 100 g for tetrahydrofolate (H_4folate), 5-methyl- H_4folate , 5-formyl- H_4folate , 10-
15 formylfolate and pteroylglutamic acid, respectively. Thus, a tenfold higher sensitivity
16 as compared to solid phase anion exchange cartridges was achieved. However,
17 affinity chromatography columns revealed a significantly higher affinity towards the
18 natural vitamers than to the racemic isotopomeric standards, which has to be
19 considered when applying the latter in stable isotope dilution assays.

20

21 *Key words:* electrospray mass spectrometry, folates, LC-MS-MS, stable
22 isotope dilution assay

23

24

1 INTRODUCTION

2 Since several years there is growing evidence that folate deficiency may increase
3 the risk of neural tube defects during pregnancies (1), coronary heart disease (2),
4 certain forms of tumors (3) and Alzheimer's disease (4). In order to prevent these
5 disorders, adequate dietary recommendations have to be based on accurate data
6 of folates in foods. However, the published data on naturally occurring food folates
7 are still in dispute as the frequently employed methods of quantitation show several
8 drawbacks.

9 The method most widely used is a microbiological assay which only provides a total
10 figure for all derivatives of folic acid (henceforth referred to as folate vitamers).
11 However, it is important to distinguish the single vitamers as they differ in terms of
12 bioavailability (5) and stability (6). For differentiation of folates HPLC methods
13 combined with fluorescence (FD) or mass spectrometric (MS) detection have been
14 developed in the past. Although HPLC-FD has been applied for many years to
15 folate analysis, it lacks specificity and, therefore, is susceptible to matrix
16 interferences. Hence more selective sample clean-up procedures such as affinity
17 chromatography on folate binding protein (7) have to be applied.

18 As coupling HPLC with mass spectrometry enables to overcome these constraints,
19 there is an emerging number of LC-MS methods in this field which focus either on
20 food or clinical matrices such as urine or blood plasma.

21 Quantitation of folates in plasma is facilitated by the fact that of all folates only the
22 5-methyl tetrahydrofolate vitamer occurs in this matrix. The first application of LC-
23 MS to folate analysis in plasma was presented by Garbis et al. (8) using
24 methothrexat as internal standard (IS). In LC-MS the addition of an IS is inevitable

1 not only to correct for recovery losses but also to compensate for variations in
2 ionization yields due to matrix interferences. A reasonable progress in LC-MS
3 methodology was the employment of IS labeled by stable isotopes such as [$^{13}\text{C}_5$]-
4 folic acid, which was first reported by Pawlosky and Flanagan (9).
5 In foods, however, quantification is hampered by the high number of vitamers. In a
6 first attempt Stokes and Webb (10) analyzed folic acid and 5-formyltetrahydrofolate
7 in fortified foods without using any IS. In order to improve accuracy we synthesized
8 [$^2\text{H}_4$]-folic acid, [$^2\text{H}_4$]-tetrahydrofolate, [$^2\text{H}_4$]-5-methyltetrahydrofolate, [$^2\text{H}_4$]-5-
9 formyltetrahydrofolate and [$^2\text{H}_4$]-10-formylfolic acid (11) and used them to develop
10 stable isotope dilution assays (SIDAs) for endogenous food folates (12).
11 In the course of our studies we found significantly lower folate contents in some
12 foods including spinach and broccoli (12) than reported earlier by laboratories using
13 HPLC-FD. In order to resolve these discrepancies the purpose of the present study
14 was, first, to improve sample clean-up by using affinity chromatography and
15 subsequently to perform a direct method comparison between SIDA and HPLC-FD.

16 **MATERIALS AND METHODS**

17 **Materials**

18 The following chemicals were obtained commercially from the sources given in
19 parentheses: ascorbic acid, bacterial protease, ethanolamine hydrochloride, formic
20 acid, hexane, hydrochloric acid, 2-mercapto ethanol, methanol, *di*-potassium
21 hydrogen phosphate, potassium hydroxide, sodium acetate, sodium azide, sodium
22 dihydrogen phosphate, sodium tetrahydroboric acid decahydrate, sodium hydrogen

1 carbonate, sodium hydroxide, sodium chloride (Merck, Darmstadt, Germany), 2-[N-
2 cyclohexylamino]ethanesulfonic acid (CHES), N-[2-hydroxyethyl]piperazine-N'-[2-
3 ethanesulfonic acid] (HEPES), sodium ascorbate (Sigma, Deisenhofen, Germany),
4 trifluoroacetic acid, folate binding protein (Fluka, Neu-Ulm, Germany),
5 dithioerythritol (Serva, Heidelberg, Germany), acetonitrile (Baker, Gross-Gerau,
6 Germany), Affi-Gel-10 (Bio-Rad, Munich, Germany). Unlabeled folate vitamers
7 (6S)-tetrahydrofolic acid ((6S)-H₄folate), (6S)-5-methyltetrahydrofolic acid ((6S)-5-
8 methyl-H₄folate) and 10-formylfolic acid were obtained from Dr. Schircks
9 Laboratories (Jona, Switzerland), (6S)-5-formyltetrahydrofolic acid ((6S)-5-formyl-
10 H₄folate) was purchased from Sigma (Deisenhofen, Germany) and folic acid was
11 from Fluka (Neu-Ulm, Germany). Rat serum was obtained from Biozol (Eching,
12 Germany).

13 [²H₄]-labeled folate standards were synthesized as reported recently (11).

14 **Solutions**

15 Extraction buffer: a mixture of aqueous HEPES (50 mmol/L) and aqueous CHES
16 (50 mmol/L) containing sodium ascorbate (2%) and 2-mercapto ethanol (0.2 mol/L)
17 was adjusted to pH 7.85. The buffer was prepared on day of use.

18 Phosphate buffer stock solution (0.1 mol/L, pH 7): a mixture of 50 mL sodium
19 dihydrogen phosphate solution (0.1 mol/L) and 80 mL dipotassium hydrogen
20 phosphate solution (0.1 mol/L) was adjusted to pH 7.

1 Wash solution I (0.025 mol/L) was prepared by diluting the phosphate buffer stock
2 solution (0.1 mol/L, pH 7) with NaCl (1 mol/L). The pH was adjusted to 7 by addition
3 of aqueous KOH (4 mol/L) or HCl (4 mol/L).

4 Wash solution II (0.025 mol/L) was prepared by diluting the phosphate buffer stock
5 solution (0.1 mol/L, pH 7) with water.

6 Elution solution: Dithioerythritol (0.02 mol/L) and trifluoroacetic acid (0.02 mol/L)
7 were dissolved in water.

8

9 **Preparation of affinity chromatography columns**

10 The affinity chromatography (AC) columns were prepared and used according to
11 Konings et al. (13).

12 For the preparation of a column with a binding capacity of approx. 4 µg folic acid,
13 folate binding protein (FBP, 3 mg) was dissolved in cold sodium hydrogen
14 carbonate solution (9 mL, 0.1 mol/L). A slurry of Affi-Gel 10 (12 mL) was transferred
15 to a glass fritted funnel and washed with three bed volumes of cold sodium acetate
16 solution (0.1 mol/L). A slight vacuum was used for draining the solution, but care
17 was taken that the gel did not run dry. The moist gel cake was transferred to the
18 FBP-sodium acetate solution within 10 minutes under gentle agitation. After stirring
19 the solution overnight at 4 °C, 1.2 mL ethanolamine was added and the stirring was
20 continued for another hour at 4 °C. The gel suspension was then filled into an
21 Econo Graph chromatography column (15 mL, Bio-Rad, Munich, Germany) with
22 sodium borate solution (0.05 mol/L) and washed twice with sodium acetate solution
23 (0.01 mol/L) and twice with phosphate buffer stock solution. When not in use, the

1 columns were stored at 4 °C in phosphate buffer stock solution containing 0.2 %
2 sodium azide.

3 The actual capacity of the columns was routinely tested by overloading them with
4 folic acid. The binding affinity of the reduced folates was tested by passing a
5 mixture of the respective commercially available (6S)-isomer and the synthetic,
6 racemic (6R,S) internal standard over the AC column and measuring the
7 concentration of the natural (6S)-vitamer before and after the elution. The analysis
8 provided an affinity factor shown in table 1 for each reduced folate which was used
9 for calculating the concentrations of these vitameres in the sample extracts.

10 **Sample Preparation**

11 **Foods**

12 Spinach, wheat bread and meat samples were purchased at local stores in the city
13 of Munich, Germany and extracted on the same day.

14 Spinach, bread and meat samples were frozen with liquid nitrogen before mincing
15 them with a blender (Privileg, Quelle, Fürth). Method comparison of SIDA to LC-FD
16 was performed by taking aliquots for each measurement from identical
17 homogenates. The aliquots designated for SIDA (1-2 g) were taken from the
18 resulting powder-like homogenate and overlaid with 10 ml of extraction buffer. [²H₄]-
19 labeled internal standards were added to the suspension in an amount to adjust a
20 mass ratio of standard to analyte ranging between 1 and 5.

21 The aliquots designated for LC-FD were overlaid with extraction buffer only and
22 extracted in the same way as the SIDA samples. For determining the rate of

1 recovery for the LC-FD procedure, sample aliquots were overlaid with extraction
2 buffer containing folates in amounts that the addition equals the endogenous
3 contents in the samples. All samples were analysed in duplicates.

4 Sample suspensions were then purged with argon, capped tightly and placed in a
5 boiling water bath for 10 min. Subsequently the extracts were rapidly cooled in an
6 ice-bath and digested with protease (3 mg/g sample) for 6 h at 37°C. After the
7 enzyme digestion, the samples were heated at 100°C for 10 min, cooled on ice and
8 spiked with 100 µL of rat serum. The deconjugation was performed at 37°C
9 overnight. At the end of the conjugase treatment, the samples were again heated at
10 100°C for 10 min, then cooled on ice and centrifuged at 6000 g for 20 min. After
11 passing the residue through a syringe filter (0.4 µm, Millipore, Bedford, MA),
12 spinach extracts were subjected to solid phase extraction clean-up, wheat bread
13 and beef extracts were purified by affinity chromatography as described below.

14

15 **Blood**

16 Blood samples were taken from a volunteer at the institute and collected into
17 vacutainers containing heparin as an anticoagulant (Sarstedt, Nümbrecht,
18 Germany). Plasma was obtained by centrifuging the blood for 10 min at 2000 g.
19 1 mL plasma was spiked with [²H₄]-5-methyl-H₄folate (20 ng) and equilibrated for 30
20 min at room temperature. The solution was then centrifuged (10 min; 2000 g) and
21 diluted with extraction buffer (2 mL) before being subjected to solid phase
22 extraction as described below.

1 **Sample Clean-up by Solid Phase Extraction (SPE)**

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

8 After applying the sample extracts, the columns were washed with 6 volumes of
9 conditioning buffer, and the folates were eluted with 2 mL of aqueous sodium
10 chloride (5 %, containing 1 % sodium ascorbate and 0.1 mol/L sodium acetate).
11 100 μ L 2-mercapto ethanol was added to each eluate and the purified extracts were
12 stored under argon at -30°C until analysis.

13

14 **Sample clean-up by affinity chromatography**

15 Bread and beef extracts were applied to the affinity columns previously equilibrated
16 with two volumes of phosphate buffer. After application, the columns were rinsed
17 with one volume of wash solution I and one volume of wash solution II, respectively.

18 Prior to the elution, the columns were washed with elution solution matching the
19 void volume of the column. The folates were then eluted with one volume of elution
20 solution into a vessel containing an aqueous solution of ascorbic acid (200 μ L,
21 25%), aqueous potassium hydroxide (41 μ L, 60 %) and 2-mercaptoethanol (5 μ L).

22 The samples for SIDA were adjusted to pH 7.5, purged with argon and stored at –

1 30 °C until measurement. The samples for HPLC-FD were checked for pH, as well,
2 filled to volume, purged with argon and stored frozen until analysis.

3 **HPLC-MS-MS and HPLC-UV Analysis**

4 The samples (100 µL) were analyzed on a spectra series HPLC system (Thermo
5 Separation Products, San Jose, CA) equipped with an Aqua C-18 reversed phase
6 column (250 x 4.6 mm; 5 µm, Phenomenex, Aschaffenburg, Germany). The mobile
7 phase consisted of variable mixtures of aqueous formic acid (0.1 %) and
8 acetonitrile, at a flow of 0.8 mL/min. Gradient elution started at 7 % acetonitrile
9 maintained for 9 min, followed by raising the acetonitrile concentration linearly to 13
10 % within 13 min and to 25 % within further 4 min. Subsequently, the mobile phase
11 was programmed to 100 % acetonitrile over 4 min before equilibrating the column
12 for 5 min with the initial mixture.

13 For MS-MS analysis the eluent was diverted to an LCQ-ion trap mass spectrometer
14 (Finnigan MAT, Bremen, Germany) set at MS-MS conditions reported recently (12).

15 For fluorescence analysis, an RF-10 AXL fluorescence detector (Shimadzu,
16 Duisburg, Germany) was coupled in-line with the UV-detector and both signals
17 were recorded simultaneously. The excitation wavelength of the fluorescence
18 detector was set at 290 nm (360 nm for 10-formylfolic acid) and the emission
19 wavelength at 356 nm (460 nm for 10-formylfolic acid). The UV-detector was
20 operated at 280 nm.

21 In contrast to chromatography of food samples, gradient elution for the analysis of
22 plasma samples (100 µL) was shortened due to lower matrix interferences. The
23 mobile phase consisted of mixtures of formic acid (0.1 %) and acetonitrile at a flow
24 rate of 0.8 mL/min. Gradient elution started at 10 % acetonitrile and was raised to

1 20 % acetonitrile within 6 min. Then, the concentration was sharply raised to 80 %
2 acetonitrile within 1 min and to 100 % within another minute. This concentration
3 was maintained for 2 min before lowering the acetonitrile concentration back to the
4 initial value and allowing the column to equilibrate for another 3 min. Each plasma
5 extract was analysed in triplicate. Amounts of the single vitamers were calculated
6 from the peak areas in the respective mass traces using the calibration functions
7 detailed recently (12).

8 The calibration for the fluorescence measurements was performed by an external,
9 four point calibration curve using standard solutions in various concentrations.

10

11 **Detection and Quantitation Limits**

12 Detection limits (DL) and quantification limits (QL) after solid-phase clean-up were
13 determined according to the method of Hädrich and Vogelgesang as reported
14 recently (12). As the low durability of AC columns restricted the number of
15 analyses, DL and QL after affinity chromatography clean-up were assessed from
16 sample chromatograms by converting the amount of internal standard added to the
17 sample to that referring to the lowest peak area considered suitable for
18 quantification (signal-to-noise ratio above 10 and 3 for QL and DL, respectively).

19

20 **RESULTS AND DISCUSSION**

21 In our previous studies on SIDA of folates (12) we found sample clean-up by solid
22 phase extraction (SPE) on strong anion exchange cartridges to be appropriate for a
23 large number of matrices. However, foods like beef or samples composed of many

1 ingredients such as bread could not be quantified due to high background noise.
2 Therefore, we prepared affinity chromatography columns in a similar way to
3 Konings (13) by coupling commercial folate binding protein (FBP) to Affigel 10 in
4 order to improve sample clean-up.

5 **Performance characteristics of the affinity chromatography columns**

6 Regarding the mass chromatograms of a wheat bread shown in figure 1, affinity
7 chromatography (AC) clean-up proved to be a very specific procedure that enabled
8 sensitive quantifications. Unfortunately, this method shows two major limitations.
9 First, we could corroborate earlier findings (13, 14) that binding capacity
10 significantly decreases after the passage of several extracts. In accordance with
11 Konings (13) reporting a 58 % reduction after 18 extracts, we found a reduction of
12 26 % and of 47 % after 12 extracts and 22 extracts, respectively. This appears to
13 be a severe limitation of this clean-up technique as FBP is quite expensive and the
14 columns obviously reach their lower useful limit after a few dozens of applications.
15 The reduction in capacity even aggravates the second drawback of AC, i.e. the
16 discrimination of certain folate vitamers. In particular, 5-formyl-H₄folate, is known to
17 exhibit a lower affinity towards the AC columns than the other vitamers, an effect
18 that is dependent on the total load of the column. Most recently, a 90 % recovery
19 for 5-formyl-H₄folate with a folate load of 25 % was reported (14), and, similarly,
20 other researchers (15, 16) recommended that the folate load should not exceed this
21 value. Therefore we ran the columns at a maximum of 30 % of their total capacity.
22 However, the useful load of the affinity chromatography columns for the unlabelled
23 vitamers is further reduced by the added isotopically labeled standards which
24 occupy folate binding sites as well.

1 In addition, AC columns revealed discrimination of racemic folate isotopomers
2 compared to their unlabeled, natural analogues. The binding affinities of the
3 racemic folate vitamers [²H₄]-H₄folate, [²H₄]-5-formyl-H₄folate and [²H₄]-5-methyl-
4 H₄folate relative to those of the natural (6S)-vitamers are presented in table 1. The
5 lowest affinity was determined for 5-formyl-H₄folate, of which the racemic
6 compound showed only 74 % affinity in comparison to the natural vitamer. From
7 these results it can be calculated that the unnatural (6R)-isomer is bound to the
8 column with an affinity of 48 % compared to the (6S)-isomer. The affinity for (6R,S)-
9 5-methyl-H₄folate (84 %) is slightly higher and that for (6R,S)-H₄folate (97 %) differs
10 only marginally from that of the natural isomer. To the best of our knowledge, only
11 Shane et al. (17) investigated the different binding affinities of reduced folates
12 towards folate binding protein. The authors only examined the behaviour of racemic
13 5-methyl-H₄folate and reported an affinity of about 80 %, which is in good
14 agreement with our data.

15

16

17 **Detection limits after solid phase and affinity chromatography clean-up**

18 Despite the beforementioned shortcomings of the affinity chromatography
19 procedure, the increase in sensitivity is markedly. Due to the more selective clean-
20 up, significantly lower detection (DL) and quantification limits (QL) could be
21 achieved. The DLs after affinity chromatography clean-up for H₄folate, 5-CH₃-
22 H₄folate, 5-formyl-H₄folate, 10-formylfolate and pteroylglutamic acid were calculated
23 to be 0.1, 0.5, 0.1, 0.08 and 0.1 µg / 100 g, which were more than 10fold lower than
24 the values gathered after solid phase extraction (1.5, 0.5, 1.2, 0.6 and 2.6 µg / 100

1 g, respectively). This enabled us to quantify complex matrices such as wheat bread
2 or samples with extremely low folate contents such as beef. Unfortunately, the
3 affinity chromatography in conjunction with SIDA does not appear to be suited for a
4 routine application as the racemic standards are discriminated and AC is more
5 expensive than solid phase extraction. However, the latter limitation may be
6 circumvented if commercially packed columns become available.

7

8 **Quantification of spinach using LC-MS-MS and LC-FD**

9 The application of both analytical techniques produced the chromatograms
10 displayed in figure 2 which illustrate that MS-MS enables unambiguous peak
11 identification and quantification. Especially 5-formyl-H₄folate can be unequivocally
12 determined in the spinach sample, whereas in the LC-FD chromatogram this
13 compound is not detectable at all. The mass ranges are almost devoid of
14 background interferences and underline the superiority of the SIDA in comparison
15 to the LC-FD method. As is evident from table 2, the amount of folates in spinach is
16 significantly underestimated by LC-FD. By applying SIDA, a 67% higher folate
17 content was acquired in the identical aliquot, although the LC-FD values were
18 corrected for the recovery values given in table 2.

19 An important reason for the lower values found by LC-FD is the fact that 5-formyl-
20 H₄folate could not be detected by LC-FD due to its weak fluorescence. In contrast
21 to this, 12.0 µg / 100 g 5-formyl-H₄folate was quantitated by SIDA. However, even if
22 this amount of 5-formyl-H₄folate was included into the total folate content gathered
23 by the LC-FD method, a significant lower value than that of SIDA was obtained.

24 The main reason for the differing total folate content is the severe difference of the

1 value for 5-methyl-H₄folate (77.2 µg / 100 g) gathered by LC-FD to the SIDA value
2 (137.0 µg / 100 g), which demonstrates the superior correction for losses by using
3 isotopically labeled standards.

4

5 **Comparative quantification of wheat bread**

6 The quantitative results of wheat bread are compiled in table 3. SIDA after affinity
7 chromatography clean-up revealed the samples to contain 5-methyl-H₄folate, 5-
8 formyl-H₄folate, H₄folate, 10-formylfolic acid and folic acid, of which the latter was
9 below quantifiable levels. An exemplary chromatogram shown in figure 2 clearly
10 shows the remarkable purity of the mass ranges, which resemble a mixture of
11 standard solutions. By LC-FD, however, neither 5-formyl-H₄folate nor folic acid was
12 detected, which was again due to the poor fluorescence of both compounds. This
13 results in a significantly different distribution of the vitamers, whereby the SIDA
14 found the main folate vitamer to be 5-formyl-H₄folate followed by H₄folate. In
15 contrast to this, the determination by LC-FD resulted in 10-formylfolate as the major
16 vitamer, followed by 5-methyl-H₄folate. In particular the value for 10-formylfolate
17 has to be viewed with caution as the low recovery below 20 % results in high
18 systematic errors, if recovery shows even small variations. These findings highlight
19 the errors, which occur in quantifying folate vitamers without using isotopomeric
20 standards.

21 Although the vitamer pattern measured by LC-FD was so different from that found
22 by SIDA, the total folate figures in wheat bread were quite similar. SIDA provided
23 a value of 19.8 µg / 100 g, whilst the LC-FD determination resulted in 16.2 µg / 100

1 g. Taken together, in case of such a complicated matrix LC-MS-MS proved to be
2 the more robust method and produced more reliable results.

3

4 **Comparative quantification of beef**

5 Meat samples proved to be a challenge for the analysis via LC-MS-MS. As the
6 folate levels in this matrix are known to be low (18), affinity chromatography clean-
7 up proved to be essential for the success of the assay. The quantitative study by
8 SIDA revealed beef to contain 0.2 µg / 100 g 5-methyl-H₄folate, 0.7 µg / 100 g
9 H₄folate and 0.3 µg / 100 g 10-formylfolic acid, whereas the values obtained by LC-
10 FD were 0.2 µg / 100 g for 5-methyl-H₄folate, 0.2 µg / 100 g for H₄folate and 0.1 µg
11 / 100 g for 10-formylfolic acid. Analogously to the foods analysed before, 5-formyl-
12 H₄folate and folic acid could not be detected by LC-FD, whereas the more selective
13 SIDA confirmed the presence of both compounds. Because of the very low levels
14 present in the matrix we could not give quantitative data for these vitamers. The
15 total folate values calculated as folic acid for both methods were 1.2 µg / 100 g for
16 SIDA and 0.7 µg / 100 g for the LC-FD; thus, the LC-FD figure in this case is quite
17 close to that of the SIDA.

18 This comparison demonstrated that the LC-FD assay only produces reliable results
19 if the matrix is not too demanding. In case of beef the analysis was facilitated
20 tremendously, as the extracts contained only few interferences in the fluorescent
21 chromatogram.

22

1 **Comparative quantification of plasma samples**

2 To evaluate the suitability of SIDA for clinical research, we used this method to
3 analyze the folate content of plasma samples in comparison to LC-FD. In
4 agreement with previous studies (7), SIDA proved the plasma samples only to
5 contained 5-methyl-H₄folate. Although small amounts of other folate vitamers were
6 reported in the literature (19), these compounds were obviously below the DL of
7 SIDA. The results of the comparison are shown in table 4. Although the values
8 were only marginally different, the coefficient of variation (CV) of LC-FD
9 determination is significantly higher than that of SIDA. This constraint is attributable
10 to interferences which are still present in the fluorescent chromatogram (figure 3)
11 although the samples were purified by solid phase extraction. On the contrary, the
12 SIDA mass chromatograms of 5-methyl-H₄folate and of the internal standard were
13 both devoid of any matrix interferences and allowed an unequivocal quantification.
14 Moreover, the mass ranges of SIDA are much clearer than those in the method
15 presented by Pawlosky et al. (20), who applied a single stage LC-MS system.
16 Using the latter equipment, only the application of AC enables unequivocal
17 quantification, as reported most recently by Hart et al. (21), who followed the
18 appearance of [¹³C₆]-5-methyl-H₄folate in plasma after oral doses of [¹³C₆]-formyl-
19 H₄folate and [¹³C₆]-folic acid. If the latter method is applied to quantify 5-methyl-
20 H₄folate in plasma by addition of labelled racemic standards, discrimination of the
21 latter substances by AC has to be considered. Compared to these single stage
22 methods, it is evident that the introduction of tandem-MS results in a markedly
23 increased specificity, and, therefore, requires less sample cleanup.

24

1 CONCLUSION

2 The method comparisons outlined in this communication show the superiority of the
3 newly developed SIDA using LC-MS-MS in contrast to the routinely applied LC-FD
4 method. The introduction of isotopically labeled standards for all analytes and the
5 improvement in selectivity and sensitivity by applying MS-MS results in a much
6 easier and more accurate quantification of folate vitamers in a wide range of
7 matrices. The need for a careful peak assignment, stressed by many authors (14) is
8 facilitated in a most comfortable way and a satisfactory chromatographic separation
9 is no longer really necessary, as characteristic mass transitions are recorded. By
10 contrast, single stage mass spectrometry is not suited to perform sensitive
11 quantifications unless affinity chromatography (AC) is used for sample cleanup. To
12 achieve maximum selectivity, the adoption of the MS-MS technique in stead of the
13 single stage MS proved to be necessary for all the matrices studied. Hence, in most
14 cases an elaborate and expensive sample clean-up by AC columns can be omitted.
15 Further improvement may be achieved by using triple-quadrupol instruments, as
16 these enable far more transitions per time, reveal a wider linearity and are less
17 susceptible to interfering ions. Therefore, HPLC run times can be shortened and a
18 higher sensitivity may be achieved.

19

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Table 1. Binding affinity of racemic folate vitamers to the affinity chromatography columns relative to the natural (6S)-vitamers. Coefficient of variation for binding affinity was 5% (mean of triplicate analysis).

Vitamer	Binding affinity [%]
(6 <i>R,S</i>)-H ₄ folate	97
(6 <i>R,S</i>)-5-Formyl-H ₄ folate	74
(6 <i>R,S</i>)-5-Methyl-H ₄ folate	84

Table 2. Folate content ^a in spinach measured by SIDA and LC-FD in µg/100g fresh weight.

	SIDA	LC-FD
5-Methyl-H ₄ folate	137.0 (3.0)	77.2 (18.5) ^b
5-Formyl-H ₄ folate	12.0 (7.1)	n.d.
H ₄ folate	15.5 (5.6)	21.5 (11.5) ^c
Sum (as Folic acid)	159.2	95.5

n.d.: not detected

^a inter-assay coefficient of variation (n=3) is given in parentheses

^b corrected for the recovery value of 43.8 ±2.1 % .

^c corrected for the recovery value of 56.3 ±3.0 %.

Table 3. Folate content ^a in wheat bread measured by SIDA and HPLC-FD in µg / 100 g fresh weight.

	SIDA	LC-FD ^a
5-Methyl-H ₄ folate	3.3 (18.8)	5.5 (2.5) ^b
5-Formyl-H ₄ folate	8.7 (13.3)	n.d.
H ₄ folate	6.4 (1.4)	1.0 (22.0) ^c
10-Formylfolate	2.3 (11.1)	10.6 ^d (2.4)
Folic acid	n.q.	n.d.
Sum (as Folic acid)	19.8	16.2

n.d.: not detected; n.q.: not quantified

^a inter-assay coefficient of variation (n=3) is given in parentheses

^b corrected for the recovery value of 84.2 ±3.3 %

^c corrected for the recovery value of 65.4 ±2.4 %.

^d corrected for the recovery value of 17.6 ±2.0 %.

Table 4. Comparison of the amount of 5-methyl-H₄folate in a plasma sample analysed by SIDA and LC-FD. The results are given in µg / L.

	SIDA ^a	LC-FD ^a
5-Methyl-H ₄ folate	5.6 (9.1)	6.8 (28.2)

^a inter-assay coefficient of variation (n=3) is given in parentheses

LEGENDS TO THE FIGURES

- Figure 1.** LC-MS-MS chromatogram (SIDA) of a wheat bread extract purified by affinity chromatography.
- Figure 2.** Chromatographic resolution of folate vitamers in a spinach extract analysed by SIDA (above) and LC-FD (below).
- Figure 3.** Comparison of a plasma extract analysed by SIDA (above) and LC-FD (below) containing 5-methyl-H₄folate as the only folate vitamer.

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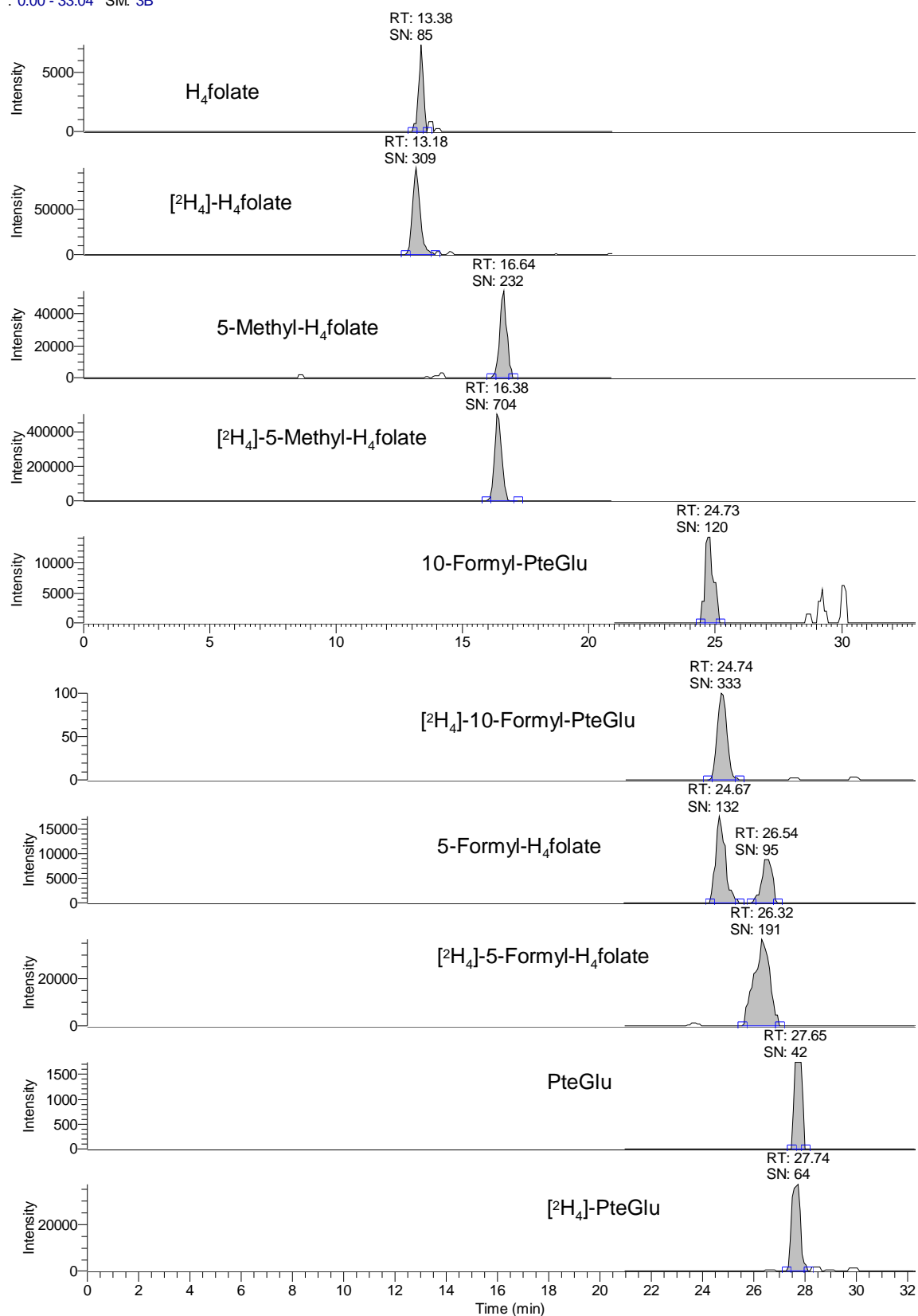
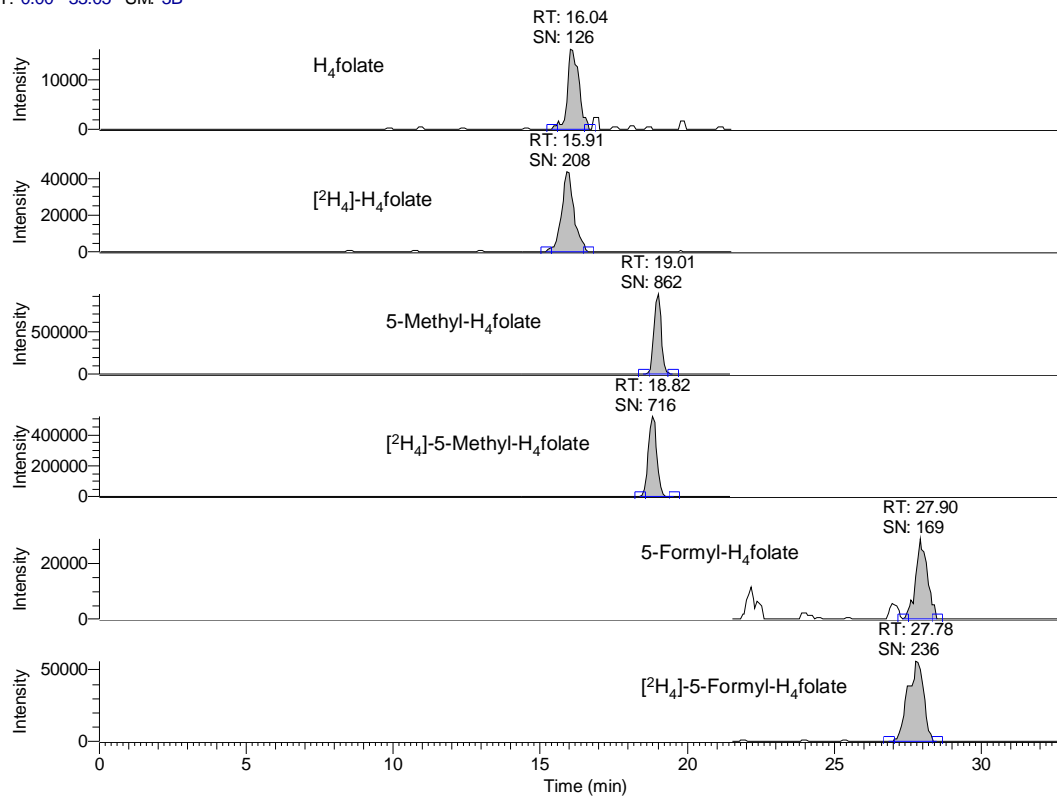


Figure 1

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RT: 0.00 - 32.98 SM: 3B

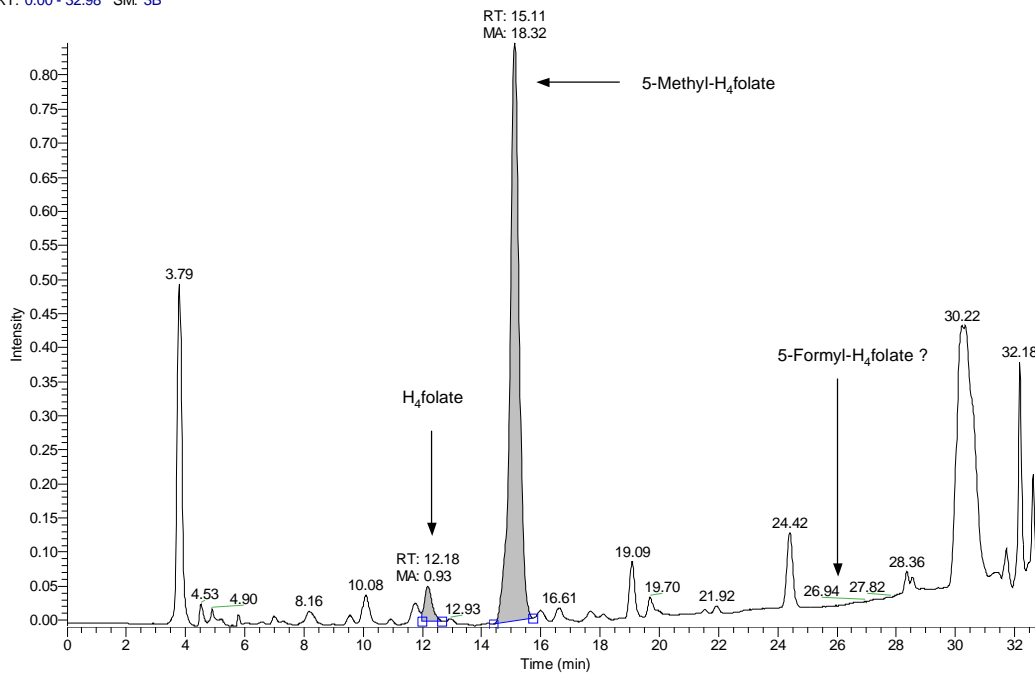
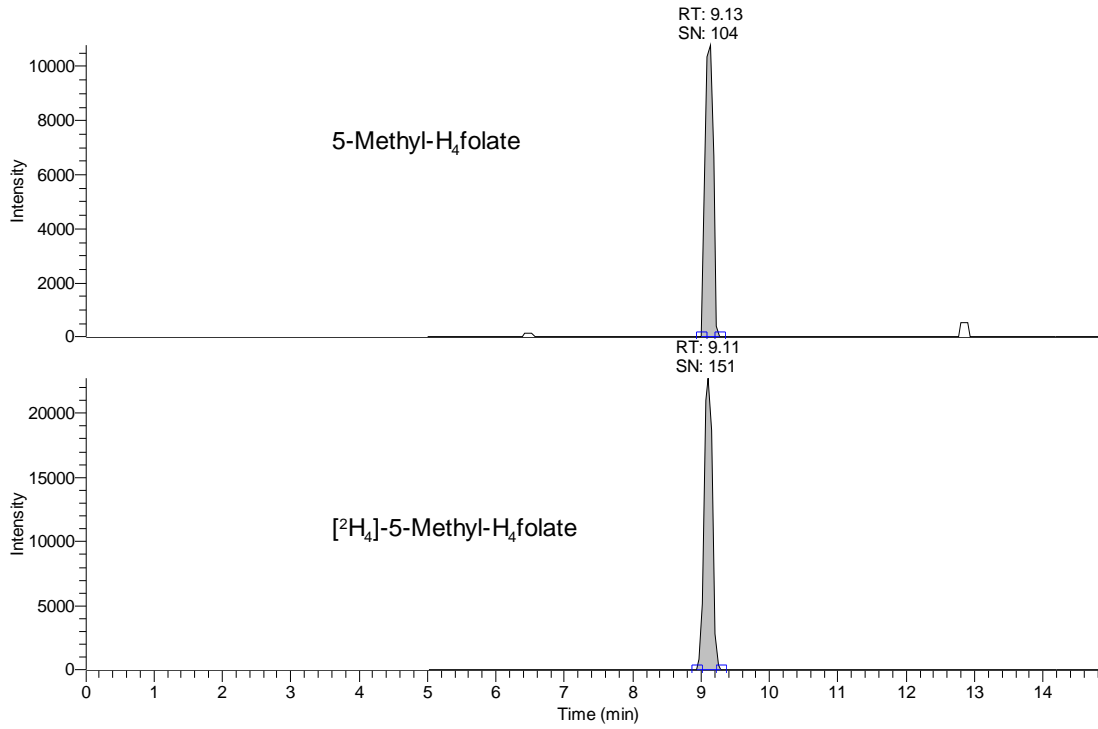


Figure 2

RT: 0.00 - 14.98 SM: 3B



RT: 0.00 - 14.98 SM: 3B

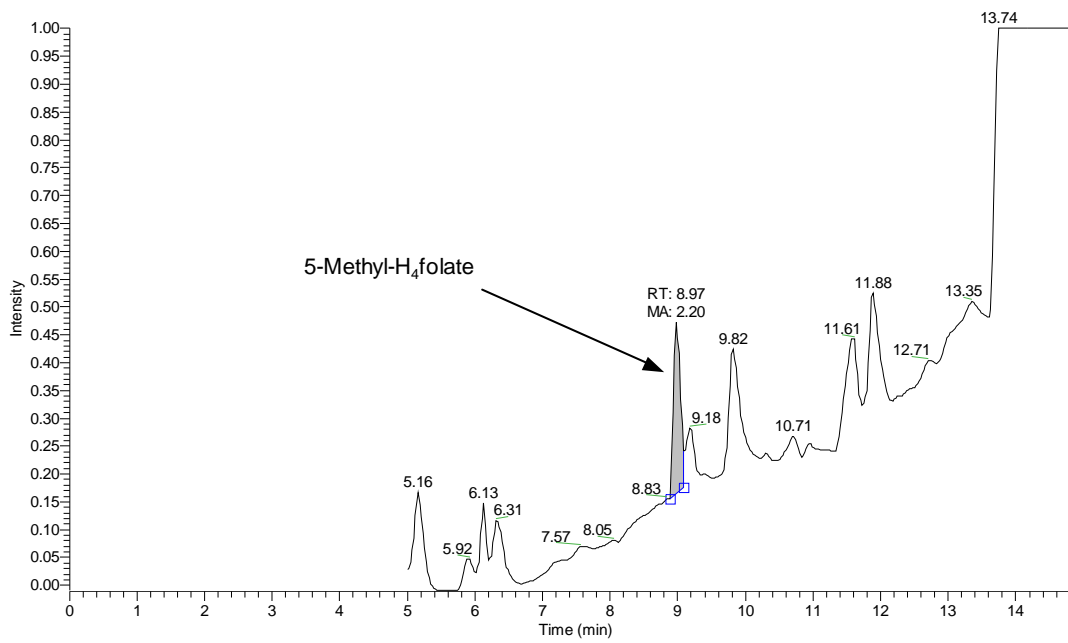


Figure 3