Simultaneous analysis of folic acid and pantothenic acid
in foods enriched with vitamins by stable isotope dilution
assays

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ABSTRACT

Folic and pantothenic acid were quantified in multivitamin products by stable isotope dilution assays using [2H₄]-folic acid and [13C₃,15N]-pantothenic acid as the internal standards. Detection was achieved by liquid chromatography-mass spectrometry which enabled unequivocal determination of the vitamins. Due to the very simple extraction procedure, analysis of the vitamins was completed within two hours. When analyzing multivitamin sweets, the intra-assay and inter-assay coefficient of variation was 3.2 % (n=5) and 3.1 % (n=5) for folic acid and 4.5 % (n=5) as well as 6.5 % (n=7) for pantothenic acid, respectively. Along with the precision data, recovery values of 99.4 % for folic acid and 103 % for pantothenic acid at addition levels of 6 mg/kg and 600 µg/kg, respectively, to starch products proved the accuracy of the new method. Application of the stable isotope dilution assay to fruit juices, whey products, cereals, sweets, pharmaceuticals, wheat flour and salt fortified with one or both vitamins revealed that for the majority of products the labelled pantothenic acid contents were exceeded by about 30 %, whereas for folic acid also significantly lower contents than the label claim were found.

Key words: folic acid, liquid chromatography – tandem mass spectrometry, multivitamin products; pantothenic acid, stable isotope dilution assay
INTRODUCTION

In the nutrition sciences evidence is accumulating that vitamins are not only essential for maintaining normal physiological functions but also to prevent from hazards resulting from oxidative stress or disorders of cell division and DNA repair. In particular the vitamers of the folate group are supposed to prevent neural tube defects, alzheimer’s disease and colon cancer. Most recently, a review highlighted that folate deficiency may induce single strand breaks and mimic radiation damages [1]. Therefore, a lot of foods are supplemented with vitamins and for cereal products, fortification with folic acid is mandatory in the USA. Analysis is commonly performed for each vitamin separately, apart from some HPLC applications to pharmaceutical multivitamin products. As pharmaceuticals contain vitamins in the mg/g range, the universal UV detection is sufficient to analyze water-soluble as well as fat-soluble vitamins [2-4]. However, in fortified foods vitamin levels are much lower, thus rendering UV detection too insensitive for a couple of compounds. For instance, pantothenic acid (vitamin B5, PA) contains no chromophore and, therefore, is often obscured by matrix interferences as it has to be detected at 200 nm. Fluorescence detection, however, although being more selective and sensitive, is restricted to thiamine, riboflavine, pyridoxine and not feasible to detect PA, folic acid, and niacin.

By contrast, mass spectrometry (MS) coupled to liquid chromatography (LC) appears to be suited to detect selectively, sensitively and universally most of the vitamins. However, as ion yields in common atmospheric pressure ionization sources show wide dispersions within one single chromatographic run, the use of internal standards is essential. As we recently developed stable isotope dilution assays (SIDA) for pantothenic acid via GC/MS [5] and for folates via LC/MS/MS detection [6], it was the goal of the current study to combine these methods into a preliminary multivitamin
method. Since SIDAs exhibit major specificity, accuracy, and sensitivity, this approach offers the perspective for a candidate reference method.

EXPERIMENTAL

Chemicals

The following chemicals were obtained commercially from the sources given in parentheses: (R)-pantothenic acid hemicalcium salt (Aldrich, Steinheim, Germany); acetonitrile, formic acid, hydrochloric acid, KHCO₃, methanol, Na HCO₃, sodium acetate, Na₂SO₄, (Merck, Darmstadt, Germany). [¹⁵N,¹³C₃]-pantothenic acid and [²H₄]-folic acid were synthesized as reported recently [5, 7].

Extraction buffer consisted of aqueous HEPES (50 mmol/L) and aqueous CHES (50 mmol/L) at pH 7.85 and contained sodium ascorbate (2 %) and 2-mercapto ethanol (20 mM).

Stable isotope dilution assay (SIDA) for the determination of free PA in foods

Tablets and sweets were ground in a mortat and breakfast cereals in a grain mill (Bosch, München, Germany). The resulting powders or flours (0.5 g) were stirred for one hour at 20 °C in extraction buffer containing calcium [¹⁵N,¹³C₃]-(R)-pantothenate (10 µg) and [²H₄]-folic acid (400 ng). To juices and whey products the labeled standards were added directly.

The extracts were filtered and, after passing through an syringe filter (0.4 µm,
Millipore, Bedford, MA, USA), analyzed by LC/MS/MS.

Liquid chromatography / mass spectrometry (LC/MS)

LC/MS and LC/MS/MS was performed by means of an LCQ (Finnigan MAT, Bremen, Germany) coupled to a spectra series high performance liquid chromatograph (Thermo separation products, San Jose, CA, USA) equipped with an Aqua C-18 reversed phase column (250 x 4.6 mm; 5 µm, Phenomenex, Aschaffenburg, Germany). 50 µL of the sample solutions were chromatographed using gradient elution with variable mixtures of aqueous formic acid (0.1 %, solvent A) and acetonitrile (solvent B), at a flow of 0.8 mL/min. After flushing the column for 9 min with 7% B, a 13-min linear gradient was programmed to 17 % B followed by a further 3-min linear gradient to 25 % B. Then, the concentration of B was raised immediately to 100 %, maintained for 5 min and subsequently brought back to the initial mixture for another 5 min to allow for column equilibration. During the first 8 min of the gradient programme, the column effluent was diverted to waste to ensure an adequate spray stability. For LC/MS/MS of pantothenic acid, the mass transitions (m/z precursor ion/ m/z product ion) 224/206 and 220/202 for labeled and unlabelled pantothenic acid, respectively, were chosen. For folic acid, the mass transitions 446/299 and 442/295 were recorded for the labeled and unlabelled isotopomer, respectively. 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 mass spectrometer operated in the positive electrospray mode with a spray needle voltage of +5 kV and a spray current of 20 µA. The temperature of the capillary was 200° C and the capillary voltage was +13 V. The sheath and
auxillary gas nitrogen nebulized the effluent with flows of 68 and 19 arbitrary units, respectively. The ion trap was operated at a helium pressure of $10^{-3}$ Torr.

Determination of response factors for LC-MS/MS

Solutions of calcium pantothenate/ calcium $[^{15}\text{N},^{13}\text{C}_3]$-pantothenate as well as folic acid / $[^2\text{H}_4]$-folic acid in extraction buffer were mixed in nine mass ratios ranging from 0.1 to 9 to give a total volume of 10 mL. Subsequently, the mixtures were subjected to LC/MS/MS as outlined before. Response factors $R_f$ were calculated as reported recently [5].

Determination of detection and quantification limits

30, 110, 220 and 560 ng of PA were added to edible corn starch (1g) and analyzed as detailed before. Each sample was analyzed in triplicates. Detection (DL) and quantification limits (QL) were calculated according to Hädrich and Vogelgesang [7]: 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. QL and DL of folic acid were determined as reported recently [6].
PRECISION AND RECOVERY

Intra-assay precision was evaluated by analyzing a multivitamin sweet as detailed before (n=5).

Recovery was determined by adding 6 µg of PA and 600 ng of FA to edible corn starch (1g) and performing SIDA as detailed before in quadruplicate analysis.

RESULTS AND DISCUSSION

LC/MS of pantothenic acid and folic acid

Recently we reported on two SIDAs, the first to quantify PA by GC/MS-detection [5] and the second to analyze folates by LC-tandem MS [6]. Both assays were based on the use of isotopomeric vitamins as the internal standards. As PA is a very polar molecule, it appeared to be also detectable sensitively by electrospray ionization (ESI)-MS. This assumption was confirmed by preliminary experiments on aqueous solutions of PA, which revealed a conceivable signal of the quasimolecular ion at m/z 220 in positive ESI-MS as depicted in fig. 1.

Besides [M + H]^+ two minor signals at m/z 242 and 461 appeared, which can be assigned to [M + Na]^+ and [2M + Na]^+, respectively. Analogously, [15N,13C3]-PA gave signals at m/z 224, 246 and 469.

As the recently developed SIDA of folates was based on LC/MS/MS due to matrix interferences [6], tandem MS was also applied to PA isotopomers in case of being necessary for unequivocal quantification. By employing collision-induced dissociation (CID) to the protonated molecule of isotopomeric PA, the spectrum shown in fig. 2
was obtained. Subsequent experiments revealed that the signal at [M-18]+ could be 
used for differentiation and quantification of the isotopomers.

The calibrating curves of different ratios of the isotopomers revealed linearity for the 
[M + H]+ in single stage and for the product ion [M-18]+ of the protonated molecule in 
MS/MS over two decades of isotope ratios.

For simultaneous LC of PA and folic acid (FA), the system for folates [Freisleben et 
al., 2003] was adopted. LC/MS in single stage MS of a cereal extract revealed a 
good peak shape for PA isotopomers, whereas the mass chromatograms of FA and 
[2H₄]-FA contained several matrix interferences as shown in fig 3. Therefore, tandem 
MS was applied, which improved significantly specificity and peak shapes as displayed 
in fig 4.

Extraction and analysis of free folic acid and free pantothenic acid

The extraction buffer according to Wilson and Horne [8], which proved to be most 
effective for folates, was also best suited for extraction of PA. Therefore, sample 
preparation for LC/MS of PA and FA proved to be very simple. After stirring the 
powdered samples for 1 h in extraction buffer containing known amounts of 
[^15N,^13C₃]-PA and [^2H₄]-folic acid at pH 5.7, the extracts only had to be filtered and 
passed through a membrane filter. Detection was achieved either by single stage or 
tandem MS. In contrast to non-fortified foods, most of the samples analyzed here 
contained only minute amounts of conjugated vitamins. Therefore, enzymatic 
liberation, on the one hand, of bound PA by phosphatase and pantetheinase [5] and, 
on the other hand, of bound FA by amylase, proteinase and deconjugase [6] was 
evitable.
Performance criteria

To evaluate whether sensitivity of LC-MS was sufficient for quantifying PA in foods, the detection limit (DL) was determined in edible starch according to the method of Hädrich and Vogelgesang [9].

The calculations resulted in a DL of 89 µg/100g and a quantification limit (QL) of 200 µg/kg for PA and a DL of 40 µg/kg for FA in starch containing foods. Recoveries were evaluated by adding 6 mg/kg of PA and 600 µg/kg of FA to edible starch and were found to be 99.4 and 103 %, respectively.

For examining precision, a multivitamin sweet was analyzed and revealed an intra-assay and inter-assay coefficient of variation of 3.2 % and 3.1 %, respectively. All performance data mentioned before are summarized in table 1.

Results of the quantifications of foods

Several foods fortified by several vitamins were surveyed to prove the suitability of the new method. Of liquid products, four fortified fruit juices and two whey products were quantified. Moreover, two samples of breakfast cereals, two sweets and one multivitamin pharmaceutical were analyzed. Finally we quantified a meal for weight reduction, a breakfast drink as well as a wheat flour and salt, the latter of which were solely fortified with folic acid. The results of the quantifications are presented in table 2.

Regarding PA, the contents ranged from 0.39 / 100g in the wheat flour to 125 mg /100g in the pharmaceutical. In nearly all products the PA label claim was exceeded
by 10 to 50 %, except one fruit juice, which contained only 60 %, one cereal
containing 240 % and one whey containing 225 % of the amounts declared on the
label. As already reported by Romera et al. [10] on infant formulas, overfortifications
by up to 260% of the PA label claim are common, which was confirmed by our data.
In contrast, the PA content of the pharmaceutical was exactly the labelled one.
Considering folic acid, the contents ranged between 20 and 8380 µg/100g. Contrary
to PA, the folate contents were much more scattered and exceeded only for 7
products the label claim, whereas 5 products contained significantly lower amounts
than labelled. Only two fruit juices and the wheat flour were well in line with the label
within a tolerance of 10 %. Remarkable discrepancies were found on the one hand
in case of the breakfast cereals, which exceeded the label claim by more than 50 %
for both vitamins. Although an overdosage is thought to be reasonable to anticipate
losses during manufacture and storage, these differences to the label appear too
high. On the other hand, the breakfast drink and the meal for weight reduction both
did not reach the labelled FA content, which was first suspected to be due to
endogenous food folates stemming from the single ingredients. To prove this
assumption, the folates were analyzed by the SIDA reported recently [6]. However, in
both products only about 6 µg/100g folates different from folic acid were found, which
did not significantly contribute to the total folate content. The LC-mass
chromatogramms of the slim meal extract is displayed in fig 3.
These findings are in quite accordance with the results of Osseyi et al. [11] who
analyzed folic acid in fortified cereal products by LC-UV. However, the data of the
latter authors ranged between 72 and 147 % of the label claim and thus showed
lower discrepancies than the products analyzed in the present study.
CONCLUSION

In particular folic acid is a common vitamin for food fortification in order to prevent the aforementioned disorders. Considering the recommended intakes of 400 µg/d and the upper limit of 1000 µg/d [12], consumers have to rely on the labelled content to make their diet meet the recommendations. However, our results indicate, that differences for FA up to 110 % above and 20 % below the label claim occur in multivitamin products. Therefore, the manufacturers are called upon adjusting the folate contents more accurately and the official laboratories upon controlling the contents more frequently.

The method presented here reveals excellent accuracy and sensitivity. Other water-soluble vitamins such as pyridoxine or niacin contain nitrogen and, therefore, should be ionizable and detectable by LC/MS. This offers the perspective to include these vitamins into the method presented here and to open inroads into a multivitamin SIDA by LC/MS, which would enable simultaneous, fast and accurate quantification of vitamins.

ACKNOWLEDGEMENT

I am grateful to Mrs. D. Fottner for her excellent technical assistance. Appreciation is also due to Mrs. I. Otte for running the LC/MS equipment.

REFERENCES


**Table 1.** Comparison of performance data of the stable isotope dilution assays (SIDA) based on LC/MS/MS to that based on GC/MS

<table>
<thead>
<tr>
<th>Performance criterion</th>
<th>Folic acid</th>
<th>Pantothenic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Detection limit</td>
<td>40 µg/kg</td>
<td>86 µg/kg</td>
</tr>
<tr>
<td>Quantification limit</td>
<td>120 µg/kg</td>
<td>240 µg/kg</td>
</tr>
<tr>
<td>intra-assay CV</td>
<td>3.2 % (n=5)</td>
<td>4.5 % (n=5)</td>
</tr>
<tr>
<td>inter-assay CV</td>
<td>3.1 % (n=5)</td>
<td>6.5 % (n=7)</td>
</tr>
<tr>
<td>Recovery</td>
<td></td>
<td></td>
</tr>
<tr>
<td>in multivitamin sweets</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Addition level: 6 mg/kg</td>
<td>99.4 % ± 2.0 % (n=3)</td>
<td>103.0 ± 6.5% (n=4)</td>
</tr>
<tr>
<td>Addition level: 0.6 mg/kg</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

n.d. not determined; CV coefficient of variation
Table 2. Analyzed and labelled contents of pantothenic acid and folic acid in foods

<table>
<thead>
<tr>
<th>mg/100g</th>
<th>Pantothenic acid</th>
<th>Folic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>analyzed</td>
<td>label claim</td>
</tr>
<tr>
<td>Fortified Fruit juices</td>
<td>3.58</td>
<td>2.50</td>
</tr>
<tr>
<td></td>
<td>2.66</td>
<td>2.00</td>
</tr>
<tr>
<td></td>
<td>3.79</td>
<td>3.00</td>
</tr>
<tr>
<td></td>
<td>1.86</td>
<td>3.00</td>
</tr>
<tr>
<td>Whey products</td>
<td>2.03</td>
<td>0.90</td>
</tr>
<tr>
<td></td>
<td>1.35</td>
<td>1.20</td>
</tr>
<tr>
<td>Breakfast Cereals</td>
<td>7.94</td>
<td>5.10</td>
</tr>
<tr>
<td></td>
<td>14.65</td>
<td>6.00</td>
</tr>
<tr>
<td>Sweets</td>
<td>23.31</td>
<td>18.00</td>
</tr>
<tr>
<td></td>
<td>36.10</td>
<td>29.70</td>
</tr>
<tr>
<td>Pharmaceutical</td>
<td>115.1</td>
<td>115.00</td>
</tr>
<tr>
<td>Breakfast drink</td>
<td>0.91</td>
<td>0.60</td>
</tr>
<tr>
<td>Meal for weight reduction</td>
<td>0.84</td>
<td>0.65</td>
</tr>
<tr>
<td>Fortified wheat flour</td>
<td>0.36</td>
<td>-</td>
</tr>
<tr>
<td>Fortified salt</td>
<td>n.d.</td>
<td>-</td>
</tr>
</tbody>
</table>

n.d. not detectable

a containing 6.1 µg/100g endogenous food folates

b containing 6.4 µg/100g endogenous food folates
Legends to the Figures

**Figure 1.** Mass spectrum of pantothenic acid (above) and $[^{15}\text{N}, ^{13}\text{C}_3]$-pantothenic acid (below) in positive electrospray ionization mode.

**Figure 2.** MS/MS spectrum of pantothenic acid (above) and $[^{15}\text{N}, ^{13}\text{C}_3]$-pantothenic acid (below) after collision-induced dissociation (CID) of the quasimolecular ions in positive electrospray ionization mode.

**Figure 3.** Single stage mass chromatograms of fortified breakfast cereals containing 7.94 mg/100g of pantothenic acid (PA) and 270 µg / 100g of folic acid (FA).

**Figure 4.** MS/MS chromatograms of fortified breakfast cereals containing 7.94 mg/100g of pantothenic acid (PA) and 270 µg / 100g of folic acid (FA).

**Figure 5.** MS/MS chromatograms of a breakfast drink containing 2.1 µg, 3.2 µg, and 14 µg / 100 g of 5-methyltetrahydrofolate, 5-formyltetrahydrofolate, and folic acid, respectively.
fig. 1
UV 200 nm

m/z 220

PA

m/z 224

[^13]C_3, ^15N]-PA

m/z 442

FA

m/z 446

[^2]H_4]-FA
UV 200 nm

PA  
SRM 220/202

SRM 224/206

FA  
SRM 442/295

SRM 446/299
5-Methyl-5-H4folate

[2H4]-5-Methyl-5-H4folate

5-Formyl-5-H4folate

[2H4]-5-Formyl-5-H4folate

FA

[3H4]-FA

UV 280 nm

SRM 474/327

SRM 478/331

SRM 460/313

SRM 464/317

SRM 442/295

SRM 446/299