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# Determination of folic acid by ion-pair RP-HPLC in vitamin-fortified fruit juices after solid-phase extraction

Dietmar E. Breithaupt*

*Institut für Lebensmittelchemie, Universität Hohenheim, Garbenstr. 28, D-70593 Stuttgart, Germany*

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**Abstract**

A sensitive and reliable method was developed for the determination of folic acid (FA) in vitamin-fortified fruit juices and fruit drinks. After solid-phase extraction clean-up with strong-anion-exchange material, FA was determined by ion-pair reversed phase high-performance liquid chromatography (RP-HPLC). Limits of detection (LOD) and quantitation (LOQ) were 0.04 and 0.06 mg/l, respectively. Average recoveries at two fortified levels (0.5 and 1.0 mg/l) were 97% in 0.1 M acetate buffer (pH 4.9) and ranged from 78 to 93% in spiked blackcurrant nectar, apple juice, and cherry nectar, with standard deviations less than 2.3%. The method was used to analyze nine commercial fruit juices and fruit drinks containing FA in the range of 0.30–1.40 mg/l. In two samples significantly less FA was found than specified. 

*Keywords:* Folic acid; Ion-pair RP-HPLC; Fruit juices

## 1. Introduction

The term 'folate' generally describes a family of compounds with chemical structures similar to synthetic pteroyl monoglutamic acid, commonly known as folic acid (FA). FA is not the physiological active form of the vitamin but is applied as a dietary supplement or for enrichment of food. Usually the folate content of food is quantified by time consuming microbiological assays, carried out as *Lactobacillus casei*-based turbidimetric assay or titrimetric methods (Official Methods of AOAC, 1995; Lim, Mackey, Tamura, Wong, & Picciano, 1998). For detection of FA in vitamin-fortified fruit juices several enzyme immunoassays are commercially available (e.g. Radiascreen© from R-Biopharm GmbH, Darmstadt, Germany) but no simple, rapid and reliable high-performance liquid chromatography (RP-HPLC) method is reported in the literature for such food so far. Therefore, we developed a clean-up procedure, with solid-phase extraction (SPE) at a strong-anion-exchange resin, and an ion-pair reversed phase high-performance liquid chromatography (ion-pair RP-HPLC) method for the determination of FA in different types of vitamin-fortified fruit juices at low milligram-per-litre levels.

## 2. Materials and methods

### 2.1. Materials

FA was obtained from Fluka [Steinheim, Germany; purity (HPLC) $\geqslant$ 97%]. A stock solution (200 mg/l) was prepared by dissolving 10.0 mg FA in 50 ml 0.1 M phosphate buffer (pH 7.0) containing 1% (w/v) sodium L-ascorbate. Aliquots (5 ml) of the stock solution were stored at −18 °C. Working standard solutions were prepared daily by dilution with 0.1 M acetate buffer (pH 4.9) containing 1% (w/v) sodium L-ascorbate. Since the presence of sodium L-ascorbate in the standard solutions hampers a spectrophotometric stability check, HPLC analysis of a 1 mg/l working standard was performed daily. The resulting data showed the stock solution to be stable at −18 °C for about 4 weeks.

All organic solvents (methanol, acetonitrile, n-hexane) and tetra-n-butylammonium hydrogen sulphate ($\geqslant$99%) were obtained from Merck (Darmstadt, Germany). Sodium L-ascorbate ($\geqslant$99%), sodium acetate (anhydrous, >99%), sodium chloride ($\geqslant$99.5%), citric acid monohydrate ($\geqslant$99.5%), and L-malic acid ($\geqslant$99.5%)

* Corresponding author. Tel.: +49-711-459-4097; fax: +49-711-459-4096.

*E-mail address:* breithau@uni-hohenheim.de

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were purchased from Fluka (Steinheim, Germany). For HPLC analysis, ultrapure water from a Milli-Q 185 plus apparatus (Millipore, Eschborn, Germany) was employed.

### 2.2. Instrumentation

#### 2.2.1. HPLC

The HPLC consists of a Hewlett-Packard HP1100 module system (Hewlett-Packard GmbH, Waldbronn, Germany) with autosampler, gradient pump, diode array detector (DAD), and column thermoregulator. An HP3D ChemStation software was used for data processing. Operating conditions were as follows: column temperature, 25 °C; flow rate, 1 ml/min; injection volume: 20 μl; UV-detection at 284 nm, 4 nm bandwidth, with reference at 500 nm, 100 nm bandwidth. Chromatographic separations were performed on an Eurospher 100 $C_{18}$ column (5.0 μm particle size, 250×4.6 mm i.d.) including a guard column ($C_{18}$, 5.0 μm particle size, 20×4.0 mm ID; both Knauer, Berlin, Germany). For gradient elution mobile phases A and B were employed. Solution A and B both contained 5 mM tetra-n-butylammonium hydrogen sulphate and 25 mM sodium chloride in water, Solution B additionally contained 1 mM potassium dihydrogen phosphate in water and 65% (v/v) acetonitrile. The following gradient was used: at the outset, the mobile phase was run isocratically for the first 10 min with 90% A and 10% B. The percentage of B was increased linearly to reach 36% after 15 min, 50% after 35 min, held for 3 min, ramped to the original composition in 2 min, and equilibrated for 5 min. Prior to HPLC analysis, all samples were filtered using Chromafil-PET-45/25 filters (Macherey-Nagel, Düren, Germany) with 0.45 μm pore size.

#### 2.2.2. SPE

SPE was performed using Chromabond-SB cartridges with strong anion exchange material (either 500 mg, 3.0 ml or 900 mg, without reservoir; Macherey-Nagel, Düren, Germany). For elution under reduced pressure (800 mbar) a Baker SPE-12G vacuum manifold (J.T. Baker, Phillipsburg, NJ) was used. The cleanup procedure was as follows: the SPE Cartridge (500 mg, 3.0 ml) was conditioned by sequential elution with two column volumes (about 6 ml each) of n-hexane, methanol, and water without allowing the column to run dry. After the final washing step about 0.5 cm supernatant were kept and a 5 ml sample transferred to the cartridge. The vacuum was adjusted to elute the sample at about 1 drop/s. When the entire extract was eluted, the cartridge was rinsed with two column volumes of water and drained completely after the last wash step. FA was eluted with sodium acetate solution (0.1 M) containing 10% (w/v) sodium chloride directly into a 5 ml volumetric flask which contained 50 mg sodium L-ascorbate until the mark was reached. Prior to HPLC analysis, all samples were membrane filtered using Chromafil-PET-45/25 filters (Macherey-Nagel, Düren, Germany) with 0.45 μm pore size.

### 2.3. Samples

Apple juice, cherry nectar (fruit content: 40%), blackcurrant nectar (fruit content: 30%), and nine commercially available vitamin-fortified fruit juices and fruit drinks (Table 1) were purchased from local supermarkets. The original pH of the samples was not modified. Turbid fruit juices and fruit drinks were centrifuged for 4 min at $10\times10^3$ *g* (Biofuge, Kendro, Osterode, Germany) and membrane filtered prior to SPE cleanup.

### 2.4. Quantitation and recovery studies

For preparing a calibration curve, appropriate volumes of the stock solution were diluted with 0.1 M acetate buffer (pH 4.9) in volumetric flasks. Three replicates of standards at five concentration levels (0.1, 0.2, 0.5, 1.0, and 2.0 mg/l) and a reagent blank were analyzed. For quantitative determination peak areas of FA in the sample chromatograms were correlated with the concentrations according to the calibration curve.

For recovery experiments, 125 μl or 250 μl FA stock solution were diluted in volumetric flasks (50 ml) with 0.1 M acetate buffer (pH 4.9), apple juice, cherry nectar, or blackcurrant nectar, respectively, and subjected to SPE cleanup. Spiked samples (0.5 or 1.0 mg/l) were protected from direct light and processed immediately. Recoveries were calculated according to AOAC methods as follows: % Recovery $=[c_F/c_A]\times100$, where $c_F$ = concentration of FA measured in the fortified sample, and $c_A$ = concentration of FA added in the fortified sample. For each fortification level three replicates were prepared. Samples were generally analyzed within 1 day.

Table 1
Determination of FA in commercial vitamin-fortified fruit juices and fruit drinks

| | Fruit content | Folic acid (mg/l) | |
|---|---|---|---|
| | | Amount labelled | Amount determined[a] |
| a | 20% (turbid) | 0.30 | 0.32±0.02 (=107%) |
| b | 50% (clear) | 0.30 | 0.11±0.02 (=37%) |
| c | 100% (turbid) | 0.60 | 0.61±0.03 (=102%) |
| d | 50% (turbid) | 1.00 | 1.22±0.05 (=122%) |
| e | 100% (turbid) | 1.00 | 1.11±0.02 (=111%) |
| f | 100% (turbid) | 1.00 | 1.49±0.03 (=149%) |
| g | 100% (turbid) | 1.00 | 1.24±0.02 (=124%) |
| h | 100% (turbid) | 1.00 | 1.49±0.05 (=149%) |
| i | 100% (turbid) | 1.40 | 0.66±0.02 (=47%) |

[a] $n=3$; values represent means±standard deviations.

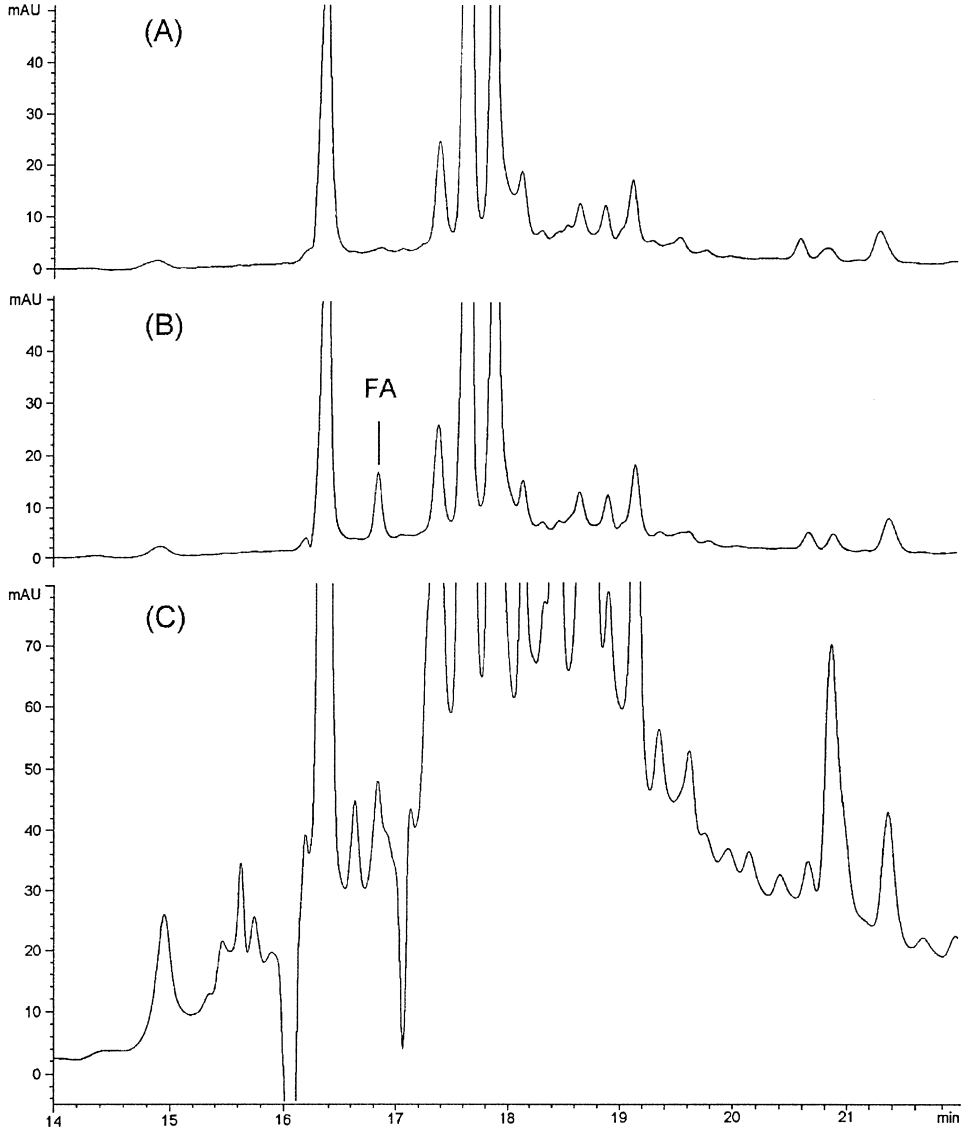


Fig. 1. High-performance liquid chromatograms of (A) unspiked cherry nectar after SPE, (B) spiked cherry nectar (1.0 mg/L) after SPE, and (C) spiked cherry nectar (1.0 mg/L), without clean up. FA, folic acid. The operating conditions are reported in Section 2.2.

If this was not possible, samples were refrigerated overnight in their final form after SPE cleanup.

## 3. Results and discussion

In contrast to reduced folate monoglutamates (e.g. tetrahydrofolate, 5-methyl- and 5-formyltetrahydrofolate) FA shows no fluorescence. Thus, UV detection has to be used for quantification of FA.

SPE, which has received considerable attention as a powerful tool for sample clean-up in recent years, was used prior to chromatographic analysis. However, fruit drinks show a very high matrix background in UV detection, even after purification by SPE. The use of gradient elution with phosphate buffer and acetonitrile for separation of the FA derivatives, which has been reported in the literature (Konings, 1999; Vahteristo, Ollilainen, & Koivistoinen, 1996; Vahteristo, Ollilainen, & Varo, 1997), was not successful in this case. Therefore, ion-pair RP-HPLC was chosen to separate FA from anionic compounds, co-eluting from the SPE column. For HPLC analysis, a gradient described earlier by Bagley and Selhub (1997) was modified and used for separation. Addition of tetra-n-butylammonium, as an ion-pairing reagent, significantly increases the retention time of FA, thus leading to a well shaped and clearly separated single peak as shown in Fig. 1B. Furthermore, the chromatogram 1A of unspiked cherry nectar obtained after SPE clean-up clearly demonstrates, that no peaks are observed which might interfere with the signal for FA. The same holds for apple juice and blackcurrant nectar. The very small peak eluting at the retention time of FA in chromatogram 1A is possibly

due to the native FA content of cherry nectar. Due to the limits of quantitation (LOQ) (see Section 3.1), it was not possible to quantify the native FA concentration of the nectar. However, for the purpose of determination of FA in vitamin-fortified juices, this error is considered acceptable.

### 3.1. Method performance

Identity of FA was confirmed by comparing retention time and UV spectrum of the respective peaks with those obtained from FA reference material (main maximum: $\lambda = 284$ nm; broad shoulder: $\lambda = 350$ nm). The calibration curve for FA diluted with 0.1 M acetate buffer (pH 4.9) was linear from 0.06–2.0 mg/l, based on peak area determination (correlation coefficient, $r = 0{,}999$). Limits of detection (LOD) and LOQ, calculated from the calibration graph according to the recommendations of the Deutsche Forschungsgemeinschaft (DFG, 1991) were 0.04 and 0.06 mg/l, respectively. Method performance was validated by analyzing three replicates of 0.1 M acetate buffer (pH 4.9), apple juice, cherry nectar, and blackcurrant nectar, fortified with FA at 0.5 and 1.0 mg/l each (Table 2). The recoveries for FA spiked at 1.0 mg/l were 97% in 0.1 M acetate buffer (pH 4.9), 92% in apple juice and cherry nectar, and 78% in blackcurrant nectar, with comparatively small standard deviations (1.2–1.7%, $n = 3$). With exception of the last mentioned level, these results lie within the acceptable recovery range of 80–110%, which is recommended at the 1 mg/l level by the AOAC International (1993). Furthermore, they show that there was no need to use an enzymatic treatment with α-amylase and protease, sometimes applied to release protein or oligosaccharide adsorbed FA (Lim et al., 1998). Beyond this, a treatment with folate conjugase to cleave glutamyl residues from polyglutamyl folates of biological samples was not necessary in this investigation.

The low recovery of FA in blackcurrant nectar may be caused by the native content of citric acid of blackcurrants, which shows an extremely high affinity to the strong-anion-exchange resin of the SPE cartridge. Due to this competing process, FA is expected not to be adsorbed quantitatively. L-Malic acid, the main organic acid of apples and cherries, obviously shows a low affinity to the employed SPE material, resulting in good recoveries for FA in apple juice and cherry nectar. This hypothesis was strengthened by results (Table 3) obtained from analyses of FA standard solutions (1.0 mg/l) additionally containing citric acid or L-malic acid [8.0 g/l, 0.1 M acetate buffer (pH 4.9)]. The concentrations of organic acids used correspond to the native content of apple juice, cherry nectar, and blackcurrant nectar (Souci, 1986). Compared to the recovery of FA in a L-malic acid solution (about 96%), the recovery in solutions containing citric acid was only about 86%.

Table 2
Recoveries of FA determined from 0.1 M acetate buffer solutions (pH 4.9) and spiked fruit juices and fruit nectars

| Sample | Fortification (mg/l) | Recovery (%)[a] |
|---|---|---|
| 0.1 M Acetate buffer (pH 4.9) | 1.0 | 97±1.2 |
| | 0.5 | 97±2.3 |
| Apple juice | 1.0 | 92±1.7 |
| | 0.5 | 93±1.2 |
| Cherry nectar (fruit content: 40%) | 1.0 | 92±1.5 |
| | 0.5 | 93±1.2 |
| Blackcurrant nectar (fruit content: 30%) | 1.0 | 78±1.7 |
| | 0.5 | 79±1.2 |

[a] $n = 3$; values represent means±standard deviations.

Table 3
Recoveries of FA (1.0 mg/l) from 0.1 M acetate buffer solutions (pH 4.9) additionally containing L-malic acid or citric acid (8 g/l each) after SPE clean up

| Sample | Cartridge size (mg) | Recovery (%)[a] |
|---|---|---|
| 0.1 M Acetate buffer (pH 4.9) | 500 | 97±1.2 |
| | 900 | 96±0.6 |
| 0.1 M Acetate buffer (pH 4.9) + L-malic acid (8 g/l) | 500 | 96±0.6 |
| | 900 | 95±0.6 |
| 0.1 M Acetate buffer (pH 4.9) + citric acid (8 g/l) | 500 | 86±1.2 |
| | 900 | 87±1.2 |

[a] $n = 3$; values represent means±standard deviations.

Neither dilution of the FA/citric acid solution prior to SPE cleanup nor using cartridges filled with higher amounts of anion-exchange material (900 mg) did enhance the recovery of FA in solutions containing high amounts of citric acid. Nevertheless, the method provides satisfactory and reproducible results for routine monitoring purposes.

### 3.2. Analysis of commercial fruit juices and fruit drinks

Table 1 summarizes the results obtained for the determination of FA in nine fruit juices and fruit drinks purchased from local supermarkets. The levels of FA detected by the present method ranged from 37 to 149% compared with the amounts labelled. Only in two cases (samples ***b*** and ***i***, Table 1) the FA concentrations were significantly lower than labelled. Sample ***b*** showed only 37%, sample ***i*** 47% of the specified FA concentration. In these cases the discrepancy between the 'Amount labelled' and the 'Amount determined' may be due to

unknown degrading reactions of added FA. Consequently, the corresponding serving sizes of samples ***b*** and ***i*** do not provide the amounts of FA, expected by the consumer. This may reduce the benefit for pregnant women, utilizing FA enriched drinks for protecting their child against folate-related diseases such as neural tube defects and anaemia.

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