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Liquid chromatography/mass spectrometry compatible approaches for the quantitation of folic acid in fortified juices and cereals using aqueous normal phase conditions

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ABSTRACT

Folic acid was separated under aqueous normal phase (ANP) conditions with Diamond Hydride™ columns and quantitated in fortified cereal and juice matrices using high performance liquid chromatography/ultraviolet absorption (HPLC-UV) and liquid chromatography/mass spectrometry (LC–MS) based methodologies. The folic acid was well-resolved from matrix components under the ANP conditions studied and allowed for the direct analyses of the fortified juices and cereals without the sample cleanup that is often required for other reported LC-based approaches. The calibration curve obtained from the LC–MS analyses demonstrated good linearity (R^2 = 0.9997) in the studied concentration range of 0.05–0.5 mg/L. The spiked flour percent recovery was 90% with HPLC-UV and 91% with LC–MS. Spiked juice percent recovery was 102% with LC–MS. However, analyses of juices were unsatisfactory either in terms of recovery or sensitivity. Therefore, analyses of juices will either need to be performed by LC–MS or, if by HPLC-UV, will require sample cleanup. Three cereals and one juice were analyzed with the methods.

1. Introduction

Folic acid (pteroyl-L-monoglutamic acid) is a member of a biologically important class of compounds known as folates and is added to many foods and beverages by what is referred to as fortification. As shown in Fig. 1, folic acid consists of a pterin ring moiety, a methylene bridge, and a *para*-aminobenzoic acid (PABA) residue with a peptide bond to a glutamic acid residue. In contrast to the synthetically produced folic acid, naturally occurring folates are typically in a reduced form and have multiple glutamyl residues [1]. These differences have important consequences that illustrate why folic acid was chosen for fortification instead of naturally occurring forms; the fully oxidized, conjugated pterin ring of folic acid makes the compound more stable than natural folates and therefore increases its shelf life. Also, ingested folates with multiple glutamyl residues must be hydrolyzed to monoglutamyl forms in order to cross the cell membrane [2]. Hence, a monoglutamyl folate has greater bioavailability than a homologous polyglutamyl one [3].

Folate metabolites play a vital role in nutrition. For instance, deficiency of folates in the diet leads to an accumulation of homocysteine, since the folate metabolite 5-methyl-tetrahydrofolate (mTHF) is required for remethylation of homocysteine to methionine by methionine synthase [4]. Elevated levels of homocysteine, in turn, have been shown to inhibit purine biosynthesis [5]. The methionine produced is needed for reaction with adenosine-5′-triphosphate (ATP) to form the methyl donor S-adenosyl methionine (SAM). Folate deficiency also leads to lowered amounts of 5,10-methylenetetrahydrofolate, which is needed for the conversion of deoxyuridine to thymidylate [6]. SAM and thymidylate play key roles in DNA methylation and synthesis respectively.

Due to the important biochemical role that folic acid plays, numerous health benefits are associated with adequate folate consumption. The most notable of these is the reported significant reduction of neural tube defects in developing embryos [7]. For this reason, folic acid fortification of grain-based products such as cereals has been mandatory in the U.S. since 1998 [8]. In addition, many juices are also being fortified, although their fortification is not mandatory in the U.S. at this time. Currently, the recommended daily value (RDV) of folic acid is 400 μg, and the folate content of a given food or beverage is listed as a percentage of the RDV per serving.

Despite the well-documented health benefits of folate consumption, some concerns have been raised regarding the fortification program. For example, in order to be converted into metabolically active tetrahydrofolate (THF), folic acid is reduced in a two-step process by dihydrofolate reductase (DHFR). The first step, reduction to 7,8-dihydrofolate, has been shown to be extremely slow due to the difficulty of reducing the highly conjugated pterin ring of folic acid [9]. The low activity of DHFR in

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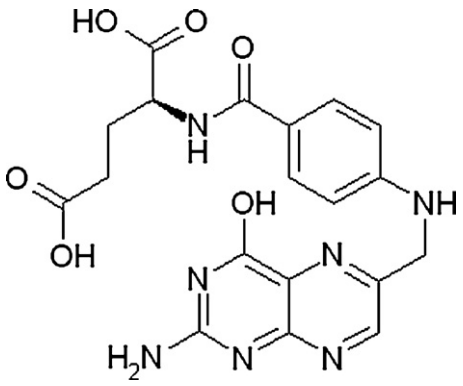

Fig. 1. Structure of folic acid.

this step means that, above a certain intake threshold, folic acid can accumulate in the body. Indeed, unmetabolized folic acid has been detected in plasma [10], breast milk [11], and even cord blood and serum of newborn infants [12]. In light of findings such as these, there has been some concern that this circulating unmetabolized folic acid could have potential detrimental effects. Along these lines, unmetabolized folic acid has shown correlation with reduced natural killer cell cytotoxicity in postmenopausal women [13].

Another concern is that folic acid fortification could impair the diagnosis of vitamin B_{12} deficiency. According to the "methylfolate trap" hypothesis [14], mTHF and homocysteine would accumulate in a B_{12}-deficient individual since vitamin B_{12} is required as a cofactor for methionine synthase [15]. Consequently, less THF would be available for DNA synthesis. This can lead to megaloblastic anemia, which is one of the primary symptoms of vitamin B_{12} deficiency. However, since folic acid is reduced by DHFR in a two-step process to THF, it can take part in DNA biosynthesis without first being converted to mTHF, thus bypassing the methylfolate trap. Therefore, an individual may have vitamin B_{12} deficiency without exhibiting megaloblastic anemia.

To verify that the correct amount of folic acid has been added to fortified products, reliable analytical methods are needed for its quantitation. These methods have included microbiological assays [16] and ion-pair [17] reversed phase (RP) HPLC techniques. However, the microbiological assay is not only time-consuming but is limited in that it only quantifies total folates [18]. If native folates are present, this would not be an appropriate analytical method for quantifying only added folic acid.

Likewise, the HPLC methods typically require laborious sample cleanup steps such as solid phase extraction (SPE) [17] or affinity chromatography [19] in order to remove interfering components from the sample matrix. However, the reported use of hydrophilic interaction chromatography (HILIC) for the quantitation of folates using LC/MS/MS eliminated the need for this type of sample pretreatment in the case of a plasma matrix; most of the interfering matrix components eluted near the void volume while the highly polar folates were substantially retained [20].

It was therefore of interest to investigate whether a similar approach would be of use in analyses of folic acid in fortified product matrices. Along these lines, we have investigated the use of aqueous normal phase (ANP) chromatography for the quantitation of folic acid in fortified juices and cereals. Unique to silica hydride-based stationary phases, ANP has been shown to be a highly effective technique in separations of polar compounds. ANP is similar to HILIC in that retention is believed to be at least partly due to analyte partitioning with a water layer adsorbed onto the stationary phase surface in both techniques. In the case of HILIC, this mechanism was first proposed by Alpert [21] and is beginning to accumulate convincing experimental evidence [22]. The difference between the two techniques is that in ANP, the hydration shell is believed to be much thinner; this is due to the lower polarity, and therefore lower hydrophilicity, of the Si–H moiety compared to the silanols or polar organic moieties responsible for water layer formation in HILIC. The lower hydrophilicity of silica hydride compared to ordinary type B silica has been demonstrated, for example, by the reversed phase retention of hydrophobic test solutes on underivatized silica hydride [23]. The advantages arising from the difference in layer thickness can be illustrated by considering the equation for the stationary phase mass transfer term H_{sp} in the van Deemter equation as modified by Snyder and Kirkland [24], which is given by

$$H_{sp} = \frac{(C_s d_f^2 u)}{D_s} \quad (1)$$

where C_s is a constant which involves stationary phase transfer, D_s is the diffusion of the solute in the stationary phase, u is the linear flow velocity, and d_f is the thickness of the film (the hydration shell in this case). From Eq. (1), it can be shown that because the value of d_f is lower in an ANP retention mechanism than in HILIC, a less significant stationary phase mass transfer term results. This contributes to a lower total plate height H. More detailed descriptions of ANP behavior and silica hydride-based materials have been published elsewhere [25–27].

In this work, Diamond Hydride™ LC columns were used for the separations. One of a wide variety of silica hydride-based stationary phases, Diamond Hydride™ materials are comprised of a small percentage of carbon (~2%) chemically bonded to a silica hydride support. The hydride surface allows for ANP retention while the hydrophobic attached carbon can be used in an RP mechanism. In this manner, the columns can be used for retention of both hydrophilic and hydrophobic analytes depending on the mobile phase composition. Under ANP conditions, they have been found to be especially useful in metabolomics applications [28–30], since a given metabolome often contains a variety of polar species which are difficult to retain and separate by conventional RP techniques.

2. Materials and methods

2.1. Materials

Folic acid and sodium L-ascorbate were obtained from Calbiochem–Behring Corp. (La Jolla, CA, USA). Ammonium formate (>97%) was from Matheson Coleman & Bell (Norwood, OH, USA). Methotrexate and phenylthiohydantoin (PTH)-glutamic acid were from Sigma–Aldrich (St. Louis, MO, USA). Deionized water (DI H_2O) was obtained from a Milli-Q™ purification system from Millipore (Bedford, MA, USA). Acetonitrile of HPLC grade was from Honeywell (Muskegon, MI, USA).

2.2. Instrumentation

2.2.1. HPLC-UV

For UV-based analyses, a Hewlett–Packard (Palo Alto, CA, USA) 1050 HPLC system comprised of an autosampler, degasser, gradient pump, and variable wavelength UV detector set at 284 nm was used. The system was interfaced with Agilent Chemstation (Santa Clara, CA, USA) software. The analytical column was 4.6 mm × 75 mm and was packed with a Diamond Hydride™ stationary phase (MicroSolv Technology Corp. Eatontown, NJ, USA) with a particle diameter of 4.2 μm. Mobile phase A was DI H_2O + 10 mM ammonium formate. Mobile phase B was 90:10 acetonitrile:DI H_2O + 10 mM ammonium formate. Both solutions were vacuum filtered through a 0.45 μm nylon filter (MicroSolv Technology Corp. Eatontown, NJ, USA). Cereal extracts (see Section 2.3), spiked flour (see Section 2.4.2), and the appropriate calibration curve solutions (see Section

Table 1
Gradients used in the analyses.

Gradient 1		Gradient 2	
Time (min)	%B	Time (min)	%B
0	100	0	100
10	90	5	90
19	50	9	50
20	100	10	100

2.4.1) were analyzed with a method consisting of an injection volume of 4 μL, a flow rate of 0.5 mL/min, and Gradient 1 (shown in Table 1). A seven minute equilibration time was allowed between runs.

Spiked apple juice (see Section 2.4.2) was analyzed with a method consisting of an injection volume of 2 μL, a flow rate of 1 mL/min, and Gradient 2 (shown in Table 1). A five minute equilibration time was allowed between runs. Analyses at both 284 and 360 nm were investigated.

The blank run (see Section 2.4.1) was subtracted from all the chromatographic data. All analyses were run in triplicate, and the respective data obtained in each case were averaged. Folic acid and methotrexate were identified by comparing their retention times in chromatograms of the calibration curve solutions with the appropriate peaks in percent recovery or sample extract chromatograms.

2.2.2. LC–MS

For MS-based analyses, an Agilent (Little Falls, DE, USA) 1200SL Series LC system, including degasser, binary pump, temperature-controlled autosampler and temperature-controlled column compartment was used. The mass spectrometer system was an Agilent (Santa Clara, CA, USA) Model 6220 MSD TOF with a dual sprayer electrospray source (ESI). The analytical column was 2.1 mm × 150 mm and was packed with a Diamond Hydride™ stationary phase with a particle diameter of 4 μm. Mobile phase A was DI H_2O + 10 mM ammonium acetate. Mobile phase B was 90:10 acetonitrile/DI H_2O + 10 mM ammonium acetate, pH 7 adjusted with ammonia. An injection volume of 1 μL, a flow rate of 0.4 mL/min, and Gradient 2 (shown in Table 1) were used. A five minute equilibration time was allowed between runs. All fortified samples, calibration curve solutions, and percent recovery solutions were analyzed with this method. All analyses were run in triplicate, and the respective data obtained in each case were averaged. Folic acid and methotrexate were identified by comparing the extracted ion chromatogram (EIC) retention time of the analyte in the calibration curve solutions with the appropriate peak in a given percent recovery or sample extract. In negative ion mode, the following ions were monitored: m/z = 440.1324 was used to monitor $(M–H)^-$ for folic acid and m/z = 453.1640 for methotrexate.

2.3. Samples

Fortified turbid orange juice was purchased from a local supermarket. For the fortified juice extract, 100 μL of the methotrexate internal standard solution (see Section 2.4.1) was diluted with untreated fortified orange juice in a 50 mL volumetric flask. A portion of this solution was centrifuged at 10,000 × g for 4 min. The supernatant was collected and vacuum filtered through a 0.2 μm nylon membrane filter from Alltech (Deerfield, IL, USA). The filtrate obtained was used for the appropriate LC–MS injections (see Section 2.2.2).

Three fortified whole wheat flour-based cereals (referred to as cereals A, B, and C) were purchased from a local supermarket. A portion of fortified cereal was ground using a mortar and pestle. A 20.0 g portion of the ground cereal was added to a beaker with a stirbar. Subsequently, 500 mL of a solution consisting of DI H_2O + 10 mM ammonium formate, 0.5 mg/L methotrexate, 0.05% (w/v) sodium L-ascorbate, and 12 mM NH_3 was quantitatively added to the beaker. The beaker was then covered with Parafilm (Pechiney Plastic Packaging, Chicago, IL, USA). The mixture was stirred for 3 h and sonicated for 30 min. Subsequently, a portion of this mixture was centrifuged at 10,000 × g for 4 min. The supernatant was then collected and filtered through a 0.45 μm nylon membrane HPLC filter prior to HPLC-UV injections (see Section 2.2.1). This process was repeated for each of the three fortified cereals. For LC–MS analyses (see Section 2.2.2), the procedure was the same except that 5.0 g of ground cereal and 0.2 mg/L methotrexate were used.

2.4. Quantitation and recovery studies

2.4.1. Calibration curve

For the calibration curve and spiked juice, a 200 mg/L stock solution of folic acid with 0.05% (w/v) sodium L-ascorbate was prepared by adding 50.0 mg folic acid and 125.0 mg sodium L-ascorbate to a 250 mL amber volumetric flask containing a portion of DI H_2O + 10 mM ammonium formate. A 1000 μL aliquot of 3 M NH_3 was added to dissolve the folic acid. The solution was diluted to the mark with DI H_2O + 10 mM ammonium formate and refrigerated when not in use. An internal standard solution of 100 mg/L methotrexate was also prepared by adding 10.0 mg methotrexate to a 100 mL volumetric flask containing a portion of DI H_2O + 10 mM ammonium formate. A 200 μL aliquot of 3 M NH_3 was added. The solution was diluted to the mark with DI H_2O + 10 mM ammonium formate and refrigerated when not in use.

From the folic acid stock solution, appropriate aliquots were taken and transferred to 50 mL volumetric flasks in order to obtain folic acid concentrations of 0.1, 0.2, 0.5, 1.0, and 2.0 mg/L for the HPLC-UV method and 0.05, 0.1, 0.2, 0.3, and 0.5 mg/L for the LC–MS method. The solutions were spiked with 250 μL of the methotrexate internal standard solution for the HPLC-UV method and 100 μL for the LC–MS method. The flasks were then each diluted to the mark with a solution consisting of DI H_2O + 10 mM ammonium formate, 0.05% (w/v) sodium L-ascorbate, and 12 mM NH_3. Blanks were prepared by omitting the folic acid spike. Aliquots of these solutions were then injected into the HPLC in triplicate. The measured peak integrals of folic acid were plotted on the ordinate, and the known concentrations of folic acid were plotted on the abscissa. Data analysis was performed with Agilent Chemstation and Microsoft Excel 2003 (Microsoft, Redmond, WA, USA). The data were fit to linear models obtained from least squares fitting computations. Analyte peak integrals were correlated with the appropriate linear model to obtain the calculated concentration of folic acid in the percent recovery solutions (see Section 2.4.2) and the fortified sample extracts (see Section 2.3).

2.4.2. Percent recovery

Non-fortified, non-turbid apple juice was purchased from a local supermarket. For HPLC-UV (see Section 2.2.1) juice recovery studies, a 200 mg/L PTH-glutamic acid solution was prepared by adding 50.0 mg PTH-glutamic acid to a 250 mL volumetric flask containing a portion of DI H_2O + 10 mM ammonium formate. A 1000 μL aliquot of 3 M NH_3 was added. The solution was diluted to the mark with DI H_2O + 10 mM ammonium formate and refrigerated when not in use. Next, 500 μL of the folic acid stock solution (see Section 2.4.1) and 250 μL of the PTH-glutamic acid solution were added to a 50 mL volumetric flask and diluted with the non-fortified untreated apple juice to give 2.0 mg/L folic acid and 1.0 mg/L PTH-glutamic acid. For LC–MS analyses (see Section 2.2.2), 125 μL of the folic acid stock solution and 100 μL of the methotrexate internal standard solution were added to a 50 mL volumetric flask and diluted with the non-fortified untreated apple juice to give 0.5 mg/L folic acid and

0.2 mg/L methotrexate. For each of the HPLC-UV and LC–MS percent recovery solutions, a portion was vacuum filtered through a 0.2 μm nylon membrane filter. The filtrates obtained were then analyzed by the specified HPLC method.

For cereal percent recovery, it is recommended to use flour of the same type as is used in the cereal [31]. As such, non-fortified whole wheat flour was purchased from a local supermarket. The solution preparation procedure for HPLC-UV analyses (see Section 2.2.1) was as follows: A 10.0 mg portion of folic acid was added to 200.0 g non-fortified whole wheat flour. This spiked flour was homogenized by mixing with a mechanical shaker for 24 h. A portion of the spiked flour was then ground using a mortar and pestle. A 20.0 g portion of this spiked flour was added to a beaker with a stirbar. Subsequently, 500 mL of a solution consisting of DI H_2O + 10 mM ammonium formate, 0.5 mg/L methotrexate, 0.05% (w/v) sodium L-ascorbate, and 12 mM NH_3 was quantitatively added to the beaker. The beaker was then covered with Parafilm. The mixture was stirred for 3 h and sonicated for 30 min. Subsequently, a portion of this mixture was centrifuged at 10,000 × *g* for 4 min. The supernatant was then collected and filtered through a 0.45 μm nylon membrane HPLC filter prior to HPLC injections. For LC–MS analyses (see Section 2.2.2), the procedure was the same except that 5.0 g spiked flour and 0.2 mg/L methotrexate were used. The expected concentrations of folic acid in the spiked flour extracts using these protocols were therefore 2.0 and 0.5 mg/L for HPLC-UV and LC–MS respectively.

3. Results and discussion

The extraction of folic acid from food matrices has typically involved several steps. First, homogenization of the sample in an appropriate extraction solvent is carried out in the presence of an anti-oxidizing agent such as sodium ascorbate or 2-mercaptoethanol. After the extraction, incubation steps with α-amylase, protease, and folate conjugase are typically employed. The amylase and protease are used to break down carbohydrates and proteins respectively in which folates may be trapped, thus leading to a higher percent recovery. However, the cereal percent recoveries obtained in this work were sufficiently high without the use of either enzyme, and therefore these steps were omitted. The conjugase breaks down naturally occurring polyglutamyl folates into monoglutamyl forms [19]. Since the focus of the present work was on quantifying only folate added due to fortification, this step was not employed either.

Solid phase extraction is then performed in typical reversed phase protocols for either food or juice extracts in order to remove compounds from the matrix which would co-elute with folic acid in the RP-HPLC runs. By performing the separation via an ANP retention mechanism, however, this step can be omitted; many of the matrix components are less hydrophilic than folic acid and therefore elute earlier in the HPLC run. In this manner, the separation acts as the sample cleanup. This process is illustrated in Fig. 2, which depicts a typical chromatogram obtained from one of the fortified cereals. Many of the sample components elute near the void volume while folic acid is highly retained, and its corresponding peak is well-resolved with respect to the other sample peaks. In contrast, separation by a reversed phase mechanism, even with the use of ion-pairing agents, is often unfeasible without prior sample cleanup due to insufficient differences in hydrophobicity between folic acid and some of the matrix components.

Even though UV detection functions at moderately high concentration levels, the efficiency and peak shape (symmetry) for folic acid are excellent, which are essential requirements for the development of an accurate and reliable quantitative method. For example, the 2.0 mg/L folic acid calibration curve solution run using the HPLC-UV method and Gradient 1 (see Table 1) gave an efficiency

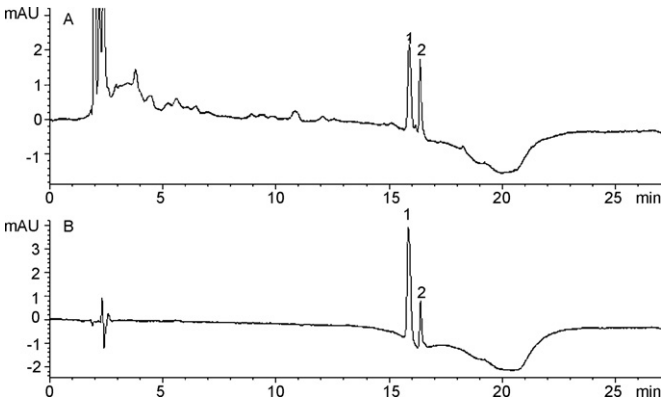

Fig. 2. HPLC-UV chromatograms of A) fortified cereal C extract and B) 2.0 mg/L folic acid calibration curve solution using Gradient 1. Peaks 1 and 2 correspond to folic acid and methotrexate respectively. See Section 2.3 for sample preparation procedure and Section 2.2.1 for chromatographic conditions.

Table 2
%RSD of folic acid retention time in various samples by HPLC-UV. *n* = 3.

Sample	%RSD
Calibration curve	
0.1 mg/L	0.05
0.2 mg/L	0.08
0.5 mg/L	0.02
1.0 mg/L	0.05
2.0 mg/L	0.03
Cereals	
Cereal A	0.14
Cereal B	0.07
Cereal C	0.03
Percent recovery	
Spiked flour	0.02

of close to 120,000 plates per meter and an asymmetry factor of 1.1. The efficiency and the asymmetry factor were greater and closer to unity respectively than those that could be estimated from published HILIC-based separations of folic acid using gradient elution [32,33]. The repeatability of the method was demonstrated by the low percent relative standard deviations (%RSD) of the folic acid retention times for multiple injections of the same sample in the case of each calibration curve solution, cereal extract, and percent recovery solution. Table 2 shows the %RSD data obtained using the HPLC-UV method.

Fig. 3 shows three overlays of the LC–MS EIC of a fortified orange juice extract. The folic acid peak in the chromatogram demonstrates

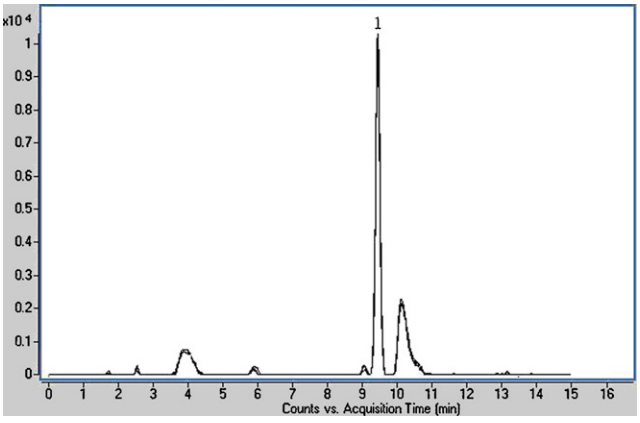

Fig. 3. Overlay of three LC–MS EICs of fortified orange juice extract at *m*/*z* = 440.1234 using Gradient 2. Peak 1 corresponds to $(M–H)^-$ for folic acid. See Section 2.3 for sample preparation procedure and Section 2.2.2 for chromatographic conditions.

Table 3
Percent recovery of folic acid in spiked, non-fortified matrices.

Sample	Concentration			Recovery (%)	
	Ideal	Measured		(UV)	(MS)
		(UV)	(MS)		
Cereals (μg/g)					
Spiked flour	50.0	45	46	90	92
Juices (mg/L)					
Spiked apple juice	0.50		0.51		102

the excellent efficiency and peak symmetry that can be achieved with an ANP separation. This can be rationalized by considering the nature of the ANP retention mechanism. As described earlier, the thin hydration shell believed to be involved in retention allows for the kinetically facile partitioning of the analyte between the stationary phase and mobile phase. This is in contrast with HILIC, where the postulated thicker hydration shell can cause long equilibration times [34] and peak broadening [35]. The use of MS as a detector adds another degree of selectivity to the analysis via the ability to obtain an EIC at a given m/z value. The LC–MS methods also showed good repeatability, with a %RSD of analyte retention times between 0.5 and 0.8.

PTH-glutamic acid was initially chosen as the internal standard. However, LC–MS data obtained from juices using this internal standard revealed that the compound was degrading in the presence of the matrix (data not shown). Therefore, the folate antimetabolite methotrexate was chosen instead as the internal standard. It has been found to be a suitable internal standard in other reported folate analyses [20], and no degradation was observed with its use.

For the LC–MS method (see Section 2.2.2) using folic acid peak area as a function of known concentration, the calibration curve data demonstrated good linearity in the studied concentration range of 0.05–0.5 mg/L, with an equation of $y = 344376x - 1128.5$ and a correlation coefficient (R^2) of 0.9997. The data was in good agreement with results using folic acid-to-methotrexate peak area ratios as the ordinate (data not shown).

The percent recovery data is given in Table 3. The recoveries for spiked juice by LC–MS and the spiked flour and juice by HPLC-UV were in an acceptable range (80–110%) for food and beverage analyses [17]. The lower percent recovery for spiked flour was likely due to incomplete homogenization of the folic acid in the flour. As such, taking a representative sample was more difficult than with spiked juice, where homogenization is much more readily achieved. Unfortunately, recovery of spiked juice by HPLC-UV at 284 nm was so low that further investigations of juices using this wavelength were not warranted (quantitative results not shown). Fig. 4 shows HPLC-UV chromatograms obtained from spiked juice at 284 and 360 nm. In Fig. 4A, the fact that the separation was good but the recovery was still low suggests that the effect the matrix had on the method of detection was the issue and not the separation. The juice matrix strongly absorbs in this region of the UV and therefore likely reduced the sensitivity of the UV-based analysis. However, Pérez Prieto et al. found that in the case of some beverages, these detrimental matrix effects could be reduced by monitoring at folic acid's lesser absorbance maximum of 360 nm [36]. In our case, it was observed that although the recovery was satisfactory at 360 nm, the sensitivity of the folic acid peak was greatly reduced compared to analyses at the global maximum of 284 nm (see Fig. 4B). Due to the low analyte signal, the method at 360 nm was not sensitive enough to study the full calibration curve range or to quantitate folic acid at typical fortification levels. Even so, the high recovery at 360 nm suggests that matrix effects at 284 nm were indeed the issue. Furthermore, percent recovery of spiked juice by LC–MS was good. Hence, it appears that sample cleanup strategies would be required for juices if the analyses are to be performed by HPLC-UV but not for LC–MS.

Table 4
Comparison of measured and labeled folic acid concentrations from fortified matrices.

Sample	Concentration		
	Labeled	Measured	
		(UV)	(MS)
Cereals (μg/g)			
Cereal A	14	13 (93%)	16 (114%)
Cereal B	13	16 (123%)	11 (85%)
Cereal C	15	17 (113%)	19 (127%)
Juices (mg/L)			
Orange juice	0.25		0.26 (104%)

Four fortified samples were analyzed with the methods. The quantitative results obtained from the analyses are shown in Table 4. In most cases, the amounts of folic acid were somewhat higher than those stated from the labels. This finding is not uncommon and has been reported for both juices and cereals [17,31,36,37]. Since some folic acid may be lost in the production process, adding slightly more than labeled would ensure that at least the minimum amount required is present. However, it is

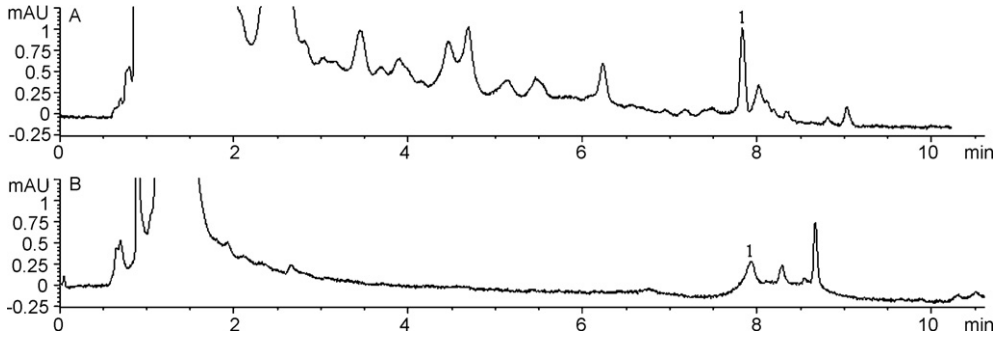

Fig. 4. HPLC-UV chromatograms of spiked, non-fortified apple juice using Gradient 2 at A) 284 and B) 360 nm. Peak 1 corresponds to folic acid. See Section 2.4.2 for solution preparation procedure and Section 2.2.1 for chromatographic conditions.

noteworthy that there are relatively large differences between the concentrations obtained from some of the cereal extracts using the two methods, while the spiked flour percent recovery data from Table 3 shows much better agreement. This is likely due to the more heterogeneous nature of a typical cereal matrix compared to that of spiked flour. Therefore, taking a representative sample was a more difficult task in the case of a cereal. In future studies, this problem could be overcome by using a larger sample size or by homogenizing the entire contents of the cereal before taking a sample.

4. Conclusions

The data have demonstrated the utility of ANP as an effective means of retaining and resolving the highly polar folic acid from other matrix components in juice and cereal extracts untreated by SPE or similar sample cleanup techniques. The calibration curves showed good linearity in the studied concentration ranges. The percent recoveries were satisfactory for spiked flour by both methodologies but only for LC–MS in the case of spiked juice. It appears that the strongly UV-absorbing nature of juices is unsuitable for quantitative analyses by HPLC-UV without prior sample cleanup. The methods were used to analyze the folic acid content of three fortified cereals and one fortified juice. Excellent repeatability with respect to analyte retention time between runs was obtained with minimal re-equilibration (5–7 min) times for gradient analyses.

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