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Short communication

Liquid chromatographic method for determining added folic acid in fortified cereal products[1]

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Abstract

Reversed-phase ion-pair high-performance liquid chromatography (HPLC) was coupled with detection by UV absorption (280 nm) for separation and quantitation of added folic acid (FA) in fortified cereal based foods. A simple and rapid liquid–solid extraction method, combined with enzymatic digestion, to recover FA from the sample matrices is also presented. The quantitation of added FA was achieved in products including corn (maize), wheat-, rice- and oat-based cereal breakfastfoods fortified at 25% and 100% of the reference daily intake (RDI). The retention time for FA was ca. 15 min, and the detection limit was 2 ng/20 μl injection for standard FA. When FA was added to unfortified samples of wheat flour at concentrations of 3.08 or 20.0 μg/g, recoveries were 93% and 96%, respectively. Comparison of HPLC results with those of a standard microbiological assay has shown quite good agreement ($r=0.998$). A solid-phase extraction clean-up procedure has also been developed for use with samples fortified with low levels of FA, where interferences may otherwise hinder quantitation.

Keywords: Cereals; Folic acid

1. Introduction

Folic acid (FA) plays an important role in human nutrition. Folate compounds prevent megaloblastic anemia, and recent studies implicate their deficiency in the etiology of neural tube defects [1,2], chronic diseases [3,4], and coronary heart disease [5,6]. Because of perceived health benefits, cereal breakfastfoods and other cereal based products in the United States have been voluntarily fortified with FA and other vitamins for several years. Now, based on recent scientific findings, the fortification of specified cereal products with FA has been made a requirement in the United States by regulation at the federal level [7]. Reliable analytical methods are, therefore, needed for quantitating added FA in cereal foods.

For quality assurance and regulatory purposes, what is especially needed are rapid and reliable methods for insuring that the correct amount of FA is being added to a cereal product. Existing methods for measuring folates in foods are designed to measure not only added FA, but also the native folates present. Native folates occur in foods at levels much lower than those used in fortification, and are present as multiple derivatives with different reduced and substituted pterin ring structures, and

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glutamic acid chains of varying length. Therefore, analytical methods designed to measure added FA simultaneously with native folates are frequently complex and very time consuming. The most common procedure for quantitating total folate in foods is the microbiological assay [8]. However, the microbiological procedure requires an extended incubation period, making it of limited use in quality assurance applications.

Several chromatographic methods have been reported for the separation and quantitation of FA and other folate derivatives in foods, and have been summarized by Hawkes and Villota [8]. Recently published methods include those of Müller [9], Vahteristo et al. [10], and Pfeiffer et al. [11], which all appear to work well for quantitating low levels of native and added folates in foods. However, these procedures use lengthy multi-enzyme extractions to release bound native folates and convert polyglutamates to monoglutamate forms, extensive sample clean-up and concentration using ion-exchange or affinity columns, and gradient elution chromatographic profiles. This complexity makes such methods of questionable use for quality control laboratories where it may be important to measure only the FA monoglutamate being added.

The goal of this project was to develop a reliable, and relatively simple and rapid method for measuring added FA in fortified cereal based foods. To achieve this goal, we desired a method that needed minimum sample clean-up and pre-treatment, and one that used isocratic elution for the chromatographic separation. The following report describes such a method.

2. Experimental

2.1. Standards

FA was purchased from Sigma (St. Louis, MO, USA). A stock FA solution was prepared by dissolving FA in dibasic potassium phosphate (pH 8–9) buffer to a concentration of 50.0 μg/ml. Working FA standard solutions were prepared by diluting the stock solution with water to concentrations within the range of 0.1–2.0 μg/ml.

2.2. Samples

Samples of commercial cereal breakfastfoods, fortified with FA to provide 25 to 100% of the reference daily intake (RDI) of 400 μg per serving, were purchased from local supermarkets. Wheat flour used to determine recovery was prepared by spiking non-fortified all-purpose wheat flour with FA at a concentration of 20.0 μg/g, or bread flour at 3.08 μg/g (equivalent to 1.4 mg/lb) (1 lb=0.453 kg), and mixing in a rotating V-shaped blender (Patterson-Kelly, East Stroudsburg, PA, USA).

2.3. Extraction

Cereal breakfastfoods were ground using a Janke and Kunkel grinder (Model A-10, Tekmar, Cincinnati, OH, USA), and sieved (40 mesh). All samples (2.000 g) were homogenized by stirring for 1 h in 50 ml of 0.1 *M* K_2HPO_4 (pH adjusted to 8–9 with KOH or H_3PO_4) containing 0.05% (w/v) ascorbate. The slurry pH was then adjusted to 6.9 with phosphoric acid, followed by α-amylase treatment (1.0 h) in a 65°C water bath to hydrolyze starch. Hydrolysis of starch has been shown to increase the recovery of folates [12]. The α-amylase was derived from *Aspergillus oryzae* (Type X-A, Sigma), and the enzyme was dissolved in distilled, filtered water to give a solution concentration of 25 mg/ml. One ml of the enzyme solution was used per gram sample. Following the enzymatic digestion, the temperature was raised to 90°C to inactivate the enzyme, the sample was cooled and centrifuged for 15 min at 5000 *g*, the volume adjusted to 50 ml, and an aliquot of the supernatant was then filtered through a 0.45-μm microporous filter before injection. If needed, a solid-phase extraction (SPE) clean-up procedure was applied to the sample extracts prior to filtration.

2.4. Solid-phase extraction

A modification of the procedure developed by Vahteristo et al. [10] was used. Sample clean-up was achieved on disposable strong anion-exchange (SAX) cartridges (Quaternary amine, Baker 7091-3). The cartridges were conditioned by wetting with 3 ml of hexane followed by 3 ml of methanol, then equilibrated with 5 ml of 0.1 *M* K_2HPO_4 buffer (pH

8–9) containing 0.05% (w/v) ascorbic acid. A 4-ml portion of the sample extract was then diluted with 2 ml of the phosphate buffer, and loaded onto the cartridge at a rate of ca. 0.6 ml/min. The cartridge was then rinsed with 2 ml of diluted (0.02 *M*) phosphate buffer. FA was eluted from the cartridge with a minimum of 4 ml of 0.1 *M* sodium acetate (pH 4.5) containing 5% (w/v) Na_2HPO_4 and 0.05% (w/v) ascorbic acid.

2.5. Chromatography

Analyses were performed using a high-performance liquid chromatography (HPLC) system consisting of a Waters Model 510 solvent metering pump (Waters, Milford, MA, USA), a Rheodyne (Cotati, CA, USA) injector with a 20-μl loop, and a Waters 486 UV–Vis detector operating at 280 nm. A Brownlee 30 mm×2.1 mm I.D. guard column with 5 μm ODS packing (Varian, Sunnyvale, CA, USA) was installed before a Microsorb-MV C_{18} analytical column (100 mm×4.6 mm I.D., 3 μm particle diameter, Varian). The optimized mobile phase was composed of (24–26%):(76–74%) methanol (v/v) in aqueous potassium phosphate buffer (0.0035 *M* KH_2PO_4 and 0.0032 *M* K_2HPO_4), pH 6.8, containing 0.005 *M* tetrabutylammonium dihydrogenphosphate (Sigma) as an ion-pairing agent. Isocratic elution at ambient temperature was used with a flow-rate of 1 ml/min, and the detector sensitivity was set at 0.01 absorbance unit full scale (a.u.f.s.). Chromatograms were recorded, and peak areas quantitated using a Hewlett-Packard (Wilmington, DE, USA) Model HP 3395A integrator.

2.6. Microbiological assay (MBA)

Extracts prepared for HPLC analysis were also subjected to MBA to determine their folate contents. Total folate contents were measured using *Lactobacillus casei* ATCC 7469 (American Type Culture Collection, Rockville, MD, USA) in bacto–FA medium (Difco Labs., Detroit, MI, USA). After incubation at 37°C for 24 h, the growth of *L. casei* was estimated by turbidimetric measurement at 640 nm.

2.7. Calculations and statistical analysis

The calibration curve for FA measured by HPLC was obtained by the external standard method in which peak areas were plotted against eight concentrations of standard FA injected. FA contents in samples were quantified by using linear regression procedures. A response curve for MBA was obtained by plotting absorbance values against FA concentrations. Folate contents in samples were determined by interpolation. Reproducibilities of both HPLC and MBA procedures were tested by estimation of the coefficient of variability from replicate analyses of samples. Differences between HPLC and MBA measurements were evaluated by one-way analysis of variance using Fisher's least significant difference (LSD). Differences were considered to be significant at $p<0.05$.

3. Results and discussion

The conditions used in this study were a modification of those used by Holt et al. [13]. The effects of various mobile phase parameters, including organic modifier concentration and pH, on the chromatographic behavior of FA were investigated. A range of pH values near neutrality (6.5–7.2) was found to provide reasonable ionization and retention of FA. The optimum separation of FA from interferences in the samples was obtained at pH 6.8, which agreed with the results of Holt et al. [13]. Methanol concentrations of 24–26% used under isocratic conditions allowed FA to be eluted with stable retention times of less than 15 min, while providing adequate resolution from sample interferences. Chromatograms of corn flakes with and without FA fortification are shown in Fig. 1, indicating that FA can be resolved from other sample components. Because of the relatively high FA levels in fortified cereal products, detection and quantitation by UV absorption at 280 nm can be achieved. Detection responses were linear ($r=0.999$) over the working standard range, with a detection limit of 2 ng/20 μl injection of standard FA.

Extraction parameters including time, temperature, buffer composition and enzymes were varied to maximize recovery. Since only FA was being mea-

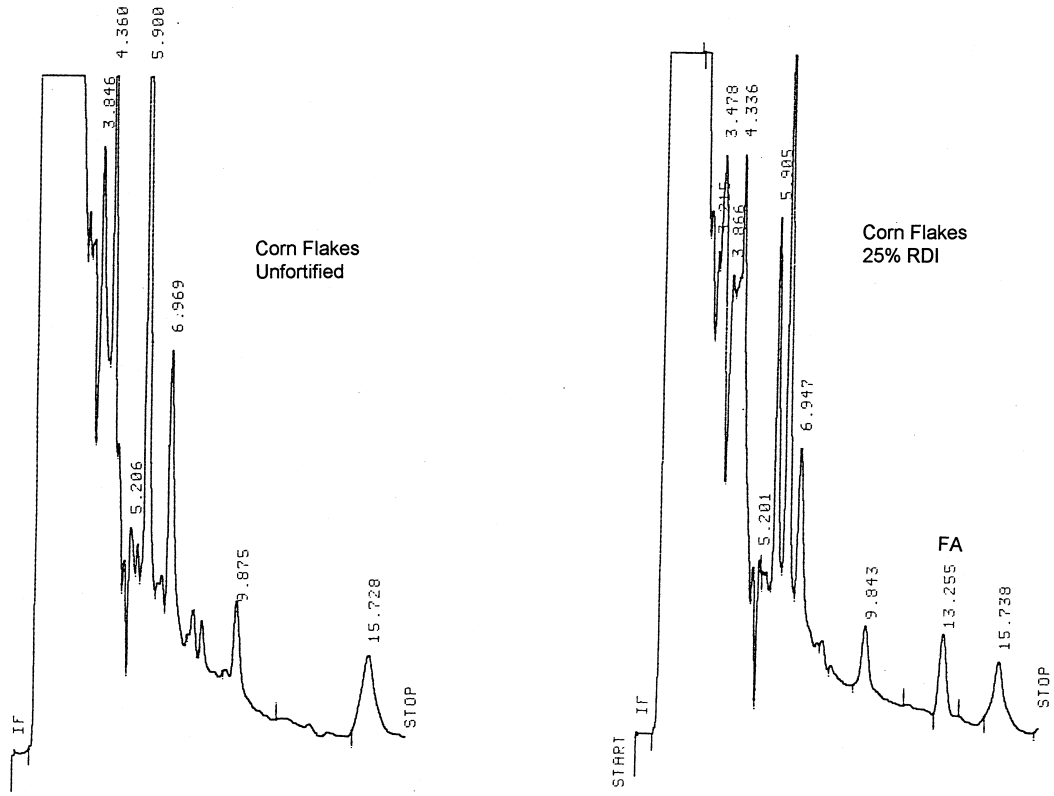

Fig. 1. Chromatograms of unfortified cornflakes (left), and cornflakes fortified with folic acid at 25% of the RDI (right). Chromatographic conditions as in Section 2.5 using methanol–aqueous phosphate buffer (26:74).

sured, the ascorbate level in the extraction solvent could be kept low (0.05%) without loss of the vitamin. This was beneficial in reducing upward shifting of the chromatogram baseline due to the effect of ascorbate coeluting with sample components. Recovery values using the optimized extraction procedure were 93±5.2% (C.V.=5.6%) for bread flour spiked at 3.08 μg/g, and 96±3.1% (C.V.=3.2%) for all-purpose flour spiked at 20.0 μg/g, indicating the validity of the extraction procedure. Treatment with α-amylase facilitated the filtration of sample extracts, but did not significantly improve FA recovery for fortified cereal breakfastfoods or wheat flour (data not shown).

A comparison of HPLC results with those of MBA, the current standard method, for 11 selected cereal breakfastfoods is summarized in Table 1. It should be noted that the HPLC method described here measures only added FA, while the microbiological procedure measures total folates, including other derivatives of the vitamin that occur naturally in cereal grains. However, the contribution of native folates in these fortified products is expected to be minimal compared to the amount of FA added during processing. In fact, quite good correlation (r=0.998) existed between the HPLC and MBA measurements. Folate values by MBA were slightly higher than those obtained by HPLC, which in turn were often higher, but not for all samples fortified at 25% of the RDI, than those of the label claim. In anticipation of some losses during processing, fortification of cereal products with a higher amount of FA than that declared on the label would be expected. The average difference between HPLC and MBA values over all fortification levels in this study was approximately 1.6 μg/g sample; however, this difference was found to be non-significant at the 0.05 level. Part of the difference is likely accounted for by

Table 1
Folic acid contents of fortified cereal breakfastfoods as determined by HPLC and MBA procedures

Product	% RDI fortification per serving[a]	*L. casei* assay[b] (μg/g)	Label claim (μg/g)	HPLC[c] (μg/g)
Toasted corn/oats/ wheat/rice flakes	100	16.7±0.7	13.4	14.3±0.7
Cornflakes	100	17.3±0.7	13.4	15.7±0.5
Whole wheat/brown rice flakes	100	17.8±0.6	13.4	15.5±0.8
Whole wheat flakes	25	4.8±0.2	3.4	3.5±0.2
Cornflakes 1	25	7.5±0.6	3.6	5.5.3±0.2
Cornflakes 2	25	4.4±0.2	3.4	2.2.7±0.1
Sweetened corn puffs	25	3.9±0.1	3.4	2.5±0.1
Crisp rice cereal	35	4.9±0.2	4.7	3.3.4±0.3
Whole grain oat flakes	25	4.1±0.3	3.2	33.3±0.1
Whole wheat flakes	Unfortified	1.5±0.1	–	nd[d]
Cornflakes	Unfortified	1.1±0.1	–	nd

[a] Serving sizes vary from 28 to 32 g.
[b] Mean±S.D. of four replicates.
[c] Mean±S.D. of three replicates.
[d] nd=Not detected.

the presence of native folates other than FA. Some of the difference may also be attributed to the presence of non-folate *L. casei* reactive substances. For a fortified breakfast cereal, Pfeiffer et al. [11] reported a difference of 0.2 μg/g between values measured by MBA and an HPLC technique that quantifies both added FA and native folates.

In the present study, the average FA contents as measured by HPLC were 15.2±0.8 μg/g for breakfast cereals fortified at 100% of the RDI, and 3.6±1.0 μg/g for those fortified at 25% of the RDI. The former result agrees well with the 15.5 μg/g total folate value reported by Pfeiffer et al. [11] for a cereal breakfastfood fortified at 100% of the RDI. Earlier work by Day and Gregory [14] reported a folate content of 3.8 μg/g for a fortified breakfast cereal having a 3.5 μg/g label claim, which agrees well with the values we obtained for samples fortified at 25% of the RDI. As expected, we did not detect FA in two samples of non-fortified corn and whole wheat flakes by HPLC, although these samples contained respectively, 1.1±0.1 μg/g and 1.5±0.1 μg/g of folate as measured by the *L. casei* assay. Based on the eleven samples, the average C.V.s were 5.2% for MBA and 3.6% for HPLC.

In most previous reports [9,11,14], purification of sample extracts before chromatography was indispensable for determining total or added folates in food products, making these procedures lengthy. In the current study dealing only with added FA in cereal-based products, SPE of FA on SAX cartridges was tested. Recovery of FA standards from the SPE cartridge ranged from 97–99%, while recoveries of FA from extracts of spiked bread flour were 86–90%. The SPE procedure was effective in eliminating some substances that eluted in the vicinity of FA, and often resulted in baseline resolution of FA (Fig. 2). However, the reproducibility of quantitation for fortified cereal breakfastfood extracts was not significantly improved by the SPE procedure. Therefore, we recommend SPE as an optional step that will likely not be needed for many products, but may be beneficial for products fortified with low levels of FA.

4. Conclusions

Reversed-phase ion-pair HPLC separation provides sufficient selectivity to resolve FA from other compounds in fortified cereal-based foods. Coupling this separation with UV detection at 280 nm provides a suitable instrumental method for determining added FA with minimal sample pre-treatment. An optional SPE procedure may be useful in eliminating interferences when quantitating products fortified at lower levels. Simplicity and relative speed are distinct

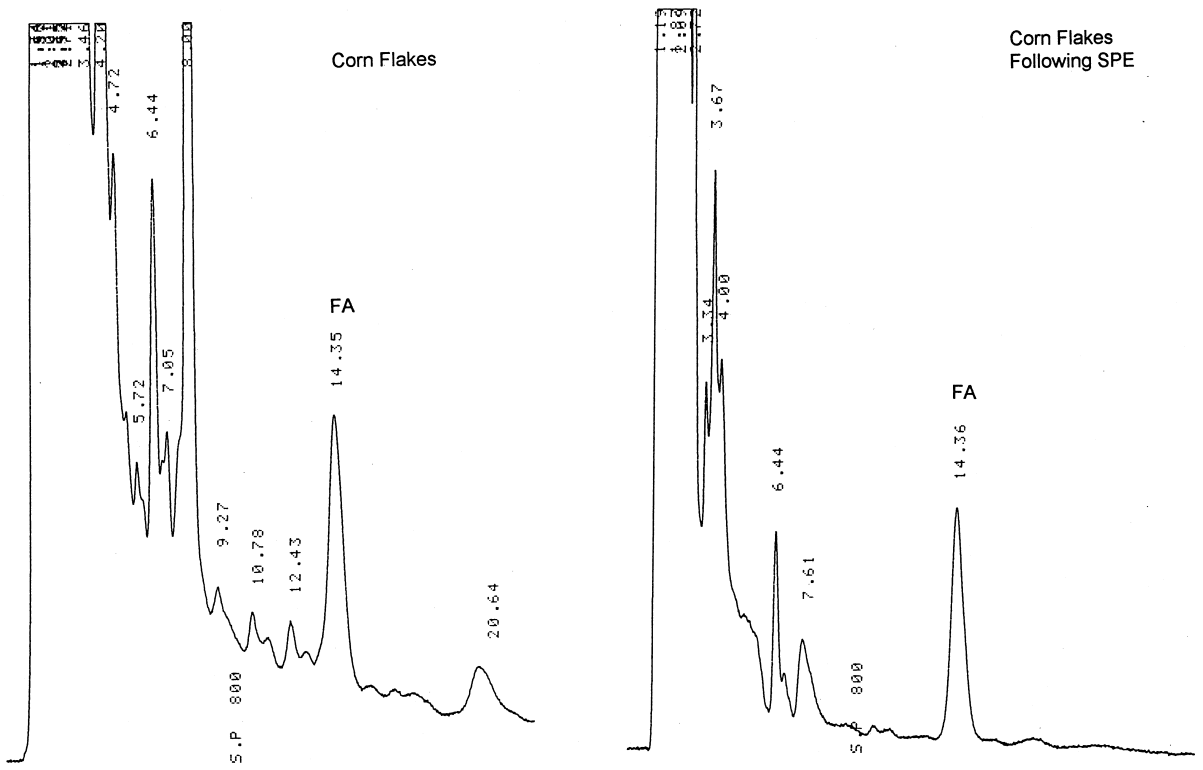

Fig. 2. Chromatograms of an extract from fortified cornflakes before (a) and following solid-phase extraction (b) with a SAX cartridge. Chromatographic conditions as in Section 2.5, with the concentration of methanol in the mobile phase at 25%.

advantages that make this procedure suitable for application in quality assurance environments.

References

[1] J. Mulinare, J.F. Cordero, J.D. Erickson, R.J. Berry, J. Am. Med. Assoc. 260 (1988) 3141.
[2] A. Czeizel, I. Dudas, New Engl. J. Med. 327 (1992) 1832.
[3] E. Giovannucci, M.J. Stampfer, G.A. Colditz, E.B. Rimm, D. Trichopoulos, B.A. Rosner, F.E. Speizer, W.C. Willet, J. Natl. Cancer Inst. 85 (1993) 875.
[4] J.B. Manson, in: B.L. Bailey (Ed.), Folate in Health and Disease, Marcel Dekker, New York, 1995.
[5] J.J. Selhub, P.F. Jacques, P.W.F. Wilson, D. Rush, I.H. Rosenberg, J. Am. Med. Assoc. 270 (1993) 2693.
[6] M.J. Stampfer, W.C. Willet, J. Am. Med. Assoc. 270 (1993) 2726.
[7] Food and Drug Administration Fed. Reg. 61 (1996) 8781.
[8] J.G. Hawkes, R. Villota, Crit. Rev. Food Sci. Nutr. 28 (1989) 439.
[9] H. Müller, Z. Lebensm. Unters. Forsch. 197 (1993) 573.
[10] L.T. Vahteristo, V. Ollilainen, P.E. Koivistoinen, P. Varo, J. Agric. Food Chem. 44 (1996) 477.
[11] C.M. Pfeiffer, L.M. Rogers, J.F. Gregory, J. Agric. Food Chem. 45 (1997) 407.
[12] S. De Souza, R. Eitenmiller, J. Micronutr. Anal. 7 (1990) 37.
[13] D.L. Holt, R.L. Wehling, M.G. Zeece, J. Chromatogr. 449 (1988) 271.
[14] B.P. Day, J.F. Gregory, J. Agric. Food Chem. 29 (1981) 374.