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Analytical Methods

Investigation of folic acid stability in fortified instant Asian noodles by use of capillary electrophoresis

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ABSTRACT

A simple, rapid procedure, using capillary zone electrophoresis (CZE), that can efficiently measure added folic acid in fortified instant fried noodles has been developed and validated. Optimum separation of folic acid was obtained on a 72 cm × 75 μm capillary using 8 mM phosphate-12 mM borate run buffer with 5% MeOH at pH 9.5, temperature of 30 °C and voltage of 28 kV. The extracts were introduced into the capillary via electrokinetic injection and the folic acid monitored at 214 nm. For quantification purposes, nicotinic acid was added as internal standard to all samples. Under these conditions the analysis required approximately 12 min. Good results were obtained for different analytical parameters including linearity, accuracy and precision. The limit of detection was calculated to be 5.3 mg/L. Prior to CZE determination, noodle samples were homogenized in the buffer solution for 1 h followed by treatment with α-amylase solution for 1 h at 65 °C or protease solution for 4 h at 37 °C. The enzyme solution was added at a concentration of 25 mg/L and then adjusted to pH 7.0. Using standard addition to eliminate the effect of sample matrix interference, results showed that a higher and more efficient recovery of added folic acid could be obtained when using α-amylase. During the four main stages of instant fried noodle manufacturing (dough crumbs, cut sheets, steaming and frying) folic acid was found to be stable with recoveries of 96–103%.

1. Introduction

Folates are generally classed as water-soluble vitamins belonging to the B group complex. As folate is synthesized only by microorganisms and plants, humans depend on a variety of dietary sources for the vitamin, including liver; fresh, dark green, leafy vegetables; beans; wheat germ; and yeasts (Francis, 1999; Lucock, 2000). Naturally occurring dietary folates consist of a reduced pteridine ring and a polyglutamate peptide that are hydrolyzed in the intestinal lumen by polyglutamate hydrolases prior to transport across the intestinal mucosa (Basu & Donaldson, 2003; Francis, 1999). Research over the past decade has shown that low or inadequate folate concentration may contribute to congenital malformations and development of chronic disorders including neural tube defects, megaloblastic anemia and elevated levels of homocysteine (Jacobs, & Wood, 2003). In Australia, it has been reported that between 3% and 5% of pregnant women were folate deficient whilst significantly higher rates of folate deficiency occur in developing countries (Soothill, 1996).

Recently it has been suggested that folic acid (FA) may be more effective in reducing neural tube defect incidence than conjugated food folate, as free folic acid is more readily absorbed (Francis, 1999). As a result, FA is now being increasingly used for food fortification purposes where it provides better bioavailability than folate from natural sources (Wright, Finglas, & Southon, 2001). FA, also known as pteroylglutamic acid is the simplest naturally occurring and most stable form of folate (Ball, 1998; Vora, Riga, Dollimore, & Alexander, 2002). It is composed of three structural moieties: a bicyclic 6-methylpterin ring, *p*-amino benzoic acid and a single molecule of L-glutamic acid, all of which have no vitamin activity (Vora et al., 2002). The chemical structure is shown in Fig. 1. Folic acid is tasteless, odourless and displays a yellow-orange crystalline appearance in the solid state. Being a photosensitive compound it is degraded in aqueous solution by sunlight, ultraviolet and visible light (Akhtar, Khan, & Ahmad, 2003). The susceptibility of folic acid to cleavage under acidic conditions and high temperatures has long been established (Arcot & Shrestha, 2005). However, the degree and rate of destruction has been shown to be influenced by the pH of the medium, reducing agents in the buffer, folate derivatives, type of buffer, and the food system (Arcot & Shrestha, 2005). Under neutral to alkaline conditions, folic acid displays increased solubility and stability particularly in

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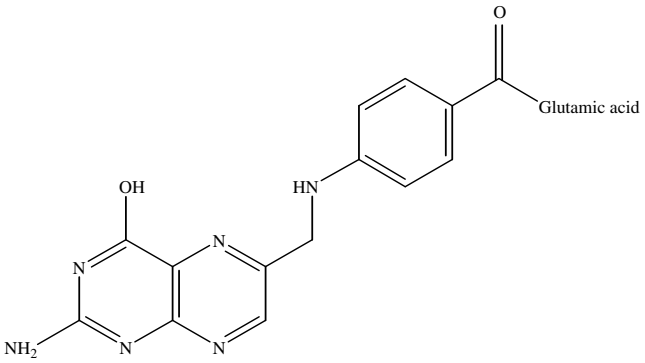

Fig. 1. The structure of pteroylglutamic acid (folic acid (FA)).

solutions of alkali hydroxides and carbonates as well as a limited number of organic solvents (Budavari, 2001). In aqueous solution folic acid is stable at 100 °C for 10 h in a pH range of 5.0–12.0 when protected from light, but becomes increasingly unstable as the pH decreases below 5.0 (Arcot & Shrestha, 2005).

Currently, three approaches exist for providing appropriate amounts of folic acid and other vitamins in the diet: consumption of fresh vegetables and legumes, fortification of staple foods, and increased consumption of supplements (Locksmith & Duff, 1998). Fortification offers several advantages over supplementation and dietary modification as it is a reliable and effective way to attain health benefits by increasing the nutrient intake of a population without relying on individual supplementation practices (Hunt & Dwyer, 2001). In January 1998, the Food and Drug Administration (FDA) implemented a program to fortify flour and cereal products with folic acid at the level of 140 μg/100 g of product (Daly et al., 1997; Neuhouser & Beresford, 2001), with the initial goal in seeking an appropriate dose of folic acid fortification. This aimed to raise the usual daily intake of folic acid to 400 μg in at least 90% of the target group (women of child bearing age) and prevent excess folic intake in non-target groups (Cho, Johnson, & Song, 2002; Neuhouser & Beresford, 2001).

To date, cereal grains and staple foods have been the main candidate for fortification as they are consumed by most women of childbearing age (Buttriss, 2005; Cornel, de Smit, & de Jong-van den Berg, 2005; Pawlosky, Hertrampf, Flanagan, & Thomas, 2003; Romano, Waitzman, Scheffler, & Pi, 1995). However, in order to ensure that the amount of fortificant in the product as purchased is not less than the amount claimed on the label, and that levels do not fall below the stated minimum, 'reasonable overages of the nutrient within the limits of current good manufacturing practice' should be typically added to fortified products (Choumenkovitch et al., 2002; Rader, Weaver, & Angyal, 2000; Wright et al., 2001).

While there is a long history of developments in Asian noodle processing (Nagao, 1996), recent emphasis has been on the enhancement of sensory attributes and satisfying consumer preferences on colour and appearance, as well as texture. Internationally, over 12% of global wheat production is utilized for noodle manufacture whilst in Australia, approximately one third of bread wheat exports are used for noodle production (Asenstorfer, Wang, & Mares, 2006; Cato, Halmos, & Small, 2006a,b; Hatcher, 2001; Wang, Kovacs, Fowler, & Holley, 2004). As a result there is a wide diversity of Asian noodle products available in the marketplace. Despite this, there have been few studies on the fortification of Asian noodles, particularly instant noodles with respect to folates and the method of analysis applied. Recently studies by Bui and Small have been conducted whereby folates were analysed in various varieties of Asian noodles by microbiological assay (Bui & Small, 2007a,b,c).

Internationally, microbiological assay has been regarded as the main choice for folate determination providing a high degree of sensitivity for a wide range of food matrices. The method requires a minimum analysis time of up to 5 days to obtain a result and is thus time consuming. By comparison, the use of analytical techniques such as high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE) offer potential as they do not demand such labour intensive preparations, provide very efficient separations, have low solvent consumption and are readily automated (Lindeberg, 1996). Whilst HPLC is usually well suited for the analysis of added folate in foods (Breithaupt, 2004; Osseyi, Wehling, & Albrecht, 1998, 2001; Póo-Prieto et al., 2006), the method sometimes lacks sufficient resolving power causing difficulties when modifying selectivity (Cheung et al., 2007). In contrast, CE offers great versatility and flexibility by providing unique sensitivity and high resolution (Frazier, Ames, & Nursten, 1999). Additionally, CE has been applied to a variety of pharmaceutical formulations and supplements, of which most have examined folate in the form of folic acid (Aurora-Prado, Silvia, Tavares, & Altria, 2004; Gomis, González, & Álavarez, 1999).

Although CE has proven useful in the analysis of many food components including vitamins (Hau Fung Cheung et al., 2007), few reports relate specifically to folic acid. Zhao, Yuan, Xie, and Xiao (2006) used capillary electrophoresis with chemiluminescence detection for the determination of naturally occurring folic acid in apple juice. Studies showed that by heating folic acid the chemiluminescence reaction between luminal and BrO^- was enhanced. For three apple juice samples, folic acid ranged between 0.33 and 0.43 mg/L. Gomis et al. (1999) employed micellar electrokinetic capillary chromatography (MEKC) for the analysis of seven water-soluble vitamins in a pharmaceutical formulation, one of which included folic acid. The limit of detection for FA was 0.08 μg/mL. (Aurora-Prado et al., 2004) developed and validated a microemulsion electrokinetic chromatography method (MEEKC) for the determination of folic acid in a commercial tablet. In this method, electrokinetic separations are achieved using buffers containing surfactant coated oil droplets. Niacin, in the form of nicotinic acid was used as an internal standard and detected at 214 nm.

Although the above methods were not directly related to the analysis of folic acid in food samples they uniformly provided good results with respect to linearity, precision and accuracy, and could be considered when developing a CE method for the analysis of folic acid in fortified instant noodles. In this context this study will focus on the stability and retention of folic acid in fortified instant fried noodles, through the development and application of a capillary electrophoretic method.

2. Experimental

2.1. Materials

All chemicals including enzyme preparations and vitamins used in product formulations and analytical procedures were of analytical grade or of the highest purity available. FA (F-7876) was used as a chemical standard for analytical purposes as well as fortification. A commercial flour (Bakers flour, from Weston Milling, VIC, Australia) was selected as it is unfortified and suited to noodle preparation. Nicotinic acid (NA), FA, sodium tetraborate (borate), di-potassium hydrogen orthophosphate (potassium phosphate, dibasic), hydrochloric acid (HCl) and α-amylase (A-3176) were obtained from Sigma–Aldrich (Sigma Chemical Co, USA). Protease (subtilisin A from *B. licheniformis*) was from Megazyme International, Ireland Ltd whilst methanol (MeOH), sodium carbonate, potassium carbonate and sodium hydroxide (NaOH) were obtained from Ajax (Ajax Chemicals, NSW, Australia). Sodium chloride (NaCl) was from BDH Laboratory Supplies, England. Working standard solutions were prepared daily by dilution of the stock

standard solutions with Milli-Q water with distilled water used for all other preparations.

2.2. Preparation of instant noodles

A number of batches of the same noodle style were made in the laboratory and the samples of these batches were analysed in triplicate. The averages of the results from the analysed data were calculated and are presented in this report, representing the value of each batch of noodles prepared. Noodle samples were prepared using procedures based on those described elsewhere (Bui & Small, 2007c; Moss, Gore, & Murray, 1987). It is specifically noted here that all steps in the preparation of noodles were carried out in subdued lighting conditions in order to minimize the potential impact of light on vitamin stability and non-specific loss due to sample handling. The ingredients used to make instant noodles were: 300.0 g flour, 105.0 g water, 0.36 g potassium carbonate, 0.24 g sodium carbonate and 3.0 g common table salt. Palm oil was used to deep fry the noodles (Auroma Pty Ltd., Australia). The procedure for making instant noodles firstly involved mixing and rolling. Subsequent to these steps, the resultant sheet was not rested but was immediately passed a further four times between the rollers to reduce the sheet thickness before cutting into noodle strands for the further preparation steps of steaming, frying and draining.

The kansui mixture (potassium and sodium carbonate) and salt was dissolved in water and this solution was added to the flour during mixing. After mixing, the resultant dough had a crumbly consistency. The dough was formed into a dough sheet by a process of folding and passing the crumbly dough through the rollers of the noodle machine (Imperia, Ambrogio di Torino, Italy) several times. For this combining step the rollers was set at the maximum gap available. Typically, three passes were required to give a uniform sheet which held together as a single dough piece. The combined sheet was allowed to rest for 30 min, covered with aluminium foil, and then sealed in a plastic bag to firstly exclude light and secondly to prevent moisture loss after resting. The thickness of the sheet was reduced stepwise by passing between the rollers of the noodle machine before cutting into strands. Fresh noodles strands were placed in a steamer and steamed over vigorously boiling water for 2 min. Then they were removed from the steamer and placed onto dry paper towel for 30 s. The noodles from the steaming step were then immediately placed into a wire basket and deep fried in palm oil for 45 s. The temperature of the oil was carefully checked and noodles were only placed into the oil once it had attained 150 °C. The fried noodles were removed from the oil using the wire basket and allowed to drain for 30 s. The noodles were transferred to absorbent paper and allowed to cool in the air flow created by a fume cupboard for 20 min prior to placing into a sealed bag or container for storage and subsequent analysis.

2.3. Fortification of noodle samples with folic acid

FA was incorporated into the formulation at a level of 0.05 g FA per 100 g fresh flour. The vitamin was dissolved in the salt solution and added during the early stage of mixing.

2.4. Preparation of running buffer

The optimized running buffer for capillary zone electrophoresis (CZE) was prepared by dissolving di-potassium hydrogen orthophosphate and sodium tetraborate in 5% (v/v) MeOH/water and Milli-Q water both at concentrations of 8 mM and 12 mM, respectively. The buffer solutions were then adjusted to a pH of 9.5 using 1 M NaOH. Solutions were stored in the refrigerator at 4 °C.

2.5. Preparation of standard solutions and establishment of the external analytical curve

FA and NA (internal standard) were separately dissolved in the running buffer to make individual FA and NA stock solutions with concentration of 300 mg/L. The FA solution was further diluted with running buffer and 2.00 mL of the NA stock solution (giving a constant I.S. concentration of 60 mg/L) to produce solutions of FA concentrations 10, 20, 30, 40 and 50 mg/L. A calibration curve was then constructed ($y = 0.015x$; y, area ratio of FA to NA; x, FA concentration mg/L, $R^2 = 0.996$).

2.6. Preparation of enzyme solutions

Each of the enzyme solutions was prepared freshly on the day of use. Protease solution was prepared by diluting 5 mL of a 50 mg/mL solution with equal amounts of water to achieve a final concentration of 25 mg/mL. The α-amylase solution was prepared by suspending 1.25 g α-amylase in 50 mL of distilled water (25 mg/L), which was then stirred gently with a magnetic stirrer for 5 min and centrifuged at 3000 rpm (1200*g*) for 10 min. The supernatant was covered with Parafilm® prior to use.

2.7. Extraction of FA from fortified instant noodle samples

The following extraction procedure was applied to the four main stages of processing with variations occurring only in the enzyme solution employed and their corresponding incubation conditions. The overall approach in preparation of sample extracts involved homogenization of 2.0 g of sample with 40.0 mL of running buffer with a magnetic stirrer for 1 h at ambient temperature. The resultant mixture was adjusted to pH 7.0 with 1 M NaOH or 1 M HCl prior to the addition of the enzyme solution and incubation in a water bath. For 2 g of sample used, a corresponding volume of enzyme solution was added (2.0 mL). After the period of incubation, samples were boiled at 100 °C for 5 min. Samples were then cooled to room temperature and adjusted to pH 9.5 using 1 M NaOH. Samples were subjected to centrifugation for 20 min at 4000 rpm (2100*g*) with the supernatant removed and filtered using a 0.45 μm polytetrafluoroethylene (PTFE) syringe filter (Biolab, AUS) before analysis by CE.

2.7.1. Incubation conditions for enzymatic extraction of FA with protease or α-amylase

Samples to be treated with the protease solution were incubated for 4 h at 37 °C whilst samples subjected to α-amylase treatment required a 1 h incubation period at 65 °C.

2.8. Preparation of standard addition samples for CE analysis

Sample extracts were prepared as mentioned in Section 2.7. A calculated volume of both standard stock solutions were directly added to a known volume of sample extract and subsequently made to the mark with run buffer. Details of the standard addition formulation are given in Table 1. It should be noted that standard addition was applied to the four stages of processing at each concentration of FA, producing a total of 16 samples to be analysed.

2.9. Preparation of internal calibration curve samples for CE analysis

Essentially, the working standards, normally made to the mark in run buffer and used as in external calibration curve, are instead prepared using the sample extract. Thus, to prepare the internal calibration curve typically, 0.5 g of unfortified dough crumb sample is homogenized for 1 h in 10 mL running buffer, which consists of a known volume of the internal standard and FA stock solution.

Table 1
Standard addition formulation for CE analysis

FA (mg/L)	*v* (sample extract) (mL)	*v* (300 mg/L NA stock solution) (mL)	*v* (300 mg/L FA stock solution) (mL)	*v* (running buffer) (mL)	Total volume (mL)
0	6.0	2.0	0	2.00	10.0
10	6.0	2.0	0.33	1.67	10.0
20	6.0	2.0	0.67	1.33	10.0
30	6.0	2.0	1.00	1.00	10.0

Table 2
Formulation for internal calibration curve standards for CE analysis

FA concentration (ppm)	*v* (300 ppm NA stock solution) (mL)	*v* (300 ppm FA stock solution) (mL)	Sample extract (mL)	Total volume (mL)
0	2.00	0	8.00	10.00
10	2.00	0.33	7.67	10.00
20	2.00	0.67	7.33	10.00
30	2.00	1.00	7.00	10.00
40	2.00	1.33	6.67	10.00
50	2.00	1.67	6.33	10.00

The mixture is then adjusted to pH 9.5 and centrifuged for 20 min at 4000 rpm (2100 × *g*). The supernatant is removed, filtered and analysed in the same manner as per Section 2.7. The same extraction process is applied to a fortified sample with the exclusion of the FA standard stock solution (extracted with buffer and internal standard). Details of the internal calibration curve standards are given in Table 2. The calibration curve is then applied to the four analysed samples, given that the unfortified and fortified samples are prepared on the same day of manufacturing.

2.10. CE apparatus and operating conditions

An Agilent HP 3D CE system (Aglient, USA) with a diode array detector (DAD) was used in all experiments. Chemstation software was used for data acquisition and analysis. Separations were performed using uncoated fused-silica capillaries with an inner diameter of 75 μm and a total length of 72 cm (50 cm effective length) (Agilent Technologies, USA). The capillary was preconditioned prior to separation by flushing under vacuum conditions (~20 " mercury vacuum) for 20 min with 1 M NaOH and 30 min with Milli-Q water before the first run and subsequently for 3 min with 0.1 M NaOH and 5 min with the run buffer prior to each subsequent run. Samples and standards were diluted using the running buffer and then were injected electrokinetically at 5 kV for 0.2 min with the following standard separation conditions: voltage at 28 kV (positive polarity), capillary temperature 30 °C, detector wavelength 214 nm and the running buffer as described in Section 2.4. At the end of the day, a final 10 min washing with Milli-Q water followed by 2 min with air was performed. All standards, sample solutions, the carrier electrolyte buffer and NaOH solution were filtered through 0.45 μm PTFE syringe filters (Labquip Tech, Australia) prior to use.

3. Results and discussion

3.1. Optimization of pH, temperature and voltage

Prior to optimizing the operating conditions, various variables including detection wavelength, use of an internal standard, and injection type, were considered. Originally, a wavelength of 275 nm was chosen as it showed the maximum absorption for FA. However upon the inclusion of the I.S. NA, which was used to improve precision, minimize injection fluctuations, dilution errors and errors during sample treatment, the I.S. exhibited minimal absorbance at the selected wavelength and so an alternative wavelength was required. As a result, a wavelength of 214 nm proved to be adequately selective for both vitamins.

In order to determine the optimum response of FA in a phosphate-borate buffer system a 3-level central composite design was carried out for three factors; voltage (26–30 kV), temperature (26–30 °C) and pH (9.0–10.0). The ranges for the three factors were chosen based upon previous studies by (Aurora-Prado et al., 2004), (Gomis et al., 1999) and (Flores, Peñalvo, Mansilla, & Gómez, 2005). The experimental design produced a total of 18 experimental combinations, all of which were performed in triplicate and evaluated with respect to the ratio of the area of FA to that of the I.S. Statistical analysis of the results obtained from the electropherograms showed that only pH was significant ($p < 0.05$) with respect to the area of the peak. By employing a running buffer at pH 9.5 and operating the system at 30 °C and +28 kV a maximum peak area relative to the I.S. was produced. The pH of the running electrolyte has a significant impact on area response of FA relative to NA. According to the p*K*s of FA (3.5 and 4.8) and the structure of the analyte, a basic buffer was used to promote ionization (Zhao et al., 2006). By employing a high pH buffer a reasonable electroosmotic flow (EOF) can be maintained in the migration direction (Desai & Park, 2005). To ensure a constant electrolyte pH at the optimized conditions the buffer was replenished by frequently changing the inlet and outlet electrolyte reservoirs every 6 runs. Rinsing of the capillary between runs using NaOH assisted in reconditioning of the inner surface to produce more consistent results.

3.2. Effect of varying concentrations of buffer species on quantitation of folic acid

Using conditions derived in Section 3.1, the composition of the buffer was investigated using a two level full factorial design whereby concentrations of phosphate and borate were varied (8–12 mM and 8–12 mM, respectively). Peaks were quantified against the I.S. as this corrected for analyte losses during sample clean-up and compensates for irreproducible introduction volumes based on the calculation of relative area ratios. A running buffer consisting of 8 mM phosphate and 12 mM borate with the optimized conditions

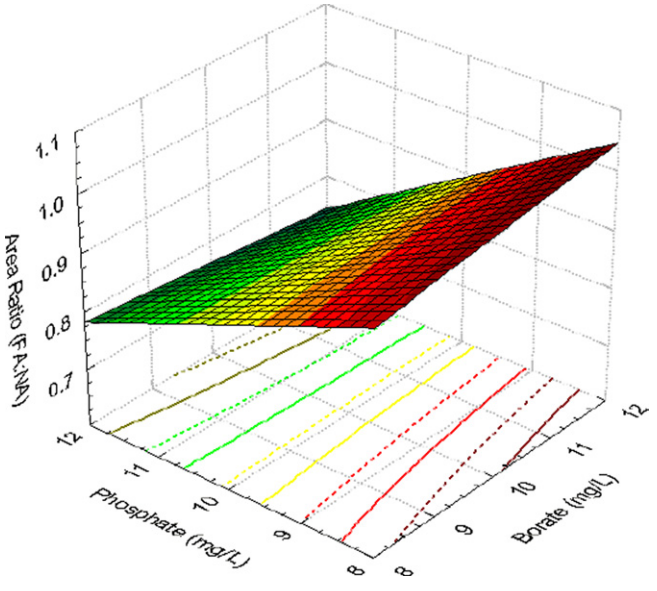

Fig. 2. Surface contour plot for the effect of ratio of buffer species.

above resulted in the highest area response of FA relative to the I.S. using the specified capillary electrophoretic system. Fig. 2 illustrates that the concentration of phosphate does not exhibit a significant affect on the area ratio in comparison to the concentration of borate, which shows a more distinct effect on the area ratio of FA–NA. The addition of 5% methanol (MeOH) gave enhanced results when compared with those found when MeOH was not used, increasing the sharpness in the peak. Hence, the final optimized conditions for the analysis of FA were comprised of a 8 mM phosphate and 12 mM borate running buffer at pH 9.5 with 5% MeOH, with the operating conditions at +28 kV and 30 °C and detection at 214 nm. With respect to the LOD, the value was calculated as 5.3 mg/L using the International Standards Organization (ISO) standard method as described by Hibbert and Gooding (2005), however based on the calculation of three times signal-to-noise, the LOD is 2.2 mg/L.

3.3. Preliminary findings for FA recovery in instant noodles without enzymatic extraction

Preliminary results at the four main stages of processing using a simple non-enzymatic method of extraction showed repeated inconsistencies and inadequate recoveries between days with values ranging as low as 70% to as high as 86%. The method employed to determine the FA content in fortified instant noodles involved the homogenization of the noodle sample at any stage of processing in a known amount of running buffer for 1 h. The mixture was then directly centrifuged, filtered and analysed by CE. Initially, it was believed that a basic form of extraction could be applied as the noodle matrix consists mainly of flour and water. However, even during the early stages of processing, at the dough crumb and cut noodle stages, where no extensive heat treatment was applied, recoveries were still insufficient. Based upon these results, it was concluded that some form of enzymatic treatment was required to liberate any bound FA from the starch-based matrix.

3.4. Liberation of FA from instant noodles: choice of enzymatic treatment for FA extraction

Essentially, three types of enzymes are used for the extraction of folates. These consist of protease, α-amylase and conjugase. Studies have shown that each enzyme can be used individually or collectively (recognized as a tri-enzymatic extraction) to liberate folates from the foods, thereby maximizing the folate activity in certain foods. Traditionally, conjugase has been the most extensively used enzyme as it converts polyglutamyl forms of folate to mono- and di-glutamates. However, in this circumstance, since folic acid already exists in the monoglutamic form conjugase was not needed. Instead, extraction with protease or α-amylase was considered given the nature of the food matrix.

3.5. Proteolytic extraction of FA in fortified instant noodles and the need for correcting sample matrix interference by standard addition

Protease was originally chosen as the enzyme of choice as it was believed that it might help to release the FA that was trapped or bound to the matrices of protein. The incubation conditions for protease extraction were chosen based on literature data (Tamura, 1998), which stated that typically a 1–6 h incubation period at 37 °C and pH 4–7 was applied. Although the suggested pH range was slightly acidic to neutral, a pH of 7 was used instead given the susceptibility of FA to acidic conditions. A pH of 7 was also used as it is within the range for the optimum activity of this particular enzyme. Although preliminary results using the proteolytic extraction for FA revealed that samples treated without enzyme exhibited lower levels of folic acid compared to those treated with protease, upon closer analysis of the results it was concluded that interferences between the sample matrix and FA may have occurred given that excessive recoveries ranging between 100% and 127% were found when calculated on a dry weight basis. It should be noted that all recoveries were calculated on a dry weight basis so to account for any differences that may occur due to the moisture content of each individual sample. The excessive recoveries were also consistently demonstrated at all four stages of processing when samples were prepared and analysed on different days. Thus, given these circumstances, the standard addition method was used for all stages of instant noodle manufacturing, to correct the matrix effect of signal enhancement. By adding the standard solution directly to the sample, the matrix effect would effectively show the same behaviour for both the sample and the spiked solution. To account for any calculation errors when extrapolating to zero concentration, a reasonable range for the standards was chosen (0–30 mg/L). Application of the proteolytic enzyme during extraction and the use of standard addition produced consistent recoveries of FA for each stage of processing. When calculated to a dry weight basis, FA exhibited excellent stability and retention during processing except at the final stage of frying. A typical electropherogram of protease extracted FA in fortified instant noodles is given in Fig. 3. With respect to the linearity and precision of standard addition with protease extraction, satisfactory correlation coefficients confirmed the response of FA was linear over the concentration range studied (Table 3). In addition, no significant differences were found between the analytical curves for the four stages of processing, which indicates that the sample matrix interference was consistent between the samples analysed. All recoveries were within the range of 100 ± 2% (mixing, recovery 101.1%; cutting, recovery 98.0%; steaming, recovery 97.9%), however in the final stage of processing (frying); the recovery was calculated as 92%. Although preliminary findings would suggest that FA is lost during this stage of processing, it was concluded that since the matrix is primarily starch-based, FA was partially liberated with protease during the final heat treatment of frying. Thus, in an attempt to improve the recovery, the experiments were repeated with α-amylase.

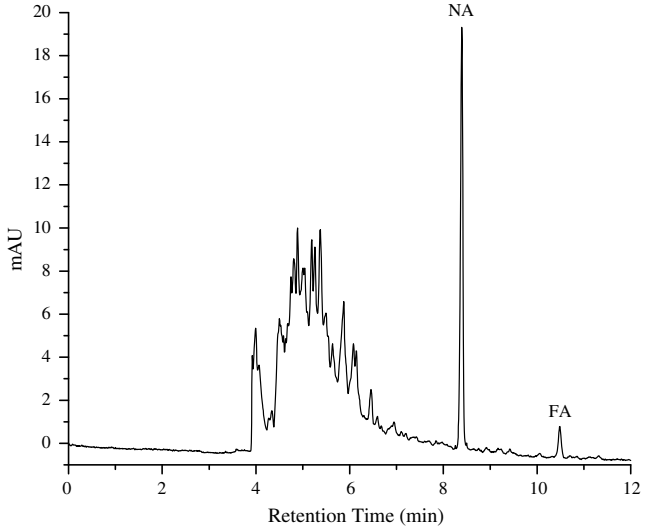

Fig. 3. Electropherogram of protease extracted FA in fortified instant noodles. Separation was performed using a buffer consisting of 8 mM phosphate, 12 mM borate, 5% MeOH/water (v/v) (pH 9.5). Voltage: 28 kV; temperature: 30 °C; UV detection was performed at 214 nm.

Table 3
Statistical data for determination of FA in extracts of instant noodles in terms of corrected peak areas for standard addition

Enzyme extraction	Processing stage	Intercept	Slope	R^2	Linear range (mg/L)
Protease	Mixing	9.11	0.0101x + 0.092	0.998	0.0–30.0
	Cutting	8.86	0.0104x + 0.0921	0.995	0.0–30.0
	Steaming	9.34	0.0097x + 0.0906	0.9981	0.0–30.0
	Frying	9.10	0.0099x + 0.0901	0.9992	0.0–30.0
α-Amylase	Mixing	12.43	0.007x + 0.087	0.996	0.0–30.0
	Cutting	11.86	0.007x + 0.083	0.994	0.0–30.0
	Steaming	10.63	0.008x + 0.085	0.998	0.0–30.0
	Frying	12.25	0.008x + 0.098	0.987	0.0–30.0

3.6. Increasing the efficiency of the extraction and FA recovery by using α-amylase

The conditions for amylolytic extraction of FA were based on a previous study (Osseyi et al., 2001) in which researchers incorporated α-amylase in their extraction method for the determination of stability and distribution of added FA and other endogenous folates during bread making. When subjected to amylolytic extraction, higher recoveries were obtained (mixing, recovery 103.4%; cutting, recovery 96.2%; steaming, recovery 100.1%; frying, recovery 97.1%), which supported the conclusion that protease did not extract all of the FA during the frying stage. By employing a 1 h incubation time at a higher temperature of 65 °C, the overall extraction procedure was reduced by 3 h. Linearity and correlation coefficients were both satisfactory (Table 3). A typical electropherogram of FA in fortified instant noodles is given in Fig. 4.

The stability of FA in instant noodles in this study varies from that found previously (Bui & Small, 2007c). In that study, FA was also used as the form of folate used for fortification, however analysis by microbiological assay revealed that the forms of total and free folate measured were unstable during the same processing conditions. Although the findings here indicate adequate stability of the folic acid fortificant, it is noted that the form of folate measured, the extraction procedure employed, and the method of analysis were all different in the previous work. In addition, several studies have also recognized that folic acid in its simplest form has shown considerable stability during far more extensive heat treatments (Arcot & Shrestha, 2005; O'Broin, Temperley, Brown, & Scott, 1975; Paine-Wilson & Chen, 1979). In order to ensure effective release of folic acid from the matrix, enzyme treatment was carried out on the food homogenate before heat treatment and centrifugation (Tamura, Mizuno, Johnson, & Jacobs, 1997). Furthermore, given that the degree and rate of destruction are dependent on the pH of the medium, the folate derivative used, the buffer type employed and the food system, all these factors have been taken into account in order to increase the stability of FA in instant noodles. For example, both the pH of the buffer and the noodle matrix are alkaline, at pH 8.0 and 9.5, respectively, which assists in stabilizing FA in aqueous solution. Moreover, phosphate has been considered the 'buffer of choice' for other routine folate assays as it has demonstrated enhanced stability towards FA.

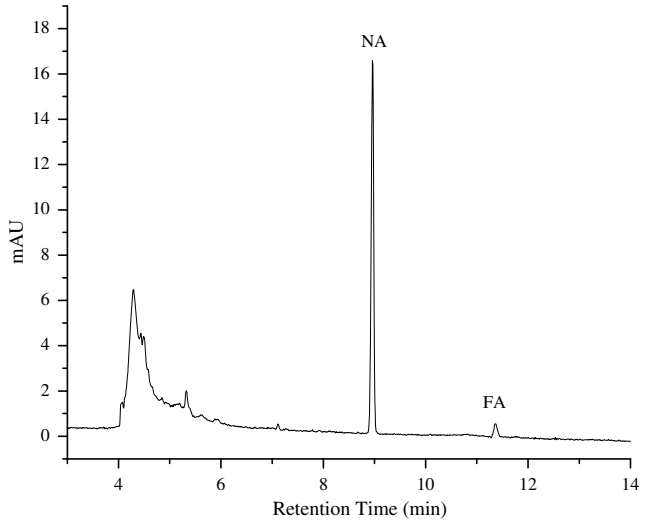

Fig. 4. Electropherogram of α-amylase extracted FA in fortified instant noodles. Separation was performed using a buffer consisting of 8 mM phosphate, 12 mM borate, 5% MeOH/water (v/v) (pH 9.5). Voltage: 28 kV; temperature: 30 °C; UV detection was performed at 214 nm.

3.7. Confirmation of standard addition method with α-amylase extraction by internal calibration curve CE analysis: effect of sample matrix interference and recovery of FA

To confirm the findings from the standard addition method with α-amylase extraction, the same incubation conditions were applied to the samples, and analysed using the 'internal calibration method' as described in Section 2.9. The purpose of this method was not only to support the findings of standard addition but to also further increase the efficiency and simplicity of the extraction method. As seen in Section 2.9, the internal calibration curve is typically prepared in unfortified dough crumbs as it is the simplest form of the noodle matrix produced among the various stages of processing. One of the main concerns however, when constructing the internal calibration curve in this type of matrix was the consistency of the matrix interference, as displayed in the standard addition method. Even though the matrix interference was shown to be consistent across each stage of processing analysed using standard addition, the same effect was also tested here for confirmatory purposes. Hence, for each stage of processing of unfortified instant noodles, a five point calibration curve was constructed in addition to the 4 fortified samples analysed. Once each recovery was calculated using their corresponding matrix and adjusted to a dry moisture basis to account for any differences in moisture content, the values were then compared. The study confirmed an important aspect of the findings made with standard addition, i.e. that any of the four matrices could be used for the construction of the internal analytical curve as the variations in the recoveries was insignificant, differing by approximately 3%. When the recoveries for the internal calibration method were compared with standard addition, the results were in good agreement, showing FA to be highly stable during processing. The recoveries for the stages of processing were 98.1%, 102.9%, 102.5% and 99.1% during mixing, cutting, steaming and frying, respectively. In all cases RSD values were below 4.5% with excellent linearity (r^2 = 0.993), between 0.0 mg/L to 50 mg/L.

Although the levels of addition of FA used here were higher than those commonly incorporated during fortification, the results confirm those reported recently (Bui & Small, 2007c) which were

obtained using a microbiological assay and demonstrated only minor losses in folic acid during processing of instant noodles. The sensitivity of the procedure reported here is currently insufficient for extracts of foods fortified at typical levels. As these would require at least a 10-fold decrease in the limit of detection, further refinements are now being investigated, along with direct comparisons with results from HPLC analyses of these samples.

4. Concluding remarks

In summary, the simple and rapid CE method of analysis, which has been validated with respect to linearity, accuracy, and precision, demonstrates the reliability of the electrophoretic procedure for its intended application. Excellent resolution was obtained between sample matrix interferences present in instant noodles and the analysed vitamin. In all cases, the standard addition method was used for the determination of FA relative to NA in the noodle extracts. The results obtained concerning linearity, recovery, precision and accuracy were highly satisfactory. FA has exhibited excellent stability throughout the processing of instant noodles, which could therefore be used for the purpose of enhancing folate intakes through fortification.

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