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Evaluating folate extraction from infant milk formulae and adult nutritionals: Enzymatic digestion *versus* enzyme-free heat treatment

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ABSTRACT

This study compares enzymatic treatments to release folic acid (FA) and endogenous 5-methyltetrahydrofolate (5-MTHF) from infant milk formulae with enzyme-free heat extraction. The limits of detection and quantitation of FA were 1.4 ng/mL and 3.1 ng/mL, respectively; 7.5 ng/mL and 16.2 ng/mL for 5-MTHF. Absolute mean recoveries were 85% (FA) and 95% (5-MTHF). The RSD of the within-run variability was 6% and the inter-day variability was 8%. Averaged measurements of FA and 5-MTHF in SRM-1849a were within the certified value range. Analysed folate levels in three brands were greater than label values, because of inherently high 5-MTHF occurring in samples. The results indicate that enzyme-free heat treatment prior to UPLC–MS/MS analysis gives better sensitivity and reduces chromatographic interferences for the determination of FA and 5-MTHF in milk formulae than enzymatic treatments. Enzyme-free heat treatment is more compatible with UPLC–MS/MS than folate extraction techniques involving the addition of enzymes to milk.

1. Introduction

Infant formula is an alternative to breast milk for providing adequate infant nutrition. According to the World Health Organisation Code, breast milk substitutes have a legitimate role to play in circumstances where a child is not breastfed and cannot access breast milk (WHO, 2007). It is important that during infancy the food supply provides optimal nutrition and minimises the exposure to potentially harmful substances (NAP, 2004). Commercial infant formulae and adult nutritionals available in the market are generally cows'- and soy-based spray-dried milk powder containing proteins, carbohydrates, fat, vitamins and minerals.

Synthetic folic acid (FA) is a fortificant used to enrich foods and beverages (Fig. 1). Often the terms FA and folate are used interchangeably. Folate is a generic term encompassing an array of naturally occurring folate vitamers including FA. In cows' milk, 5-methyltetrahydrofolate (5-MTHF) is the most abundant naturally occurring monoglutamate vitamer (Fig. 1). Polyglutamate vitamers are also found in addition to this monoglutamate folate (Forssen, Jagerstad, Wigertz, & Witthoft, 2000). Endogenous folate vitamers are present in a reduced rather oxidised state. The oxidised and most stable form is FA, a term commonly used to refer to pteroylmonoglutamic acid that occurs naturally in very low concentrations. Folate vitamers are sensitive to oxygen, heat and ultra-violet (UV) light. Any oxidative folate degradation results in p-aminobenzoyl-glutamic acid, pterins, dihydrofolate and folic acid (Strandler, Patring, Jägerstad, & Jastrebova, 2015). It has been reported that folate binding protein (FBP) present in milk shows a protective effect against folate degradation (Strandler et al., 2015). As an essential water soluble B vitamin, folate vitamers act as co-enzymes in single-carbon transfer reactions, playing crucial roles in the synthesis and methylation of DNA and amino acid metabolism (Combs, 2012). Because of the important roles of folate in metabolic pathways, infant formula is supplemented with FA at levels greater than those of endogenous milk folates. Folate vitamers are associated with etiologies of chronic diseases and birth defects (Combs, 2012). In particular, there is a correlation between increased intake of FA and reduced prevalence of neural tube defects (Czeizel & Dudas, 1992).

In the past, determination of folate using microbiological assay required either heat treatment to release folate from its binding proteins (Gregory, 1989) or enzymatic deconjugation of polyglutamate chains into monoglutamate derivatives (Tamura, 1990) to extract folate from food. The enzyme pteroylpolyglutamate

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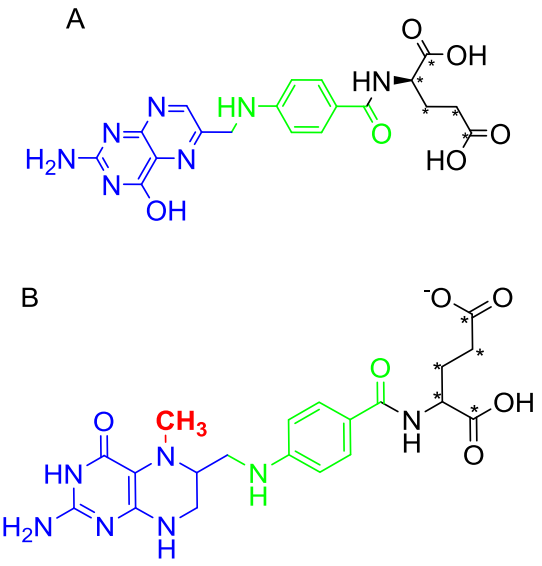

Fig. 1. The structures of FA (A) and 5-MTHF (B) are composed of a pteridine ring or 6-methylpterin (blue-coloured), para-aminobenzoic acid (green-coloured) and glutamic acid moiety. The methyl group in 5-MTHF is highlighted in red colour. Labelled FA and 5-MTHF were used as internal standard, ^{13}C atoms in the glutamic acid moiety are denoted by asterisks (*).

hydrolase, used as deconjugase can be sourced from rat serum, hog kidney and chicken pancreas (Ramos-Parra, Urrea-López, & Díaz de la Garza, 2013). Additional treatment with α-amylase is regarded as essential in starch- or glycogen-containing food to release folate bound to polysaccharides (Černá & Káš, 1983). Tri-enzyme treatment was first advocated in 1990 (Martin, Landen, Soliman, & Eitenmiller, 1990) and, subsequently, it received widespread adoption (De Souza & Eitenmiller, 1990; Pfeiffer, Rogers, & Gregory, 1997; Tamura, Mizuno, Johnston, & Jacob, 1997), because of the higher yields of measurable folate achieved in foods than heat/deconjugase treatment alone. Relevant to this study is that tri-enzyme digestion has been performed to determine total folate in infant formulae and adult nutritionals (Szpylka, DeVries, Cheney, & House, 2012). However, it has been argued that the tri-enzyme treatment does not necessarily increase the folate release compared to deconjugase treatment alone (Hyun & Tamura, 2005; Pfeiffer et al., 1997), and other factors, such as food matrix type and the differences in the stability of individual folate vitamers, might affect analyte yield (Hyun & Tamura, 2005; Pfeiffer et al., 1997).

Previous experimental evidence suggests that enzymatic digestion is not required for determination of vitamin levels in dietary supplements and fortified foods (Goldschmidt & Wolf, 2010). This is consistent with our 2013 study, where sample preparation using a simple cold extraction was adequate to release FA from fortified flour (Chandra-Hioe, Bucknall, & Arcot, 2013). Another study also showed that no enzymatic treatment was necessary to determine FA in infant formulae (Zhang et al., 2014). In this study, we have determined FA and 5-MTHF in adult nutritional and infant formulae, and partly re-validated our previously published UPLC–MS/MS measurement method (Chandra-Hioe, Bucknall, & Arcot, 2011), originally used for analysis of fortified bread. Initially, enzyme-free heat treatment was compared with enzymatic treatments to extract FA and 5-MTHF from whey-based milk formula.

2. Materials and methods

2.1. Standard solutions and reagents

Standard FA and 5-MTHF were supplied by Schircks Laboratories (Jona, Switzerland). Stable isotope labelled [$^{13}C_5$]-FA and [$^{13}C_5$]-5-MTHF used as internal standards (IS) were purchased from Merck-Eprova (Schaffhausen, Switzerland) and stored at -85 °C. Both IS were synthesized with ^{13}C labelled sites within the glutamic acid structural moiety (Fig. 1). Stock standard solutions (100 μg/mL) were prepared under UV-free light in 0.1 mol/L phosphate buffer (pH 6.1) containing 1% (w/v) ascorbic acid and 0.1% (v/v) 2,3 dimercapto-1-propanol (Patring, Johansson, Yazynina, & Jastrebova, 2005) as antioxidants to minimise folate degradation. Concentrations of standard solutions were verified and calculated from UV absorbance measurements (SpectraMax M2, Molecular Devices, Sunnyvale, CA) using the Beer-Lambert Law (Pfeiffer et al., 1997; Zhang et al., 2003). Aliquots of stock standard solutions (1 mL) were flushed with nitrogen gas before being stored at -85 °C for use within 3 months.

Hydrochloric acid 32% (10.2 M), HPLC grade acetonitrile and formic acid were acquired from Univar, Ajax-Finechem (Sydney, Australia). Purified water (≤0.1 μS/cm) was sourced from a Milli-Q system (Millipore, Bedford, MA, USA). α-Amylase from *Bacillus licheniformis* was purchased from MP Biomedicals (Solon, OH, USA), protease *Subtilisin A Bacillus licheniformis* from Megazyme (Wicklow, Ireland) and rat serum from Innovative Research (Novi, MI, USA). L-ascorbic acid, and 2,3 di-mercapto-1-propanol were obtained from Sigma-Aldrich (Sydney, Australia). Standard Reference Materials SRM 1849 and SRM 1849a (Infant/Adult Nutritional formula), were acquired from National Institute of Standards and Technology (NIST, Gaithersburg, MD, USA).

2.2. Sampling

FA fortified samples of adult nutritional (hydrolysed corn starch based; n = 4) formulae and infant milk formulae (whey, casein and soy based; n = 14) were purchased, as available, from various supermarkets and pharmacies in Sydney (Australia). According to the Codex Standard for infant formulae and formulae for special medical purposes intended for infants, FA is added at the minimum level of 2.5 μg/100 kJ (Codex., 2011). Samples (in duplicate) were reconstituted according to each manufacturer's recommendation.

Aliquots (1 mL) of 0.1 M phosphate buffer (pH 6.1) containing IS were pipetted into centrifuge tubes. IS was added at the beginning of extraction to compensate for degradation losses occurring during folate extraction (Ringling & Rychlik, 2013). Therefore, degradation was not assessed quantitatively. Reconstituted formulae (2 mL) were then added to extraction buffer and vortexed. Folate was extracted using enzyme-free heat treatment (detail in Section 2.3). The nominal individual concentrations of both [$^{13}C_5$]-FA and [$^{13}C_5$]-5-MTHF in the final mixture was 0.7 μg/mL.

2.3. Enzyme-free heat treatment

The sample tubes were placed in a boiling water bath for 15 min, cooled on ice and centrifuged (Heraeus Multifuge X3R, Thermo Fisher Scientific, Australia), at 10,000*g*, 4 °C for 15 min. Supernatants were decanted and filtered using Whatman 41 ashless filter paper.

2.4. Enzyme treatments

One brand of whey-based infant formula (S26 Newborn formula, Aspen Nutritional Australia/Nestlé) was selected to evaluate and compare the effects of various enzyme treatments on folate extraction. The tri-enzyme approach involved serial incubation of reconstituted milk samples with α-amylase, protease and rat serum. Di-enzyme treatments to extract folate were performed through incubation with either α-amylase and rat serum, or protease and rat serum. In cows' milk, carbohydrate is present mostly as lactose and other simple sugars (Chávez-Servín, Castellote, &