Voluntary food fortification with folic acid in Spain: Predicted contribution to children’s dietary intakes as assessed with new food folate composition data

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**A B S T R A C T**

The Spanish market offers a significant number of folic acid (FA) voluntarily fortified foods. We analysed FA and (6S)-5-methyltetrahydrofolinic acid ((6S)-5-CH$_3$-H$_4$PteGlu) content in ready-to-eat cereals (RTEC) (n = 68) and cow's milk (n = 25) by a previously validated affinity chromatography–HPLC method. Contribution to potential FA intakes for children aged 2–13 years, was assessed using food consumption data from a representative nationwide study, folate Recommended Dietary Intakes (RDI), and Upper Levels (UL). Results showed that at all food fortification levels obtained, fortified products provided more than tenfold FA than (6S)-5-CH$_3$-H$_4$PteGlu. For RTEC, the high fortification level provided 6–21% per serving, of RDI and ≤32% of ULs at 90th percentile of RTEC consumption (P90). Milk products fortified at the higher level reached on average 54–136% of RDI per serving and only exceeded UL at P90 of milk consumption in children aged 2–5 years.

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1. Introduction

Folic acid (FA) is the synthetic form of a water soluble vitamin group also known as folate or folates. In the past decade it has achieved great attention because of the finding that it can prevent Neural tube Defects (NTDs) in a high proportion (Czeizel, Bártfai, & Bánhidy, 2011), as well as for other potential benefits on cardiovascular health (McCully, 2007), neurocognitive status (Reynolds, 2006) and some types of cancer (Alonso-Aperte, Gonzalez, Poo-Prieto, & Varela-Moreiras, 2007; Kim, 2006; Varela-Moreiras, Gonzalez, & Alonso-Aperte, 2005). Recently, the European Food Safety Authority (EFSA, 2009) and a number of researchers worldwide have focused on the risk-benefit analysis of inadequate intakes of folic acid (Dragsted, Renwick, Verhagen, Flynn, & Tuijtelaars, 2009; Verkerk, 2010). While some of the benefits of an optimal folate status in humans are extensively studied, it remains unknown to what extent a lifetime exposure to high doses of FA can be detrimental (Smith, Kim, & Refsum, 2008). Up to date, deleterious effects of FA have only been described with high doses of the synthetic form of the vitamin, and in animal or in vitro studies (Butterworth & Tamura, 1989; Smith et al., 2008; Troen et al., 2006).

Vitamin requirements and status comprise different issues depending on the vulnerability of the population group that we focus on (Lawrence et al., 2009; Smith et al., 2008). Women at a fertile age are clearly a target for optimal folate intake, and these needs are addressed by means of different public health policies in order to increase their intakes. The US and Canada governments were the first to adopt a mandatory fortification policy for flours and cereal derivatives back in 1998 (IOM., 1998) and currently almost 75 countries worldwide have implemented this strategy. However, in Spain only voluntary fortification of processed foods is taking place at present (EFSA, 2009; Verkaik-Kloosterman, 2009). The Spanish market offers an important number of FA voluntarily fortified products as found in our previous work, and we developed the first database for commercialized FA fortified foods, which included up to six different food groups and a total of 260 products (Samaniego-Vaesken, Alonso-Aperte, & Varela-Moreiras, 2009). Cereals and derivatives was the most commonly fortified group (52% of total recorded products). Addition of FA overages to these products seems to be a common practice, as we observed in ready-to-eat breakfast cereals (RTEC) and dairy products, when analysed total folate was compared to values declared on labels (Samaniego-Vaesken, Alonso-Aperte, & Varela-Moreiras, 2010). Another leading fortified food group were dairy products (17%) which mainly included cow’s milk and yogurt (Samaniego-Vaesken et al., 2009).

Dietary folate intakes in Spanish children are insufficient as found by the enKid Study, which was conducted nationwide amongst a representative sample of children and adolescents aged 2–24 years (Serra-Majem et al., 2001). In addition, Serra-Majem et al. published a meta-analysis of available nutritional studies in Spanish children and adolescents aged 4–18 years, and described
a risk of inadequate intakes (defined as <2/3 of folate RDI) specially in adolescent females (13–18 years, n = 1270) (Serra-Majem, Ribas Barba, et al., 2001). Insufficient folate intake was also observed in Spanish adults (Varela-Moreiras et al., 2010) and the elderly (Mila Villarroel, Abellana Sangrà, & Farran Codina, 2009), whose intakes of naturally rich sources of folates, such as green leafy vegetables and some legumes, were not meeting recommendations.

Amongst this significant number of voluntarily fortified products currently available in the Spanish market, a great proportion are targeted at children by means of product marketing and labelling, mainly RTEC and dairy products (milk, yogurt, etc.) (Samaniego-Vaesken et al., 2009). However, there is a lack of updated FA data for processed products in food composition tables and databases (Bouckaert et al., 2010) given the difficulty of keeping up to date with the ever growing market, and the fact that methods of folate analysis involve a great difficulty (Finglas et al., 2006). In a previous work we analysed a representative sample of FA fortified RTEC by trienzyme extraction and microbiological assay (Samaniego-Vaesken et al., 2010); but data obtained by this methodology, while being one of the most widely used in food total folate analysis (Arcot & Shrestha, 2005), seems controversial in the literature, as a number of researchers suggest the use of individual analysis of folate vitamers as a better approach to assessing population folate intakes (Konings et al., 2001).

For all stated, our aim was to further analyse FA fortified food products, including ready-to-eat breakfast cereals and milk products, by extracting, purifying and quantifying their two main folate vitamers by HPLC, and to assess their contribution to potential FA intake in children aged 2–13 years.

2. Materials and methods

The analysis of folic acid fortified food products involved three key steps: trienzyme extraction and deconjugation of folates from the food matrix, their purification and concentration by affinity chromatography with folate binding protein (FBP), and finally separation and quantification of vitamers by HPLC, as previously described in the literature (Bagley & Selhub, 2000; Martin, Landen, Soliman, & Eitenmiller, 1990; Póo-Prieto et al., 2006). Added FA and (6S)-5-methyltetrahydropteroylmonoglutamate ((6S)-5-CH₃-H₄PteGlu) were analysed. (6S)-5-CH₃-H₄PteGlu is the main natural folate vitamer in the selected food samples: RTEC (Póo-Prieto et al., 2006) and milk (Forsssén, Jägerstad, Wigertz, & Witthöft, 2000). Folate vitamers were quantified by HPLC coupled with fluorescence and UV detection. Resulting data was used to calculate theoretical intakes of folic acid and folate in children aged 2–13 years. Intakes were then assessed by comparing them to age-specific folate intake recommendations in Spain (RDI) (Moreiras, Carvajal, Cabrera, & Cuadrado, 2011) and to the folic acid Upper Intake Limits (EC, 2000). Published Spanish food consumption data from the enKid Study (Serra-Majem, García-Closas, et al., 2001) provided children’s intakes, in grams per day, of the counterpart unfortified foodstuffs.

2.1. Chemicals and reagents

2.1.1. Folate standards

Folic acid and (6S)-5-CH₃-H₄PteGlu disodium salt were obtained from Sigma (Spain) in analytical grade. Standard stock solutions were prepared separately in 1% ascorbic acid, protected from light and stored at −80 °C. Concentrations were calculated by measuring absorbance of folic acid at λ = 282 nm with a spectrophotometer (Beckman DU-650, EE UU) and by molar extinction coefficient (27,600 l mol⁻¹ cm⁻¹ for (6S)-5-CH₃-H₄PteGlu). 31,700 l mol⁻¹ cm⁻¹ value was used at λ = 290 (Blakley, 1969).

2.1.2. Folate binding protein (FBP)

Bovine milk FBP was purified from a commercially available whey protein concentrate by the method by Bagley and Selhub (Bagley, Selhub, Sutton, Wagner, & McCormick, 1997). Affi-Gel 102 from Bio-Rad Laboratories (Hercules, CA, USA), was used as stationary phase for FBP purification. Trienzyme extracts from samples were purified with 1 ml FBP columns coupled with Affi-Gel 10 (Bio-Rad).

2.1.3. Enzymes

Rat plasma obtained from Charles Rivier Laboratories (France) was used as folate conjugase source. It was treated with a strong anion exchange resin (AG1X8, Bio-Rad, Spain) in order to eliminate endogenous folate (Horne, Krumdieck, & Wagner, 1981). Deconjugation efficacy of the enzyme was tested by the protocol by Wright, A.J.A (personal communication) in which folate polyglutamates from a yeast extract (Becton Dickinson, Spain) were subjected to deconjugation and quantified as folate monoglutamates by microbiological assay (Samaniego-Vaesken et al., 2010). α-Amylase from Bacillus sp. (EC.3.2.1.1) and protease from Streptomyces griseus type XIV (EC.3.4.24.31) were acquired from Sigma (Spain) and prepared in water at a final concentration of 20 and 2 mg/ml respectively. All enzyme preparations were filtered in sterile conditions and stored at −20 °C. New enzyme batches were prepared on a weekly basis. Trienzyme blank extractions were also performed to check for endogenous folate contribution by purifying and quantifying by HPLC, and also by the microbiological assay with chloramphenicol-resistant Lactobacillus casei ssp. rhamnosus (NCIB 10463) (O’Broin and Kelleher, 1992). External quality control was achieved by assaying aliquots of serum folate International Standard (IS 03/178 NIBSC) from the National Institute for Biological Standards and Control (Hertfordshire, UK).

2.1.4. Reagents

Tris–HCl, sodium ascorbate, trifluoroacetic acid, 2-mercaptoethanol, piperazine, potassium phosphate dibasic, potassium phosphate monobasic, and concentrated phosphoric acid (> 85 wt.%) were purchased from Sigma (Spain) in analytical grade. Ditioeritritol was obtained from Bio-Rad Labs. (Spain) and HPLC grade acetonitrile from Panreac (Spain).

2.1.5. Food samples and storage

Fortified food products were purchased at local supermarkets and retail stores from Madrid Region, Spain, and consisted of pre-packaged ready-to-eat breakfast cereals (RTEC, n = 68) of different food matrices (wheat, rice, oat, mixed) and toppings (dried fruit, nuts, chocolate); and heat-sterilised cow milk (n = 25) with diverse fat content (whole, semi-skimmed, skimmed). One randomly selected package of each product was acquired. Samples were stored at room or refrigeration temperature until individual processing (grinding and/or homogenising) and finally aliquots were flushed with nitrogen and frozen at −20 °C. Samples were thawed, extracted, purified and analysed in duplicate. A 0.5–1 g sample of RTEC was weighed and ca. 1 ml of milk were diluted to a final 15 ml volume for trienzyme extraction. Label data such as recommended serving, ingredients, nutritional values and a photo of the product were recorded in our food composition database (Samaniego-Vaesken et al., 2009).

2.2. Folic acid and folate quantification in fortified ready-to-eat cereals and milk

FA and (6S)-5-CH₃-H₄PteGlu in extracted and purified samples were separated and quantified by HPLC with fluorescence (295 nm excitation and 360 nm emission wavelengths) and UV detection at 280 nm, as described in the work by Póo-Prieto et al.
An HPLC system (Beckman Coulter, Inc., UK) composed of a binary bomb, ultra violet diode array and fluorescence detector (Shimadzu, RF-551) was used. Separation was achieved with a reverse phase ODS-Hypersil column (Keystone Scientific, 250 × 4.6 mm, 5 μm) preceded by a C18, 3 mm column (SecurityGuard, Phenomenex).

2.2.1. Quality controls

The Standard Reference Material 1846, a FA fortified milk-based infant formula powder from NIST (National Institute of Standards and Technology) (Sharpless et al., 1997), was used as an external quality control for intra and inter assay precision. Internal quality control food samples from pooled trizymatic extracts at three fortification levels (ca. 30, 100 and 450 μg folic acid/100 g food product) were prepared, stored at −20 °C and assayed on a weekly basis to account for repeatability.

2.2.2. Method recovery

Recovery from food folate extraction and purification processes, as well as FBP activity was evaluated by addition of [3,5,7,9-3H] folic acid diammonium salt tracer (69 Ci/mmol), from Moveereck Biochemicals (Brea, CA, US) to food samples. Additionally, known concentrations of the folate standards (4 μg/g folic acid and 0.04 μg/g (6S)-5-CH3-H4PteGlu) were added to unfortified commercial wheat flour which was subjected to the same phases of analysis as food samples.

2.3. Estimation of folic acid and folate intake in children

Folic acid fortified foods were categorised into four fortification levels, which were defined by calculating the percentage of FA Recommended Daily Allowances (%RDA) (“Council Directive 90/496/EEC of 24 September 1990 on nutrition labelling for foodstuffs,”) per serving (in grams or millilitres) as declared label and FA values on each product. Defined values for each level were set as follows: Level 1: < 33, Level 2: 33.1–51, Level 3 51.1–70 and Level 4: ≥ 70.1 μg of FA/serving (Table 1).

Average and percentile population intake data of the counterparts of the analysed food groups (RTEC and milk, in grams or millilitres per day), were obtained from the enKid Study results (Aranceta Bartrina & Serra-Majem, 2000). For the present work, selected population groups were boys and girls aged 2–5, 6–9 and 10–13 years. Predicted folic acid and (6S)-5-CH3-H4PteGlu daily intakes in micrograms (μg) per day, were calculated by the following equation: 

\[ X = \left( \frac{Q \times F}{100} \right) \]

where \( Q \) is the amount of ingested food in grams or millilitres of fortified food product, and \( F \) is the amount of ingested food in grams or millilitres.

Age-specific folate Recommended Dietary Intakes (RDI) for the Spanish population, reviewed by Moreiras et al. (2011), and folic acid tolerable Upper Intake Levels (UL) published by the European Commission, Health and Consumer protection Directorate (EC, 2000), were used as reference for the calculation of adequate or excessive vitamin intakes. For the assessment of RDI, FA and (6S)-5-CH3-H4PteGlu were considered separately; folate RDI values for children are: 150 μg (2–5 years), 200 μg (6–9 years) and 300 μg (10–13 years) (Moreiras et al., 2011). Percentage of RDI (%RDI) were calculated as follows:%RDI = \( \frac{F \times 100}{RDI} \), where: \( F \) is the folic acid or the (6S)-5-CH3-H4PteGlu quantity ingested daily by means of the assessed fortified product and RDIy is Folate Recommended Dietary Intake for each age-group. Only the synthetic vitamer, FA, was used to compare with UL values for children: 300 μg (2–5 years) 400 μg (6–9 years) 600 μg (10–13 years) (EC, 2000). Percentage of UL (%UL): %UL = \( \frac{F \times 100}{ULy} \), where: \( F \) is the FA quantity potentially ingested daily from the assessed fortified product and ULy are the FA tolerable Upper Intake Levels for each age-group.

3. Results and discussion

3.1. Folic acid and folate content in fortified products

A total of 93 FA fortified food products from 18 different commercial brands were analysed, and values were used for the calculation of potential intakes. Method performance was considered acceptable according to AOAC criteria (AOAC, 1998). Recovery from folate standards, calculated as average ± standard deviation of three independent determinations, was 85.3 ± 6.2% for FA and 83.5 ± 4.1% for (6S)-5-CH3-H4PteGlu after subtraction of endogenous contribution from matrix (23 ± 0.5 μg/100 g, CV = 2.2%). Linearity for folic acid and (6S)-5-CH3-H4PteGlu standards was between 0.04 and 1.32 μg/ml, \( r^2 = 0.993; \) and 0.005–0.115 μg/ml, \( r^2 = 0.9961, \) respectively. Method precision was measured by intra- and inter-assay coefficients of variation (CV) for the Standard Reference Material. Intra-assay results for FA and (6S)-5-CH3-H4PteGlu concentrations were expressed as the CV of the peak areas of both vitamers in five samples extracted in parallel with fortified food samples and run separately on the same day, providing average values ± standard deviation, of 91.3 ± 6.17 μg FA/100 g (CV = 7%) and 9 ± 0.54 μg (6S)-5-CH3-H4PteGlu/100 g (CV = 6%). Inter-assay precision was determined by extraction and analysis of SRM samples extracted over five consecutive days, resulting in values of 88 ± 7.81 μg FA/100 g (CV = 9%) and 9 ± 1.08 μg (6S)-5-CH3-H4PteGlu/100 g (CV = 12%).

In Table 1 we present a summary of the results expressed as average micrograms of FA and (6S)-5-CH3-H4PteGlu per 100 grams or millilitres, each food group segmented four fortification levels.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Fortification levels, folic acid and (6S)-5-methyltetrahydropteroylmonglutamate ((6S)-5-CH3-H4PteGlu) contents in analysed food products.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Folic acid Fortification Level</strong>&lt;sup&gt;a&lt;/sup&gt; (μg/serving)&lt;sup&gt;b&lt;/sup&gt;</td>
<td><strong>N</strong></td>
</tr>
<tr>
<td>Ready-to-eat breakfast cereals (RTEC) (35 g serving)</td>
<td>Level 1 (&lt; 33)</td>
</tr>
<tr>
<td></td>
<td>Level 2 (33.1–51)</td>
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<td></td>
<td>Level 3 (51.1–70)</td>
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<td></td>
<td>Level 4 (&gt; 70.1)</td>
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<tr>
<td>Cow's milk&lt;sup&gt;e&lt;/sup&gt; (UHT) (200 ml serving)</td>
<td>Level 3 (51.1–70)</td>
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<tr>
<td></td>
<td>Level 4 (&gt; 70.1)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Calculated as percentage of folic acid Recommended Daily Allowances (200 μg) per serving.

<sup>b</sup> As indicated by the manufacturer on products label. N: number of independent food products included in each folic acid fortification level and analysed by trienzyme extraction, purification and HPLC quantification. (6S)-5-CH3-H4PteGlu: (6S)-5-methyltetrahydropteroylmonglutamate.

<sup>c,d</sup> Values expressed as average ± standard deviation of two replicates from each N sample comprising independent trienzyme extraction, purification and HPLC analysis per replicate.

<sup>e</sup> For this food group no fortification Level 1 or 2 products were found at the market. UHT: Ultra-High-Temperature processed milk.
Contents of FA found in fortified RTEC are comparable to those of Rader et al. who analysed 28 different breakfast cereals in the USA back in 2000, few years after mandatory fortification was implemented (Rader, Weaver, & Angyal, 2000). However, their data refers to total folate values instead of FA only. In a previous study, we found overages (defined as higher than declared values), analysing RTEC by using microbiological assay (Samaniego-Vaesken et al., 2010), but our HPLC data shows lower FA values in some but not all cases (data not published). Konings et al. also showed lower folate values when quantification was performed by HPLC compared to microbiological assay, and this was the case for different food groups (Konings et al., 2001). On the other hand, Poó-Prieto et al. found a number of lower FA levels than those declared in cereal products commercialised in the USA (Póo-Prieto et al., 2006) using the same extraction and quantification methodologies as in the present work.

Spanish researchers studied a number of FA fortified milk products using HPLC as quantification method, and they found lower levels than ours, similar to the declarations on the products labels (Perez Prieto, Cancho Grande, Garcia Falcon, & Simal Gandara, 2006). A study published by Johnston et al. also using the extraction method by Martin et al. (Martin et al., 1990) but with quantification of total folates by the microbiological assay (Johnston, Diriendo, & Tamura, 2002), found similar levels to ours for milk and derivatives.

3.2. Estimation of folic acid and folate intakes in the enKid Study population

Effectiveness of nutritional policies such as a fortification program relies on adequately reaching target population groups in order to meet their recommended intakes. In a recent publication by Yeung et al., the authors studied the contributions of the three main sources of folic acid in the USA: RTEC, supplements and mandatory fortification in children from the National Health and Nutrition Examination Survey (NHANES) (Yeung et al., 2011). They found that only children who consumed FA supplements or a combination of the three sources, could be potentially exposed to intakes above the UL.

While the situation in Europe is rather different, as mandatory fortification is not implemented (EFSA, 2009), the increasing processed foods market and the addition of vitamins puts forward a great interest on the potential contributions to both recommended or excessive intakes.

In Fig. 1 we present the results obtained when assessing the percentage of folate Recommended Intakes (%RDI) according to children's RTEC intake and applying the highest fortification level found in our analysis of 68 different samples of RTEC. For average RTEC consumption levels, highest fortification level (Level 4) RTEC provided a range from 6% to 21% of folate RDI. Predicted contribution to folate RDI was highest for boys aged 2–5. When focusing on the highest percentile of consumption (P90) for RTEC, boys aged 6–9 years could have a potential intake >60% of their folate RDI, while girls from the same age only reach 42%. In contrast, estimated contribution of the main natural folate vitamer ((6S)-5-CH3-H4Pte-Glu) in analysed RTEC products reached less than 1% of children's folate RDI.

Age-specific tolerable Upper Intake Level (UL) values were used as indicators of potential excessive intakes when percentage contribution was ≥100%. Fig. 2 shows that for RTEC consumption, fortification Level 4 average intakes reached a maximum of 10% UL for boys aged 2–5 years, and when higher level of consumption (P90) is considered, intakes in boys aged 6–9 reach ≤31.6% of UL. In this case, only FA values were used as UL apply only for the synthetic form of the vitamin and not for natural folates (EC, 2000).

High level fortification milk (Level 4) provided on average 136% and 130% of RDI for boys and girls aged 2–5, respectively (Fig. 3). At the higher level of milk consumption (P90) these values reached 252% and 234% of RDI. As expected, children aged 2–5 years were the only subgroups to potentially exceed folic acid UL by intake of fortified milk products (Fig. 4); obtained values were 26% and 17% above UL, for boys and girls at the 90th percentile of intake, respectively.

According to the authors of the enKid Study (Aranceta Bartrina & Serra-Majem, 2000; Serra-Majem, García-Closas, et al., 2001), a high percentage of selected population-age groups may be at risk of folate deficiency as a result of their dietary habits (low vegetable, legume and fruit intakes). Our results suggest that at current FA fortification levels, folate requirements can be achieved by the
intake of a single fortified food product such as milk (Fig. 3). Average intake of fortified cow’s milk in children aged 2–9 could be expected to provide between 92% and 135% of the RDI.

Children’s folic acid UL are an extrapolation from adults values, based on body weight, as there is no evidence of deleterious effects available for this population group (IOM, 1998). Potential consequences of excessive intakes are, to date, unknown (Smith, 2010). Children’s eating patterns are of great concern today, because not only they can lead to excess in other areas of nutritional balance (sugars, fats, sodium), but could also negatively affect the intake of natural folate sources (green vegetables, fruits, legumes).

4. Conclusion

Voluntary fortification of specific processed foods could be a useful strategy to improve folic acid intakes amongst Spanish children. Nonetheless, our results suggest that while some age groups could achieve optimal intakes through current FA fortification levels, younger children at higher consumption levels could be exposed to excessive FA intakes, especially with milk products. Concerns regarding unknown consequences of excess underline the importance of adequate target group identification, and warrant further research in this field.
Fig. 4. Predicted contribution of high level folic acid fortified cow’s milk (Level 4) to folic acid tolerable Upper Intake Levels (UL) for children aged 2–13 years, as estimated from the enKid Study food consumption data (Aranceta Bartrina & Serra-Majem, 2000). Cow’s milk folic acid fortification Level 4: ≥70 μg folic acid/declared serving, on average 53.9 ± 22.6 μg/100 g. Folic acid tolerable Upper Intake Levels (UL) reviewed by the European Commission, Health & Consumer protection Directorate: 300 μg (2–5 years: 400 μg (6–9 years: 600 μg (10–13 years) (EC, 2000)). %UL = (F / Ul) * 100 where: F is the folic acid quantity potentially ingested daily from the assed fortified product and Ul is the folic acid tolerable Upper Intake Levels for each age-group.

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