2015-12-01

Folate Stability in Fortified Corn Masa Flour, Tortillas, and Tortilla Chips

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A thesis submitted to the faculty of
Brigham Young University
in partial fulfillment of the requirements for the degree of

Master of Science

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December 2015

ABSTRACT

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Neural tube defects (NTDs) occur at higher rates in Hispanic populations in the USA. Such populations would benefit from folic acid fortification of corn masa flour (CMF). This study evaluated folate stability in fortified CMFs and products made from the flours, tortillas and tortilla chips. There was no significant loss of folate during the six-month shelf-life of fortified tortilla CMF and tortilla chip CMF. There was a 13% loss ($P < 0.05$) of folate during tortilla baking and no loss during tortilla chip frying. Both tortillas and tortilla chips showed significant folate losses over the two-month shelf-life for these products, with a 17% loss in fortified tortillas and 9% loss in tortilla chips. Folate in fortified CMFs, tortillas and tortilla chips is relatively stable and comparable to the stability of folate in wheat flour and breads.

Keywords: corn, corn masa flour, folic acid, stability, fortification, tortilla, chip

ACKNOWLEDGEMENTS

A special thanks to my graduate committee Dr. Oscar Pike, Dr. Michael Dunn, Dr. Laura Jefferies and Dr. Chad Hancock for the opportunity to work on this project and all of the hours of work they contributed to giving feedback and reviewing the project. Special thanks also to the undergraduate research assistants, especially Muriel Johnson, Nate Camp, Grace Kim, Jason Kim, Parker Dunn and Brad Bae, for all of the many hours helping with analysis of folic acid and sampling products. Thank you to Azteca Milling for providing flour for my research, March of Dimes, for funding the stability research, as well as Analytical Resource Lab in Orem, Utah and Rancho Markets in Provo, Utah for use of their equipment, facilities and time.

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1. INTRODUCTION

Spina bifida and anencephaly are neural tube birth defects (NTDs) affecting 300,000 children a year globally. Spina bifida is a disease that is caused when the neural tube of a fetus fails to close properly, commonly because the mother did not have an adequate folate intake during spinal development of the fetus. It is estimated that about 75% of these cases are caused by maternal folate deficiency during the first month of pregnancy (Oakley 2002). Many pregnancies are unplanned and many women do not know they are pregnant during this critical period. For this reason, in 1992 the United States (USA) Public Health Service recommended that all women capable of becoming pregnant consume 400µg of folate a day to decrease the risk of NTDs (Berry et al 2010; Boulet et al 2009; De-Regil et al 2010). Even after substantial effort to promote this recommendation, only 30-40% of women of childbearing age report taking a daily supplement (Heseker 2011). The Hispanic population in the USA has a higher incidence of spina bifida, especially when the mother has recently immigrated (Au et al 2008; Canfield et al 2009). The reasons for the disparity are not fully understood but it has been associated with education, income, acculturation, genetics and diet (Fleischman and Oinuma 2011).

To compensate for lack of folate in the diet of most of the general population, some cereal grains are fortified with folic acid (FA) in the USA (Berry et al 2010; Crider et al 2011). In 1996, FA fortification was allowed in white flour and rice; and in 1998, standards of identity for fortified versions of these products were changed to require FA addition at a level of 140µg FA/100 g. As a result of this FA fortification requirement, the incidence of spina bifida in the USA was reduced by 22.9% between October 1998 and December 1999 (Boulet et al 2009; Imbard et al 2013). However, even after this fortification requirement, Hispanic women continued to have a higher incidence of NTDs than non-Hispanics (Fleischman and Oinuma, 2011). One possible reason for this is the widespread use of corn masa flour (CMF) and related products among the Hispanic population in the USA, in lieu of wheat and rice products.

It has been suggested that fortification of CMF with FA would significantly reduce the incidence of spina bifida in the Hispanic population (Osterhues et al 2013). Many Latin American countries, including Mexico, Costa Rica and El Salvador, currently fortify CMF with FA. As a result, these countries have seen a decrease in the incidence of spina bifida and other NTDs (Orioli et al 2011). There is not standard of identity for CMF in the USA and current food additive regulations do not allow FA fortification of CMF. In order for the Food and Drug Administration (FDA) to approve addition of FA to CMF, a food additive petition must be submitted showing that there is a documented need; there is a reasonable certainty that the addition of FA to CMF and corn masa products would result in no harm to the general public; and food technology and manufacturing issues such as the proper amount to be added, homogeneity during processing, and stability of FA during processing and storage, must be considered and evaluated (Fleischman and Oinuma 2011).

A common product made from corn masa is tortillas, which can be manufactured using either of two methods. The traditional method is to first cook corn kernels with lime (calcium hydroxide) and to steep them overnight. The cooking liquor is then discarded and the corn is washed. The product of steeping (nixtamal) is ground into masa using volcanic mill stones and then used directly to make tortillas or other products. The second method is to dry the masa into masa flour for storage and transport, then to rehydrate the CMF to masa, which is then used to make tortillas or other products. Tortillas are made in industry using a tortilla machine that forms tortilla rounds, and conveys them through a triple pass oven where they are baked into tortillas (Dunn et al 2008). One major concern with this process is that the alkaline treatment may cause the pH to be high enough to negatively impact the stability of added FA. In this respect, the stability of folate in CMF products cannot be compared to wheat products such as bread.

Tortillas made by the traditional method and fortified right before grinding, with a micronutrient premix including thiamin, riboflavin, niacin, FA, iron and zinc, had significant losses of folate during grinding nixtamal into masa and smaller losses during baking and reheating of tortillas (Burton et al 2008). A study by Dunn et al (2008) indicated traditionally-made, fresh masa tortillas only retained 20% of the theoretical fortified level of FA. The commercial production step resulting in the greatest total folate (TF) loss was the holding of hot, freshly ground, fortified masa (73% loss after 4 hours) prior to baking (Chapman et al 2010). Heat from baking, and iron addition did not affect TF amount in fortified corn masa dough. There is no reported research on the stability of added FA during frying of tortilla chips.

The purpose of this research was to determine the stability of TF in CMF fortified with 154 µg FA/100g, during flour storage, during baking into tortillas and frying into tortilla chips, and during storage of tortillas and tortilla chips.

2. MATERIALS AND METHODS

2.1. Materials

Six 22.7 kg bags from each of two lots (representing different production days) and twelve bags from a third lot of unfortified commercial CMF for making tortillas (Premium Corn Masa Flour #30) were provided by Azteca Milling LP (Plainview, TX USA). This unfortified tortilla CMF contained the following ingredients: ground corn treated with lime, propionic acid, guar gum, cellulose gum, benzoic acid, phosphoric acid, and enzymes. This product is distributed by Azteca Milling in the Southwest region of the USA, which is the area of greatest corn tortilla consumption. The third lot with twelve bags was divided in half, with one half being fortified and one half used as an unfortified control. Also, four 22.7 kg bags from one lot of unfortified tortilla chip CMF (Chip Delight, White Corn #6) were provided, comprised solely of white corn cooked with lime water. The lot was divided in half, with half being fortified and the other half used as an unfortified control. CMFs were stored for one month in ambient conditions in Orem, UT until they were fortified at the commencement of this study, after which they were stored at 22 ^{o}C and 65% rh. FA (>99.9% purity) was provided by DSM Nutritional Products (Parsipanny, NJ USA).

2.2. CMF Fortification

For the purposes of stability evaluation, it was important that the FA be distributed as homogenously as possible throughout the CMF so that stability changes over time could be distinguished from sample-to-sample variation. Commercial fortification is known to result in excessively large variation within and between bags. Consequently, a preliminary study was conducted where zinc oxide (having a particle size similar to FA powder) was added to 136 kg tortilla CMF and 45.4 kg chip CMF using the blending procedure detailed below. The mixture was blended for 15 min in a commercial multi-directional blender (NOAH, model HD-1000, Shenzhen City, China) and tested for homogeneity by taking a sample from every 13.6 kg of tortilla CMF and every 4.5 kg chip CMF, as the blender was discharged. ZnO was used due to its stability during processing (since it is elemental in nature) and because it could be measured using inductively coupled plasma atomic emission spectroscopy (ICP), which has a low analytical coefficient of variation. This mixing procedure provided a coefficient of variation (CV) of 1.1% when mixing 136 kg of tortilla CMF and 2.8% when mixing 45.4 kg of chip CMF. A CV under 10% was considered adequately homogenous (Herrman and Behnke 2014). This preliminary study indicated the blending method would be sufficient to achieve a homogenous mixture of FA in the CMF.

Each lot of CMF was fortified independently. Fortification took place by adding FA at a level of 154 µg/100g to each lot of flour in the multi-directional blender. This fortification level was based on the 140 µg/100g FA added to wheat flour, with an additional 10% to allow for some loss. Six 22.7-kg bags (136 kg total) of unfortified tortilla CMF were blended together during fortification. Two 22.7-kg bags (45.4 kg total) of unfortified chip CMF were blended together during fortification in a smaller model of the same mixer (NOAH, model HD-200, Shenzhen City, China).

An amount of 0.2097 g of FA was added to enough corn starch (for the purpose of facilitating dispersion) to total 30.0000 g and shaken by hand in a small plastic bag for 2 min. This mixture was then combined with 1.36 kg of tortilla CMF and shaken by hand in an 18.9 L pail for 2 min. This FA-CMF premix was then divided into 5 equal parts and 1 part was added between every 22.68 kg bag of flour as 6 bags were added to the stationary commercial multi-directional blender (NOAH, model HD-1000, Shenzhen City, China). Once all the flour and premix were loaded, it was blended for 15 min, returned to the 22.68 kg kraft plastic-lined bags that the CMF came in, and the tops were rolled over and resealed with packing tape before transferring the bags to an environmental control chamber (Environmental Growth Chambers, Chagrin Falls, OH, USA) for storage at 22ºC and 65% rh for up to 6 months (the expected commercial shelf life). The process was repeated for each of the other two lots. This same mixing process was followed for the tortilla CMF control bags, except that no FA was added. Fortification of chip CMF was carried out in a smaller model HD-200 mixer, and followed the same protocol scaled down by one third to result in a 45.4 kg lot of fortified chip CMF.

2.3. Tortilla Production

Two 4.5 kg portions from each lot of tortilla CMF, which had previously been stored for 3 months (the midpoint of the shelf life), were taken to a local tortilla production facility to be made into tortillas. Each 4.5 kg portion of tortilla CMF was combined with 6.1 kg of water and mixed for 5 min in a large planetary mixer to make a batch of dough. The dough was then put through the tortilla machine which rolled and cut the dough into 14 cm tortillas, and then baked them in a triple pass oven at 290ºC for a total of 48 sec. Tortillas were cooled for 90 sec on a cooling conveyor before being placed into low density polyethylene bags (1.0 kg capacity) sealed with a twist tie. The tortillas were then stored at 22ºC and 65% rh for 2 months, a common shelf life for this product.

2.4. Tortilla Chip Production

Two 4.5 kg portions of fortified and unfortified chip CMF, which had been previously stored for 3 months, were taken to a small local tortilla production facility to be made into tortilla chips. Each 4.5 kg portion of chip CMF was mixed with 7.7 kg of water in a large planetary mixer for 5 min to make a batch of dough. The dough was then made into tortillas as described above. After baking, the tortillas were cut into quarters and chilled 10 min in a freezer before frying. Tortillas were fried in a continuous fryer (Superior Food Machinery Inc, Pico Rivera, CA, USA) in 177 ºC vegetable oil for 95 sec. After cooling, tortilla chips were put into polypropylene bags (0.45 kg capacity) and sealed with a twist tie. Chips were then stored at 22ºC and 65% rh for 2 months, which is a common shelf life for this product.

2.5. Sample Preparation for Vitamin Analysis

Newly Fortified CMF. A 15 g sample was taken from every 13.6 kg of flour as the blender was discharged and the samples were mixed together and served as the initial 0-month sample for each lot. Three 20g sub-samples were taken from the composite and shipped over-night on ice-packs to NP Analytical (St. Louis, MO, USA) for moisture and TF analysis.

Stored CMF. Bags (22.7kg) of CMF were removed from storage at 22 °C and 65% rh after 3 and 6 months. Each bag of flour was transferred to a ribbon-blender (Aaron Process Equipment Company, Model IMB5, Bensenville, IL, USA) and mixed for 15 min. Ten 50g samples were collected from the ribbon blender, using a flour trier, following a mixer sampling plan (Herrman and Behnke, 2014). The ten individual samples were combined in an inflated plastic bag and mixed well to form a composite sample for each lot. Three 20g sub-samples were taken from the composite and shipped over-night on ice-packs to NP Analytical (St. Louis, MO, USA) for moisture and TF analysis.

Tortillas and Tortilla Chips. For each time point (0 and 2 months), the contents of an entire plastic bag of tortillas or tortilla chips was ground using a food-processor (Hamilton Beach, 70590, Southern Pines, NC, USA) on the second of ten settings for 30 sec. Three 20g sub-samples of the ground material from each bag were placed in separate plastic Whirl-pak sample bags, and shipped over-night on ice-packs to the analytical testing laboratory for moisture, fat (chips only), and TF analysis. Unused portions were repackaged in Whirl-pak sample bags and held frozen at -62 °C as retention samples until the above analyses were completed.

2.6. pH, Moisture and Fat Analysis

Active acidity (pH) was determined in CMF using the AOAC Method 943.02. Ten g of CMF were weighed into a clean, dry Erlenmeyer flask and suspended in 100mL of boiled water.

Samples were then allowed to sit for 30 min, shaking frequently. The pH was measured using an electrode potentiometer calibrated using buffered solutions of pH 4 and 7.

To report results on a db, moisture content of all samples were determined gravimetrically using a combination of AOAC Methods 925.10 and 930.15.

Crude fat was measured to make folate data between chip flour and chips comparable. Following AOAC Method 922.06, samples were treated with concentrated hydrochloric acid to free fats and oils. The fat was then extracted with a mixture of ethyl and petroleum ether, which was subsequently volatilized, leaving the fat. The fat was dried, weighed, and quantitated as percent (w/w) fat.

2.7. Total Folates

Vitamin results were determined on a wb and then db values were calculated, based on moisture content of each sample. Endogenous folates and added FA were extracted using the trienzyme extraction AOAC Method 2004.05 for cereals and cereal foods. Extracted folates were quantified using an Autoturb turbidimetric microbiological method, based on the observation that *Lactobacillus casei* ATCC 7469 requires FA for growth. A basal medium, nutritionally complete in all respects except for folate, was used as the diluent for extracts from standards and samples. The mixture was incubated and the growth response of the bacterial cultures was measured as percent light transmittance on the Autoturb. A dose-response line was constructed and the sample concentrations were calculated. An unfortified wholemeal wheat flour standard (Community Bureau of Reference Certified Reference Material, Sigma-Aldrich, St. Louis, MO, USA), as well as a 154 µg/100g fortified tortilla CMF sample (internal laboratory standard), were analyzed along with each set of samples run at a given time.

2.8. Statistical Analysis

The structure of each experiment was a full factorial design with tortilla CMF from three different manufacturing lots (one of which was split into a control and fortification treatment) and one lot of chip CMF split into control and treatment. A multi-way mixed model analysis of variance (ANOVA) was used for the statistical analysis. The mixed models were used to account for the correlation in the observations from the three lots and the bags of tortilla CMF within the lots. The dependent variable for this analysis was the TF levels in CMF after storage. The independent variables included lot, storage time, and fortification level. A blocking factor by lot was also used in the analysis for CMF storage, and by batch for product storage. Suspected outlier values from bags with coefficients of variation >5% were tested using a modified Z-score test (NIST, 2013), to determine whether they were significant. Twelve data values receiving Z-scores >3.5 were considered significant outliers and were excluded from the final statistical data analysis, resulting in some bags only having 2 analytical repetitions instead of 3.

3. RESULTS AND DISCUSSION

3.1. pH, Moisture and Fat

The pH of the tortilla CMF averaged 4.20 for unfortified samples and 4.23 for fortified samples. Chip CMF, both unfortified and fortified, had an average pH of 5.84. Although it has been established that pH is not a major factor in folate degradation, there was concern that the expected basic pH of the CMF after being treated with calcium hydroxide might impact folate stability (Chapman et al 2010). However, despite the alkaline process, the pH of the tortilla and chip CMF was within the range of stability for FA (Eitenmiller 1998). The lower pH of the tortilla CMF is presumably due to the acidic preservatives added. Tortilla CMF used in the Southwest region of the USA usually contains preservatives. Chip CMF did not have preservatives and was expected to have a higher pH, but was not far removed from the pH 6 - 6.5 expected value stated by the manufacturer (Azteca, Evansville, IN, USA).

The average moisture content of the tortilla CMF was 11.7 ± 0.294% (±*SD*), which was nearly identical to the chip CMF moisture content of 11.5 ± 0.08%. Tortillas averaged 51.0 ± 0.690% moisture, with insignificant change over time, whereas moisture content of the tortilla chips was 1.1 ± 0.916% at time 0 and 3.8 ± 0.677% after two months. The average fat content of the chip CMF was 3.9 ± 0.286%, which increased to 25.6 ± 1.08% after frying into tortilla chips.

3.2. Total Folate

Stored CMF. Folate levels in tortilla and chip CMF, measured over the 6-month shelf-life are presented in Table 1. The expected/theoretical level of folate in the CMF after fortification – the sum of added FA plus the endogenous folate in the control flour – was calculated to be 176 and 178 µg/100g (db) for tortilla CMF and chip CMF respectively. This was calculated by adding the amount of endogenous folate and the 143 µg/100g of added FA, then converting to db. At 0 months, the TF content of the tortilla flour was measured to be 167µg/100g of CMF. This value is lower than the theoretical value. Possible explanations include incomplete extraction or other analytical errors, nonhomogeneity, sampling errors or weighing errors. At 6 months the TF in tortilla CMF was 188 µg/100g, but this was not a statistically significant increase ($P > 0.05$). Most importantly there was no significant loss of TF during storage of fortified tortilla CMF over the 6 month storage period. The statistically significant increase in the endogenous folate content of unfortified tortilla CMF, from 12.9 µg/100 g at 0 months to 13.7 µg/100 g after 6 months, is not considered practically significant. Fortified chip CMF had an initial TF content of 159 µg/100g. This is also lower than the theoretical value, presumably for one or more of the reasons mentioned for the lower than expected tortilla flour value. After 6 months of storage, the TF content was 155 μg/100g, which was not a significant loss. The lack of change in folate content over time is not surprising given the reported stability of FA in dry form (Ranhotra and Keagy 1995). This agrees with previous research that has shown that FA is stable for over 6 months in fortified cereals, including corn meal and corn grits, and vitamin-mineral premixes at ambient temperature (Gujska and Majewska 2005; Ranhotra and Keagy 1995; Berry et al 2010).

Processing. There was a 13% loss of folate during baking tortilla CMF into tortillas as seen in Table 2. Table 2 also shows that in the case of fortified chips, there was a 24% increase in TF. (Values for fortified tortilla CMF in Table 2 only represent samples taken from the same lot that was used to make tortillas. Values for chip CMF and products were calculated on a fat free dry weight basis rather than db only; hence these values are different from their counterparts in Table 1.) A possible explanation for the greater TF value in the fried chips, as comparted to starting flour, may be a potential increase in extraction rate of folate from the food matrix after frying. More importantly, it does not appear that TF was negatively impacted by frying at high temperatures. This finding is supported by the observation of Wieringa et al (2014) who found that for all fortified rice types evaluated, frying the rice kernels before boiling resulted in no significant loss of FA compared to boiled rice that was not pre-fried. Also, Cheung et al (2008) showed a similar increase in FA (105% recovery) following frying of Asian noodles, indicating that frying also tended to increase recovery in their study, and resulted in no significant loss.

The stability of TF during processing of CMF products in this study is similar to the stability reported by Gujska and Majewska (2005) who observed that both added (200 μg/100g) and natural folate had losses between 12-20% during wheat bread production. Similar results were also observed by Anderson et al (2010) who reported a loss of 20-30% during baking of bread. Since neither tortilla nor tortilla chip production involve long exposure to heat, it would be expected that losses during processing would be lower in such products, compared to bread which is baked much longer. Burton et al (2008) observed a 33% loss of TF during production of tortillas from the time of fortification through baking, using the traditional method of making tortillas from fresh nixtamal. The traditional method produces substantial heat during the grinding of nixtamal to masa and involves the holding of hot masa until it is used. These differences may account for the greater loss of TF using the traditional method. These changes in fortified CMF and tortillas are also similar to those reported in studies in wheat flour and wheat breads. Swindler et al. (2013) reported 24% loss of native folate in unfortified wheat flour after 6 months of storage at room temperature, and no significant loss of added folic acid in white enriched wheat flour over the same time. Cort et al. (1976), on the other hand observed about a 19% loss of folic acid in fortified wheat flour over 6 months. Omar et al. (2009) reported 32% loss of native folate, and 15% loss of added folic acid during baking of fortified wheat baladi bread. Gujska and Majewska (2005) observed about a 19-21% loss of added folic acid during baking of wheat and rye yeast breads, and no loss of endogenous folates during breadmaking – although yeast production of folate offset endogenous losses that might have been present. Anderson et al. (2010) also reported a loss of 17-30% of added folic acid during baking of fortified white pan bread, 25-40% loss in fortified wholemeal bread, and 20-30% loss in fortified brown soda bread. The Anderson study is particularly relevant, as it shows that in wheat bread processing, addition of alkali (in the form of baking soda) did not increase folic acid loss over and above the losses observed in the non-alkaline products. This also agrees with Chapman et al. (2010) where pH between 5 and 9 did not have an effect on folate stability in tortillas using the traditional production method.

Stored Tortillas and Tortilla Chips. Table 3 presents data for finished products prepared from fortified and unfortified flours. Both tortillas and tortilla chips showed statistically significant TF losses over the two-month shelf-life for these products. Fortified tortillas stored for two months at 22ºC and 65% rh showed a 13% loss of TF. Unfortified tortillas showed an even greater relative loss, with a 44% decrease in endogenous folate. Similarly, fortified chips lost 9.3% of TF, while unfortified chips lost 32% of endogenous folate. Determination of fortification levels in such products should take into account these losses that occur during storage. This is the first report of folate stability during storage of corn masa products manufactured using fortified CMF.

4. CONCLUSION

Since there is no significant loss of TF during storage of fortified CMF up to 6 months and losses in product manufacturing and storage appear to be no greater than wheat flour products, it can be concluded that no overage would be needed to account for losses of folate during storage of dry CMF. FA used to fortify CMF is sufficiently stable during baking and frying under the conditions of this study to make a significant contribution of TF in the final product without the use of high fortification overages. The fortification level of 154µg/100 g of CMF is also sufficient to deliver 138 µg (the TF folate value of fortified tortillas after 2 months of storage) of TF per 100 g of product at the end of the product's shelf life.

In addition to these encouraging results, commercial fortification of CMF would be easy to implement. Wheat flour is already fortified in the USA and the same fortification process used for wheat flour can be used to fortify CMF.

Further studies could determine the stability of FA during in-home preparation of CMF. Finally, many of tortilla production facilities in the USA produce tortillas and tortilla chips using the traditional tortilla process rather than rehydrating CMF as observed in this study. FA fortification of CMF would not affect products made using the traditional method.

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TABLES AND FIGURES

Table 1: Total folate content (db) of corn masa flour during 6 month shelf life

Flour Type	0 Month (μg/100g)	3 Month (μg/100g)	6 Month (μg/100g)	Loss (%)
Unfortified Tortilla	13 ± 0.2	13 ± 0.2	14 ± 0.2**	-6.2
Fortified Tortilla	167 ± 8.0*	176 ± 7.6	188 ± 7.7	-13
Unfortified Chip	15 ± 0.2	14.0 ± 0.2	ND***	4.8
Fortified Chip	159 ± 5.1	157 ± 5.4	155 ± 6.2	2.5

* ± SEM

**indicates significant difference from 0 Month ($P < 0.05$). n=6 for fortified flours; n=2 for unfortified flours

***Not Determined

Table 2: Total folate content (db) before and after processing of tortillas and tortilla chips

Product	3 Month Flour (µg/100g)	0 Month Product (µg/100g)	Loss (%)
Unfortified Tortillas	13 ± 4.9	21 ± 4.7	-62
Fortified Tortillas	181 ± 4.5*	158 ± 4.7**	13
Unfortified Tortilla Chips†	15 ± 3.4	18 ± 3.4	-20
Fortified Tortilla Chips†	156 ± 4.2	194 ± 3.4**	-24

* ± SEM

**indicates significant difference from 3 Month Flour ($P < 0.05$). n=6 for fortified tortilla flour; n=2 for chip flours and unfortified tortilla flour; n=4 for products

†fat-free basis

Table 3: Total folate content (db) of tortillas and tortilla chips during 2 month shelf life

Product	0 Month (µg/100g)	2 Month (µg/100g)	Loss (%)
Unfortified Tortillas	21 ± 1.3	12 ± 1.2**	44
Fortified Tortillas	158 ± 1.7*	138 ± 1.7**	17
Unfortified Tortilla Chips†	18 ± 0.9	12 ± 0.9**	32
Fortified Tortilla Chips†	194 ± 4.5	176 ± 4.5**	9.3

*± SEM
**Indicates significant difference from 0 Month ($P < 0.05$) n=4
†fat-free basis

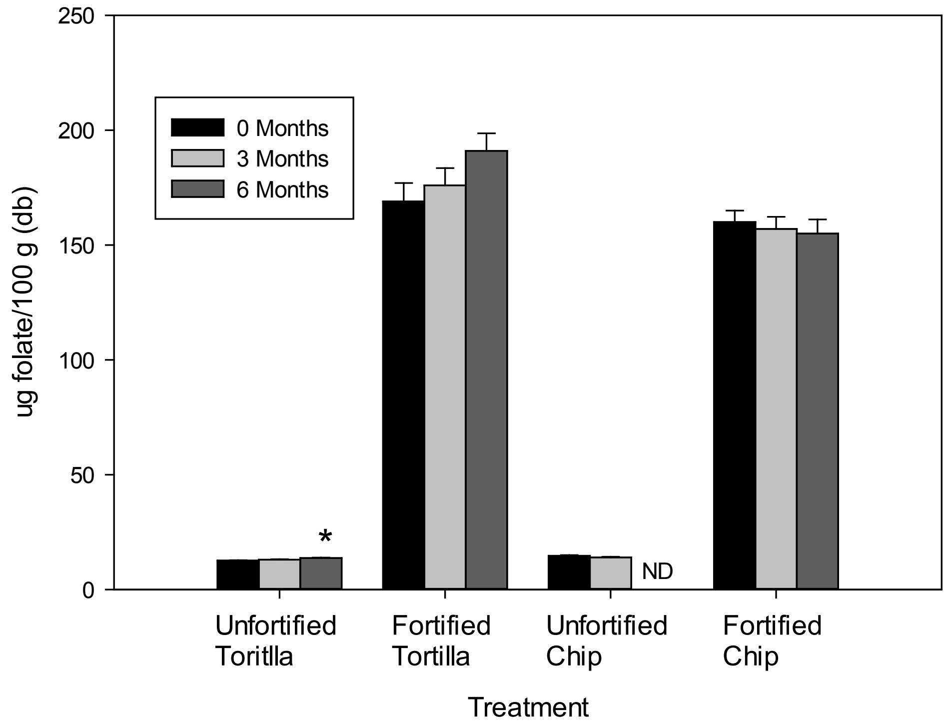

Figure 1. Folate content of corn masa flour during 6 months of storage. ± SEM; n = 6 for fortified tortilla flour; n = 2 for all other flours; * Indicates significant difference from time 0 within treatments ($P < 0.05$). ND = Not Determined.

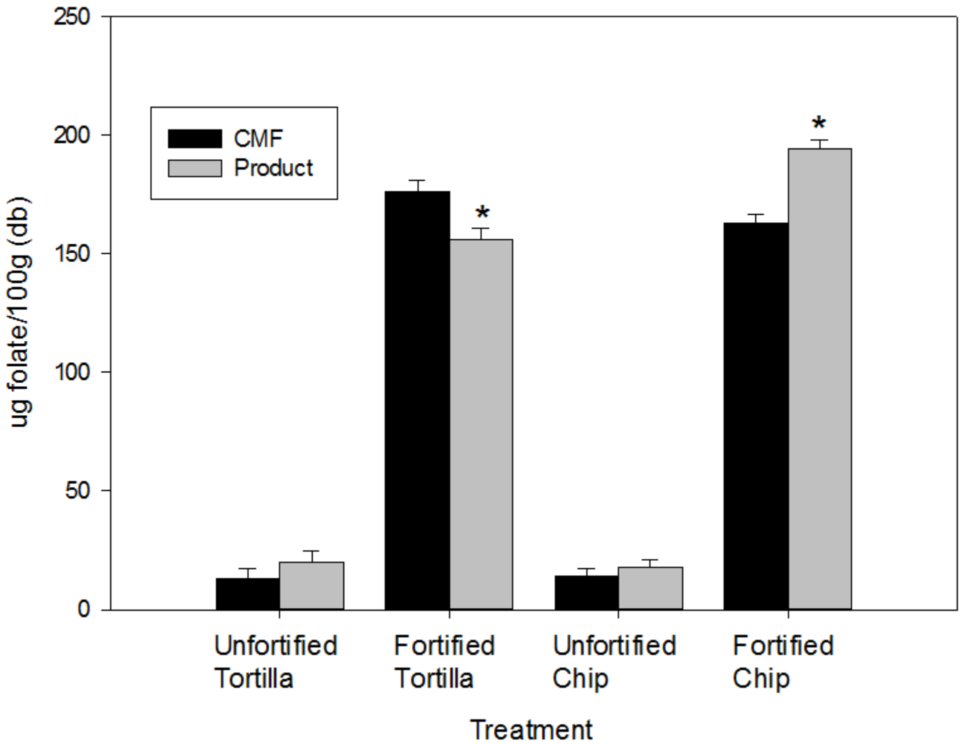

Figure 2. Folate content before and after processing tortilla and tortilla chips. ± SEM; Chip values are measured on a fat free dry weight basis; n = 6 for fortified tortilla flour; n = 2 for chip flours and unfortified tortilla flour; n = 4 for products; * indicates significant difference from flour within treatments ($P < 0.05$)

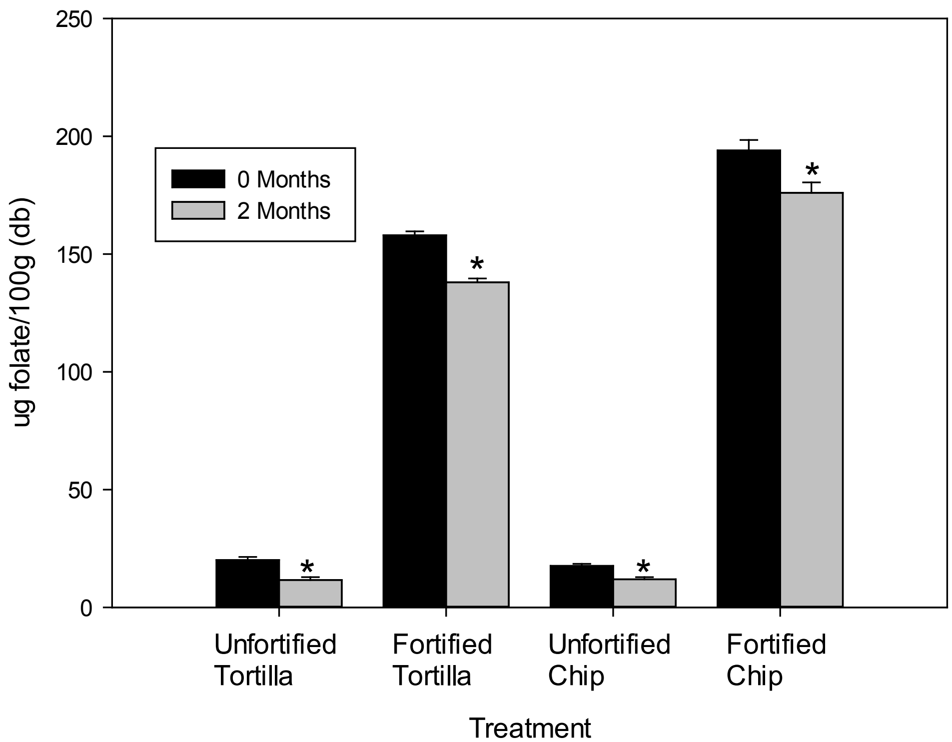

Figure 3. Folate content of tortillas and tortilla chips during 2 months of storage. ± SEM; chip values are measured on a fat free dry weight basis; n = 4; * indicates significant difference from 0 months within treatments ($P <$ 0.05).

APPENDIX

A. LITERATURE REVIEW

Effects on Health

As a result of folate fortification in 1998, the average increase of intake of folate for non-supplement users in the United States was 190 μg/day (Choumenkovitch et al 2002). This is almost twice as much as the targeted 100 μg/day increase. Similar increases were seen in supplement users exposed to fortification. The prevalence of folate intake under the EAR (Estimated Average Requirement) was reduced from 50% to 7%. The prevalence of folate intake over the UL only increased significantly in supplement users (from 1.3 to 11.3%).

Several studies have observed the reduction of neural tube defects from folate fortification of flour (Castillo-Lancellotti et al 2013). The largest observed decreases in neural tube defects due to fortification occurred in Costa Rica (58%), Canada (49%) and Argentina (49.7%). The largest reductions of spina bifida were observed in Costa Rica (61%), Canada (55%), and Chile (55%). The smallest reduction of neural tube defects and spina bifida were both in the United States (15.5% and 3.4%) respectively. In the United States, the highest reduction was seen in the non-Hispanic black population which has the lowest prevalence of neural tube defects (Boulet et al 2008). Costa Rica, Canada, Argentina and Chile experienced the most reduction of anencephaly after folate fortification (68, 58, 57 and 50% respectively). The lowest reduction was observed in South Africa (9.8) and in African Americans in the United States. Costa Rica also fortifies maize flour, milk and rice in addition to wheat flour. The results are variable depending on the integrity of each individual country's surveillance system. Fortification programs in Latin America have seen decreases in the prevalence of neural tube defects ranging from a 33 to 59% decrease (Barboza-Arguello et al 2015; Rosenthal et al 2014). In Jordan, the fortification of cereals foods was established to increase the average intake of folate among women of childbearing age by 30-70% without posing a risk to the general public (Amarin and Obeidat 2010). A dose of 150 μg/100 g was used. Fortification of folate in cereal products resulted in a 49% overall decrease in the incidence of neural tube defects.

Researchers predicting the effect of folate fortification of flour on socioeconomic residences and ethnic groups in Guatemala found that fortification of flour alone would have no effect on the neediest population (Imhoff-Kunsch et al 2007). Among all households surveyed, the per capita of wheat flour purchased was very low. Purchase of ready-made breads was much higher than purchase of wheat flour but lowest in the poor, rural and indigenous households. The contribution of wheat flour to the EAR and RDA in poor households was only 5 and 4% respectively. For urban households it was much higher (78 and 62% respectively). The flour fortification program is still very useful but other products should also be fortified to provide folate to populations that will not be affected as much by flour fortification. From 1999 to 2004, the incidences of spina bifida and anencephaly have decreased in the United States (Boulet et al 2008). The prevalence of spina bifida and anencephaly is 40% higher in Hispanic babies than in black non-Hispanic babies and 30% than white non-Hispanic babies. Multiple studies have shown that the Hispanic population in the United States has a higher incidence of neural tube defects than the non-Hispanic white and non-Hispanic black populations (Williams et al 2005; Yang et al 2007). This is predicted to be due to genetic factors as well as diet.

In the NHANES over 25% of women aged 15-44 years reported eating CMF products. More of these women were Hispanic (60%) than non-Hispanic white, and non-Hispanic black. Within the total USA population over 50% of Hispanics reported eating a food that contained CMF within the last two days while only about 20% of non-Hispanics did. According to a model developed by Hamner et al (2009), the fortification of FA in CMF would result in almost a 20% increase in daily folate intake in Mexican American women. Based on this model, the percentage of Mexican American women who would be receiving less than 400µg/day would decrease from 85% to 78%. Almost no change in folate intake was observed for non-Hispanic populations. When applying the model to the women that reported eating products that contained CMF in the survey, there was an estimated 33.9% increase in folate intake if the CMF had been fortified. However, this study has limitations because researchers had to predict which reported products contained CMF. The researchers also assumed that all CMF would be fortified with 140µg/100g.

Another study observing the effect of acculturation showed that women who had less acculturation (based on language preference) were more likely to report eating foods that contain CMF. These women also had a lower median daily folate intake (Hamner et al 2013b). The relative predicted increase, according to the model, of folate intake in Mexican America women who spoke mainly Spanish is 30.5% while Mexican American women who spoke mainly English is only 8.3%. The percentage of Mexican American women that are currently getting the daily recommended amount of folate is 13% which is estimated to increase to 19% with corn masa fortification. The percentage of Non-Hispanic women getting the daily recommended amount of folate is only estimated to increase by 1%. The percentage of women with lower acculturation getting the daily recommended amount of folate is expected to increase by 8.2%, and the percentage of women with higher acculturation, by 2.6%. This shows that using CMF would target the population in most need of intervention without affecting other parts of the population.

Tinker et al (2013) predicted the effect of fortifying CMF with folate (140µg/100g) on the incidence of neural tube defects. It was estimated that there would be a reduction of spina bifida by 6% (95% CI: 0-19%) and a 4% (95% CI: 0-15%) reduction in anencephaly among

Hispanic women in the United States. Although this doesn't seem to be significant because the target population is a small proportion of the overall population, if folate fortification of CMF was required, it is estimated that 40 babies from Hispanic mothers would be saved from spina bifida annually as well as 20 babies from non-Hispanic white mothers. Given the total lifetime direct cost of a child born with spina bifida is so high ($560,000) an additional 40 healthy babies born each year could provide a substantial return on the investment of fortifying CMF with FA. This model was also used to predict the increase in individuals who will be exceeding the UL for folate if CMF were to be fortified with FA (Hamner et al 2013a). There was no evidence that any population in the United States would have a higher percentage of people consuming levels of folate over the UL (1 mg/day) for adults and children. The only factor associated with exceeding the UL is use of supplements.

Adverse effects of excess folate intake include masking a vitamin B12 deficiency (Yetley and Rader 2004). There are also concerns that excess FA will increase seizures in pregnant women with epilepsy. Recently, other links with excess FA intake have been seen in colorectal cancer (Crider et al 2011; Lucock and Yates 2009). FA taken before the development of cancer has been shown to decrease the incidence of colorectal cancer but facilitates the progression of precancerous lesions if they are already present. However, since 1998, the incidence and mortality of colorectal cancer have declined overall (Imbard et al 2013). There is also evidence to suggest that the UL should be increased (Crider et al 2011; Oakley 2002). The main function of folate is to participate in single carbon transfers (Crider et al 2012). One cellular pathway that depends on these transfers includes DNA methylation which is critical to normal gene function. Folate deficiency has been shown to be mutagenic by causing the incorporation of uracil into DNA because thymine can't be synthesized. It was estimated that 700µg/day with 6µg/day of

B12 was required ensure the stability of the human genome. The average western diet contains only 100µg/day of folate. Since the UL (upper limit) of folate is set at 1000µg/day, the limit that has been placed on fortification is a point that is too low to help most of the population to reach 700µg/day (Crider et al 2011; Oakley 2002). It was suggested that the upper limit should be raised and the maximum fortification dose be raised to 240µg/100g. It was also suggested that products be fortified with B12 in addition to folate to deal with the risk of folate masking a B12 deficiency. However, there was not enough data to determine the safety and an appropriate amount of the vitamin B12 to fortify with folate for the FDA to accept the recommendation (Yetley and Rader 2004). The FDA used a safe upper limit intake of 1000 µg/day of folate when evaluating the proposal to fortify cereal grains. Through these considerations the FDA decided on a level of 140 µg/100 g of FA in cereal-grain products. This was estimated to add 80-100 µg/day in the target population but kept the estimated intake of those taking supplements to 800-880 µg/day.

Folate Stability in Non-Corn Masa Products

Natural folate stability during cooking of major sources of folate in the UK diet was investigated by McKillop et al (2002). Spinach, broccoli and potatoes were boiled; spinach and broccoli were steamed; and beef was grilled. Folate in potatoes and beef was fairly stable during cooking with no significant loss in either. When boiled, spinach and broccoli lost about 50% folate. No significant loss was observed in broccoli or spinach when it was steamed. It is believed that the high starch in potatoes and protein in beef were protective of folate and excess water used in boiling caused a greater amount of leaching than other methods.

Vitamin losses during processing are usually less severe in grains in comparison to the processing of fruits and vegetables (Ranhotra and Keagy 1995). Added FA also appears to be more stable than natural folate in flour during storage. Good stability has also been shown in corn meal and corn grits.

FA in fortified cereals and in vitamin-mineral premixes has been shown to be stable enough to significantly contribute to total folate content over 6 months at ambient temperature (Berry et al 2010). Following storage of fortified flour and grains, FA stability during baking of bread products is high. Added and endogenous folate was stable enough during wheat bread production to be a significant contribution to total folates when added at 0.2mg/100g (Gujska and Majewska 2005). There was a 12-20% decrease during production. In another study, looking at several types of breads, about 50% was lost during production with 20-30% being during baking (Anderson et al 2010). A third study also found loss of added FA to be about 20% during baking (Osseyi et al 2001). Endogenous folate appeared to be quite stable with a slight increase observed due to folate production by yeast during the fermenting stage of production. While it appears that endogenous folate is very stable during bread production, the loss of endogenous folate is actually masked by the production of folate by yeast during fermentation (Gujska and Majewska 2005). A study using the sponge and dough method found an increase in endogenous folate of 72% during the fermenting stage (Osseyi et al 2001). Over the entire bread making process there was a 16% increase in total folates.

Rye bread can be made with yeast or with a sourdough starter (Gujska et al 2009). Rye bread made with yeast rather than starter had higher folate content because of folate produced by yeast during fermentation. The loaves were then stored at -18ºC. Folates did not decrease significantly for 5 weeks. There was a 25% loss in bread made with yeast and 38% loss in bread made with a sourdough starter after 16 weeks of frozen storage.

Storage of pseudo cereals (amaranth, buckwheat and quinoa) showed a loss of folate during 3 months of storage ranging from 18.6 to 45.8% (Schoenlechner et al 2010). Staple foods (bread, cookies and noodles) prepared from pseudo cereals all contained a considerable amount of folate with quinoa products having a high level of folate. Folate loss was highest in bread with an average loss of 50.8%. Noodles lost 23.9% of the original folate while cookies lost only 15.8%.

To deal with the instability of FA, L-5-Methyltetrahydrofolic acid was encapsulated and added with ascorbate in hopes of improving stability. A modified starch from waxy maize was used to encapsulate the FA because of its resistance to oxidation (Liu et al 2013). Addition of sodium ascorbate also improved the recoverability of FA during the encapsulation process. Encapsulation of FA made blending into flour easier because of the larger mass to be blended. The flour was then used to make bread. A higher core-to-wall ratio, (meaning there was more FA encapsulated in less starch), correlated with a higher recovery rate indicating more stability. Microencapsulation as well as addition of sodium ascorbate significantly increased the recovery rate of FA after baking and storage of bread.

Retention of FA in fortified rice during cooking was tested for 3 types of fortification (coating, cold extrusion, and hot extrusions) and 5 types of cooking, which included soaking, frying, and washing steps as well as using excess water or just enough for the rice to adsorb it all (Wieringa et al 2014). Overall retention for the different cooking methods ranged from 61% to 75% showing that the added FA was reasonably stable during all cooking methods.

Corn Tortillas

Heat, pH and iron did not affect folate stability in corn masa production in a study by Chapman et al (2010). Encapsulating folate increased its stability. The most folate is lost during holding of hot masa during commercial production with a 14% higher retention than unencapsulated folate (144 vs 97.9mg/100g). However, endogenous folate was not lost during the whole process. This is only a problem when making tortillas from wet masa where freshly ground masa is used to make tortillas. This isn't a problem when making tortillas from CMF because there is no heat involved except during the short baking time of the tortillas and frying of the chips.

Tortillas were fortified with a micronutrient premix including thiamin, riboflavin, niacin, FA, iron and zinc. Significant losses of folate occurred in grinding nixtamal to masa and smaller losses during baking and reheating of tortillas (Burton et al 2008). Fortified tortillas were slightly less stretchable than unfortified tortillas and had a slight blue green color from ferrous fumarate which was used as an iron source. There was no preference for fortified or unfortified tortillas over unfortified tortillas in a sensory evaluation. Encapsulation and minimizing the time holding hot masa will improve FA stability in corn tortillas. Similar results were seen in tortillas fortified with only thiamin, riboflavin, iron and zinc by Rosado et al (2005).

FDA Folate Guidelines

As of 1998, wheat flour is required to be fortified with FA. The guidelines the FDA used to make this decision are as follows (Yetley and Rader 2004). A nutrient may be added to a food to correct a dietary insufficiency recognized by the scientific community if sufficient information is available to identify the nutritional problem and the affected population; the food used to supply the nutrient is likely to be consumed in quantities that will make a significant contribution to the diet of the population in need; the nutrient added to a food is stable in the food under customary conditions of storage, distribution, and use; the addition of the nutrient is not likely to create an imbalance of essential nutrients; the nutrient is physiologically available from the food; there is reasonable assurance that consumption of the fortified food will not result in an excessive intake of the nutrient, considering cumulative amounts from other sources of the nutrient in the diet.

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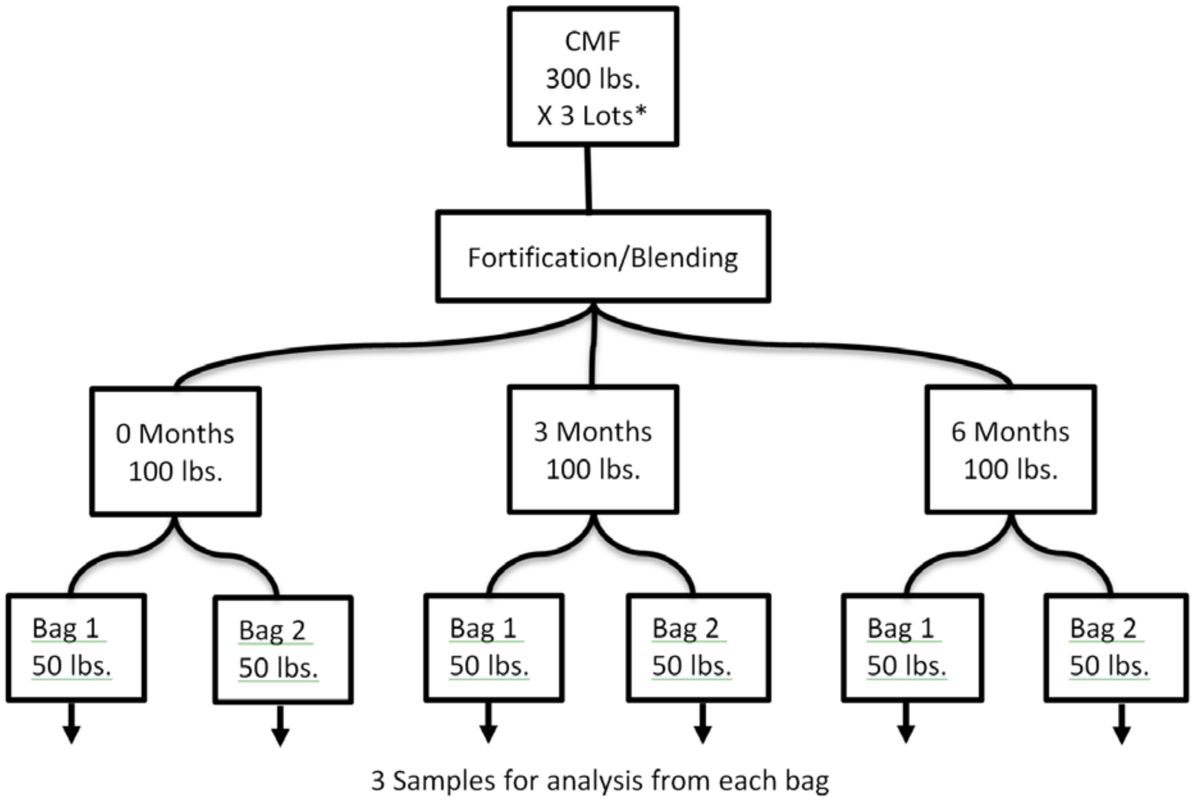
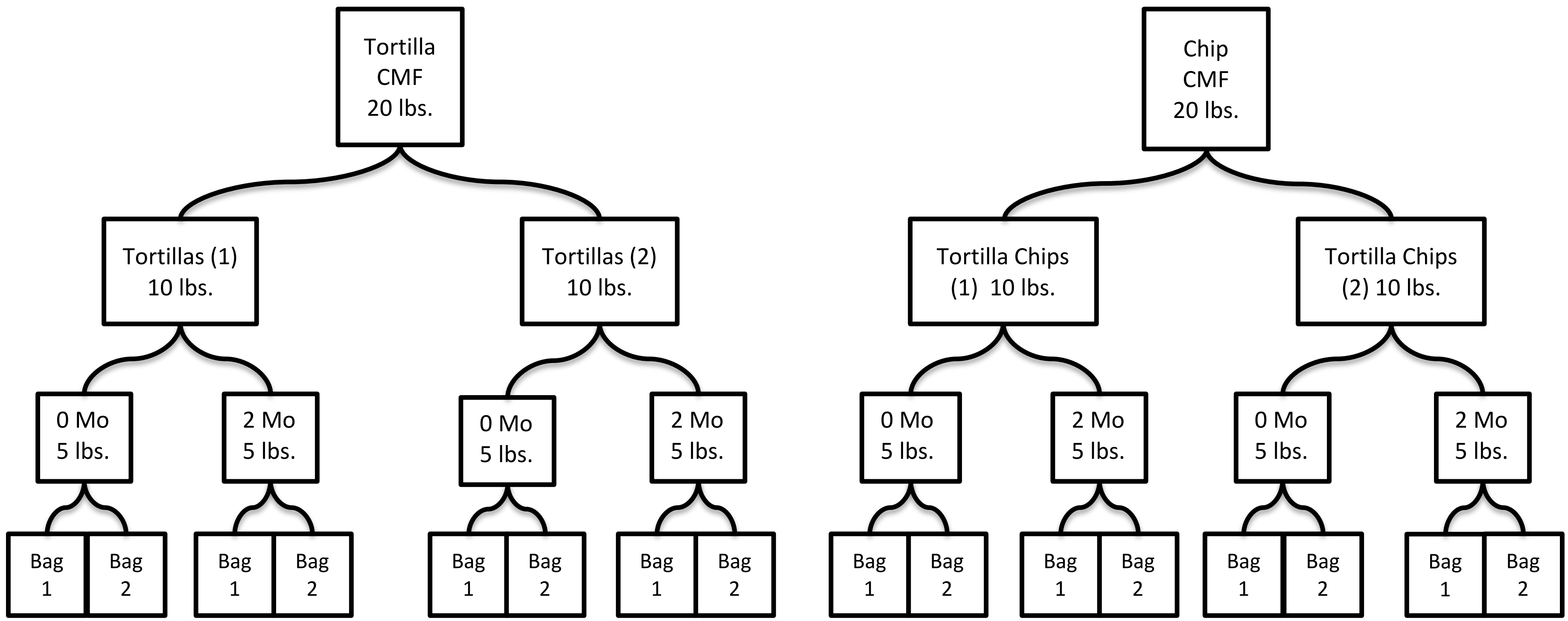
B. STUDY DESIGNS



*Six additional unfortified bags from one lot will be placed into storage and sampled and analyzed following the same protocol.

Figure 4: Study 1 Design – Folate stability in corn masa flour



*Also, tortillas and chips from one lot of unfortified CMF will be placed into storage and sampled and analyzed following the same protocol.

Figure 5: Study 2 Design – Folate stability in tortillas and tortilla chips

C. NP ANALYTICAL FOLATE DETERMINATION CALCULATIONS

NP uses an excel spreadsheet to do the following calculation:

1.1.1. Average the duplicate %T values for each working standard, for the 0.10 mL dilutions and the 0.15 mL dilutions (dilutions from two different-sized loops on Autoturb diluter).

1.1.2. Plot two standard curves on linear graph paper, one for the 0.10 mL dilutions and one for the 0.15 mL dilutions, graphing average %T versus µg/mL FA in the working standards.

1.1.3. Using the %T for each sample, read from the appropriate 0.10 mL or 0.15 mL standard curve, µg/mL FA in the sample solutions.

1.1.4. Average the four sample solutions values, in µg/mL FA.

1.1.4.1. Only values which do not vary by more than $\pm$ 10% from the sample solution average are acceptable for calculation.

1.1.4.2. At least three out of four values must be acceptable.

1.1.4.3. No sample value should be used that falls below one half of the concentration of the lowest working standard (0.0025 µg/mL FA).

1.1.5. Calculate ppm FA activity in the samples by the following formula:

FA activity (ppm) = [(C) (V) (D)]/W

Where: C = Average µg/mL FA from standard curve

V = Extraction Volume, mL

W = Sample Wt, g

D = Dilution, (Final Volume, mL/ Aliquot Pipetted, mL)

D. FOLATE LOSS CALCULATIONS

$\%\ loss\ during\ storage$

$$= \frac{product\ folate\ time\ 0\ (db)\ - product\ folate\ time\ X\ (db)}{product\ folate\ time\ 0\ (dwb)} \times\ 100$$

$$\%\ loss\ during\ baking = \frac{CMF\ folate\ (db\dagger) - tortilla\ folate\ (db)}{CMF\ folate\ (dwb)} \times 100$$

$$\%\ loss\ during\ frying = \frac{CMF\ folate\ (mfffb\ *) - tortilla\ chip\ folate\ (mfffb)}{CMF\ folate\ (mfffb)} \times 100$$

$$folate\ dwb\ (dry\ weight\ basis) = \frac{folate\ (wb\ **)}{100 - \%\ moisture} \times 100$$

$$folate\ mfffb\ (moisture\ free, fat\ free\ basis) = \frac{folate\ (wb)}{100 - \%\ moisture - \%\ fat} \times 100$$

† db = dry weight basis

*mfffb = moisture free fat free basis

**wb=wet weight basis (as is)

E. COMMON COMMERCIAL FORTIFICATION PROCESSES

A premix feeder delivers a specified quantity of micronutrients, which is introduced into the flour stream by pneumatic conveyance. In this case, the premix is blown by positive pressure or sucked by a vacuum through a pipe into the flour collection conveyor. Alternatively, the premix may simply drop by gravity directly into the flour stream as it flows through the conveyor.

In either case, the premix typically enters the flour stream at least 3 meters from the discharge end of the flour collection conveyor to ensure adequate blending. The mixing and turning of the flour stream during conveyance should allow adequate dispersion of micronutrients throughout the flour mass under these conditions. Alternatively, baffles can be installed downstream from the conveying pipe to improve mixing and homogenization of the micronutrient premix in the flour stream prior to depositing the fortified flour into storage silos or packing hoppers.

The batch fortification used in this study provided a much longer mixing time than would be observed commercially. However, folate in dry form -- mixed in a light-impermeable, stainless steel blender -- should not be adversely affected by this additional mixing time. Samples were evaluated following mixing to ensure that no significant losses resulted from blending in the manner indicated in the blending protocol for this study.

F. HOMOGENEITY STUDY

To determine the homogeneity capability of the intended mixers, a preliminary trial was conducted with 136 kg of tortilla CMF, blended in a commercial multi-directional blender (NOAH, model HD-1000, Shenzhen City, China) and 45.4 kg of chip CMF, blended in a smaller NOAH HD-200 multi-directional mixer. A small amount of zinc oxide (which appeared to have a similar particle size to FA) -- equivalent in weight to the amount of FA to be added (0.2097g for tortilla CMF and 0.0699 g for chip CMF) was added to 30 g of corn starch and shaken for 2 min. in an inflated plastic bag. The mixture was then combined with 1.63 kg of CMF and shaken by hand in a 5 gallon bucket for 2 min. The ZnO-CMF premix was then split into 5 parts and one part was added between every 22.7 kg bag of flour as the six bags were added to the mixer. FA premix for chip CMF was prepared similarly, but scaled down by one-third. Once all the flour and premix were loaded into the mixer, they were blended for 15 min. Zinc analysis by inductively coupled plasma atomic emission spectrum, for 10 consecutive samples taken from the discharge run in duplicate, indicated that the mixer provided a CV of 7.8% for Chip CMF and 1.1% for tortilla CMF. The objective was to obtain a CV <10%, so this was considered to be an acceptable protocol for both flours.

G. STATISTICAL OUTPUT

```
                    The SAS System
              Analysis for Tortilla Flour
            15:13 Friday, September 11, 2015

                 The Mixed Procedure

                  Model Information

Data Set                       WORK.IN
Dependent Variable             folate
Covariance Structure           Variance Components
Estimation Method              REML
Residual Variance Method       Profile
Fixed Effects SE Method        Model-Based
Degrees of Freedom Method      Containment

               Class Level Information

   Class    Levels    Values

   lot           3    1 2 3
   time          3    0 3 6
   bag           2    1 2

                     Dimensions

          Covariance Parameters             3
          Columns in X                      6
          Columns in Z                     12
          Subjects                          1
          Max Obs Per Subject              54


               Number of Observations

      Number of Observations Read              54
      Number of Observations Used              50
      Number of Observations Not Used           4


                  Iteration History

Iteration    Evaluations    -2 Res Log Like       Criterion

        0              1       437.86394384
        1              3       436.87920523      0.00002864
        2              1       436.87390926      0.00000018
        3              1       436.87387760      0.00000000


             Convergence criteria met.
```

The SAS System
Analysis for Tortilla Flour
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The Mixed Procedure

Covariance Parameter Estimates

Cov Parm	Estimate
lot	0
lot*time	76.6791
Residual	571.98

Fit Statistics

-2 Res Log Likelihood	436.9
AIC (smaller is better)	440.9
AICC (smaller is better)	441.2
BIC (smaller is better)	439.1

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
time	2	4	1.77	0.2818
bag	1	40	4.44	0.0414

Least Squares Means

Effect	time	Estimate	Standard Error	DF	t Value	Pr > |t|
time	0	167.10	8.0098	4	20.86	<.0001
time	3	176.17	7.5721	4	23.27	<.0001
time	6	187.89	7.7041	4	24.39	<.0001

The Mixed Procedure

Model Information

Data Set	WORK.IN
Dependent Variable	folate
Covariance Structure	Diagonal
Estimation Method	REML
Residual Variance Method	Profile
Fixed Effects SE Method	Model-Based
Degrees of Freedom Method	Residual

Class Level Information

Class	Levels	Values
time	3	0 3 6
bag	2	1 2

Dimensions

Covariance Parameters	1
Columns in X	6
Columns in Z	0
Subjects	1
Max Obs Per Subject	18

Number of Observations

Number of Observations Read	18
Number of Observations Used	16
Number of Observations Not Used	2

Covariance Parameter Estimates

Cov Parm	Estimate
Residual	0.1570

Fit Statistics

-2 Res Log Likelihood	18.2
AIC (smaller is better)	20.2
AICC (smaller is better)	20.6
BIC (smaller is better)	20.7

The SAS System
Tortilla flour control
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The Mixed Procedure

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
time	2	12	9.21	0.0038
bag	1	12	0.00	0.9501

Least Squares Means

Effect	time	Estimate	Standard Error	DF	t Value	Pr > |t|
time	0	12.9187	0.1783	12	72.45	<.0001
time	3	12.7213	0.1783	12	71.34	<.0001
time	6	13.6833	0.1617	12	84.60	<.0001

--------------------------------- lot=1 ---------------------------------

The Mixed Procedure

Model Information

Data Set	WORK.IN
Dependent Variable	folate
Covariance Structure	Diagonal
Estimation Method	REML
Residual Variance Method	Profile
Fixed Effects SE Method	Model-Based
Degrees of Freedom Method	Residual

Class Level Information

Class	Levels	Values
time	3	0 3 6
bag	2	1 2

Dimensions

Covariance Parameters	1
Columns in X	6
Columns in Z	0
Subjects	1
Max Obs Per Subject	23

Number of Observations

Number of Observations Read	23
Number of Observations Used	23
Number of Observations Not Used	0

Covariance Parameter Estimates

Cov Parm	Estimate
Residual	229.21

Fit Statistics

-2 Res Log Likelihood	165.0

AIC (smaller is better)	167.0
AICC (smaller is better)	167.2
BIC (smaller is better)	167.9

The SAS System
Analysis for Chip Flour
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The Mixed Procedure

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
time	2	19	0.09	0.9141
bag	1	19	0.04	0.8346

Least Squares Means

Effect	time	Estimate	Standard Error	DF	t Value	Pr > |t|
time	0	158.59	5.0588	19	31.35	<.0001
time	3	156.50	5.3527	19	29.24	<.0001
time	6	155.33	6.1808	19	25.13	<.0001

Differences of Least Squares Means

Effect	time	time	Estimate	Standard Error	DF	t Value	Pr > |t|
time	0	3	2.0922	7.3650	19	0.28	0.7794
time	0	6	3.2589	7.9871	19	0.41	0.6878
time	3	6	1.1667	8.1764	19	0.14	0.8880

Differences of Least Squares Means

Effect	time	time	Adjustment	Adj P
time	0	3	Tukey-Kramer	0.9566
time	0	6	Tukey-Kramer	0.9127
time	3	6	Tukey-Kramer	0.9888

---------------------------------- lot=2 -----------------------------------

The Mixed Procedure

Model Information

Data Set	WORK.IN
Dependent Variable	folate
Covariance Structure	Diagonal
Estimation Method	REML
Residual Variance Method	Profile
Fixed Effects SE Method	Model-Based
Degrees of Freedom Method	Residual

Class Level Information

Class	Levels	Values
time	2	0 3
bag	2	1 2

Dimensions

Covariance Parameters	1
Columns in X	5
Columns in Z	0
Subjects	1
Max Obs Per Subject	12

Number of Observations

Number of Observations Read	12
Number of Observations Used	12
Number of Observations Not Used	0

Covariance Parameter Estimates

Cov Parm	Estimate
Residual	0.3505

Fit Statistics

-2 Res Log Likelihood	20.8
AIC (smaller is better)	22.8

--------------------------------- lot=2 ----------------------------------

The Mixed Procedure

Fit Statistics

AICC (smaller is better)	23.4
BIC (smaller is better)	23.0

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
time	1	9	4.00	0.0766
bag	1	9	0.19	0.6711

Least Squares Means

Effect	time	Estimate	Standard Error	DF	t Value	Pr > |t|
time	0	14.7167	0.2417	9	60.89	<.0001
time	3	14.0333	0.2417	9	58.07	<.0001

------------------------------- Lot=1 -----------------------------------

The Mixed Procedure

Model Information

Data Set	WORK.IN
Dependent Variable	Folate
Covariance Structure	Variance Components
Estimation Method	REML
Residual Variance Method	Profile
Fixed Effects SE Method	Model-Based
Degrees of Freedom Method	Containment

Class Level Information

Class	Levels	Values
Prod_Batch	2	1 2
Time	2	0 2
Bag	2	1 2

Dimensions

Covariance Parameters	2
Columns in X	5
Columns in Z	2
Subjects	1
Max Obs per Subject	23

Number of Observations

Number of Observations Read	24
Number of Observations Used	23
Number of Observations Not Used	1

Iteration History

Criterion	Iteration	Evaluations	-2 Res Log Like
	0	1	133.48290252
0.00000000	1	1	133.48290252

Convergence criteria met.

---------------------------------- Lot=1 ----------------------------------

The Mixed Procedure

Covariance Parameter
Estimates

Cov Parm	Estimate
Prod_Batch	0
Residual	33.2770

Fit Statistics

-2 Res Log Likelihood	133.5
AIC (Smaller is Better)	135.5
AICC (Smaller is Better)	135.7
BIC (Smaller is Better)	134.2

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Time	1	19	67.92	<.0001
Bag	1	19	0.32	0.5778

Least Squares Means

Effect	Time	Estimate	Standard Error	DF	t Value	Pr > |t|
Time	0	157.70	1.7428	19	90.49	<.0001
Time	2	137.83	1.6653	19	82.77	<.0001

------------------------------- Lot=2 ----------------------------------

The Mixed Procedure

Model Information

Data Set	WORK.IN
Dependent Variable	Folate
Covariance Structure	Variance Components
Estimation Method	REML
Residual Variance Method	Profile
Fixed Effects SE Method	Model-Based
Degrees of Freedom Method	Containment

Class Level Information

Class	Levels	Values
Prod_Batch	2	1 2
Time	2	0 2
Bag	2	1 2

Dimensions

Covariance Parameters	2
Columns in X	5
Columns in Z	2
Subjects	1
Max Obs per Subject	23

Number of Observations

Number of Observations Read	24
Number of Observations Used	23
Number of Observations Not Used	1

Iteration History

Iteration	Evaluations	-2 Res Log Like	Criterion
0	1	121.95652003	
1	1	121.95652003	0.00000000

Convergence criteria met.

---------------------------------- Lot=2 -----------------------------------

The Mixed Procedure

Covariance Parameter Estimates

Cov Parm	Estimate
Prod_Batch	0
Residual	18.7004

Fit Statistics

-2 Res Log Likelihood	122.0
AIC (Smaller is Better)	124.0
AICC (Smaller is Better)	124.2
BIC (Smaller is Better)	122.6

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Time	1	19	24.76	<.0001
Bag	1	19	6.32	0.0211

Least Squares Means

Effect	Time	Estimate	Standard Error	DF	t Value	Pr > |t|
Time	0	20.5663	1.3064	19	15.74	<.0001
Time	2	11.5750	1.2483			

---------------------------------- Lot=1 ----------------------------------

The Mixed Procedure

Model Information

Data Set	WORK.IN
Dependent Variable	Folate
Covariance Structure	Variance Components
Estimation Method	REML
Residual Variance Method	Profile
Fixed Effects SE Method	Model-Based
Degrees of Freedom Method	Containment

Class Level Information

Class	Levels	Values
Prod_Batch	2	1 2
Time	2	0 2
Bag	2	1 2

Dimensions

Covariance Parameters	2
Columns in X	5
Columns in Z	2
Subjects	1
Max Obs Per Subject	24

Number of Observations

Number of Observations Read	24
Number of Observations Used	24
Number of Observations Not Used	0

Iteration History

Iteration	Evaluations	-2 Res Log Like	Criterion
0	1	178.35857358	
1	1	178.30487691	0.00000000

Convergence criteria met.

The SAS System
Analysis for Chip Product
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--------------------------------- Lot=1 ----------------------------------

The Mixed Procedure

Covariance Parameter Estimates

Cov Parm	Estimate
Prod_Batch	6.3896
Residual	203.49

Fit Statistics

-2 Res Log Likelihood	178.3
AIC (smaller is better)	182.3
AICC (smaller is better)	183.0
BIC (smaller is better)	179.7

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Time	1	20	9.91	0.0051
Bag	1	20	3.35	0.0819

Least Squares Means

Effect	Time	Estimate	Standard Error	DF	t Value	Pr > |t|
Time	0	194.00	4.4891	20	43.22	<.0001
Time	2	175.67	4.4891	20	39.13	<.0001

---------------------------------- Lot=2 ----------------------------------

The Mixed Procedure

Model Information

Data Set	WORK.IN
Dependent Variable	Folate
Covariance Structure	Variance Components
Estimation Method	REML
Residual Variance Method	Profile
Fixed Effects SE Method	Model-Based
Degrees of Freedom Method	Containment

Class Level Information

Class	Levels	Values
Prod_Batch	2	1 2
Time	2	0 2
Bag	2	1 2

Dimensions

Covariance Parameters	2
Columns in X	5
Columns in Z	2
Subjects	1
Max Obs Per Subject	24

Number of Observations

Number of Observations Read	24
Number of Observations Used	24
Number of Observations Not Used	0

Iteration History

Iteration	Evaluations	-2 Res Log Like	Criterion
0	1	113.66734877	
1	1	113.66734877	0.00000000

Convergence criteria met.

--------------------------------- Lot=2 ----------------------------------

The Mixed Procedure

Covariance Parameter Estimates

Cov Parm	Estimate
Prod_Batch	0
Residual	9.5150

Fit Statistics

-2 Res Log Likelihood	113.7
AIC (smaller is better)	115.7
AICC (smaller is better)	115.9
BIC (smaller is better)	114.4

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Time	1	20	20.68	0.0002
Bag	1	20	1.74	0.2023

Least Squares Means

Effect	Time	Estimate	Standard Error	DF	t Value	Pr > |t|
Time	0	17.6083	0.8905	20	19.77	<.0001
Time	2	11.8817	0.8905	20	13.34	<.0001

The SAS System
Analysis for Tortilla Flour
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The Mixed Procedure

Model Information

Data Set	WORK.IN
Dependent Variable	Folate
Covariance Structure	Variance Components
Estimation Method	REML
Residual Variance Method	Profile
Fixed Effects SE Method	Model-Based
Degrees of Freedom Method	Containment

Class Level Information

Class	Levels	Values
Lot	2	1 2
Batch	2	1 2
Bag	2	1 2
source	2	Flour Tortillas

Dimensions

Covariance Parameters	2
Columns in X	27
Columns in Z	2
Subjects	1
Max Obs Per Subject	49

Number of Observations

Number of Observations Read	49
Number of Observations Used	44
Number of Observations Not Used	5

Iteration History

Iteration	Evaluations	-2 Res Log Like	Criterion
0	1	312.79278030	
1	1	380.72120400	0.00000000

Convergence criteria met but final hessian is not positive definite.

The SAS System
Analysis for Tortilla Flour
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The Mixed Procedure

Covariance Parameter Estimates

Cov Parm	Estimate
Lot	1.341E17
Residual	238.15

Fit Statistics

-2 Res Log Likelihood	380.7
AIC (smaller is better)	384.7
AICC (smaller is better)	385.1
BIC (smaller is better)	382.1

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Lot	1	0	1065.00	.
Batch	1	36	0.00	0.9541
Lot*Batch	1	36	0.05	0.8255
source	1	36	2.67	0.1111
Lot*source	1	36	11.44	0.0017
Batch*source	1	36	0.00	0.9541
Lot*Batch*source	1	36	0.05	0.8255

Least Squares Means

Effect	source	Lot	Estimate	Standard Error	DF	t Value	Pr > |t|
Lot*source	Flour	1	181.00	4.4549	36	40.63	<.0001
Lot*source	Tortillas	1	157.57	4.6723	36	33.72	<.0001
Lot*source	Flour	2	12.7200	4.8801	36	2.61	0.0132
Lot*source	Tortillas	2	20.8917	4.6723	36	4.47	<.0001

Differences of Least Squares Means

Effect	source	Lot	source	Lot	Estimate	Standard Error	DF
Lot*source	Flour	1	Tortillas	1	23.4333	6.4557	36
Lot*source	Flour	1	Flour	2	168.28	6.6076	36

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The Mixed Procedure

Differences of Least Squares Means

Effect	source	Lot	source	Lot	Estimate	Standard Error	DF
Lot*source	Flour	1	Tortillas	2	160.11	6.4557	36
Lot*source	Tortillas	1	Flour	2	144.85	6.7561	36
Lot*source	Tortillas	1	Tortillas	2	136.68	6.6076	36
Lot*source	Flour	2	Tortillas	2	-8.1717	6.7561	36

Differences of Least Squares Means

Effect	source	Lot	source	Lot	t Value	Pr > |t|
Lot*source	Flour	1	Tortillas	1	3.63	0.0009
Lot*source	Flour	1	Flour	2	25.47	<.0001
Lot*source	Flour	1	Tortillas	2	24.80	<.0001
Lot*source	Tortillas	1	Flour	2	21.44	<.0001
Lot*source	Tortillas	1	Tortillas	2	20.68	<.0001
Lot*source	Flour	2	Tortillas	2	-1.21	0.2343

The SAS System
Analysis for Chips Flour
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The Mixed Procedure

Model Information

Data Set	WORK.IN
Dependent Variable	Folate
Covariance Structure	Variance Components
Estimation Method	REML
Residual Variance Method	Profile
Fixed Effects SE Method	Model-Based
Degrees of Freedom Method	Containment

Class Level Information

Class	Levels	Values
Lot	2	1 2
Batch	2	1 2
source	2	Chips Flour

Dimensions

Covariance Parameters	2
Columns in X	27
Columns in Z	2
Subjects	1
Max Obs Per Subject	48

Number of Observations

Number of Observations Read	48
Number of Observations Used	44
Number of Observations Not Used	4

Iteration History

Iteration	Evaluations	-2 Res Log Like	Criterion
0	1	293.25601940	
1	1	293.25601940	0.00000000

Convergence criteria met but final hessian is not positive definite.

The SAS System
Analysis for Chips Flour
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The Mixed Procedure

Covariance Parameter Estimates

Cov Parm	Estimate
Lot	0
Residual	138.72

Fit Statistics

-2 Res Log Likelihood	293.3
AIC (smaller is better)	295.3
AICC (smaller is better)	295.4
BIC (smaller is better)	293.9

Type 3 Tests of Fixed Effects

Effect	Num DF	Den DF	F Value	Pr > F
Lot	1	0	1940.13	.
Batch	1	36	1.67	0.2043
Lot*Batch	1	36	1.34	0.2551
source	1	36	32.17	<.0001
Lot*source	1	36	23.67	<.0001
Batch*source	1	36	1.67	0.2043
Lot*Batch*source	1	36	1.34	0.2551

Least Squares Means

Effect	source	Lot	Estimate	Standard Error	DF	t Value	Pr > |t|
Lot*source	Chips	1	194.00	3.4000	36	57.06	<.0001
Lot*source	Flour	1	156.00	4.1642	36	37.46	<.0001
Lot*source	Chips	2	17.6083	3.4000	36	5.18	<.0001
Lot*source	Flour	2	14.7000	3.4000	36	4.32	0.0001

Differences of Least Squares Means

Effect	source	Lot	source	Lot	Estimate	Standard Error	DF	t Value
Lot*source	Chips	1	Flour	1	38.0000	5.3759	36	7.07
Lot*source	Chips	1	Chips	2	176.39	4.8084	36	36.68

The SAS System
Analysis for Chips Flour
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The Mixed Procedure

Differences of Least Squares Means

Effect	source	Lot	source	Lot	Estimate	Standard Error	DF	t Value
Lot*source	Chips	1	Flour	2	179.30	4.8084	36	37.29
Lot*source	Flour	1	Chips	2	138.39	5.3759	36	25.74
Lot*source	Flour	1	Flour	2	141.30	5.3759	36	26.28
Lot*source	Chips	2	Flour	2	2.9083	4.8084	36	0.60

Differences of Least Squares Means

Effect	source	Lot	source	Lot	Pr > |t|
Lot*source	Chips	1	Flour	1	<.0001
Lot*source	Chips	1	Chips	2	<.0001
Lot*source	Chips	1	Flour	2	<.0001
Lot*source	Flour	1	Chips	2	<.0001
Lot*source	Flour	1	Flour	2	<.0001
Lot*source	Chips	2	Flour	2	0.5491