OPTIMIZATION OF ENZYME EXTRACTIONS FOR TOTAL FOLATE IN CEREALS AND DETERMINATION OF TOTAL FOLATE IN BREAKFAST CEREALS AND SNACK FOODS

by

SUNGEUN CHO

(Under the Direction of Ronald R. Eitenmiller)

ABSTRACT

The trienzyme digestion including protease, α-amylase, and conjugase for the extraction of total folate from cereals was optimized using response surface methodology. CRM121 wholemeal, oat, triticale, and buckwheat flours were used to suggest an optimized digestion. Significant effects were determined for the incubation time of α-amylase ($p < 0.05$) and conjugase ($p < 0.01$), while Pronase® digestion was not significant ($p > 0.01$) for extraction of total folate from the cereals. An optimum trienzyme digestion time of Pronase®, α-amylase, and conjugase are 1 h, 2.5 h, and 6 h, respectively. A differential assay of folic acid and total folate is used to determine μg DFE in breakfast cereals (676 to 2184 μg DFE/100g) and snack samples (15 to 394 μg DFE/100g). To provide better information to customers, μg DFE values should be declared on labels. Also, all assayed values of breakfast cereals exceeded label declarations. Thus, exact labels on breakfast cereals will be needed.

INDEX WORDS: Folic acid; Total folate; Trienzyme extraction; Cereals; RSM; Optimization; Breakfast cereals; Snacks

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DEDICATION

To my beloved father, Hyungu Cho, mother, Hyangja Shin who offered me unconditional love and support, and to my beloved brother's family, Hangwoo Cho, Yunjeong Choi, and Youngki Cho.

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CHAPTER 1

INTRODUCTION

'Folate', a family of water-soluble vitamers, plays a crucial role in nucleic acid synthesis and protein metabolism (Davidson and others 1979). While 'folate' is the term used to refer to all naturally occurring forms in foods, 'folic acid' refers to a synthetic form, monoglutamate, which is the most oxidized, stable, and easily absorbable form (Hoffpauer and Bonnette 1998). Legumes (peanuts, cowpeas, peas, etc.), leafy greens, some fruits, vegetables (broccoli, cauliflower), citrus (orange juice), liver, egg yolk, wheat germ, yeast, and fortified breakfast cereal products are considered as good folate sources (Eitenmiller and others 2008; Ohrvik and Witthoft 2008; Arcot and Shrestha 2005).

Wills recognized folate deficiency in 1931 through the study of Indian women who prevalently had a pernicious anemia. The biological roles of folate account for its deficiency. Folate deficiency increases the risk for congenital neural tube defects including spina bifida due to the restriction of cell division (Kamen 1997; Czeizel and Dudas 1992; MRC Vitamin Research Group 1991; Selhub and others 1995). Also, its deficiency produces megaloblastic anemia and is associated with dementia and Alzheimer's disease (Zittoun 1993; Ball 2004; Seshadri and others 2002). Additionally, folate deficiency may increase the risk of cancer, arteriosclerosis, or stroke (Green and

Jacobsen 1995; Boushey and others 1995; Wald and others 2002; Casas and others 2005; Choi and others 2002; Blount and others 1997).

To assist women of child bearing age in meeting an intake of 400μg folate/day to reduce their risk of having a pregnancy affected with a neural tube birth defect, many types of cereal-grain products are required to be fortified with folic acid, since January 1, 1998 (Food and Drug Administration (FDA) 1996; Department of Health and Human Services, Public Health Service 1992). The fortification level is 140μg/100g of cereal-grain products (Rader and others 2000; Department of Health and Human Services, Public Health Service 1992). The Centers for Disease Control and Prevention (CDC) in Atlanta, Georgia indicates that the rate of neural tube defects dropped by 25 percent in the United States after the fortification program started (CDC 2004). In addition to the decreased rate of neural tube defects, a 12 percent reduction in cleft palate occured (Canfield and others 2005) and prevalence of orofacial clefts declined from 85.2 to 80.2 per 100,000 births (Yazdy and others 2007).

However, fortification may harm some people because of the excessive high concentration of total folates and unmetabolized folic acid (Smith and others 2008). Overdose of folic acid may decrease natural killer cell cytotoxicity and the response to antifolate drugs (Troen and others 2006; Dervieux and others 2005). Also, it may increase risk of cognitive impairment and anemia with poor vitamin B_{12} status in elderly and of insulin resistance and obesity in their children in pregnant women (Reynolds 2006; Yajnik and others 2008). Additionally, it may facilitate progression and growth of preneoplastic cells and subclinical cancers (Kim 2007; Kim 2003; Kim 2006a; Kim 2006b; Ulrich and Potter 2006). Thus, reliable databank values for the folate content of

foods are important to estimate and evaluate the adequacy of folate intakes of populations, formulate experimental diets in folate nutrition studies, and to develop dietary recommendations (Gregory 1998).

However, most breakfast cereals contain folate content higher than the amount required by federal regulations, 25% to 100% of the daily value (DV) of 400μg (Rader and others 2000; Whittaker and others 2001; Yang and others 2007; USDA 2003); thus, it is important to have not only reliable databank values but also exact label claims for folate safety.

Among several methods for the determination of folate such as bio-specific methods or chromatography, a microbiological assay relying on bacterial growth has been the best and most widely used method for the past half century (Tamura 1998; Hawkes and Villota 1989). Folate extraction includes enzymatic digestions to liberate folates from the cellular matrix and deconjugate polyglutamates to simpler measurable forms (Arcot and others 2002; Shrestha and others 2000). DeSouza and Eitenmiller (1990) developed a trienzyme digestion procedure including protease (EC 3.4.24.31) and α-amylase (EC 3.2.1.1) digestion in addition to the traditional treatment with folate conjugase to increase measurable folate from complex food matrices (DeSouza and Eitenmiller 1990). Since the trienzyme method was developed, many papers reported a significant increase in total folate concentrations in foods extracted with the trienzyme treatment compared with the values from folate conjugase treatment alone (Martin and others 1990; Tamura 1997; Pfeiffer 1997; Rader and others 1998).

AOAC Official Method 2004.05, Total Folates in Cereals and Cereal Foods, Microbiological Assay, Trienzyme Method, accepted First Action 2004 is applicable to

the assay of total folate including folic acid in cereals and enriched cereal grain products (De Vries and others 2005). The trienzyme digestion is universally applicable to the assay of total folate in all kinds of food (Eitenmiller and others 2008).

To save time and cost for microbiological assay, Chen and Eitenmiller (2007) used response surface methodology (RSM) to optimize the trienzyme digestion for folate extraction from vegetables. While the digestion times of AOAC Official Method 2004.05. are 3 h, 2 h, and 16 h for protease, α-amylase, and conjugase, respectively, they optimized them to 1.5 h, 1.5 h, and 3 h, respectively. Additionally, the shorter optimized digestion time yields higher folate values than the trienzyme digestion parameters employed in AOAC Official Method 2004.05 (Chen and Eitenmiller 2007). Unfortunately, the optimized digestion time may not be applicable to other types of food, because folate extraction is largely dependent on food matrices.

The objectives of this study were 1) to determine folate content in breakfast cereals and snack food which are highly fortified and whose labels are usually underestimated to provide reliable data for folate safety, and 2) to optimize the trienzyme digestion time for folate extraction from cereals for more accurate values, time savings and reduced cost.

CHAPTER 2

LITERATURE REVIEW

Folate and Folic Acid

'Folate' refers to a family of water-soluble vitamers that plays an important role in nucleic acid synthesis and protein metabolism (Davidson and others 1979). While 'folate' is the term used to refer to all forms of its derivatives which occur naturally as polyglutamates in foods, 'folic acid' refers to a synthetic form, monoglutamate, which is the most oxidized, stable, and easily absorbable form (Hoffpauer and Bonnette 1998). 'Folic acid' and 'folate' are commonly used interchangeably, but the widespread use of folic acid in fortification of foods and the preparation of vitamin supplements is due to its stability and availability (Gregory 1989). Legumes (peanuts, cowpeas, peas, etc.), leafy greens, some fruits, vegetables (broccoli, cauliflower), citrus (orange juice), liver, egg yolk, wheat germ, yeast, and fortified breakfast cereal products are considered as good folate sources (Eitenmiller and others 2008; Ohrvik and Witthoft 2008; Arcot and Shrestha 2005).

Physiochemical properties of folates

The heterocyclic compounds based on the pteroic acid structure conjugated with one or more L-glutamates linked through the γ-carboxyl of the amino acid are considered as folate. Folic acid (pteroylglutamic acid) contains one glutamic acid residue (Eitenmiller

and others 2008). The molecular weight of folic acid is 441.4Da. It is more stable in alkaline than acidic environments (Keagy 1985). Tetrahydrofolate (H_4 folate), which is the active coenzyme form of the vitamin, is one of the reduced forms of folic acid. Figure 2.1 (Eitenmiller and others 2008) shows the structure of folic acid and structural relationships of folates.

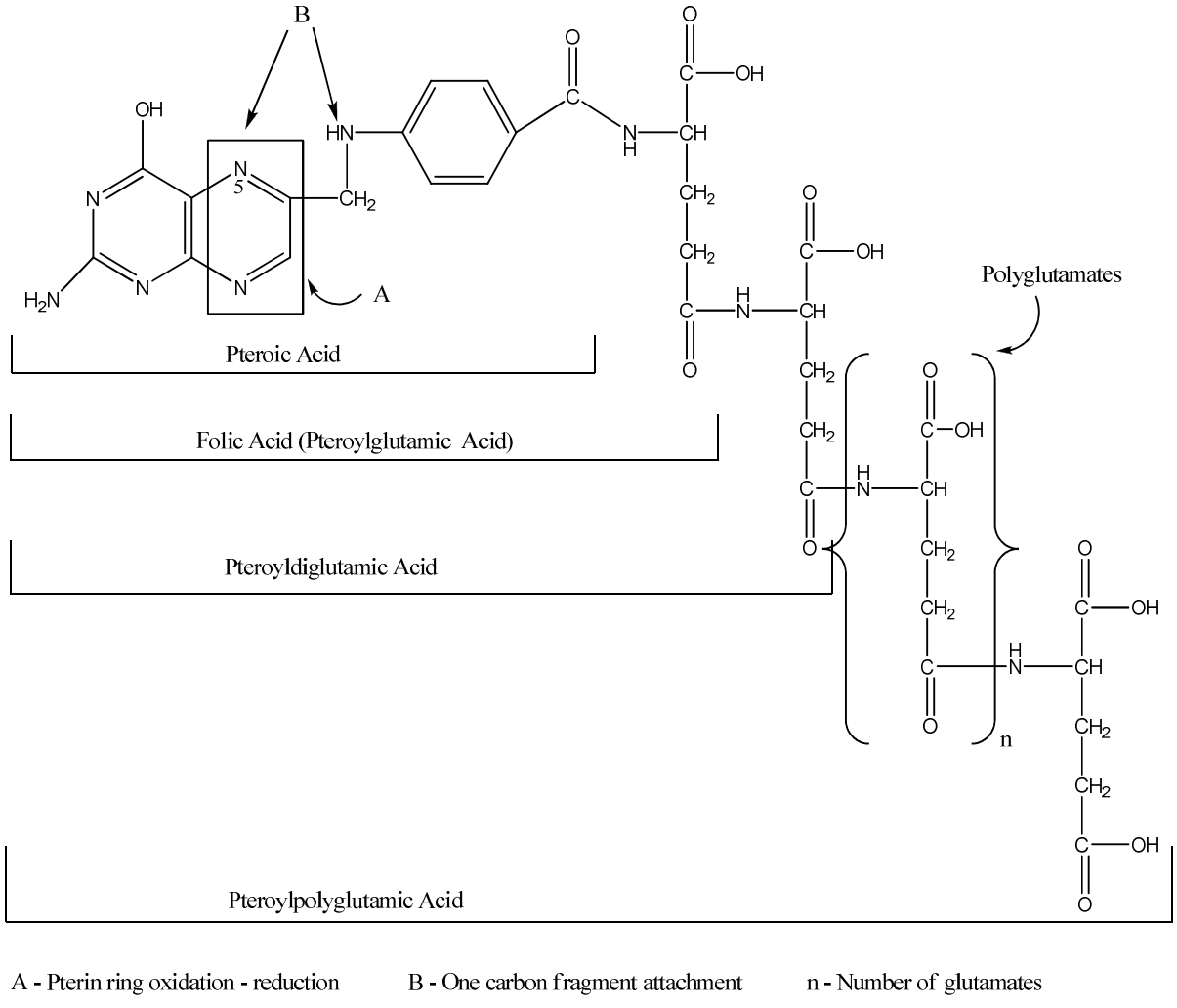

Figure 2.1 Structural relationships of the folates

That folic acid is susceptible to degradation under acidic conditions, light, and high temperature has long been established (Dick and others 1948; Stokstad and others 1947). Its stability varies by the pH of the medium, reducing agents in the buffer, folate derivatives, type of buffer, and the food system. Folates can have much greater stability in the presence of ascorbic acid or other reducing agents (Arcot and Shrestha 2005). Lactose, protein and iron are also able to reduce the partial pressure of oxygen within the model system; therefore, they can contribute to the stability of the vitamin (Day and Gregory 1983).

Dietary Reference Intakes

Dietary Reference Intakes (DRIs) for folate include 4 reference values for the intake of nutrients by Americans and Canadians: (1) Recommended Dietary Allowance (RDA), (2) Estimated Average Requirement (EAR), (3) Adequate Intake (AI), and (4) Tolerable Upper Intake Level (UL). The EAR is the amount of nutrient that is sufficient to satisfy the requirement of 50% of the population. The RDA is defined as the average daily dietary intake level adequate to meet the nutrient requirement of nearly 98% of the population. An AI is defined as the quantity of intake consumed by groups with no evidence of nutrient inadequacy. The AI is determined when data are insufficient to estimate an EAR. The UL is the highest level of daily nutrient intake not likely to be harmful to the majority of the population (Bailey 1998). Table 2.1 shows DRIs for folate in μg dietary folate equivalents (DFEs). DFEs will be discussed in the following section.

Table 2.1 Dietary Reference Intakes for Folate

Life Stage	DRI (μg DFE/day)	Life Stage	DRI (μg DFE/day)
Infants (months)		Males & Females (years)	
0-6	65	9-13	**300**
7-12	80	14+	**400**
Children (years)			
1-3	**150**	Pregnancy	**600**
4-8	**200**	Lactation	**500**

Bold type: Recommended Dietary Allowance: ordinary type: adequate intake (AI)
DFE – dietary folate equivalent
Source: Institute of Medicine (1998)

Dietary Folate Equivalents

Dietary Folate Equivalents (DFEs) account for differences in bioavailability of synthetic folic acid used in supplements and fortification compared to naturally occurring food folate. Synthetic folic acid without food has nearly 100% bioavailability and its estimated bioavailability with food is approximately 85%. Food folate represents 50% bioavailability. Therefore, folic acid is 1.7 times (85/50) more bioavailable than food folate when a mixture of folic acid and food folate is injested (Gregory 1997; Pfeiffer and others 1997; Cuskelly and others 1996; Wei and others 1996; Sauberlich and others 1987). The following formula is to calculate μg DFEs in this situation (DHHS/PHS/FDA 1996):

$$\mu g\ DFEs = \mu g\ food\ folate + 1.7 X \mu g\ folic\ acid \quad (eq\ 2.1).$$

Also, DFEs may be expressed by different relationships (Suitor and Bailey 2000):

1 μg DFE = 1.0 μg food folate = 0.6 μg folic acid added to foods = 0.5 μg folic acid taken without food.

1μg folic acid as a fortificant = 1.7 μg DFE.

1μg folic acid as a supplement, fasting = 2.0 μg DFE.

The use of the μg DFE is recommended for planning and evaluating the adequacy of folate intake by the human (Suitor and Bailey 2000).

Folate Deficiency

Wills recognized folate deficiency in 1931 through the study of Indian women who prevalently had a pernicious anemia. “Vitamin M” was used for the antianemia factor (Day and others 1938). Mitchell and co-workers isolated folic acid from spinach in 1941 and the term “folic” was derived from the Latin “Folium” for leaf. Factors of folate deficiency are insufficient intake, defective absorption, abnormal metabolism, or conditions such as drug therapy, leading to increased requirements. General symptoms of marginal deficiency include tiredness, irritability, and decreased appetite (Machlin and others 1994).

The biological roles of folate account for its deficiency. One of them is that folate is needed to produce and maintain new cells, especially during periods of rapid cell division and growth such as infancy and pregnancy. For example, the biologically active form, tetrahydrofolate is an essential coenzyme in the biosynthesis of nucleotides. Its deficiency causes the reduction of the concentration of nucleic acids for DNA synthesis. Thus, when folate intake is inadequate, congenital neural tube defects including spina bifida can occur due to the restricting cell division (Kamen 1997; Czeizel and Dudas 1992; MRC Vitamin Research Group 1991; Selhub and others 1995). Also, folate deficiency produces megaloblastic anemia due to the involvement in DNA synthesis and cell division affecting most clinically the bone marrow which is a site of rapid cell

turnover. Since RNA and protein synthesis are not hindered, abnormally large blood cells called megaloblasts produced in bone marrow or in the blood result in megaloblastic anemia. Megaloblastic anemia is also associated with vitamin B_{12} deficiency because it plays a key role in DNA synthesis like folate (Zittoun 1993; Ball 2004). In 1945, folic acid was first recognized as a cure for megaloblastic anemia (Machlin and others 1994). Additionally, folate deficiency has been linked to increased concentrations of serum homocysteine. Elevated plasma homocysteine in the circulation is implicated as an independent risk factor for coronary artery disease, such as arteriosclerosis, and stroke (Green and Jacobsen 1995; Boushey and others 1995; Wald and others 2002; Casas and others 2005). Low folate status is also associated with dementia and Alzheimer's disease (Seshadri and others 2002). Altered methylation of DNA that may have effects on gene expression and uracil-induced genomic instability is associated with low folate status, too. Both of these outcomes may increase cancer risk (Choi and others 2002; Blount and others 1997)

Fortification with Folic Acid

In the United States, many types of cereal-grain products are required to be fortified with folic acid, since January 1, 1998 (Food and Drug Administration (FDA) 1996). The ruling was established to assist women of child-bearing age in raising their intake of folate to reduce their risk of having a pregnancy affected with a neural tube birth defect (Department of Health and Human Services, Public Health Service 1992). The fortification levels (Table 2.2) are intended to help women of child-bearing age meet an intake of 400μg folate/day. The U.S. Public Health Service recommendation is

140μg/100g of cereal-grain products (Rader and others 2000; Department of Health and Human Services, Public Health Service 1992).

Table 2.2 Fortification Levels of Cereal-Grain Products with Folic Acid

Enriched cereal-grain products	Folic acid μg/100g	Products fortified
Part 136 bakery products		
Enriched breads, rolls, buns	95	All
Part 137 cereal flours and related products		
Enriched flour	154	All
Enriched corn meals	154-220	All
Enriched farina	154-220	All
Enriched rice	154-308	All
Part 139 macaroni and noodle products		
Enriched macaroni products	198-264	All
Enriched noodle products	198-264	All

Source: Rader, JI, and others (1998)

Using data from 23 birth defect registries that cover about half of United States births with extrapolation of the findings to the rest of the country, the Centers for Disease Control and Prevention (CDC) in Atlanta, Georgia indicates that the rate of neural tube defects dropped by 25 percent in the United States after the fortification program started (CDC 2004). In addition to the decreased rate of neural tube defects, a 12 percent reduction in cleft palate occured (Canfield and others 2005) and prevalence

of orofacial clefts declined from 85.2 to 80.2 per 100,000 births (Yazdy and others 2007).

Since the U.S. started the fortification program, many countries such as Canada, Mexico, Chile, and Hungary have started fortification programs (Cornel and others 2005). Beginning in 1998, Canada started the implementation of folic acid food fortification and the fortification program has been a success. De Wals and others found that the rate of neural tube defects plummeted by 46 percent; the prevalence of neural tube defects was 1.58 per 1,000 births before fortification and the number dropped to 0.86 per 1,000 births after fortification (De Wals and others 2007). In Chile, the Chilean Ministry of Health legislated for folic acid fortification in 2000. There were 51% and 46% decreases in the birth prevalence rates of spina bifida and anencephaly, respectively in the years 2001 and 2002 (Lopez-Camelo and others 2005). In 2004, the National Health Surveillance Agency (ANVISA) mandated the fortification of corn meal and wheat flour with folic acid at the level of 0.15mg/100g (ANVISA 2004)

Also, the UK Standing Advisory Committee on Nutrition (SACN) has recommended the implementation of mandatory fortification of flour with folic acid (SACN 2006) and the UK Food Standards Agency (FSA) has recommended folic acid be added to flour or bread (FSA 2007).

Folate Health Benefits

Heart Health

Voluntary food fortification is associated with a substantial increase in dietary intake. A potential health benefit of increased folic acid intake is decreased risk of

cardiovascular disease (CVD) via homocysteine lowering (Hoey and others 2007). Homocysteine is a sulphur-containing amino acid produced during the metabolism of methionine. In healthy individuals, plasma total homocysteine (tHcy) is between 5 and 10μmol/L (Jamaluddin and others 2007). Hoey and others showed that the group with the highest fortified food intake (median intake: 208 μg/d folic acid) has 2 μmol /L lower tHcy concentrations in serum than in the nonconsumers of fortified foods (0 μg/d folic acid) (Hoey and others 2007). tHcy is very responsive to intervention with folate, B_6, B_{12} and riboflavin required for its metabolism (Homocysteine Lowering Trialists' Collaboration 1998; Eussen and others 2005; McKinely and others 2001; McNulty and others 2006). The B vitamins are considered as important regulators of homocysteine metabolism in the body (Larsson and others 2008). However, a number of secondary prevention trials published recently did not show a benefit of tHcy lowering therapy on CVD events generally (The Vitamin Intervention for Stroke Prevention Randomized Controlled Trial 2004; The Heart Outcomes Prevention Evaluation 2 Investigators 2006; The Norwegian Vitamin Trial 2006). Also, observational studies on folate and other B vitamins related to stroke risk have showed inconsistent findings. Randomized clinical trials examining the effects of supplemental folic acid and other B vitamins on stroke incidence among individuals with preexisting cardiovascular or renal disease have produced conflicting results (Giles and others 1995; Bazzano and others 2002; Al-Delaimy and others 2004; He and others 2004; Van Guelpen and others 2005; Virtanen and others 2005; Zeitlin and others 1997; Bazzano and others 2006; Wang and others 2007).

Substantial evidence exists linking elevated blood total homocysteine concentrations to increased risk of ischemic stroke, although many studies have failed to show the potential benefit of folate in decreasing the risk of CVD (Folsom and others 1998; Homocysteine Studies Collaboration, 2002; Fallon and others 2003; Iso and others 2004; Wald and others 2002). Randomized controlled trials have established that supplementation with folate (natural dietary folate or the synthetic folic acid) alone or in combination with vitamins B_6 and B_{12} significantly reduces blood homocysteine concentrations (Brouwer and others 1999; Homocysteine Lowering Trialists' Collaboration 2005; Bønaa and others 2006; Lonn and others 2006). A recent meta-analysis of randomized controlled trials indicates that a high folate intake improves endothelial function, which could potentially reduce the risk of CVD (de Bree and others 2007). Larsson and others in 2008 found that high doses of folic acid were associated with a statistically significant lower risk of cerebral infraction. Findings from the study suggest that a high dietary folate intake may reduce the risk of cerebral infraction in men (Larsson and others 2008).

Cancer

Colorectal cancer

Folate functions coenzymatically in the synthesis of thymidylates and the methylation of DNA and protein by providing one-carbon units. Folate deficiency associated with DNA instability and hypomethylation results in human carcinogenesis (IOM 1998; Molloy and Scott 2001; Duthie and others 2000; Goelz and others 1985). The association between dietary folate and colorectal cancer has been investigated

(Glynn and others 1996; Kato and others 1999; Ma and others 1997; Ma and others 1999). In the review paper of epidemiologic studies of folate and colorectal neoplasia in 2002, Giovannucci showed that folate status is inversely associated with colorectal cancer (Giovannucci 2002). Also, Sanjoaquin and others in 2005 concluded that there is a modest inverse association between dietary folate and colorectal cancer in their meta-analysis of prospective cohort and case-control studies and dietary folate had more inverse association than total folate did (Sanjoaquin and others 2005). Of the prospective cohort studies published, one observed that dietary folate intake had more inverse association with the risk of colorectal cancer among smokers (Larsson and others 2005). One other prospective study observed the association between folate, vitamin B_6, multivitamin supplements, and colorectal cancer risk especially in women (Zhang and others 2006). While the study did not find dietary folate to be associated with colorectal cancer, the association between intake of folate and vitamin B_6 and colorectal cancer was observed among women not taking supplements. In general, prospective cohort studies support a role of folate in modulating colorectal carcinogenesis and overall the epidemiological evidence suggests that increased dietary folate intake may reduce the risk of colorectal cancer (Baron and others 1998; Giovannucci and others 1993; Martinez and others 2004; Bingham and others 2005). However, other confounding nutrients may be a factor for the inverse association (Bingham and others 2005). Polymorphisms in folate-metabolizing enzymes may also modify CRC risk in relation to folate intake (Sanderson and others 2007). Also, Sanderson and others mentioned that folic acid supplementation may help prevent the development of new cancer and it may promote the progression of existing

premalignant/preneoplastic and neoplastic lesions (Ulrich and Potter 2006; Sanderson and others 2007). They recommended further research should give consideration not only to supplemental form but also to the timing of exposure and dose of folic acid used which are associated with the possible dual effects (Sanderson and others 2007).

Breast cancer

Water-soluble vitamins such as folate, vitamin B_6, and B_{12} have been extensively evaluated to show the association with breast cancer risk. Lin and others in 2008 reviewed published papers about water-soluble vitamins and breast cancer. They organized the research into two groups (Lin and others 2008); case-control studies showing an inverse association between folate or other water-soluble and breast cancer (Shrubsole and others 2001; Levi and others 2001; Ronco and others 1999; Freudenheim and others 1996; Graham and others 1991;Negri and others 2000; Lajous and others 2006), and prospective cohort studies showing the inverse association among women consuming alcohol (Sellers and others 2001; Zhang and others 1999; Rohan and others 2000; Baglietto and others 2005; Tjonneland and others 2006) and among current smokers (Cho and others 2003). However, most studies found no consistent support for an overall association between intakes of folate or other vitamins and breast cancer risks (Cho and others 2003; Sellers and others 2001; Zhang and others 1999; Rohan and others 2000; Feigelson and others 2003; Baglietto and others 2005; Tjonneland and others 2006; Lewis and others 2006; Larsson and others 2007; Lin and others 2008). Lin and others recommended that additional research is needed to elucidate the role of folate in breast cancer development (Lin and others 2008).

Alzheimer's disease

Observational studies have found that high homocysteine (Hcy) levels are inversely associated with an increased risk of some types of dementia; e.g.) Alzheimer's disease (AD) (Seshadri and others 2002; Malouf and others 2003; McIlroy and others 2002; McMahon and others 2006; Ravaglia and others 2005; Wolters and others 2005) and serum folate is inversely associated with Hcy levels (Clark and others 2003; Hoey and others 2007). While Durga and others (2007) found small improvements in information processing in the folate group by comparison to a placebo, folate supplementation has not shown significant effects on overall cognition in cognitively normal younger or older adults despite lowering Hcy levels in some double-blind placebo-controlled trials (Durga and others 2007; Bryan and others 2002; McMahon and others 2006). Clarke and others (2003) and Sommer and others (2003) did not find significant effects in subjects with dementia in their double-blind studies. However, it is important to find out whether there is a relationship of levels of Hcy, folate and vitamin B_{12} with dementia or not, because they can represent modifiable risk factors for dementia. Understanding the mechanisms will help form treatment strategies for dementia (Köseoglu and Karaman 2007). Connelly and others in 2008 studied the relationship between folic acid supplementation and response to cholinesterase inhibitors (ChI) based upon criteria from the National Institute Clinical Excellence (NICE 2006). ChI is the first line treatment for AD. Takeda and others in 2006 reported the effectiveness of ChI treatment over a placebo in treating cognition, function and behavior, but NICE has questioned cost effectiveness. Connelly and others in the pilot double-blind study concluded that supplementation of ChI with folic acid may be a better

therapeutic in patients with AD; thus, folic acid supplementation could significantly improve the cost effectiveness of treating AD with ChI (Connelly and others 2008). Further studies to establish whether the risk of AD might be influenced by folic acid supplementation are needed.

Health Risk of Too Much Folic Acid

Folic acid is generally considered safe because it is usually excreted in urine when its intake is in excess of metabolic requirements. For guidance, a tolerable upper intake level (UL) for adults has been set at 1000μg/ day, while there is no UL for natural folate because there is no documented health risk related for folate intake from food. Table 2.3 shows ULs for folic acid for children and adults (Institute of Medicine 1998).

Table 2.3 Tolerable Upper Intake Levels for Folic Acid for Children and Adults

Age (Years)	Males and Females (μg/day)	Pregnancy (μg/day)	Lactation (μg/day)
1-3	300	N/A	N/A
4-8	400	N/A	N/A
9-13	600	N/A	N/A
14-18	800	800	800
19+	1000	1000	1000

Source: Institute of Medicine (1998)

Since mandatory fortification of food with folic acid started in the U.S., many countries have been considering whether to adopt this program because fortification will raise not only the concentration of total folates but also unmetabolized folic acid in the body (Quinlivan and Gregory 2003; Pfeiffer and others 2005). Smith and others discussed that the excessive high concentration of total folates and unmetabolized folic

acid may cause the following possible harm to some people (Smith and others 2008): (1) decrease in natural killer cell cytotoxicity and in response to antifolate drugs (Troen and others 2006; Dervieux and others 2005), (2) increased risk of cognitive impairment and anemia with poor vitamin B_{12} status in elderly and of insulin resistance and obesity in their children in pregnant women (Reynolds 2006; Yajnik and others 2008), (3) facilitation of progression and growth of preneoplastic cells and subclinical cancers (Kim 2007; Kim 2003; Kim 2006a; Kim 2006b; Ulrich and Potter 2006). Since there may be possible bad effects caused by a high intake of folic acid from fortified food or dietary supplements, nations considering fortification should be cautious (Smith and others 2008).

Importance of Exact Values of Products

Reliable databank values for the folate content of foods are important because the values can be used for estimating and evaluating the adequacy of folate intakes of populations and for formulation of experimental diets in folate nutrition studies. Also, they can be components in the development of dietary recommendations (Gregory 1998). However, some fortified foods, especially breakfast cereals contain folate contents higher than the amount required by federal regulations (Rader and others 2000; Whittaker and others 2001). Breakfast cereals have been the primary sources of folic acid intake and they are highly fortified to meet 25% to 100% of the daily value, 400μg (Yang and others 2007; USDA 2003); thus, exact label values are essential for folate safety.

Analytical Methods

Microbiological Assay with Trienzyme Extraction

For the determination of food folate for the past half century, a microbiological assay relying on turbidimetric bacterial growth has been the most widely used method (Tamura 1998; Hawkes and Villota 1989). *Lactobacillus casei* (*L. casei* (ssp. *rhamnosus*), ATCC 7469), *Enterococcus hirae* (ATCC 8043), and *Pediococcus cerevisae* (ATCC 8081) are three organisms which can be used for this assay. Among these three, *L. casei* spp. *rhamnosus* is the most common microorganism for the determination of food folate because of its similar responses to a wide variety of folate derivatives (Tamura 1998). Also, it does not respond to pteroic acid, a common folate degradation product, but only to natural folate forms (Voigt and Eitenmiller 1978). *L. casei* ssp. *rhamnosus* has greater responses to γ-glutamyl folate with lesser than three glutamic acid residues compared to *Enterococcus hirae* and *Pediococcus cerevisae*. It does not respond to folate with greater than three glutamates (Goli and Vanderslice 1989).

Folate extraction includes enzymatic steps to liberate folates from the cellular matrix and deconjugation from polyglutamates to mono- or other simpler measurable forms, mostly as pteroylglutamic acid (Arcot and others 2002; Shrestha and others 2000). Low estimates of folate content will result if liberation from the food matrices is not complete (Shrestha and others 2000). In the 1980s, a number of researchers reported that treatment with conjugase usually results in incomplete in release of bound folates. DeSouza and Eitenmiller (1990) developed a trienzyme digestion procedure to increase measurable folate from complex food matrices. The trienzyme treatment

includes protease (EC 3.4.24.31) and α-amylase (EC 3.2.1.1) digestion in addition to the traditional treatment with folate conjugase. Martin and others (1990), Tamura (1997), Pfeiffer (1997), and Rader and others (1998) reported a significant increase in total folate concentrations in foods with trienzyme treatment compared with the values from folate conjugase treatment alone (Shrestha and others 2000).

Bio-Specific Methods

Various biospecific procedures such as enzyme protein binding assay (EPBA), radiolabeled protein binding assays (RPBA), enzyme-linked immunoabsorbent assay (ELISA), and radioimmunoassay (RIA) were developed for folate analysis because they have several advantages over microbiological assay (MA) and high performance liquid chromatography (HPLC). They are faster, cheaper, and less subject to variation. Also, they have a high sample throughput (Finglas and others 1988b; Hawkes and Villota 1989; Mandella and DePaola 1984). RPBA based on isotope dilution procedures and EPBA are the assays which use vitamin binding proteins naturally occurred with isotope labels or enzyme labels. Also, ELISA and RIA are based on the specific interaction of an antibody with its antigen (Finglas and Morgan 1994; Eitenmiller and others 2008)

RPBA methods are available for folate analysis in blood and serum (Hawkes and Villota 1989) and they are suitable for food analysis when the primary form in the food is 5-CH_3H_4 folate. However, there still can be significant false response from components in the food other than folate owing to nonspecific binding (Stralsjo and others 2002;

Stralsjo and others 2003). Due to general lack of confidence for ligand binding assays for food analysis, RPBA methods should be considered only complimentary to other analytical methods (Stralsjo and others 2002; Stralsjo and others 2003; Etienmiller and others 2008).

EPBA utilizing a folate-binding protein-peroxidase conjugate for food analysis was first reported by Finglas and others. The authors compared folate values in 14 raw and cooked vegetables from EPBA with MA and concluded that there was a good agreement between the two methods. Also, they emphasized that the calibration curve for folic acid must be used (Finglas and others 1988a; Finglas and others 1988b). Arcot and others used EPBA for the analysis of fortified cereal foods and its principle was the degree of binding of folic acid to the surfaces of microwells. Also, the authors concluded that there was a good correlation between MA and EPBA for the folic acid values and that they could get higher degree of regression and repeatability with the calibration curve for folic acid. However, the 95% confidence interval for the lower and upper limit of agreement was high (Arcot and others 2002).These studies demonstrated that EPBA was simple, quicker, and cheaper than MA.

Immunoassays are highly sensitive and specific owing to the specific interaction of an antibody with its target and a high affinity interaction occuring even in complex matrices (Finglas and Morgan 1988). Immunoassays are based on the almost same principle as EPBA except that antibodies are used instead of naturally occurring vitamin-binding proteins (FInglas and Morgan 1994). Several studies have been done to prove that ELISA or RIA procedures are suitable for food analysis, but there are still

drawbacks such as high specificity for folic acid and unsuitability for the quantitation of other folate vitamers (Keagy 1985).

Biospecific procedures are limited because they generally do not provide a measure of the total folate content in foods. These kits generally show much lower response to folate derivatives other than folic acid. Thus, there can be underestimation of the natural folate content of foods (Arcot and Shrestha 2005). Also, Bio-Rad radioassay produces much lower results in serum samples due to $5CH_3THF$ underrecovery than the new isotope-dilution liquid chromatography-tandem mass spectrometry method (LC-MS/MS) or MA. The National Health and Nutrition Examination Surveys (NHANES) used the Bio-Rad radioassay for 25 years to measure total folate concentration, but NHANES stopped using it in 2007 (Fazili and others 2007). Only the LC-MS/MS or MA assays are now approved for serum analysis.

High Performance Liquid Chromatography (HPLC)

HPLC methods are used to separate, identify, and quantitate various folate derivatives in foods (Shin and others 1975; Day and Gregory 1981; Gregory and others 1984; Vahteristo and others 1996; White and others 1991; Selhub and others 1988; Selhub 1989; Seyoum and Selhub 1993; Bagley and Selhub 1997; Finglas and others 1993). Separating and quantifying different forms of folates and minimum interference from the enzymes are considered as the advantages of HPLC (Arcot and others 2002).

There are two individual steps in HPLC techniques: 1) Separation and purification of deconjugated extract, and 2) detection and quantification of eluted monoglutamates (Gregory 1989). Sample preparation and purification are very crucial

steps due to the complexity of the various natural forms of folates (Blake 2007). For extraction, pH 4.5-7.8 buffers containing ascorbic acid or 2-mercaptoethanol to reduce oxidative losses are commonly used (Arcot and Shrestha 2005). Various techniques for separation and purification have been introduced such as ion-exchange chromatography using small columns of DEAE-Sephadex A-25 (Gregory and others 1984), weak anion-exchange column (Reed and Archer 1976; Schieffer and others 1984; Goli and Vanderslice 1992), a strong anion exchanger (Clifford and Clifford 1977; Vahteristo and others 1996a; Vahteristo and others 1996b; Osseyi and others 1998), and cation-exchanger (Gregory and others 1982; Duch and others 1983). Also, several HPLC analyses used affinity chromatography utilizing immobilized milk folate-binding protein (Selhub and others 1980; Gregory and Toth 1988; Selhub and others 1988; Ruggeri and others 1999; Pffeiffer and others 1997; Rosalia and others 2006).

The need for sensitive detection techniques was emphasized since the low concentrations of folate present in most foods or other tissues (Vahteristo and others 1996a). Ultraviolet absorbance (280nm), fluorescence, electrochemical techniques, and microbiological assay (MA) of collected fractions are commonly using to detect eluted folate derivatives (Gregory 1989).

However, HPLC has several limitations, even though its potential for future analysis of folates in food is very high. One of them is the demanding sample clean-up procedure prior to final injection (Arcot and Shrestha 2005). While some researchers have reported good agreement for total folate values between HPLC and microbiological methods, in the review paper for folate analysis, Arcot and Shrestha reported consistently lower values (less than 50%) for total folate determined by HPLC

in their laboratory (Ginting and Arcot 2004). Similar results were shown in the European Interlaboratory study in 2001 for establishing validated HPLC and microbiological methods to determine folates (Kariluoto and others 2001). The authors found that the values determined by HPLC were much lower (30 – 40%) than the ones determined by microbiological assay. The lower folate results might be due to the limitation of HPLC detectors to identify some of the folate derivatives and complex sample extraction and purification procedures resulting in the loss of sensitive folate compounds (Arcot 2005).

Liquid Chromatography with Mass Spectrometry

Liquid Chromatography with mass spectrometry (LC-MS) is now commonly used for folate analysis (Arcot and Shrestha 2005). The technique is able to acquire more accurate data for total folate and even coenzyme constituents. In 2001, Pawlosky and Flanagan compared the precision and accuracy of LC-MS folic acid quantification in a cereal matrix with values obtained through microbiological assay and there was a good agreement between the values (Pawlosky and Flanagan 2001). Also, Frazili and others reported that there was good correspondence between the sum of folate species determined by isotope-dilution liquid chromatography-tandem mass spectrometry method (LC-MS/MS) and total folate determined by MA in serum samples (Fazili and others 2007).

Freisleben and others used stable isotope dilution assays using deuterated isotopmers as internal standards. To improve the specificity, they used seleted reaction monitoring and most data obtained for a wide food matrix were acceptable except for broccoli (Freisleben and others 2003a). Stable isotope dilution LC-MS/MS assays have

shown excellent results for folic acid-folate analysis in a range of matrices (Thomas and others 2003; Pawlosky and others 2003; Rychlik and others 2003; Freisleben and others 2003a; Freisleben and others 2003b; Pfeiffer and others 2004; Rychlik 2004; Rychlik and Mayr 2005; Rychlik 2006; Gutzeit and others 2008). It provides the more accurate separation of several folate forms and can be considered as a reference procedure specifically for validating LC procedures with UV and/or fluorescence detection (Arcot and Shrestha 2005; Blake 2007). For example, in 2008, Gutzeit and others adopted a stable isotope dilution assay to quantify folate vitamers in sea buckthorn berries and related products using fourfold labeled folate isotopologues of the folate derivatives as the internal standards and reversed-phase liquid chromatography electrospray ionization mass spectrometry (LC-ESI-MS/MS). They found a total degradation of tetrahydrofolate and 5-formyltetrahydrofolate in the generated juice due to the technological processing of the berries and approximately unchanged content of 5-methyltetrahydrofolate during processing the berries to a concentrate (Gutzeit and others 2008).

While LC analysis has the capability to quantify the specific folate forms which are not obtainable by other methods, the use of LC-MS remains out of reach of some laboratories due to its high cost.

AOAC International Official Methods

The Association of Official Analytical Chemists (AOAC) official methods used for compliance purposes are microbiological methods using either *E*. *hirae* (*S*. *faecium*)

ATCC No. 8043 or *L. casei* ssp. *rhamnosus* ATCC No. 7469. At present, there is no AOAC official method using LC for folate assay.

AOAC official method 944.12, Folic Acid (Pteroylglutamic Acid) in Vitamin Preparations, Microbiological Method, AOAC Official Methods of Analysis, 45.2.03. specifies use of the response of *E. hirae* for measurement of folic acid in pharmaceutical products formulated with folic acid. *E. hirae* does not respond to 5-CH_3-H_4 folates or other coenzyme forms and thus is not designed for assay of total folates (Eitenmiller and others 2008).

AOAC official method 922.05, Folic Acid (Pteroylglutamic Acid) in Infant Formula, Microbiological Method, AOAC Official Methods of Analysis, 50.2.21. uses *L. casei* and is only for the assay of folic acid in infant formula. The method uses a single-enzyme digestion with chick pancreas conjugase (folate conjugase; γ-glutamyl-carboxypeptidase; pteroylpoly-γ-glutamyl hydrolase; EC 3.4.22.12) to liberate mono- or diglutamates from naturally occurring folypolyglutamates and so measures folate originating from natural ingredients (Rader and others 1998; De Vries and others 2005). The conjugase digestion step is important since most foods contain polyglutamyl forms of the vitamin, which need to be hydrolyzed to simpler forms that will promote growth of *L. casei* (Rader and others 1998).

AOAC Official Method 2004.05, Total Folates in Cereals and Cereal Foods, Microbiological Assay, Trienzyme Method, accepted First Action 2004 is applicable to the assay of total folate including folic acid in cereals and enriched cereal grain products (De Vries and others 2005), and the method using the trienzyme digestion is close to AACC Methods 86-47 (AACC 2000; DeVries and others 2001). The trienzyme digestion

being used as such for the past decade is universally applicable to the assay of total folate in all kinds of food (Eitenmiller and others 2008).

Response Surface Methodology

Statistically-designed optimization studies are used to confirm previous effects and interactions, estimate specific curvature or quadratic effects, and determine optimal settings for critical factors (Strobel and Sullivan 1999). Response surface methodology (RSM) is a powerful experimental design tool, statistical-based technique used to optimize processes or production (Montgomery 1991). RSM is a collection of mathematical and statistical techniques which use quantitative data in an experimental design (Giovanni 1983). RSM, originally described by Box and Wilson (1951), has been successfully used for developing, improving, and optimizing processes because it enables evaluation of the effects of several process variables and their interactions on response variables (Myers and Montgomery 2002). Also, it decreases the numbers of experimental trials needed to evaluate multiple parameters and their interactions. Thus, it is less laborious and more time-saving than other techniques used to optimize processes (Mudahar and others 1989; Batisuti and others 1991; Shieh and others 1996; Vega and others 1996). The optimization of the extraction using RSM, by establishing a mathematical model, provides a clearer picture as a visual aid to evaluate the effects of various factors on extraction and locates the region where the extraction is optimized (Banik and Pandey 2008).

Lee and others first used RSM to optimize extraction procedure to quantify vitamin E in tomato and broccoli (Lee and others 2000). After RSM was successfully

applied in the vitamin E extraction procedure, Chen and Eitenmiller (2007) used RSM to optimize the trienzyme digestion for folate extraction from vegetables. The trienzyme digestion time was optimized to 1.5 h, 1.5 h, and 3 h for Pronase®, α-amylase, and conjugase, respectively. The authors reported that the condition of the much shorter, optimized digestion time showed higher folate values than the trienzyme digestion parameters (3 h, 2 h, and 16 h for Pronase®, α-amylase, and conjugase, respectively) employed in AOAC Official Method 2004.05 (Chen and Eitenmiller 2007).

CHAPTER 3

MATERIALS AND METHODS

Samples

USDA breakfast cereal and snack samples

30 breakfast cereal samples and 28 snack samples for determination of folate contents were collected through the United States Department of Agriculture National Food and Nutrient Analysis Program (USDA/NFNAP). The NFNAP designed by United States Department of Agriculture is a 5-year research program with a goal of updating and improving the quality of food composition data of key foods in the USDA National Nutrient Database for Standard Reference (Pehrsson and others 2003; Haytowitz and others 2002; Haytowitz and others 2000). Food samples through the sampling program of NFNAP are nationwide, homogenized, and representative for nutrient analysis (Chun and others 2006).

CRM121 (wholemeal flour) & non-enriched cereals

Certified reference materials (CRMs) and standard reference materials (SRMs) are important to validate the accuracy of nutrient data and are used for several purposes: 1) to facilitate evaluating the accuracy of the whole assay system while an analytical method is developed or implemented, 2) to determine whether a method

is under control during routine use, 3) to provide traceable values assigned to an in-house control material, or 4) as a reference sample to evaluate inter-laboratory variability (Phillips and others 2007). In the NFNAP, CRMs have been used broadly for monitoring the accuracy of nutrient assays for a wide range of foods (Phillips and others 2006). From nine analytical laboratories, a total of 2554 values were acquired for over 100 different components for 259 certified or reference nutrient concentrations in 26 CRMs (Phillips and others 2006).

CRM121 (wholemeal flour), European Commission Certified Reference Material, was purchased from Resource Technology Corporation, Laramie, WY, U.S.A. for the optimization of enzyme extractions for folate in cereals. Also, 4 non-enriched cereals purchased from Purcell Mountain Farms were used for the optimization study: oat meal flour, triticale flour, buckwheat flour, and whole wheat flour.

Collected samples were stored at -50˚C. The samples were thawed at room temperature before the assay. Also, the samples were mixed to ensure homogeneity with a spatula right before weighing.

Stock Solution

Stock Solution (200μg/mL)

Twenty milligrams of USP (US Pharmacopoeia) folic acid, used for the standard, was accurately weighed. The folic acid standard was mixed with 20mL of 95% (v/v) ethanol and 50mL of deionized water in a 200mL Pyrex conical flask. To dissolve the folic acid, the pH of the solution was adjusted to 10.00 with 0.1N NaOH and then the pH

was adjusted to 7.00 with 0.05N HCl. After making the solution volume up to 100mL, it was transferred to 10mL tubes with screw caps to prevent evaporation of ethanol. The tubes were stored in a refrigerator at 4˚C. The standard is very stable but should be replaced after 4 months. Before using, the purity of the stock solution was checked to verify the concentration.

Purity of standard stock solution

Phosphate buffer (0.1M) was prepared (13.61g KH_2PO_4/100mL) and the pH was adjusted to 7.00 with 4N potassium hydroxide. The 0.1M, pH 7.0 phosphate buffer was used to dilute the standard stock solution in a 1:20 ratio. Using the buffer as a blank, the absorbance of the diluted stock solution was measured at 282 nm using a 1-cm quartz cuvette. With the measured values of the standard stock solution and the blank, the purity was calculated by the following equations:

$$E^{1\%}_{1cm}\,(\text{Extinction Coefficient}) = 10(A_{std}-A_{blank})/C \quad (\text{eq } 3.1)$$

where C = concentration of diluted standard stock standard (mg/mL),

$$\text{Purity of standard} = 100\,(\text{calculated } E^{1\%}_{1cm} / \text{reference } E^{1\%}_{1cm}) \quad (\text{eq } 3.2)$$

where reference $E^{1\%}_{1cm}$ = 611.7 (table 3.1).

Table 3.1 Physical Properties of Folic Acid

Substance	Molar mass	Formula	Absorbance		
			λ_{max}	$E^{1\%}_{1cm}$	Solvent
Folic Acid	441.4	$C_{19}H_{19}N_7O_6$	282	611.7	Phosphate buffer, pH 7.0

(*Vitamin Analysis for the Health and Food Sciences, Eitenmiller & Landen Jr.,* p417)

Example:

When weighing 0.02g (20mg) USP folic acid in 100mL total volume,

C of diluted stock solution by 1:20 = (20mg/100mL)/20 = 0.01 mg/mL

A_{std} = 0.672, A_{blank} = 0.068

$E^{1\%}_{1cm}$ = 10(A_{std}-A_{blank})/C

= 10(0.672-0.068)/0.01=604

Purity of standard = 100 (the calculated $E^{1\%}_{1cm}$ /table $E^{1\%}_{1cm}$) = 100(604/611.7) = 98.7%

The true concentration of stock solution = (20mg/100mL)(98.7/100)

= 197.4μg/mL

Preparation of Chicken Pancreas Acetone Powder for γ-Glutamyl Hydrolase (Conjugase) Digestion

Fresh chicken pancreas provided by the Poultry Science Department, University of Georgia, was first cut into small pieces and then a mortar and pestle were used to grind the pancreas with dry ice. Small particles of frozen chicken pancreas were added to about 5 volumes of cold acetone cooled by dry ice. The mixture was homogenized with a homogenizer at 6500 rpm for 3 min (PRO300A, PRO Scientific Inc, Monroe, CT, U.S.A.). Cheesecloth was placed over a Büchner funnel containing filter paper (Fisherbrand®, Cat: 09-795D, 11.0cm) and the acetone containing chicken pancreas slurry was filtered with the aid of suction. The fine material on the Büchner funnel was washed several times with cold acetone and then air-dried. Before use a solution of the chicken pancreas powder (conjugase solution, 5mg/mL) was prepared in 0.1M phosphate buffer pH 7.8. The chicken pancreas powder was stored in a freezer at -5°C.

Assessment of the enzyme activity of the chicken pancreas powder is provided in the following section.

Measurement of Chicken Pancreas Conjugase Activity

Pteroyltetra-γ-L-glutamic acid (MW 828.7) was used to prepare the standard solution (0.001mg/100mL). One tenth milliliter of the standard solution (1μg/0.1mL) was added into twelve flasks containing 30mL of deionized water and 30mL of 0.1M phosphate buffer pH 7.8. Four milliliters of chicken pancreas conjugase solution (5mg/mL of 0.1M, pH 7.8 phosphate buffer) were added in the flasks. Digests were removed at 10 min intervals during the first hour and heated for 5 min for inactivating the conjugase. Digests were removed at 15 min intervals during the second hour and heated for 5 min for inactivating the conjugase. After microbiological assay, the values of measurable folate were obtained (Table 3.2). After 60 min, 1μg of measurable folates was recovered (Table 3.3). Measurable folates include pteroylmonoglutamic acid, pteroyldiglutamic acid, and pteroyltriglutamic acid with an average molecular weight of 570.5; thus, 0.00003μmol/min (0.03nmol/min) per twenty milligrams of chicken pancreas conjugase was calculated for the unit of chicken pancreas conjugase. The activity could be calculated in nano katal (nkat) which is the SI unit for enzyme activity (1 katal (kat) = 1mol/s, 1μmol/min = 16.67 nkat). One katal is the amount of enzyme that converts 1 mole of substrate per second. The activity of twenty milligrams of chicken pancreas conjugase was 0.0005 nkat (0.5 picokatal).

Table 3.2 Measurable Folate Released from Pteroyltetra-γ-L-glutamic Acid

Time (min)	Measurable foate (μg)
10	0.60
20	0.71
30	0.85
40	0.97
50	0.98
60	1.01
75	1.02
90	1.00
105	0.99
120	1.00

Table 3.3 Calculation of chicken pancreas conjugase activity (μmol/min/20mg of chicken pancreas)

Time (min)	Total folate (μg)	μmol/min
10	0.60	1.05×10^{-4}
20	0.71	6.23×10^{-5}
30	0.85	4.97×10^{-5}
40	0.97	4.25×10^{-5}
50	0.98	3.44×10^{-5}
60	**1.01**	**2.95×10^{-5}**
75	1.02	2.38×10^{-5}

Bold type: time when 100% of measurable folate obtained

Trienzyme Extraction Method

Enzyme Control (blank)

To determine how much of the enzymes contribute for the growth of *L.casei* ssp. *rhamnosus* (ATCC 7469), a control was used. The control without food samples went through all steps of the trienzyme extraction procedure.

Accuracy

Certified reference materials (CRM)

The accuracy of an analytical method shows the closeness of test results to the true or accepted value. Bias is the quantitative term describing the difference of the reported value from the accepted value (AOAC 2002). Positive bias indicates that the analytical value was less than the accepted one, while negative bias defines that the analytical value was higher than the accepted one. Accuracy can be evaluated by analyzing standard reference materials or certified reference materials (AOAC 1998). CRM 121, wholemeal flour was used for this study to evaluate accuracy.

Recovery

Recovery confirmed the accuracy of this method when the value of the recovery was in the recovery limits (about 70 to 125%) for acceptable assays (AOAC 2002). The following equation caculated the recovery (%) (AOAC 2002):

$$R\ (\%) = [(Cs - Cp) / Ca] \times 100 \quad \text{(eq 3.3)}$$

where R (%) is the percent recovery of added standard; *Cs* is folate concentration in the spiked sample; *Cp* is folate concentration in the unspiked sample; and *Ca* is the amount of folic acid standard added.

Samples were spiked with folic acid at approximately 100% levels of the estimated values of the samples, based on the AOAC guidelines for recovery evaluation (AOAC 1998). A working standard solution 0.2μg/mL (0.1mL stock solution/100mL deionized water) was prepared.

Precision

Precision was measured using an internal quality control (IQC) sample by monitoring the repeatability or the intra-laboratory precision (%RSD_r). Pillsbury all-purpose, bleached, enriched flour was used as IQC for this study.

Control Charts

Control charts are used for monitoring quality and contain a center line (mean) and two other horizontal lines (upper control limit and lower control limit). The limits for this study were ± 10% of the mean values. When the data points fall within these limits, control charts ensure that the system remains in control and the results can be accepted (NIST/SEMATECH 2003).

0.1M, pH 7.8 Phosphate Buffer

0.1M phosphate buffer containing L-ascorbic acid (1%, w/v) was prepared (2.68g $Na_2HPO_4 \cdot 7H_2O$ (sodium phosphate, dibasic, heptahydrate, EMD Chemicals Inc., SX0715-1)/100mL deionized water) and its pH was adjusted to 7.8 with 20% (w/v) NaOH.

Trienzyme extraction for total food folate

The trienzyme extraction used in this study followed AOAC Official Method 2004.05, Total Folates in Cereals and Cereal Foods, and Microbiological Assay-Trienzyme Procedure (AOAC 2005). Samples (0.5~1.0 g) were weighed into 125mL Erlenmeyer flasks and 20mL of 0.1M, pH 7.8 phosphate buffer and 30mL of deionized

water were added into the flasks. Aluminum foil was used to cover the flasks to prevent evaporation. The flasks were preheated at 100˚C for 15 min and then cooled to room temperature. Ten milliliters of the phosphate buffer and 1mL of Pronase® (2mg/mL of deionized water, Calibiochem, nr 53702, San Diego, CA, U.S.A.) were added, followed by incubation at 37˚C for 3h. The sample was heated at 100˚C for 3 min after the Pronase® digestion to inactivate the enzyme. One milliliter of freshly prepared α-amylase (20mg/mL of deionized, Fluka, nr 10065, St. Louis, MO, U.S.A.) and 0.5mL of toluene were added. The mixture was incubated at 37˚C for 2h. Chicken pancreas conjugase solution (5mg/mL of 0.1M, pH 7.8 phosphate buffer) was stirred for 10 min to ensure the homogeneity of the solution. The solution was filtered through glass wool and 4mL of the filtered solution were added. After 16 h incubation at 37˚C, the digests were heated at 100˚C for 3 min, cooled, and adjusted to pH 4.5 with 20% (w/v) HCl. The sample digests were taken to a volume of 100mL with deionized water and filtered through ashless filter paper (Whatman® No. 1, Cat: 1001-185, 18.5cm).

Extraction procedure for folic acid

From the traditional trienzyme digestion for total folate, Pronase® and conjugase digestion steps are eliminated for the extraction of folic acid (Chun and others 2006). Except the elimination of two enzymes, other steps are exactly same with the extraction procedure for total folate by AOAC Official Method 2004.05 and AACC Method 86-47.

Hexane extraction

Lipids can stimulate the growth of *L. casei* subsp. *rhamnosus* (ATCC 7469). Therefore, hexane was used for fat extraction if samples contained more than 5% of fat. Hexane (20 mL) was added, and after 15min, removed by pipet. Nitrogen flush was used to remove residual hexane (Eitenmiller and Landen Jr. 1999).

Internal Quality Control

In the IUPAC Harmonized Guidelines for IQC, internal quality control (IQC) is defined as a set of procedures undertaken to monitor continuously the operation and the results of measurements for determining whether results are reliable enough to be released (Thompson and Wood 1995). The purpose of IQC is the elongation to validate the analytical method and is continuously checking the accuracy of analytical data obtained from day to day in the laboratory; the word 'internal' in IQC implicates that repeatability conditions are acquired (Taverniers 2004).

Pillsbury all-purpose, bleached, enriched flour purchased at the local grocery was used as the internal quality control sample through out the study. To ensure homogeneity, two bags (5 lb) of the flour were combined and thoroughly mixed. Aliquots were transferred to 4 ounce leak-proof Nalgene bottles with a polyethylene screw cap and stored at 4˚C.

Assay of the quality control sample for total folate and folic acid followed the trienzyme extraction procedure.

Microplate Assay

A standard microbiological assay using *L. casei* subsp. *rhamnosus* (ATCC 7469) was used, according to the procedures outlined by Tamura (1990) and Chen and Eitenmiller (2007).

Preparation of L. casei *subsp.* rhamnosus

Every week, *L. casei* subsp. *rhamnosus* (ATCC 7469) was transferred on a new Lactobacilli agar slant. After 24h-incubation at 37˚C, the slant was stored in the refrigerator at 4˚C. It can be used for 5 days as inocula for the microplate assay.

Lactobacilli Agar AOAC (Difco, Cat: 2005-04-30)

Lactobailli agar (4.8g) was weighed and 100mL deionized water were added. The solution was boiled for 2-3 min and cooled slightly. Ten milliliters of the agar solution were dispensed into screw cap tubes and the tubes were autoclaved at 121˚C for 15min. To produce a slant, the tubes were placed at an angle while cooling. They were stored in the refrigerator at 4˚C.

Lactobacilli Broth AOAC (Difco, Cat: 2002-06-30)

The Lactobacilli broth solution was made with 3.8g of Lactobacilli broth in100mL deionized water. Lactobacilli broth was prepared similarly to Lactobacilli agar. After boiling for 2-3 min, the lactobacilli broth (10mL) was dispensed into screw cap tubes. The tubes were autoclaved at 121˚C for 15min, cooled to room temperature and stored at 4˚C.

Depletion media (Lactobacilli broth: media = 1:1)

Depletion media consisted of a 1:1 mixture of Lactobacillus broth (3.8g/100mL) and Folic Acid Casei Medium (9.4g/100mL). After boiling for 2-3 min and cooling to room temperature, 10mL of the media were dispensed into screw cap tubes. The tubes were autoclaved at 121˚C for 15min and cooled to room temperature. They were also stored in the refrigerator at 4˚C.

Set-up of the microplate assay

Working standard

The working standard was prepared as follows: 1) 0.1mL of the standard stock solution (200μg/mL) was taken and diluted to 100mL with deionized water using a 100mL volumetric flask and 2) 1mL of the first dilution (0.2μg/mL) was diluted to 100mL with deionized water using a 100mL volumetric flask, again (2ng/mL). The working standard was freshly made for every analytical batch.

Inocula

Using a 3-to-5-day-old slant culture on Lactobacilli Agar AOAC media, *L. casei* subsp. *rhamnosus* cells were transferred into a depletion media and incubated at 37˚C for 6h. For the microplate assay, the incubated depletion broth was used as the inoculum.

Sample dilution

To obtain growth spanning the concentration range of the standard curve, the filtered sample extracts were diluted to ensure that the estimated folate contents of samples were in the range of 0-30μg/100g. For example, if one sample has about 150μg total folate per 100g, a 1:5 dilution was used; 1mL of the filtered sample extraction and 4mL of deionized water were mixed. This dilution step is very important and ensures that assayed concentrations are on the linear range of the standard curve.

Preparation of media, water, ascorbic acid solution, and sample

Folic Acid Casei Medium (9.4g/100g) was prepared by boiling 2-3min and cooling to room temperature. It was sterilized by filtration through a disposable sterile filter system – Corning (Cat: 430767, Fisher Cat: 09-761-1) designed for the filtration of cell culture media, biological fluids and other aqueous solutions.

Deionized water was filtered through the disposable sterile filter system to prepare sterile water.

The ascorbic acid solution (0.5g/5mL) was prepared.

Autoclave

Five to ten milliliters of the sample extracts were transferred into test tubes after a dilution was made, if needed. The test tubes were loosely capped and autoclaved at 121˚C for 5 min. An Erlenmeyer flask (150mL) and a measuring cylinder (100mL) covered with aluminum foil were autoclaved with the samples.

Microplate procedure

The bench was wiped with 70% alcohol prior to set-up of the microplates. All the procedures were completed near the flame of a Bunsen burner to decrease chances of microbial contamination. The 96-well microplates (Figure 3.1) were opened and labeled on the cover. Sterile water was transferred into a reservoir and 300μL of the water was added in row H (blank) of the microplate and all other wells were filled with 150 μL using a 12-channel pipetter. The working standard was filtered into a reservoir, using a syringe and a sterilized syringe filter. The sterilized working standard was pipetted into well G1 and G2. 150μL of the sample solution were added to the two wells through G3 to G12. Control, recovery, and QC were also added to G3 to G12. Each of samples was pipetted into two wells. The row G was mixed by pipetting 3 times using the mix program of the 12-channel pipetter and dilutions were made by pipetting 150μL of solutions from row G to F. Serial dilutions were made until row A with the same mixing and pipetting to next well. After mixing row A, final pipetted 150μL were removed. In this way, the concentration of G is 2 times that of F, and the concentration of F is 2 times that of E, and so on. To prepare the media, 15mL per plate were measured by the sterilized measuring cylinder and transferred into the sterilized flask. Prepared ascorbic acid solution filtered using a syringe and a sterilized syringe filter was added (50mg/100mL of the media) and the incubated inoculum culture was taken with a 1mL pipette and added into the flask (1 drop/5mL of the media). The flask containing media, ascorbic acid solution, and inoculum culture was well shaken and transferred to a reservoir. 150μL of the prepared media were added to each well from row A to G. After

placing in Ziploc bags to prevent evaporation, these plates were incubated at 37°C for 24-28 h.

	1	2	3	4	5	6	7	8	9	10	11	12	
A	A-1	A-2	A-3	A-4	A-5	A-6	A-7	A-8	A-9	A-10	A-11	A-12	A
B	B-1	B-2	B-3	B-4	B-5	B-6	B-7	B-8	B-9	B-10	B-11	B-12	B
C	C-1	C-2	C-3	C-4	C-5	C-6	C-7	C-8	C-9	C-10	C-11	C-12	C
D	D-1	D-2	D-3	D-4	D-5	D-6	D-7	D-8	D-9	D-10	D-11	D-12	D
E	E-1	E-2	E-3	E-4	E-5	E-6	E-7	E-8	E-9	E-10	E-11	E-12	E
F	F-1	F-2	F-3	F-4	F-5	F-6	F-7	F-8	F-9	F-10	F-11	F-12	F
G	G-1	G-2	G-3	G-4	G-5	G-6	G-7	G-8	G-9	G-10	G-11	G-12	G
H	H-1	H-2	H-3	H-4	H-5	H-6	H-7	H-8	H-9	H-10	H-11	H-12	H
	1	2	3	4	5	6	7	8	9	10	11	12	

Figure 3.1 A diagram of a 96-well microplate

Microplate reading

After incubating for 24-28h, the plates were taken and each well was mixed by pipetting 3 times from low concentration (row A) to high concentration (row G). A Bio-Rad Benchmark Microplate Reader (Benchmark, Bio-Rad Laboratories, Inc., Hercules, CA, USA) was used to read the plates using the Microplate Manager® (ver. 5.2) and the 595nm absorbance filter. After running, raw data with unknown concentration were acquired. The absorbance of the standard (G1 and G2) was recommended to be higher than 0.9 to check maximal growth.

Data analysis

The standard concentrations were calculated by editing the standard concentration as 0.2 for concentration of S7 and 2 for the dilution factor. For the sample, unknown dilution was edited according to the sample dilution made. Standard curve was assembled by regression using Logistic 4PL, Linear-Linear Transformation, and Linear-Linear in Axis Transformation to check whether standard curve was acceptable. Once unknown concentration report was obtained, it was exported to Microsoft Excel.

Calculation of recovery

The recovery rate was calculated by the following formula:

$$R = [(Cs - Cp) / Ca] \quad \text{(eq 3.4)}$$

where R is the recovery rate of added standard; *Cs* is folate concentration in the spiked sample; *Cp* is folate concentration in the unspiked sample; and *Ca* is the amount of folic acid standard added.

Calculation of samples

After control and recovery were calculated, the sample data was corrected with by subtracting control and recovery values. At 1:1 dilution of sample, the control value should be subtracted from the sample values. At sample dilution levels greater than the control, the control is insignificant in the final calculation. Also, sample data should be corrected by the following formula:

$$\text{Corrected data} = \text{sample data/recovery rate} \quad \text{(eq 3.4)}$$

With the corrected data, the mean and standard deviation of each sample were calculated. The standard deviation between sample values at different dilutions should

be less than 10% of the mean (AOAC 2000). Outliers were discarded and the final concentration of each sample was determined.

Response Surface Methodology

Experimental Design

A fractional 3-level-3-factor experimental design with three replicates at the centerpoint was used to investigate effects of three independent variables (Pronase® digestion time, X_1; α-amylase digestion time, X_2; and conjugase digestion time, X_3) on the dependent variable (folate content, Y) for cereal samples including CRM121 (wholemeal flour), oat flour, buckwheat flour, and triticale flour. The digestion time of each enzyme (independent variables) were coded at three levels (-1, 0, and 1) and the digestion time of each level was selected on the basis of preliminary experiments. Response surface design for CRM121, wholemeal flour was shown in Table 3.4. The complete experimental design consisted of 15 experimental points.

Table 3.4 Response Surface Design and Experimental Data

	Variables (incubation time, h)			Folate (μg/100g)
Treatment no. [1]	Pronase®	α-Amylase	Conjugase	CRM121
1	1(1)[2]	3(1)	4(0)	48.3
2	0.25(-1)	3(1)	4(0)	48.3
3	1(1)	1(-1)	4(0)	45.6
4	0.25(-1)	1(-1)	4(0)	46.0
5	0.5(0)	3(1)	6(1)	51.8
6	0.5(0)	3(1)	2(-1)	46.4
7	0.5(0)	2(-1)	6(1)	46.4
8	0.5(0)	2(-1)	2(-1)	42.8
9	1(1)	2(0)	6(1)	49.1
10	1(1)	2(0)	2(-1)	46.0
11	0.25(-1)	2(0)	6(1)	51.4
12	0.25(-1)	2(0)	2(-1)	44.6
13	0.5(0)	2(0)	2(0)	48.9
14	0.5(0)	2(0)	2(0)	48.8
15	0.5(0)	2(0)	2(0)	47.8

[1] Treatments were run in a random order.
[2] (-1), (0), and (1) are coded levels

Data Analysis

For the optimization study, data analysis was conducted to predict the following second-order polynomial model through regression analysis and analysis of variance (ANOVA) by Statistical Analysis System, SAS (Version 9.00, SAS Institute Inc., Cary, NC, U.S.A.):

$$Y = \beta_0 + \beta_i X_i + \beta_{ii} X_i^2 + \beta_{ij} X_i X_j \quad \text{(eq 3.5)}$$

To create response surface and contour plots, Sigma Plot (Version 9.0 Systat Software, Inc., San Jose, CA, U.S.A.) was used.

For comparing means, Minitab Statistical Software (Version 15 Minitab Inc., PA, U.S.A.) was used and pairwise comparison tests with Tukey's method at the 5% level.

Model Verification

The certified value of CRM121 was compared to the analytical values with the optimized extraction. The predicted values of oat flour, buckwheat flour, triticale flour, and whole wheat flour were compared to the analyzed values with the optimized extraction. Breakfast cereal samples were assayed and the values with the optimized extraction were compared to the data obtained with AOAC Official Method 2004.05 to confirm that the optimized extraction will work for highly fortified cereal products.

CHAPTER 4

RESULTS AND DISCUSSION

Method Validation

Accuracy and precision were measured to confirm that the analytical method, microplate assay with trienzyme extraction was suitable for total folate and folic acid analysis of cereal products and snack food. Also, limit of detection and limit of quantitation of the trienzyme extraction combined with microplating techniques are 0.3μg/100g and 0.6μg/100g, respectively (Chen and Eitenmiller 2007).

Accuracy

CRM121, wholemeal flour

For this study, Certified Reference Material (CRM) 121, wholemeal flour was used with a certified value of 50 ± 7μg/100g. Ten trials were performed with cereal product samples; the mean value was 48 ± 2.5μg/100g within the range of a certified value. According to AOAC's Peer Verifed Program, the acceptable % RSD of CRM121, wholemeal flour (0.00005% analyte) should be less than the range of 11 to 15. The obtained % RSD is 5.2 (Table 4.1); thus, it shows an acceptable trueness of the analytical procedure. The method trueness can be shown through % of accepted value and bias which is the difference between certified value and analytical value and is affected by systematic error (Table 4.1). The control chart for total folate in CRM121

(wholemeal flour) shows the values of ten trials are within a distance of ±10% of mean (Figure 4.1).

The % of accepted value which can indicate the agreement between analytical and accepted values can be used to evaluate accuracy (LaCroix and Wolf 2002; Rolim and others 2005).

Recovery

When the test sample containing added analyte is carried through the entire assay, the analyte is recovered and the percentage of the analyte represents the recovery. To evaluate accuracy, recovery can be used. For quantitative methods, recovery may indicate trueness of the methods (AOAC 1998).

A total of 16 recoveries were completed thorough this study. The spiked level was approximately 100% of the estimated values of the test sample. Using USDA nutrient databank values, the values of the test samples were estimated. The recovery values are considered as a primary method validation parameter and recoveries are necessary to correct sample data to represent true values, which are calculated from sample data divided by recovery rate.

The recoveries were within the acceptable range of recovery limits provided by AOAC (AOAC 2002) ranged from 94 to 105%, confirming the accuracy of this method (Table 4.2). If a recovery exceeds 100%, a data correction is not done.

Repeatability Precision

Pillsbury all-purpose, bleached, enriched flour (enriched flour)

Enriched flour was analyzed for total folate and folic acid each time for 16 trials. Figure 4.2 and 4.3 show the quality control charts for total folate and folic acid, respectively. For total folate in enriched flour, the mean value was 182 ± 4.4 μg/100g. The value of each trial was within the control line, ± 10% of the mean. The mean value for folic acid in enriched flour was 156 ± 7.0 μg/100g. Also, the values of all trials were within the control line, ± 10% of the mean. Those two control charts indicate that this analytical method had good repeatability throughout the study. Percent RSD_r values were 2.4 and 4.4 for total folate and folic acid, respectively.

Optimization of Enzyme Extractions for Folate in Cereals

For the optimization study, 4 unfortified cereals including CRM121, wholemeal flour, oat flour, triticale flour, and buckwheat flour were used. The certified value for CRM121, wholemeal flour is 50 ± 7μg/100g, and the values for tiriticale and buckwheat flours from USDA Nutrient Databank are 74 ± 6.4 and 54 ± 6.8μg/100g, respectively. The value of oat flour found from Fineli® (Finnish Food Composition database) maintained by National Public Health Institute of Finland is 46μg/100g as analyzed by HPLC. Also, breakfast cereals which are highly fortified with folic acid, having only 4-45μg/100g of food folate (Table 4.22), were used to compare the optimized extraction to AOAC Official Method 2004.05.

Predicted Second-Order Polynomial Models

Experimental data for assay of the 4 cereals (Table 4.3 to 4.6) were fitted to the second-order polynomial equation (eq 3.5) by the response surface regression (RSREG). The estimated values of constant coefficients (Table 4.7 to 4.10) were used for predicting the regression models.

The second-order polynomial models for CRM121, wholemeal flour, oat flour, triticale flour, and buckwheat flour were:

for CRM121, wholemeal flour,

$$Y = 32.4 + 6.3X_1 + 5.7X_2 + 2.3X_3 – 1.6X_{11} – 1.2X_{22} – 0.1X_{33} – 0.1X_1X_2 – 1.1X_1X_3 + 0.2X_2X_3 \quad \text{(eq 4.1)},$$

for oat flour,

$$Y = 29.7 + 5.2X_1 + 5.8X_2 + 3.2X_3 – 2.4X_{11} – 1.5X_{22} – 0.2X_{33} – 0.5X_1X_2 – 0.2X_1X_3 - 0.05X_2X_3 \quad \text{(eq 4.2)},$$

for triticale flour

$$Y = 38.7 + 7.9X_1 + 36.0X_2 + 4.4X_3 – 1.0X_{11} – 27.2X_{22} – 0.2X_{33} + 1.8X_1X_2 – 1.6X_1X_3 + 0.8X_2X_3 \quad \text{(eq 4.3)},$$

and for buckwheat flour

$$Y = 29.6 + 8.4X_1 + 18.8X_2 + 2.1X_3 – 5.0X_{11} – 11.3X_{22} – 0.1X_{33} + 0.6X_1X_2 – 0.4X_1X_3 + 1.2X_2X_3 \quad \text{(eq 4.4)}$$

where X_1 is the Pronase® digestion time; X_2 is the α-amylase digestion time, and X_3 is the conjugase digestion time.

ANOVA tables (Table 4.11 to 4.14) indicate that the predicted models are all significant at 1% level and each R^2, the coefficient of determination, is over 0.95 which is sufficient to represent the 'goodness-of-fit' of the regression model. In other words, there is an actual relationship between the response (total folate) and the significant variables. *P*-values and *t*-values determined the significance of each coefficient. The higher significance of the corresponding coefficient is indicated by decreasing *P*-values and larger *t*-test values (Karthikeyan and others 1996). From all the predicted models, β_2 and β_3, constant coefficients of incubation time of α-amylase and conjugase had small *P*-values and large *t*-values (Table4.7 to 4.10) and β_1, constant coefficient of incubation time of Pronase® had large *P*-value (0.32, 0.46, 0.36, and 0.29 for CRM121, oat flour, triticale flour, and buckwheat flour, respectively). Therefore, it can be said that the linear effects of incubation time of α-amylase and conjugase are significant, while the linear effect of incubation time of Pronase® is not. For quadratic effects, each β_{22} of the predicted models for all cereals except triticale flour are significant ($p < 0.1$) (Table 4.7 to 4.10), although the quadratic models show little significance; triticale flour shows significance not only on linear model ($p < 0.01$) but also on quadratic model ($p < 0.05$). Especially, β_{33} is highly significant ($p < 0.01$). Therefore, having the quadratic effects of variables yields better R^2.

From the Table 4.15 to 4.18, incubation time of α-amylase ($p < 0.05$) and conjugase ($p < 0.01$) show significant effects on the response (total folate) whereas, Pronase® digestion was not significant ($p > 0.1$) for the optimization of enzyme extractions for folate in cereals.

Analysis of Response Surface

The three dimensional representation of the response surface is the graphical representations of the regression equation, showing the optimum values of the variables where the response is maximized (Tanyildizi and others 2005). Figures 4.4 to 4.7 show the relationship between the incubation time of α-amylase and conjugase (significant, independent variables) and total folate (dependent variable), holding the insiginificant, independent variable constant (Pronase® = 1 h) (Figure 4.4 to 4.7). It can be seen in the Figures, 4.4 to 4.7 that over 80% of maximum folate can be analyzed within the least incubation time of α-amylase and conjugase (coded at -1 level). Also, α-amylase and conjugase of CRM121, wholemeal flour and triticale flour show a slight decline after the maximum points. α-Amylase digestion of buckwheat flour shows a similar trend. In the similar study of Chen and Eitenmiller (2008), it was found that longer incubation can result in decreased folate values, most likely from destruction of folate by increasing the exposure of folate to oxidation and other deleterious conditions potentially present in the extraction media.

Optimization

Using the RSM results for the 4 cereals with different matrice compositions and levels of total folate, a generalized optimized digestion of 0.7 h for Pronase®, 2.5 h for α-amylase, and 5.9 h for conjugase can be suggested. In order to derive this suggested digestion suitable for most cereals, the longest incubation time obtained for each enzyme was taken from Table 4.7 that ensures maximal release of total folate. Thus, for Pronase® and conjugase, the optimized incubation time can be 1h and 6 h, respectively.

This digestion is much shorter than the 3h and 16 h times required for AOAC Official Method 2004.05. However, the α-amylase digestion time should be 0.5h longer than that required in the AOAC method, increasing the digestion from 2 to 2.5h.

Model Verification

For model verification, oat, buckwheat, triticale, and whole wheat flours were analyzed by both optimized digestion and AOAC Official Method 2004.05. The total folate values by optimized extraction of enzyme in 4 cereals had similar values which were close to the estimated values (USDA Nutrient Databank 2006; Fineli® 2008) by AOAC Official Method 2004.05 (Table 4.20). There was no statistically significant difference between these two methods.

To confirm that the optimized incubation time for cereals can be used for highly fortified cereal products, 3 breakfast cereals which have different levels of total folate were used. This comparison also did not show any significant difference between optimized and AOAC Official Method 2004.05 incubation times (Table 4.21).

Determination of Folate Contents in Breakfast Cereals and Snack Food

Breakfast Cereals

In the United States, breakfast cereals are the one of the primary sources of folic acid intake (Yang and others 2007); therefore, reliable labels are needed to know how much folate is taken from breakfast cereals, especially for some people who may be affected by high folic acid intakes. Table 4.22 shows the folate contents in 12 kinds of breakfast cereals (n=30). Breakfast cereals are highly fortified with folic acid to meet

25% to 100% of the daily value (DV), 400μg per serving size. The assayed data was used to calculate to compare the labeled values per serving size (Table 4.23). The analyzed values had a range from 34% to 100% of DV per serving size, while the labeled values were either 25% or 50% of DV per serving size. All the assayed values of 12 kinds of breakfast cereals exceeded label declarations by 112% to 200% (Table 4.23 and Figure 4.8). Breakfast cereals might be fortified with overages of folic acid to ensure label values are met at the end of shelf life. Thus, higher assay values than labeled ones are often encountered. However, the analyzed values of some of the cereals are 2 times of the label declaration (Honey Bunches of Oats, Honey Roasted or Rice Crispies). It can be said that the folate contents may be much higher than the labels. Also, studies about folate degradation in breakfast cereals will be needed to have exact label declarations for folate safety.

Also, it is my opinion that μg DFE should be declared on breakfast cereal labels. In the breakfast cereals, only 4-45 μg/100g of food folate was found, while 355-1322 μg/100g of added folic acid was found; thus, μg DFE is much higher than the total folate levels due to the higher bioavailability of synthetic folic acid used in fortification compared to food folate (Table 4.22). Dietary reference intake for folate is 400 μg DFE/day and sample data gives 239 to 655 μg DFE per serving size (Table 4.23). The total folate concentration was much smaller with a range from 137 to 401 μg. Use of μg DFE values on food labeles would provide more detailed information to the customer.

Snack Foods

Determination of total folate and folic acid in 28 samples of snack food were analyzed by AOAC Official Method 2004.05 and modified AACC Method 86-47. The total folate in snack samples was determined with a range of 15 to 250 μg/100g. Except triscuits and one chocolate chip cookie, 16 to 205 μg of folic acid were found; chocolate chip cookies had lowest level of folic acid (Table 4.25). Folic acid in the snack samples most likely originates from the enriched flour used in the formulation.

Also, μg DFE was calculated and ranged 15 to 394 μg DFE. Some of the highly fortified snacks such as Keebler Townhouse Crackers, Saltine Crackers and Wheat Thins can be a good source of folate consumption. However, there is no information about folate on the labels. The content of folate and μg DFE in snacks should be on the labels.

Table 4.1 Comparison of the Certified Value of Total Folate in CRM121 (Wholemeal Flour) to the Analytical Value

Sample	μg/100g		Bias[b]	% RSD[c]	% of accepted value
	Certified value[a]	Analytical value			
CRM 121 (wholemeal flour)	50 ± 7	48 ± 2.5	1.9	5.2	96 ± 5.0

[a] combination of results provided by European Commission and collaborating laboratories, values meet European Commission criteria for certification. [b] bias = certified value – analytical value. [c] % RSD = [standard deviation (SD)/mean]*100

Table 4.2 Recoveries of folic acid from cereal products and snack food

Sample (n)	Recovery (%)	Recovery limits (%)[a]
Cereals (n = 2)	102	70 to 125
Breakfast cereals (n=6)	101 ± 3.7	75 to 125
Snack food (n=8)	101 ± 3.2	70 to 125

[a] recovery limits for acceptable assays are provided by AOAC Intl. (AOAC 2002).

Table 4.3 Response Surface Design and Experimental Data for CRM121, Wholemeal Flour

Treatment no.[1]	Variables (incubation time, h)			Folate (μg/100g)
	Pronase®	α-Amylase	Conjugase	CRM121
1	1(1)[2]	3(1)	4(0)	48.3
2	0.25(-1)	3(1)	4(0)	48.3
3	1(1)	1(-1)	4(0)	45.6
4	0.25(-1)	1(-1)	4(0)	46.0
5	0.5(0)	3(1)	6(1)	51.8
6	0.5(0)	3(1)	2(-1)	46.4
7	0.5(0)	1(-1)	6(1)	46.4
8	0.5(0)	1(-1)	2(-1)	42.8
9	1(1)	2(0)	6(1)	49.1
10	1(1)	2(0)	2(-1)	46.0
11	0.25(-1)	2(0)	6(1)	51.4
12	0.25(-1)	2(0)	2(-1)	44.6
13	0.5(0)	2(0)	4(0)	48.9
14	0.5(0)	2(0)	4(0)	48.8
15	0.5(0)	2(0)	4(0)	47.8

[1] Treatments were run in random order
[2] (-1), (0), and (1) were coded levels

Table 4.4 Response Surface Design and Experimental Data for Oat Flour

Treatment no.[1]	Variables (incubation time, h)			Folate (μg/100g)
	Pronase®	α-Amylase	Conjugase	Oat
1	1(1)[2]	2(1)	4(0)	46.5
2	0.25(-1)	2(1)	4(0)	46.4
3	1(1)	0(-1)	4(0)	41.6
4	0.25(-1)	0(-1)	4(0)	40.1
5	0.5(0)	2(1)	6(1)	48.1
6	0.5(0)	2(1)	2(-1)	41.9
7	0.5(0)	0(-1)	6(1)	44.7
8	0.5(0)	0(-1)	2(-1)	38.2
9	1(1)	1(0)	6(1)	48.5
10	1(1)	1(0)	2(-1)	41.2
11	0.25(-1)	1(0)	6(1)	48.4
12	0.25(-1)	1(0)	2(-1)	40.0
13	0.5(0)	1(0)	4(0)	44.3
14	0.5(0)	1(0)	4(0)	45.7
15	0.5(0)	1(0)	4(0)	46.1

[1] Treatments were run in random order
[2] (-1), (0), and (1) were coded levels

Table 4.5 Response Surface Design and Experimental Data for Triticale Flour

Treatment no.[1]	Variables (incubation time, h)			Folate (μg/100g)
	Pronase®	α-Amylase	Conjugase	Triticale
1	1(1)[2]	1(1)	4(0)	66.7
2	0.25(-1)	1(1)	4(0)	65.1
3	1(1)	0.25(-1)	4(0)	61.6
4	0.25(-1)	0.25(-1)	4(0)	61.3
5	0.5(0)	1(1)	6(1)	70.4
6	0.5(0)	1(1)	2(-1)	59.8
7	0.5(0)	0.25(-1)	6(1)	64.8
8	0.5(0)	0.25(-1)	2(-1)	55.8
9	1(1)	0.5(0)	6(1)	68.3
10	1(1)	0.5(0)	2(-1)	63.7
11	0.25(-1)	0.5(0)	6(1)	69.1
12	0.25(-1)	0.5(0)	2(-1)	60.4
13	0.5(0)	0.5(0)	4(0)	64.8
14	0.5(0)	0.5(0)	4(0)	66.4
15	0.5(0)	0.5(0)	4(0)	67.5

[1] Treatments were run in random order
[2] (-1), (0), and (1) were coded levels

Table 4.6 Response Surface Design and Experimental Data for Buckwheat Flour

Treatment no.[1]	Variables (incubation time, h)			Folate (μg/100g)
	Pronase®	α-Amylase	Conjugase	Buckwheat
1	1(1)[2]	1(1)	4(0)	49.1
2	0.25(-1)	1(1)	4(0)	48.6
3	1(1)	0.25(-1)	4(0)	41.3
4	0.25(-1)	0.25(-1)	4(0)	40.5
5	0.5(0)	1(1)	6(1)	51.9
6	0.5(0)	1(1)	2(-1)	42.8
7	0.5(0)	0.25(-1)	6(1)	44.8
8	0.5(0)	0.25(-1)	2(-1)	39.6
9	1(1)	0.5(0)	6(1)	48.5
10	1(1)	0.5(0)	2(-1)	42.3
11	0.25(-1)	0.5(0)	6(1)	47.7
12	0.25(-1)	0.5(0)	2(-1)	40.1
13	0.5(0)	0.5(0)	4(0)	46.8
14	0.5(0)	0.5(0)	4(0)	45.1
15	0.5(0)	0.5(0)	4(0)	45.2

[1] Treatments were run in random order
[2] (-1), (0), and (1) were coded levels

Table 4.7 Regression Coefficients of the Predicted Second-Order Polynomial Model of CRM121, Wholemeal Flour

Constant Coefficient [a]	Estimate value	Standard error	Computed t-value	Pr > I t I
β_0	32.4	3.7	8.7	0.0003
β_1	6.3	5.7	1.1	0.3211
β_2	5.7	2.1	2.8	0.0401
β_3	2.3	1.0	2.2	0.0810
β_{11}	-1.6	3.6	-0.4	0.6834
β_{22}	-1.2	0.4	-2.7	0.0419
β_{33}	-0.1	0.1	-1.0	0.3482
β_{12}	-0.1	1.1	-0.1	0.9091
β_{13}	-1.1	0.6	-2.0	0.0970
β_{23}	0.2	0.2	1.1	0.3482

[a] β_0 intercept & β_1, β_2, and β_3 constant coefficients of incubation time of Pronase®, α-amylase, and conjugase, respectively.

Table 4.8 Regression Coefficients of the Predicted Second-Order Polynomial Model of oat flour

Constant Coefficient [a]	Estimate value	Standard error	Computed t-value	Pr > I t I
β_0	29.7	3.4	8.8	0.0003
β_1	5.2	6.6	0.8	0.4638
β_2	5.8	1.7	3.4	0.0201
β_3	3.2	1.2	2.6	0.0462
β_{11}	-2.4	4.5	-0.5	0.6156
β_{22}	-1.5	0.5	-2.8	0.0374
β_{33}	-0.2	0.1	-1.1	0.3125
β_{12}	-0.5	1.4	-0.4	0.7145
β_{13}	-0.2	0.7	-0.3	0.7872
β_{23}	-0.05	0.3	-0.2	0.8700

[a] β_0 intercept & β_1, β_2, and β_3 constant coefficients of incubation time of Pronase®, α-amylase, and conjugase, respectively.

Table 4.9 Regression Coefficients of the Predicted Second-Order Polynomial Model of triticale flour

Constant Coefficient [a]	Estimate value	Standard error	Computed t-value	Pr > I t I
β_0	38.7	4.4	8.8	0.0003
β_1	7.9	7.3	1.1	0.3262
β_2	36.0	7.3	5.0	0.0043
β_3	4.4	1.3	3.3	0.0220
β_{11}	-1.0	4.8	-0.2	0.8512
β_{22}	-27.2	4.8	-5.7	0.6545
β_{33}	-0.2	0.1	-1.6	0.0024
β_{12}	1.8	3.8	0.5	0.0827
β_{13}	-1.6	0.8	-2.2	0.3288
β_{23}	0.8	0.8	1.1	0.1741

[a] β_0 intercept & β_1, β_2, and β_3 constant coefficients of incubation time of Pronase®, α-amylase, and conjugase, respectively.

Table 4.10 Regression Coefficients of the Predicted Second-Order Polynomial Model of buckwheat flour

Constant Coefficient [a]	Estimate value	Standard error	Computed t-value	Pr > I t I
β_0	29.6	3.6	8.2	0.0004
β_1	8.4	7.1	1.2	0.2884
β_2	18.8	7.1	2.7	0.0451
β_3	2.1	1.1	2.0	0.1067
β_{11}	-5.0	4.8	-1.0	0.3531
β_{22}	-11.3	4.8	-2.3	0.0667
β_{33}	-0.1	0.1	-1.0	0.3774
β_{12}	0.6	3.8	0.2	0.8747
β_{13}	-0.4	0.7	-0.5	0.6292
β_{23}	1.2	0.7	1.7	0.1572

[a] β_0 intercept & β_1, β_2, and β_3 constant coefficients of incubation time of Pronase®, α-amylase, and conjugase, respectively.

Table 4.11 Analysis of Variance for the Second-Order Response Surface Model of CRM121, Wholemeal Flour

Source of variation	df	Sum of squares
Model	9	78.5[a]
Linear	3	68.8[a]
Quadratic	3	5.9[b]
Cross-product	3	3.8[b]
Lack of fit	3	2.8[b]
Pure error	2	0.8
Total error	5	3.6
R^2		0.956

[a] significant at 1% level. [b] not significant

Table 4.12 Analysis of Variance for the Second-Order Response Surface Model of Oat Flour

Source of variation	df	Sum of squares
Model	9	153.7[a]
Linear	3	143.8[a]
Quadratic	3	9.6[b]
Cross-product	3	0.3[b]
Lack of fit	3	3.9[b]
Pure error	2	1.6
Total error	5	5.5
R^2		0.966

[a] significant at 1% level. [b] not significant

Table 4.13 Analysis of Variance for the Second-Order Response Surface Model of Triticale Flour

Source of variation	df	Sum of squares
Model	9	216.6[a]
Linear	3	166.2[a]
Quadratic	3	42.4[b]
Cross-product	3	8.0[c]
Lack of fit	3	2.5[c]
Pure error	2	3.8
Total error	5	6.3
R^2		0.972

[a] significant at 1% level. [b] significant at 5% level. [c] not significant.

Table 4.14 Analysis of Variance for the Second-Order Response Surface Model of Buckwheat Flour

Source of variation	df	Sum of squares
Model	9	192.8[a]
Linear	3	180.0[a]
Quadratic	3	8.7[b]
Cross-product	3	4.1[b]
Lack of fit	3	4.6[b]
Pure error	2	1.8
Total error	5	6.4
R^2		0.968

[a] significant at 1% level. [b] not significant

Table 4.15 Analysis of Variance Showing Significance of the Variables on Responses of CRM121, Wholemeal Flour[1]

Independent variables	df	Sum of squares
Pronase®	4	3.5[a]
α-Amylase	4	30.6[b]
Conjugase	4	48.7[c]

[a] not significant. [b] significant at 5% level. [c] significant at 1% level

[1] Pronase® = digestion time at 2mg/mL, a-amylase = digestion time at 20mg/mL, and conjugase = digestion time at 20mg/4mL

Table 4.16 Analysis of Variance Showing Significance of the Variables on Responses of Oat Flour[1]

Independent variables	df	Sum of squares
Pronase®	4	1.4[a]
α-Amylase	4	50.4[b]
Conjugase	4	102.8[b]

[a] not significant. [b] significant at 1% level.

[1] Pronase® = digestion time at 2mg/mL, a-amylase = digestion time at 20mg/mL, and conjugase = digestion time at 20mg/4mL

Table 4.17 Analysis of Variance Showing Significance of the Variables on Responses of Triticale Flour[1]

Independent variables	df	Sum of squares
Pronase®	4	8.5 [a]
α-Amylase	4	70.3[b]
Conjugase	4	146.3[b]

[a] not significant. [b] significant at 1% level.

[1] Pronase® = digestion time at 2mg/mL, a-amylase = digestion time at 20mg/mL, and conjugase = digestion time at 20mg/4mL

Table 4.18 Analysis of Variance Showing Significance of the Variables on Responses of Buckwheat Flour[1]

Independent variables	df	Sum of squares
Pronase®	4	3.4[a]
α-Amylase	4	89.3[b]
Conjugase	4	104.2[b]

[a] not significant. [b] significant at 1% level

[1] Pronase® = digestion time at 2mg/mL, a-amylase = digestion time at 20mg/mL, and conjugase = digestion time at 20mg/4mL

Table 4.19 Ridge Maximum Analysis with Predicted Maximum Folate

Cereals	Pronase®	α-Amylase (h)	Conjugase	Maximum folate[1] (μg/100g)
CRM121	0.5[a]	2.5[b]	5.7[c]	51
Oat flour	0.6[a]	1.4[c]	5.8[c]	49
Buckwheat flour	0.7[a]	0.8[c]	4.6[c]	52
Triticale flour	0.6[a]	0.7[c]	5.9[c]	71

[a] not significant. [b] significant at 5% level. [c] significant at 1% level.
[1] estimated values with optimized extraction time

Table 4.20 Comparison of Folate Contents from Cereals Measured by the Optimized Extraction and AOAC Method 2004.05.

Cereals	Optimized digestion[a]	AOAC Method 2004.05.
	Folate (μg/100g ± SD)	
Oat flour	48 ± 2.0	47 ± 2.7
Buckwheat flour	53 ± 4.0	57 ± 1.0
Triticale flour	72 ± 4.1	71 ± 5.4
Whole wheat flour	46 ± 3.9	43 ± 2.4

[a] not significantly different at 1% level with AOAC Method 2004.05.

Table 4.21 Comparison of Folate Contents from Breakfast Cereals Measured by the Optimized Extraction and AOAC Method 2004.05.

Breakfast cereals	Optimized digestion[a]	AOAC Method 2004.05.
	Folate (μg/100g ± SD)	
Honey bunches of oats, honey roasted	1224 ± 41.9	1206 ± 23.8
Apple Jack	552 ± 42.0	547 ± 17.2
Malt-o-meal golden puffs	625 ± 18.2	612 ± 29.5

[a] not significantly different at 1% level with AOAC Method 2004.05.

Table 4.22 Determination of Folic Acid, Total Folate, Food Folate, and μg Dietary Folate Equivalent (μg DFE) in Breakfast Cereals

Product	μg/100g ± SD			μg/100g	
	Folic acid	Total folate	Food folate[a]	μg DFE	Mean μg DFE
Lucky Charms (n=4)	1112 ± 53.5	1147 ± 30.5	35	1925	1806
	1029 ± 58.7	1062 ± 54.8	33	1782	
	1015 ± 39.1	1045 ± 63.2	30	1756	
	1015 ± 83.6	1049 ± 27.3	34	1760	
Cheerios (n=1)	758 ± 14.3	803 ± 11.8	45	1334	1334
Kix (n=2)	1005 ± 35.7	1040 ± 18.3	35	1744	1767
	1036 ± 86.7	1065 ± 49.9	29	1790	
Cinnamon Toast Crunch (n=2)	573 ± 51.8	616 ± 18.1	43	1017	997
	553 ± 38.5	590 ± 54.4	37	977	
Frosted Flakes (n=4)	399 ± 11.2	431 ± 22.4	32	710	755
	422 ± 35.1	459 ± 36.0	37	754	
	444 ± 25.2	477 ± 6.5	33	788	
	434 ± 20.9	465 ± 27.3	31	769	
Rice Crispies (n=2)	737 ± 28.3	746 ± 67.5	9	1262	1301
	783 ± 25.1	792 ± 41.3	9	1340	
Froot Loops (n=4)	533 ± 21.4	552 ± 28.4	19	925	878
	486 ± 30.6	509 ± 8.3	23	849	
	514 ± 30.8	531 ± 30.8	17	891	
	488 ± 26.6	505 ± 44.9	17	847	
Raisin Bran (n=3)	401 ± 26.0	435 ± 23.0	34	716	676
	355 ± 31.1	391 ± 20.7	36	640	
	375 ± 30.8	409 ± 9.6	34	672	
Apple Jacks (n=2)	497 ± 34.8	528 ± 24.4	31	876	855
	475 ± 10.1	502 ± 17.0	27	844	
Malt-O-Meal Golden Puffs (n=3)	614 ± 18.8	618 ± 25.9	4	1048	1026
	555 ± 28.0	560 ± 11.9	5	949	
	632 ± 20.4	638 ± 39.0	6	1080	
Grape Nuts (n=1)	631 ± 58.3	663 ± 37.6	32	1105	1105
Honey Bunches of Oats, Honey Roasted (n=2)	1322 ± 68.7	1348 ± 51.7	26	2273	2184
	1212 ± 57.2	1237 ± 23.8	25	2085	

[a] food folate = total folate – folic acid.

Table 4.23 Comparison of Labeled Value to Assayed Value of Folate per Serving Size

Products(g)[a]	Total folate (μg)		% of daily value (400μg)	
	Labeled	Assayed	Labeled	Assayed
Lucky Charms (27)	200	290	50	73
Cheerios (28)	200	225	50	56
Kix (30)	200	316	50	79
Cinnamon Toast Crunch (31)	100	187	25	47
Frosted Flakes (30)	100	137	25	34
Rice Crispies (33)*	100	254	25	63
Froot Loops(29)	100	152	25	38
Raisin Bran (59)	150	243	38	61
Apple Jacks (28)	100	144	25	36
Malt-O-Meal Golden Puffs (27)	100	163	25	41
Grape Nuts (29)	100	192	25	48
Honey Bunches of Oats, Honey Roasted (30)*	200	401	50	100

[a] serving size. [b] dietary folate equivalents. * highly fortified with folic acid compared to what labels say.

Table 4.24 Analysis of μg DFE per serving size of breakfast cereals

Products(g)[a]	Assayed total folate (μg)	Assayed μg DFE[b]	% of DRI (400 μg DFE)
Lucky Charms (27)	290	488	122
Cheerios (28)	225	374	100
Kix (30)	316	530	133
Cinnamon Toast Crunch (31)	187	309	77
Frosted Flakes (30)	137	227	57
Rice Crispies (33)	254	429	107
Froot Loops(29)	152	255	64
Raisin Bran (59)	243	399	100
Apple Jacks (28)	144	239	60
Malt-O-Meal Golden Puffs (27)	163	277	69
Grape Nuts (29)	192	320	80
Honey Bunches of Oats, Honey Roasted (30)	401	655	164

[a] serving size. [b] dietary folate equivalents

Table 4.25 Determination of Folic Acid, Total Folate, Food Folate, and μg Dietary Folate Equivalent (μg DFE) in Snack Food

	μg/100g ± SD			μg/100g	
Product	Folic acid	Total folate	Food folate[a]	μg DFE	Mean μg DFE
Oatmeal Cookies[1] (n=3)	50 ± 4.3 57 ± 1.4 52 ± 1.5	60 ± 5.1 64 ± 1.5 61 ± 3.5	10 7 9	95 104 97	99
Oatmeal Cookies[2] (n=3)	50 ± 4.2 52 ± 1.8 52 ± 5.2	60 ± 2.2 64 ± 5.1 63 ± 1.2	10 12 11	95 100 99	98
Oatmeal Cookies[3] (n=3)	24 ± 0.8 32 ± 1.8 34 ± 0.9	47 ± 5.1 49 ± 2.7 54 ± 2.9	23 17 20	64 71 78	71
Keebler Townhouse Crackers (n=2)	105 ± 3.0 128 ± 6.7	120 ± 3.4 144 ± 10.1	15 16	194 234	214
Triscuit, Reduced Fat (n=3)	N/D[a] N/D N/D	36 ± 2.2 30 ± 2.8 32 ± 1.0	36 30 32	36 30 32	33
Triscuit, Original (n=3)	N/D N/D N/D	38 ± 2.7 32 ± 2.7 32 ± 2.4	38 32 32	38 32 32	34
Wheat Thins, Reduced Fat (n=2)	148 ± 5.0 150 ± 6.8	179 ± 12.7 186 ± 6.0	31 36	283 291	287
Wheat Thins, Original (n=1)	176 ± 2.5	206 ± 14.3	30	329	329
Chocolate Chip Cookies[4] (n=2)	16 ± 1.6 19 ± 0.8	38 ± 3.0 38 ± 1.0	22 19	49 51	50
Chocolate Chip Cookies[5] (n=1)	N/D	15 ± 1.0	15	15	15
Oreos (n=3)	48 ± 2.2 47 ± 2.9 42 ± 2.1	74 ± 4.8 71 ± 4.4 66 ± 2.9	26 25 25	108 104 95	102
Saltine Crakers[6] (n=1)	122 ± 3.6	150 ± 6.1	28	235	235
Saltine Crakers[7] (n=1)	205 ± 14.0	250 ± 16.0	45	394	394

[1] Nabisco Honey Maid. [2] Pepperidge Farms Soft Bakes. [3] Other/Store Brand. [4] Chips Ahoy Chewy.
[5] Entenmanns Original Recipe. [6] Sunshine Original. [7] Nabisco Original Premium.
[a] food folate = total folate – folic acid. [b] ND: Not Detected (< 0.05μg/100g).

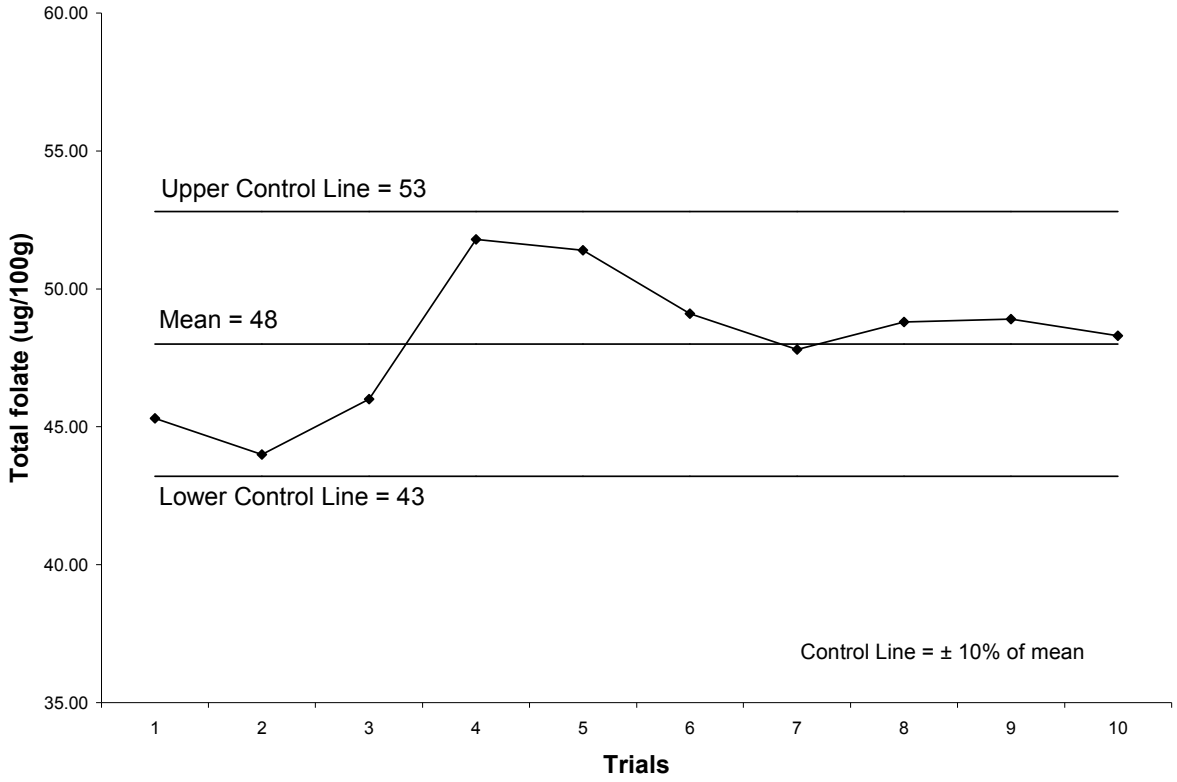

Figure 4.1 Control Chart for Total Folate in CRM 121, Wholemeal Flour

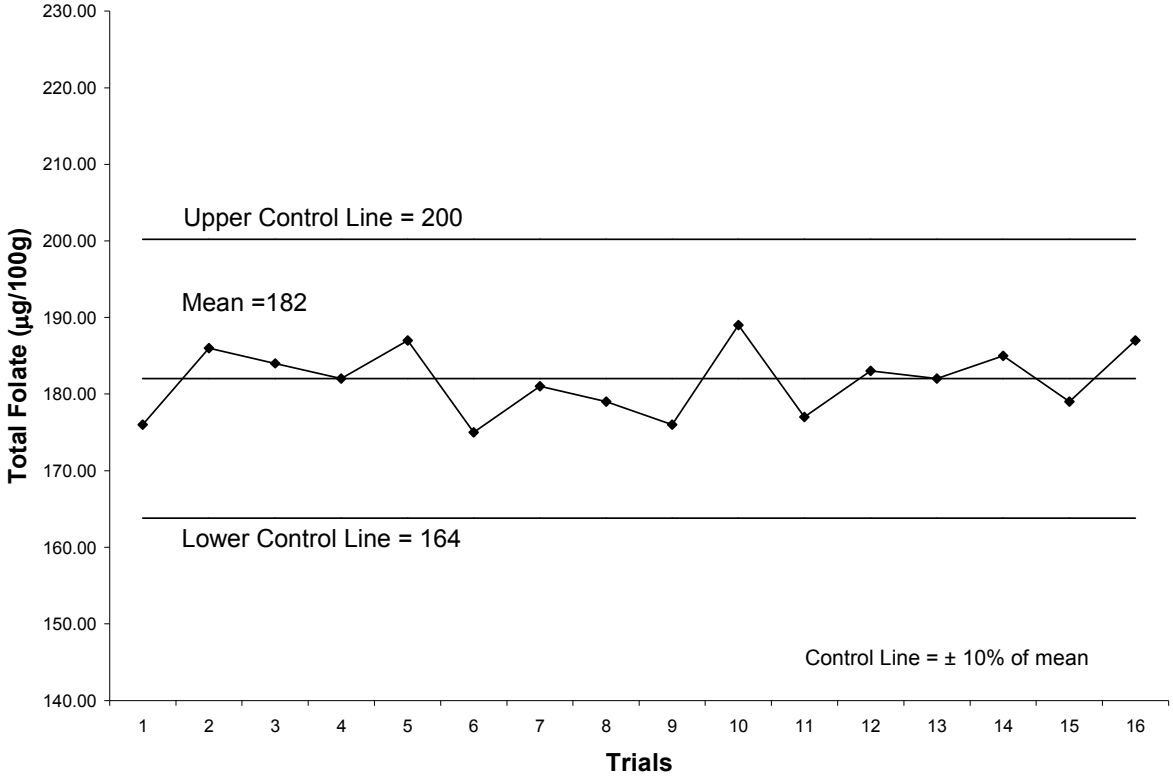

Figure 4.2 Control Chart for Total Folate in Quality Control, Pillsbury All-Purpose, Bleached, Enriched Flour

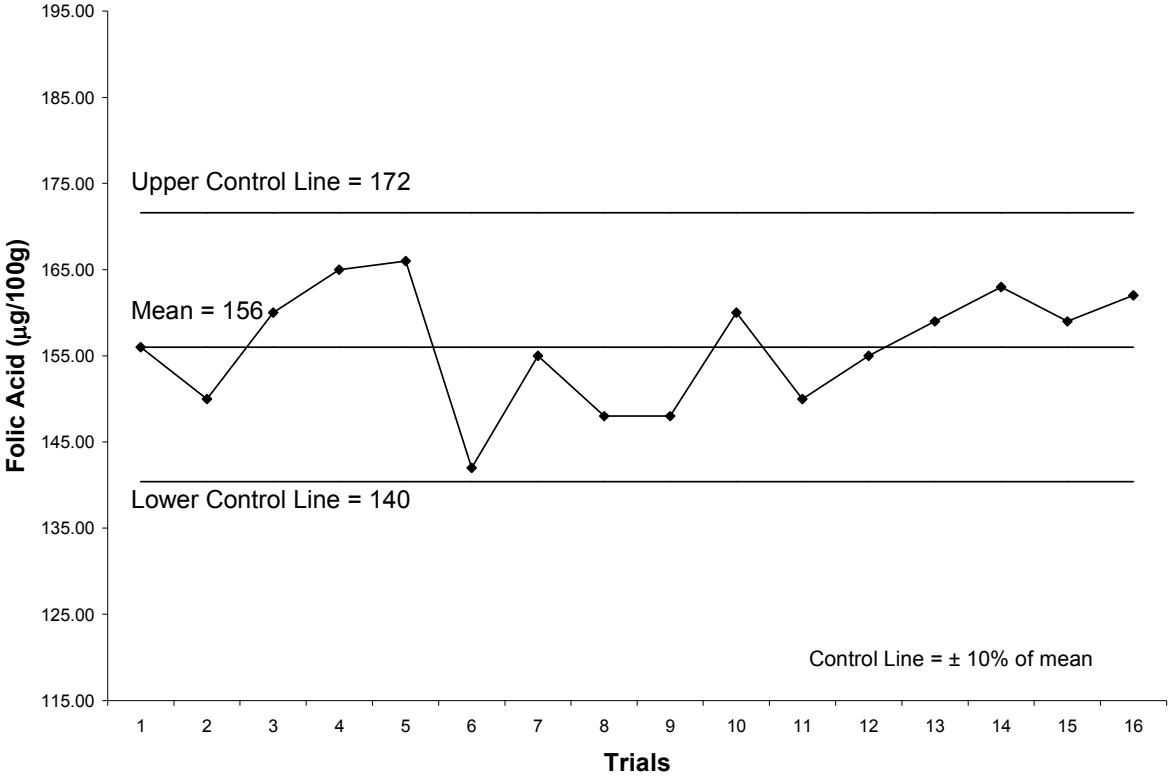

Figure 4.3 Control Chart for Folic Acid in Quality Control, Pillsbury All-Purpose, Bleached, Enriched Flour

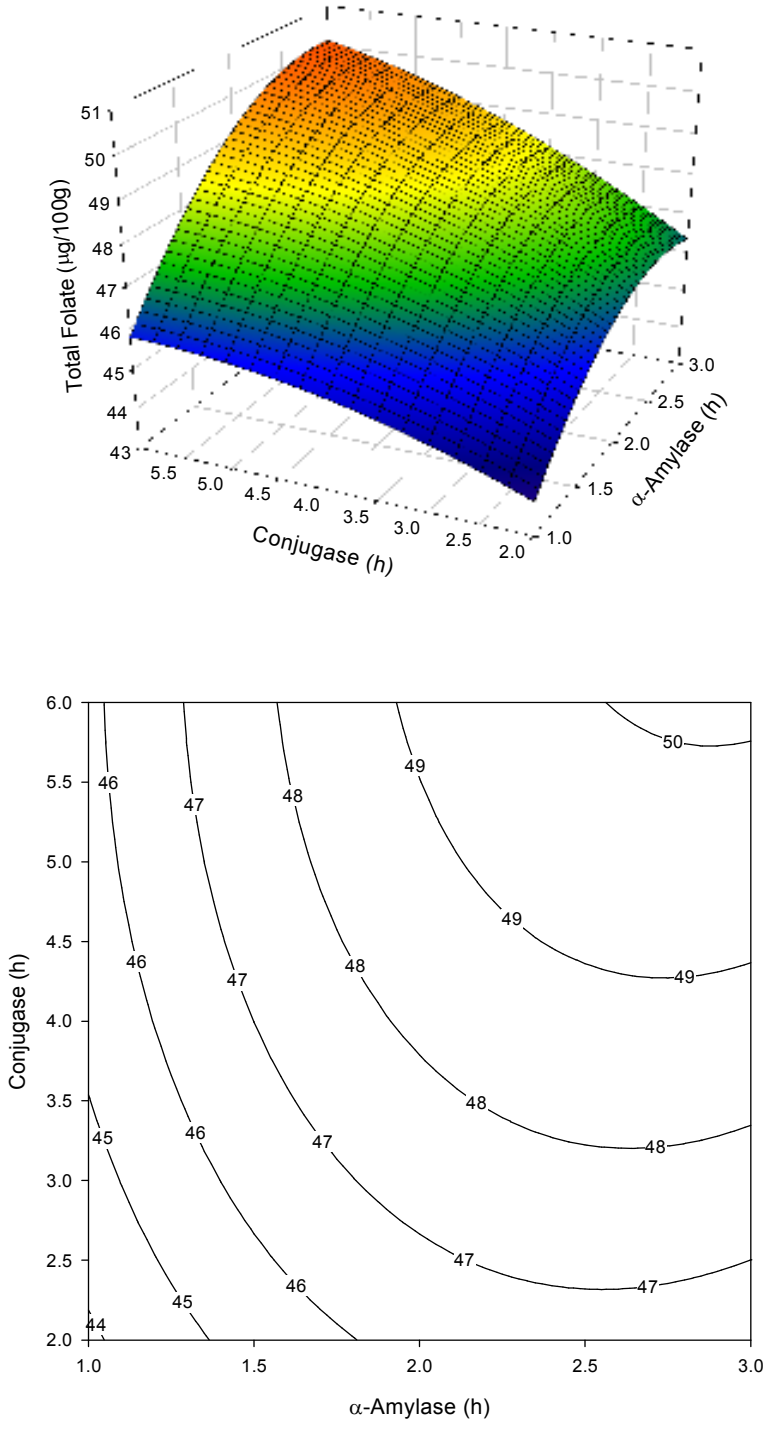

Figure 4.4 Response Surface and Contour Plot for the Effects of α-Amylase and Conjugase Digestion Time on Total Folate Assay of CRM121, Wholemeal Flour

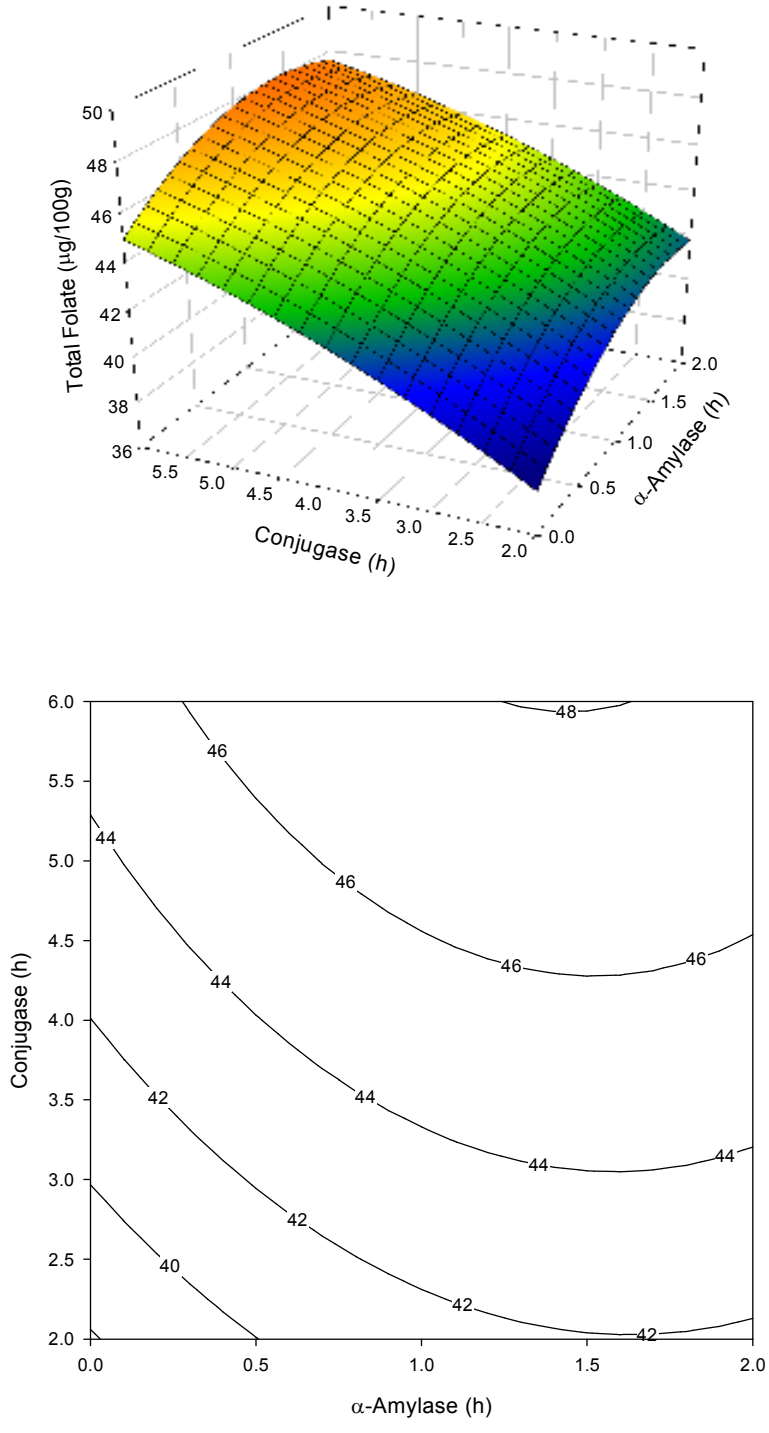

Figure 4.5 Response Surface and Contour Plot for the Effects of α-Amylase and Conjugase Digestion Time on Total Folate Assay of Oat Flour

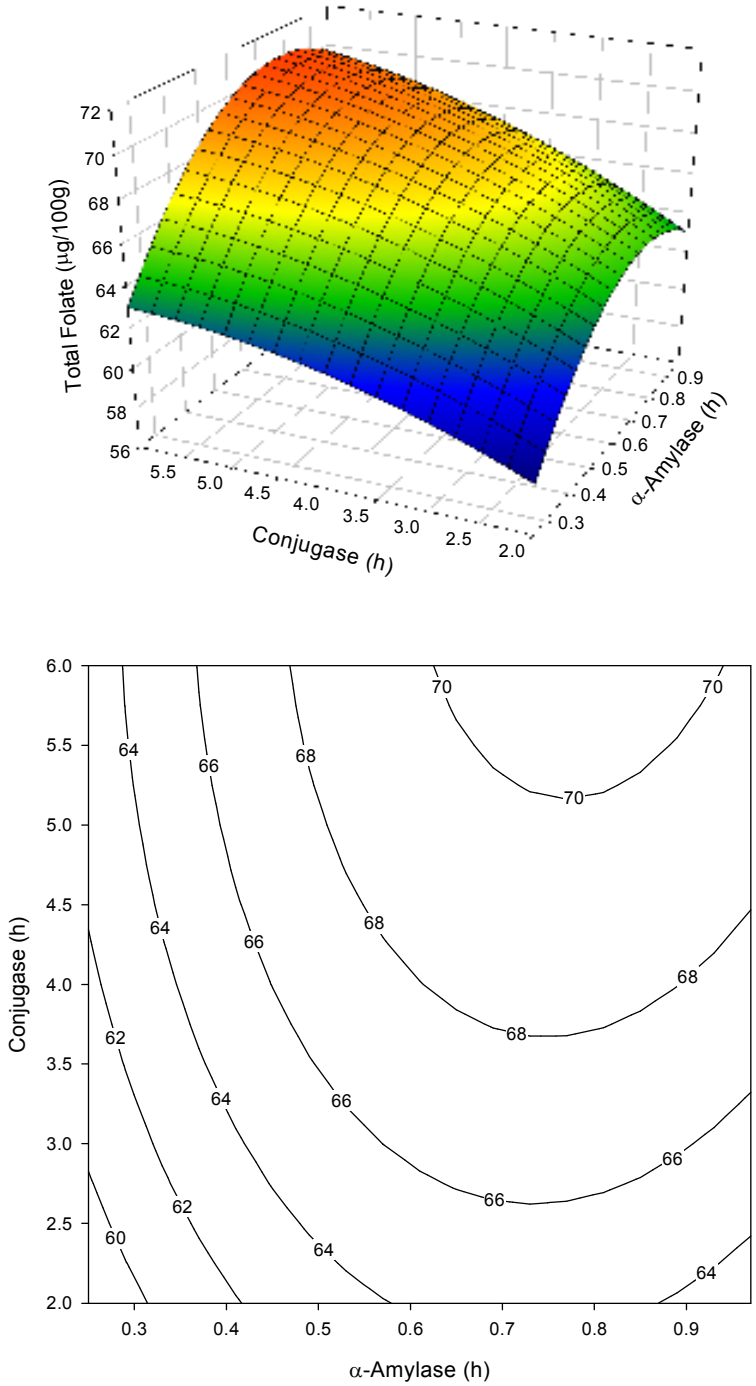

Figure 4.6 Response Surface and Contour Plot for the Effects of α-Amylase and Conjugase Digestion Time on Total Folate Assay of Triticale Flour

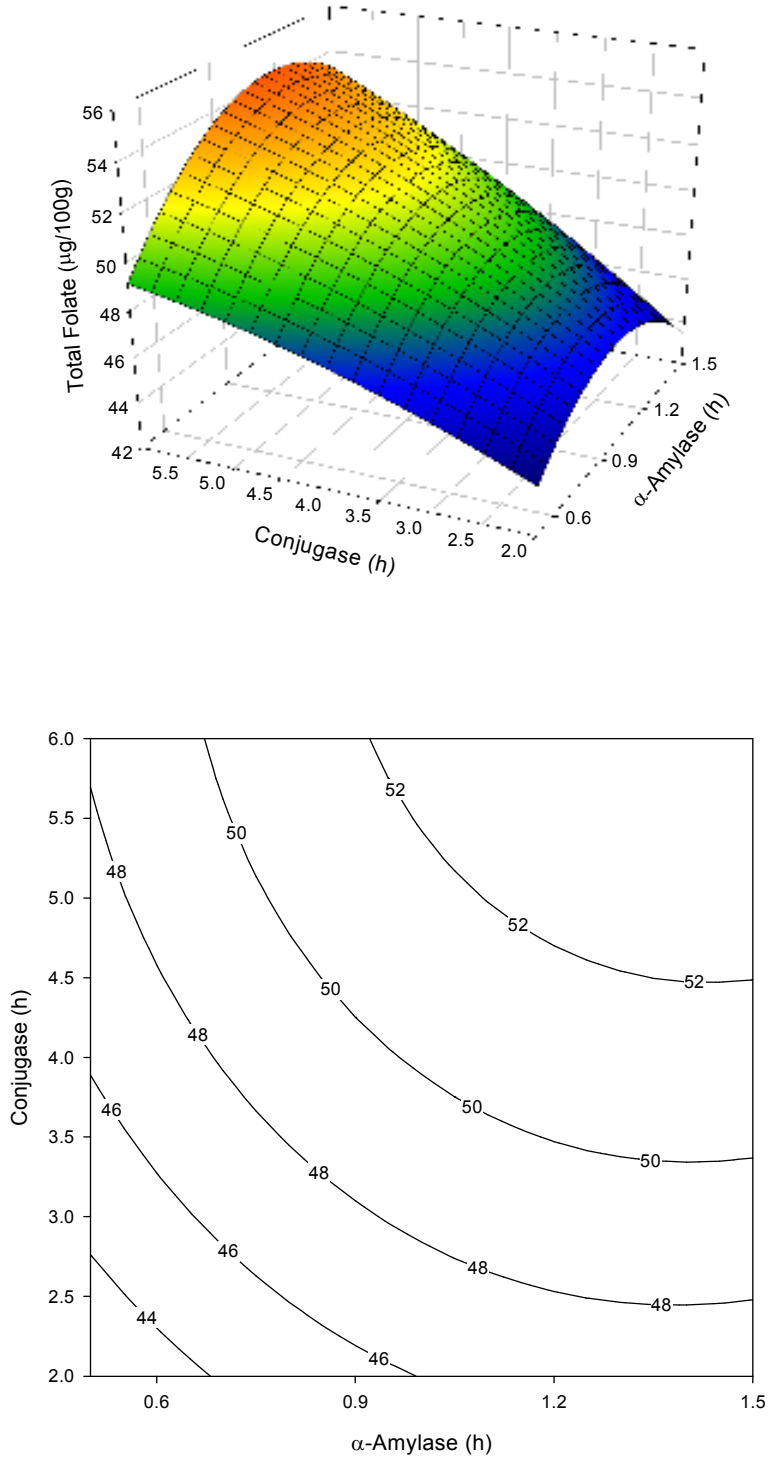

Figure 4.7 Response Surface and Contour Plot for the Effects of α-Amylase and Conjugase Digestion Time on Total Folate Assay of Buckwheat Flour

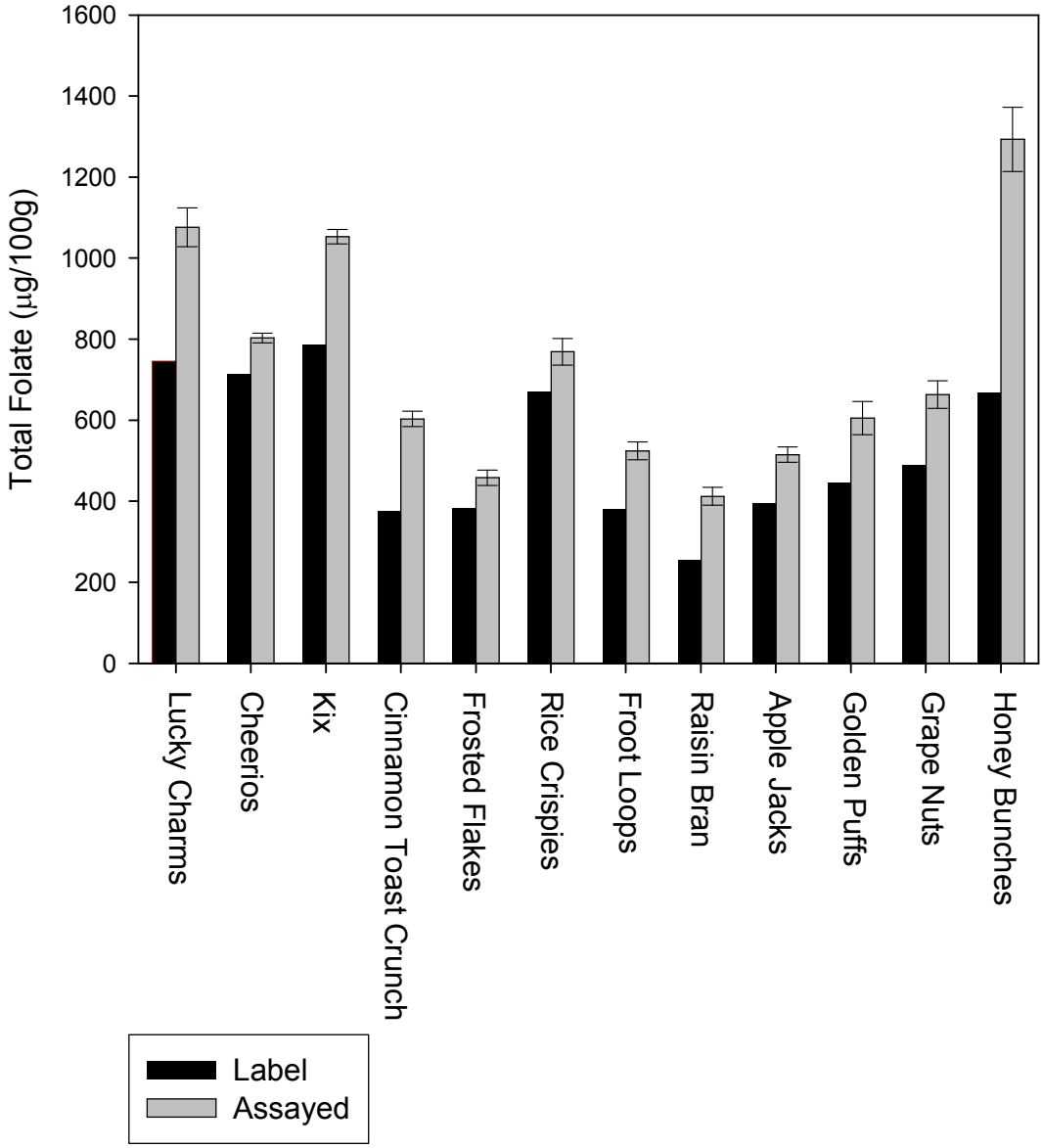

*Figure 4.*8 Comparison of Total Folate Contents of Breakfast Cereals between Labeled and Assayed Values with Standard Deviation

CHAPTER 5

CONCLUSION

The incubation time of trienzyme for AOAC Official Method 2004.05, is 3h, 2h, and 16h for Pronase®, α-amylase, and Conjugase, respectively. However, Chen and Eitenmiller (2007) found that 1.5h, 1.5h, and 3h for Pronase®, α-amylase, and Conjugase, respectively would be enough to release measurable folates in vegetables. Thus, this study was performed to find an optimum trienzyme incubation time for cereals to save time and cost. The generalized optimized incubation time for cereals was 1h, 2.5h, and 6h for Pronase®, α-amylase, and conjugase. The digestion of Pronase® and conjugase was much shorter than the incubation time for AOAC Official Method 2004.05. However, the α-amylase digestion time was 0.5h longer than AOAC Official Method. A differential assay of folic acid and total folate was used to determine folate contents of breakfast cereals and snack food. μg DFE which is not on the labels is calculated because synthetic folic acid has higher bioavailability than food folate; dietary reference intake for folate is 400 μg DFE/day. 676 to 2184 μg DFE/100g in breakfast cereals and 15 to 394 μg DFE/100g in snack samples were determined. μg DFE values will provide better information of folate content in food products to customers. Also, analyzed values of breakfast cereals exceeded label declarations by 112% to 200% more of the labeled values. Exact label values of breakfast cereals should be needed.

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