FOLIC ACID, TOTAL FOLATE AND FOOD FOLATE IN PIZZA AND OTHER FAST FOODS

By

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(Under the Direction of Ronald R Eitenmiller)

ABSTRACT

Folic acid and total folate was determined to allow calculation of food folate and the µg of Dietary Folate Equivalent (µg DFE) in fast foods and pizza. The total folate was measured after trienzyme extraction using microbiological assay by *Lactobacillus casei spp. rhamnosus*. Pizzas (125) and various fast foods (137) were studied. Quality control studies showed that accuracy, trueness and recovery of the analytical method met required standards. The folic acid content in pizzas ranged from 36-73 µg/100g, total folate from 69-109 µg/100g and µg DFE ranged from 94-161. In fast foods, the folic acid content ranged from 9-43 µg/100g, total folate from 37-73 µg/100g and µg DFE from 44-91. Retention upon baking was quiet high for all pizza types except for folic acid in cheese pizza. Calculation of nutrient density showed that pizza and fast foods are excellent sources of folate.

INDEX WORDS: Folic Acid, Total folate, Food folate, DFE, Pizzas, Fast foods, Retention, Moisture content,

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DEDICATION

To my beloved husband and to be born daughter

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I thank my loving husband for his unconditional love and encouragement, for putting up with me through thick and thin. I thank my parents for always giving me the best in life and my in-laws for their constant support and encouragement.

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CHAPTER 1

INTRODUCTION

Fast food consumption in the United States has been dramatically increasing since the 1970's. Pizza consumption alone has soared by 115 percent between 1977 and 1994 (Putnam and Allhouse, 1999). The percentage of energy intake from consumption of pizza among adolescents has increased from 1.6 to 4.7% between 1977 and 1996 and from 1.4 to 3.8% for young adults (Neilsen et al., 2002). There are approximately 61,269 pizzerias in United States, and Americans eat 100 acres of pizza per day or 350 slices per second (http://www.pizzaware.com). Each person in America eats an average of 46 slices or 23 pounds of pizza per year and approximately 3 billion pizzas are sold each year. According to the Gallop poll, children between ages 3 and 11 prefer pizza over all other groups for lunch and dinner. Pepperoni is one of the favorite toppings and mozzarella cheese usage on pizza represents 30% of the total mozzarella cheese output. Since fast food consumption in the United States seems to be growing every year, efforts are increasing by the United States Department of Agriculture (USDA) to improve the nutrient database for these foods.

The USDA maintains the National Nutrient Database for Standard Reference (SR) which is the major source of food composition data in the United States. It is the foundation of most food composition databases in the public and private sectors. The new version, Release 17(SR17), contains data for about 6,839 food items and 128 food components (USDA Nutrient Data Laboratory, Standard Reference 17, 2004). This nutrient database is used in monitoring nutrient intakes in the National Food and Nutrition Surveys conducted by the USDA and the U.S

Department of Health and Human Services (HHS). The Nutrient Data Laboratory (NDL) of the USDA has used a key word approach to select foods for nutrient analysis for over 15 to 20 years (Hepburn, 1987; Haytowitz et al., 1996). This approach allows the NDL to contribute significant amounts of information on the nutrient composition of foods of nutrients of public health interest. The NDL in cooperation with the National Heart, Lung and Blood Institute of National Institute of Health has initiated the National Food and Nutrient Analysis Program (NFNAP) to improve the quality of the nutrient databank.

Folic acid is an important water soluble vitamin that functions as a co-factor in single-carbon metabolism reactions, amino acid interconversions, synthesis and repair and functioning of DNA. The importance of adequate folate nutriture was well recognized in the past decade as significant to the reduction of neural tube defects (NTD) (in infants) and in prevention of cardiovascular disease by lowering the plasma homocysteine concentrations. Several clinical studies revealed that the risks of occurrence of NTD in infants are decreased by the fortification of food with folic acid to improve the mothers' folate status prior to conception (Honein et al., 2001; Williams et al., 2002; Gucciardi et al., 2002). Beginning January, 1998, the FDA implemented mandatory fortification of all enriched cereal grain products at levels ranging from 0.43 mg to 1.4 mg per pound of product with folic acid to prevent the NTD in infants (FDA, 21 CFR parts 136, 137 &139). After the initiation of fortification, there was a critical need to update the old databases in the nutrient databank to allow better calculation of the dietary folate intakes for nutrition monitoring and food safety evaluations. The FDA is interested in continually monitoring both the effectiveness and safety of fortification practices since it has the responsibility for safety of the food supply.

Since then, the Institute of Medicine introduced the µg Dietary Folate Equivalent (µg DFE) (IOM, 2002). Since the calculation of µg DFE requires analytical determination of folic acid and food folate, further problems were presented to the nutrition community. Older analytical data almost always presents total folate values and not independent values for folic acid and food folate. Therefore, calculation of µg DFE was problematic.

A survey of the literature indicated that little information is available on the folic acid and the food folate content of various fast foods containing enriched flour as a primary ingredient. The purpose of this study was to determine the folic acid and the total folate content in food samples containing enriched flour collected through the USDA's NFNAP using the microbiological method and the trienzyme treatment. The specific objectives of this study were:

(1) To determine the folic acid and the total folate levels in various fast foods with emphasis on pizza and to calculate food folate from the analytical data. This approach allows the calculation of the µg DFE in the food.

(2) To determine the effect of baking on the retention of the folic acid and the total folate in pizzas.

(3) To contribute information for updating the USDA National Nutrient Database.

CHAPTER 2

LITERATURE REVIEW

Since the initial discovery in India by Willis that yeast extracts could prevent macrocytic anemia in pregnant women, folate has been implicated in numerous disease states. Folate gets its name from the Latin word "folium" for leaf, since it was first extracted from spinach (Wills, 1931).

Folate and Folic Acid

Folate refers to natural forms that occur in the food. Folic acid is the synthetic form found in vitamin supplements and fortified foods. Some of the natural sources of folate include leafy green vegetables (like spinach and turnip greens), fruits (like citrus fruits and juices), dried beans and peas and berries; all natural sources of folate (USDA Nutrient Data Laboratory, Standard Reference 17, 2004). Folate is the general term used for this vitamin, and it exists in many chemical forms (Wagner, 1996). Synthetic folic acid is more stable form, has a simpler chemical structure and is absorbed more easily by the body. Folate is absorbed at about 50% efficiency, while folic acid is absorbed at 85%-95% efficiency (Bailey, 2004).

Structure of Folic Acid

Folic acid (Figure 2.1) is 2- amino –4 hydroxy-6-methylene aminobenzoyl L-glutamic acid pteridine. Folate is a complex organic compound found in the liver, yeast and other substances while folic acid is synthetic. Pteroic acid 4-{(pteridin –6-ylmethyl) amino} benzoic acid is the parent compound. It contains a pterin core ring structure which is conjugated to para-

aminobenzoic acid via a methylene bridge to form pteroic acid (Bailey, 1995). The carboxy group of the para-aminobenzoic acid is bound via a peptide to the a –amino group of the glutamate to form folic acid. Folate and folic acid are the preferred synonyms for polypteroylglutamate and pteroylglutamic acid (single glutamic acid), respectively (Bailey, 1995). The term folate refers to the large group of heterocyclic compounds that are based on the pteroic acid structure conjugated with two or more L-glutamates linked through the gamma carboxyl of the amino acid. The pterin ring portion may be in the oxidized state as in pteroylglutamic acid or may be reduced to dihydrofolate (DHF) or tetrahydrofolate (THFA). The pteroylglutamates and their corresponding acids are named after the number of glutamate residues attached: for example, pteroyldiglutamic acid with two glutamates and pteroylpolyglutamate when more than two glutamates are attached. The active co-enzyme forms of pteroylglutamic acid are N-5 methyl, N-5 or N-10 formyl, N-5 formimino,N-5,10 methylene, and N-5,10 methenyl folate, all of which have one carbon unit at N-5 or N10 or between N-5 or N-10 of the pterin ring (Eitenmiller and Landen, 1998). Although various forms exist, only the reduced forms are biologically active. Folates in foods are labile; being extremely sensitive to oxidation, light, heat, and extremes of pH. The level of instability differs with the individual vitamers. Gregory (1989) reviewed the physical and chemical properties of folic acid and folate in detail. Gregory (1989) summarized the solubility characteristics. For example, pteroic acid is less soluble than folic acid over most of the pH scale; whereas, the solubility of folic acid depends on the polar hydrophilic character of the a-carboxyl group of glutamic acid. At neutral to alkaline pH levels, polyglutamyl folates are more anionic than folic acid due to the presence of additional ionizable a-carboxyl groups of glutamic acid.

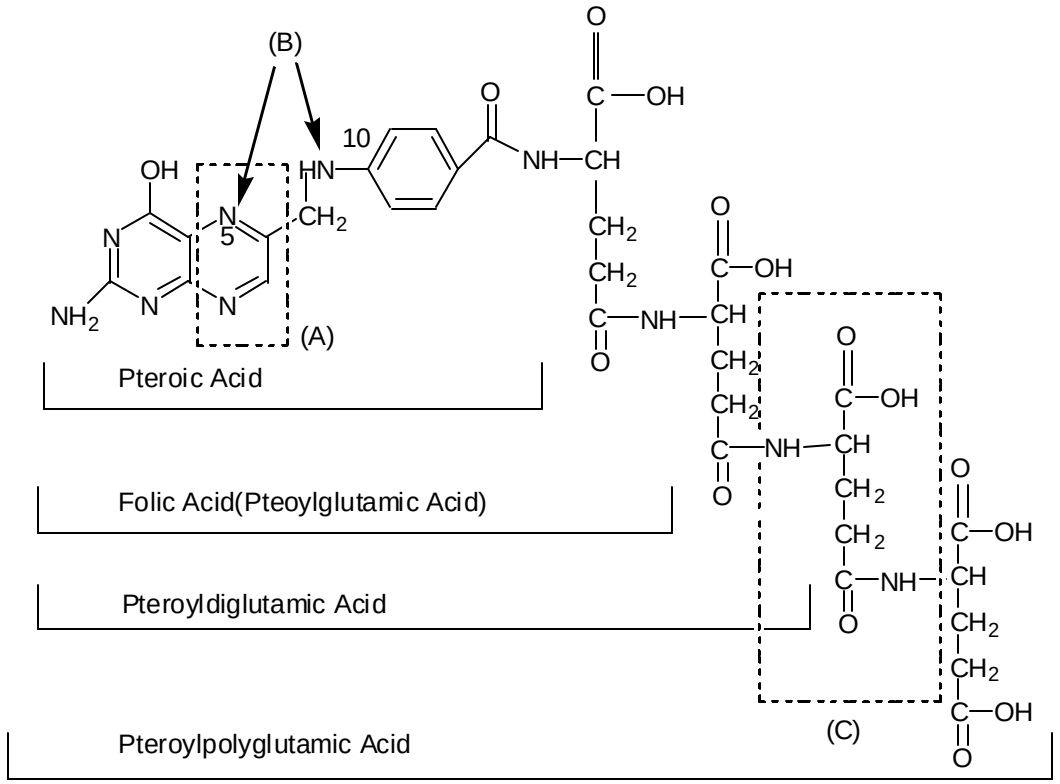


Figure 2.1
Structural Relationship of Folates and Folic acid.

Function of Folate

Folate functions in single-C metabolism. The various forms act as acceptors or donors of single C-units. They are primarily involved as coenzymes in the transfer of single carbon units (IOM, 2002) (methyl, methelene, methenyl, formyl, formimino groups). Folate coenzymes are involved in various metabolic reactions, including amino acid interconversions such as conversion of histidine to glutamic acid, serine to glycine and homocysteine to methionine. In

roles related to the cell division, they function in the synthesis, repair, and functioning of the DNA (Wagner, 1996). Folate also helps to maintain the nervous system and functions of the intestinal tract (Wagner 1996; IOM, 2002). Folate plays a vital role in the reproduction of cells in the fetus and is highly essential whenever there is a rapid proliferation of cell growth, especially during pregnancy, infancy and child growth. Lastly, it acts as the carbon carrier in the formation of the "heme" and is essential in the formation of the red blood cells.

Clinical Effects of Inadequate Intake

Clinical symptoms of folate deficiency are manifested by morphological changes in the cells of the hematopoietic system. Megaloblastic anemia is the major clinical manifestation of folate deficiency resulting in slowed DNA synthesis (Herbert and Coleman, 1979). Since folate helps in the cell division, localized deficiency may be implicated in the initiation of cancer. Several studies reported deficiency of folic acid as a cause for abortions. Inadequate folate intake first leads to a decrease in serum folate concentration, leading to a decrease in the erythrocyte folate concentration. The concentration of homocysteine increases and leads to megaloblastic changes in the bone marrow and other tissues with rapidly dividing cells characterized by impaired DNA synthesis. Then macrocytic anemia develops because of reduced erythrocyte count. Eventually, all three measures of anemia (hematocrit hemoglobin concentration and erythrocyte concentration) are depressed. According to several studies conducted by Eicher et al. (1971) and Hoffbrand et al. (1966), the serum folate level in the blood directly leads to the decrease in the erythrocyte folate concentration. Symptoms of weakness, fatigue, difficulty concentrating, irritability, headache, palpitations and shortness of breath appear in the advanced stages of anemia. Some of these symptoms may be milder in some elderly patients.

Importance of Folate in Health and Disease

Prevalence of Folate Deficiency

Since folate plays an important role in various physiological functions of the body, deficiency leads to serious symptoms. World wide interest in folate nutrition has increased since the discovery of it's relation with various diseases, including anemia, cardiovascular diseases, neural tube defects (NTD) and cancer (Massaro and Rogers, 2002). Neural tube defects result from the failure of the neural tube to close during the first four weeks of gestation (Massaro and Rogers, 2002). These birth defects can result in various disabilities after birth that can also lead to infant mortality (Yetley and Rader, 2004). Since the closure of the neural tube occurs between 18 and 27 days after conception, the defect may occur before the knowledge of conception. Approximately, 2500 infants are born each year with NTD'S among the 4 million births in the United States (Flood et al., 1992). Additionally, 1500 fetuses with NTD'S are aborted therapeutically after detection by the prenatal diagnosis, and an unknown number is lost due to spontaneous abortion in early pregnancy. The occurrence of the NTD'S vary with a wide range of factors including genetics, geography, socioeconomic status, month of conception, race, nutrition, and maternal health, including maternal age and reproductive history (Flood et al., 1992). Although the mechanism of action of folate in influencing the risk of NTD is poorly understood, the evidence of the benefits of folic acid addition to the diet has led several organizations in United States and around the world to recommend fortification programs (Centers for Disease Control, Food and Drug Administration, Bio Med Central, Canada). The most common defects of NTD include neuroblastoma, spina bifida and anencephaly.

Neuroblastoma is characterized by the formation of a malignant tumor in the brain. It is also the most commonly diagnosed malignant tumor of infancy since the development of the tumor occurs when the baby is still in the uterus (Gurney et al., 1997; Gao et al., 1997). This defect occurs in children younger than 5 years of age. The prevalence of this disease is one in 6000 to 7000 children (Bernstein et al., 1992). The aggressive nature of this tumor makes this disease the most common cause of cancer-related death among children 1 to 4 years old (Young et al., 1986). During the past decade, maternal folate status has been implicated in the development of fetal pathologic conditions. For example, periconceptional intake of folic acid, 0.4 to 4 mg/day, can prevent most cases of NTD (Czeizel et al., 1992; Wald et al., 2001). Two recent case-control studies also showed a lower risk of childhood acute lymphoblastic leukemia (ALL) and primitive neurectodermal tumors in association with use of maternal folic acid supplement tablets in pregnancy (Thompson et al., 2001). Prenatal multivitamin supplementation also was associated with a lower risk of childhood brain tumors and neuroblastoma. (Olshan, 2002)

Spina bifida is a condition where the spinal cord is exposed (meningomyelocoele). It results from the failure of the spine to close properly during the first month of the pregnancy. In several cases the spinal cord protrudes through the back and is covered by only a thin membrane. Babies born with spina bifida grow into adulthood with various disabilities including mental disabilities and paralysis (Mitchell et al., 2004).

Anencephaly is the absence of the brain. It results from failure of fusion in the cranial region of the neural tube (Mitchell et al., 2004). However, the definitive cause is not known in most cases. Up to 70% of spina bifida cases could be prevented by periconceptional folic acid supplementation. Inadequate intake of natural folate, or its synthetic form, folic acid, before and

during early pregnancy is associated with an increased risk of spina bifida and anencephaly (Mitchell et al., 2004). Some of the case-control studies, randomized clinical trials, and community-based interventions with vitamin supplements have shown that the failure to consume folic acid supplements or folic acid-containing multivitamins increases the risk of having an affected child by two to eight fold (Wald et al., 2001). Moreover, the risk of having a child affected by a NTD is indirectly related to both folic acid intake (from dietary sources and supplements) and maternal folate status (Moore et al., 2003; Wald et al., 2001).

Although mechanism of action of folic acid in influencing the risk of NTD is poorly understood, the evidence of the benefits of folic acid supplementation is indisputable. Several clinical studies have been carried out that revealed the risk of occurrence of NTD was decreased by the periconceptional supplementation of folic acid (Werler et al., 1993;Laurance et al., 1981; Czeizel et al., 1993; Smithells et al., 1981; Vergel et al., 1990; MRC Vitamin Study,1991). Evidence also suggested that consumption of multivitamins with folic acid also reduced the occurrence of NTD (Czeizel et al., 1992; Bower et al., 1989; Milunsky et al., 1989; Werler et al., 1993; Shaw et al., 1995).

Folate and Heart Disease

Homocysteine is an amino acid which is an intermediary in the metabolic pathway of methionine. The only source of homocysteine in humans comes from methionine in the dietary proteins of animal origin. Methionine is the only essential sulfur containing amino acid. Methionine and homocysteine have common regulatory mechanisms and metabolic functions, since they interconvert into one another. Homocysteine is metabolized by remethylation or trans-sulfuration (De la Calle et al., 2003). The total homocysteine in the plasma consists of four

forms; 1% of it circulates as free thiol, 80% is present as a disulphide bound to plasma proteins and 20% forms dimerhomocysteine or homocysteine mixed disulfide (Ueland, 1995). Since one of the functions of the folate involves in the conversion of homocysteine to methionine, it has a vital role in cardiovascular health. A reciprocal relationship exists between blood homocysteine and water soluble vitamins in the blood (particularly folate). The possibility that the mechanism by which supplemental folic acid acts to reduce the risk of NTD also involves the reduction of serum homocysteine levels (Wald et al., 2001; Steegers et al., 1995). Recent meta analysis showed that folic acid was the most effective homocysteine lowering agent compared to vitamin B6 and vitamin B12. In a study by Wald et al. (2001), 0.8 mg/day of folic acid could to produced maximum reduction in plasma homocysteine levels. Addition of vitamin B-12 to folic acid had an additional homocysteine lowering effect. Because of this, folic acid supplements, especially when in combination with vitamins B6 and B12, may offer a preventative measure against cardiovascular diseases. (Brattstron et al., 1988 and Schorah et al., 1998).

It has been calculated that 9% of male and 54% of female coronary artery deaths in the United States (around 50,000 deaths/year) could be prevented by mandatory fortification of grain products with 350 mg folic acid /100 g food (Motulsky et al., 1996). According to Boushey et al.(1995) elevated levels of homocysteine in the blood have been regarded as a risk factor in developing coronary heart diseases(CHD) and in some cases leads to death from CHD (Anderson et al., 2000). The homocysteine lowering effect of folic acid was found to plateau at daily doses of 0.4 –0.5 mg (Doshi et al., 2002). Evidence also suggests that these daily doses could be achieved by intake of fortified cereals (Malinow et al., 1998).

Folate and Cancer

Folate metabolism is linked to cancer on-set through the concept of localized folate deficiency. The investigation of the possible protective role of folate in carcinogenesis has been reviewed by (Glynn and Albanes, 1994). A possible mechanism of cancer prevention lies in the fact that folate helps in DNA synthesis, especially in methylation reactions as well as in DNA repair. Diminished folate status is associated with higher risk of carcinogenesis (Bailey, 1995). In humans, hypomethylation of DNA has been observed in colorectal cancers (Glynn and Albanes, 1994). Some of the most current findings on folate and cancer development indicate that low levels of folate are a risk factor for breast cancer when associated with high alcohol intake (Zhang et al., 1999). Folate antagonists have been used in the cancer treatment for nearly half a century, and methotrexate, which was discovered prior to 1950, remains the most widely used chemotherapeutic agent (Seeger et al., 1949; Allegra et al., 1990). Methotrexate is also used in treatment of rheumatoid arthritis which is administered along with folic acid to prevent the toxicity of methotrexate.

Fortification

Fortification of the food supply is an effective way to improve the overall nutrition of the general population.The FDA established standards of identity for several enriched cereal grain products.These standards required fortification of various vitamins at specific levels. Cereal grain products were chosen as the delivery vehicles for several vitamins and minerals, since they are the staple food of most of the population in the U.S and also provide a significant percentage of the daily energy intake. Food fortification has the advantages of reaching the target population without changing dietary patterns.

Proposing Fortification

Fortification of food with folic acid was a difficult decision since a safety assurance had to be made before adding the vitamin to the staple food of 290 million Americans who could be exposed to an excess amount of vitamin in high dose. The first proposal for the fortification of food with folic acid started in the 1973. Since then, various studies were conducted for the safe intake of the folic acid. After careful review, in September, 1992, the U.S Public Health Service (PHS)issued a recommendation that "all women of child bearing age in the United States consume 0.4 mg of folic acid per day to reduce their risk of a neural tube defect in pregnancy." The Centers for Disease Control and Prevention issued a recommendation for the use of folic acid to reduce the NTD in pregnancy (MMRW, 1992). The U.S PHS recommendation proposed three possible ways for women to consume folic acid; increased dietary folate intake, supplement use and the use of fortified food. Since it was difficult to change the dietary patterns in women and it was assumed that less than 25% percent of women of child bearing age would comply in taking folic acid supplements everyday, fortification of enriched cereal grain products with folic acid was recommended by the FDA.

The mandatory fortification of folic acid to all the foods has some limitations. Apart from the target population receiving the right dosage, the non target population could be overexposed to the vitamin. Thus, the general dietary guidelines for fortification were set up in 1993 (Federal Register, 1993). FDA proposed an amended food additive regulation; wherein, folic acid is allowed to be added only to certain foods that have a standard of identity and to limit addition to non standardized foods like breakfast cereals and dietary supplements (Feinleib et al., 2001).

S*afety Upper Intake Level of folate*

Based on the scientific evidence, 1000 µg of folate was set as a safe upper limit of intake by the Institute of Medicine in 1998. Thus ,the PHS recommended that "the intake of women of child bearing age should not exceed intakes of 1000 µg per day, since above this level the folic acid masks the symptoms of pernicious anemia caused by vitamin B12 deficiency" (Yetley et al., 2004). A study by Bailey et al. (1995) showed that there is a risk of masking of pernicious anemia in patients who were treated with 1000 µg of folic acid per day. After peer review of the available data, FDA concluded that if the folate intakes were less than 1000 µg daily in patients with vitamin B12 deficiency; then, the risk level was minimal. According to a recent recommendation, in countries with prevalent vitamin B12 deficiency, the food should be fortified with folic acid and also vitamin B12 (Freire et al., 2004).

Dietary Reference Intakes (DRIs)

Recommendations for intake of folate are given in the Dietary Reference Intakes developed by the Institute of Medicine of the National Academy of Sciences (IOM,2002). Dietary reference intakes is the general term for a set of reference values used for planning and assessing nutrient intake for healthy people. Four important types of reference values included in the DRIs are Recommended Dietary Allowances (RDA), Adequate Intakes (AI), Estimated Average Intake (EAR) and Tolerable Upper Intake Levels (UL). The RDA recommends the average daily intake that is sufficient to meet the nutrient requirements of nearly all (97-98%) healthy individuals in each age and gender group. An AI is set when there is insufficient scientific data available to establish a RDA. The AIs meet or exceed the amount needed to maintain a nutritional state of adequacy in nearly all members of a specific age and gender

group. The EAR is the daily intake value that is estimated to meet the requirement of half of the healthy individuals. The UL, on the other hand, is the maximum daily intake unlikely to result in adverse health effects (IOM, 2002)

µg Dietary Folate Equivalent (µg DFE)

The RDAs for folate are expressed in a term called the µg Dietary Folate Equivalent (µg DFE). The µg DFE was developed to help account for the differences in absorption of naturally occurring dietary folate and the more bioavailable synthetic folic acid. Since many studies have proved that folic acid taken with food is 85 percent bioavailable while food folate is only 50 percent bioavailable. Folic acid taken with food is, therefore, 1.7 (85/50) times more bioavailable than folate. When a mixture of folic acid and food folate is taken, µg DFE are calculated by the following formula:

µg of DFE provided = µg of food folate + (1.7*µg of folic acid).

According to Gregory (1997), supplements of folic acid when consumed on an empty stomach are nearly 100 percent bioavailable. Hence, compared with food folate, only half as much of folic acid is necessary if taken on an empty stomach. Therefore, 1 µg of DFE provided = 1 µg of food folate = 0.5µg of folic acid taken on an empty stomach = 0.6 of folic acid taken with meals. The RDA for folic acid according to the Institute of Medicine after fortification is given in the following tables. Table 2.1 lists the RDA of the folate for children and adults expressed in µg DFE .Table 2.2 lists the UL of folate for children and adults expressed in µg DFE.

Table 2.1

Recommended Dietary Allowances for Folate for Children and Adults

Age (years)	Males and Females (µg DFE /day)	Pregnancy (µg DFE /day)	Lactation (µg DFE /day)
1-3	150	N/A	N/A
4-8	200	N/A	N/A
9-13	300	N/A	N/A
14-18	400	600	500
19+	400	600	500

Table 2.2

Tolerable Upper Intake Levels for Folate for Children and Adults

Age (years)	Males and Females (µg DFE /day)	Pregnancy (µg DFE /day)	Lactation (µg DFE /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

The final focus was to increase the intake by the target population of women of child bearing age and to ensure safe intakes for all the other age and gender groups (Yetley et al., 2004). The health claim for dietary supplements in relation to the neural tube defects was authorized in 1994. On March 17,1996, the FDA issued a final rule effective from January 1st of 1998 that required all flour and enriched cereal grain products to be fortified with folic acid at levels ranging from 0.43 mg to 1.4 mg per pound of the product **(**FDA, 21 CFR parts 136,137&139**).** Amendments of standards of identity were recognized for enriched grain products for addition of folic acid (Federal Register,1996). Similarly, the fortification was

mandated in some countries around the world including Canada, which mandated the fortification in 1998 (Canada Gazette Part II, 1998), and Australia.

Postfortification Effects

According to the results of the study conducted by Honein et al. (2001) to check the prevalence of the NTD after fortification, the birth prevalence of NTD decreased by 19 percent after mandatory fortification when compared to those before fortification. Williams et al. (2002) studied the prevalence of the NTD like the spina bifida and anencephaly from 1995 (before fortification) to 1999(after fortification). Combined data from 24 population based surveillance programs showed that the prevalence of spina bifida and anencephaly decreased by 31% from pre to post fortification periods. A population based study was carried out in Nova Scotia after the mandatory fortification program was implemented in Canada. In this study, Persad et al. (2002) took into account all the live births, still births and terminated pregnancies and reported that the incidence of NTD decreased by 54% after the initiation of mandatory fortification program. Gucciardi et al. (2002) studied based the hospital data on terminated pregnancies and data from the Canadian anomalies surveillance from 1986 to 1999. They reported that the incidence of the NTD decreased from 26.2 per 10000 births in 1995 to the 8.6 per 10000 births in 1999. The incidence of the NTD declined by 53% which was attributed to folic acid fortification. Anderson et al. (2004) studied 2481 patients with CHD who underwent angiography between 1994-2004. Comparison was between patients divided into prefortification and post fortification periods. They found that the homocysteine levels decreased significantly after the implementation of fortification and found homocysteine to be an independent risk factor for mortality. A study in Australia by Flood et al. (2001) suggested that the folate levels in an elderly

population after fortification was greater than the recommended safe upper intake level of 1000 µg and suggested the inclusion of vitamin B12 along with folic acid. Beresford and Boushey (1997) reviewed 14 intervention studies, 2 metabolic studies and one observational study that included folic acid supplementation to reduce homocysteine levels. The results demonstrated an effect of various doses of supplementation to reduce the homocysteine levels.

Nutrient Data Laboratory

Knowledge of food and nutrient composition profiles has been important to various types of research related to health and well being of the population. The Nutrient Data Laboratory (NDL) is one of the seven units in the Beltsville Human Nutrition Research Center of the Agricultural Research Service (ARS) which is a part of the USDA (http://www.ndl.usda.gov/fric/foodcomp/bulletins/ndl.info). The NDL has a major role in development of an authoritative nutrient database for the United States. The major role of the NDL is to maintain the National Nutrient Data Bank that contains a wide range of nutrients or food components to support nutrition research, monitoring and policy development (Haytowitz et al., 2002). The USDA Nutrient Database for Standard Reference (SR) is the major source of the food composition data in the United States which provides the foundation for most of the food composition databases in the public and private sectors. New versions of the database are annually released (Standard Reference 17, 2004). The latest version is Release 17(SR17) which provides data for 6,839 food items and 128 food components. It includes data for all food groups and nutrients published in 21 volumes of the "Agricultural Handbook 8". This data is used to update the nutrient databases used in monitoring nutrient intakes in the National Food and Nutrition Surveys conducted by the USDA and HHS (Haytowitz et al., 2002). A key foods

approach was first developed by the NDL in the mid 1980's using the data from the USDA's 1985-1986 Continuing Survey of the Food Intakes by Individuals (CSFII) and the 1987-1988 Nationwide Food Consumption Survey.

The NDL in cooperation with the National Heart, Lung and Blood Institute of NIH has initiated the National Food and Nutrient Analysis Program (NFNAP) in 1997 to improve the quality and quantity of the nutrient data in the USDA's databank (Haytowitz et al., 2002). The NFNAP is an integrated system that identifies food and nutrients, food sampling, sample preparation, chemical analysis, and data dissemination. The main aims of NFNAP are to evaluate the existing data, to identify the key foods and nutrients for analysis, development of the nationally based sampling plans, and complete analysis of the samples and to compile and calculate the representative food composition data (Pehrsson et al., 2003). A sampling plan was developed for the foods that had poor data or if the foods or the methods have changed (Pehrsson et al., 2003). Among the target of over 1000 foods, 500 of them have been analyzed under NFNAP. Five hundred foods were analyzed up to spring 2002 which have been included in the databank. The main focused foods during the year 2000 and 2001 were fresh fruits and fast foods. Their target was to complete 1000 foods by 2003-2004.Some of the other foods that have been analyzed under NFNAP include eggs and fast foods (Pehrsson et al., 2003). The fast foods that were analyzed included hamburgers, cheese burgers, French fries, chicken sandwiches ,fish sandwiches, shakes, carbonated beverages, and coffee. These results are presently undergoing the quality control review prior to their inclusion in the database. NFNAP also analyzed some foods to check the unit to unit variability which include; cheese pizza both frozen and cooked analyzed for all nutrients; pepperoni pizza both frozen and cooked analyzed for proximates, minerals, fortification nutrients, fatty acids and vitamin E; white bread for proximates minerals

and fortification nutrients; eggs for proximates, minerals and fatty acids; milk all nutrients and all fat levels and selected fast foods for proximates, minerals and fatty acids. A future report is supposed to examine their variability in detail (Pehrsson et al., 2003).

The Food Survey Research group at the USDA has implemented a survey Nutrient Database for Trends Analysis to use with the food composition surveys in the U.S. In this system, the databank is updated each time changes are made to food itself or when there is an improvement in the available data that causes the database value to change (Anderson et al., 2001). The data on food composition is essential for nutrient intake planning for individuals or populations. Food composition tables provide information about the different nutrients and the kilocalorie values of various foods. The food composition tables are not easily understood by the consumer and not all kinds of foods are included. Hence, the NFNAP is in the process of analyzing more foods to be included in the database.

Need for Updating Old Databases

The importance of adequate folate nutriture was well recognized in the past decade in the reduction of NTD and in prevention of the cardiovascular disease by lowering the plasma homocysteine concentrations. Since, several studies proved the role of folic acid in preventing the NTD (Honein et al., 2001; Williams et al., 2002; Gucciardi et al., 2002), the FDA implemented mandatory fortification of all enriched cereal grain products with 0.43 mg to 1.4 mg per pound of the product with folic acid beginning January, 1998, to prevent NTD (FDA, 21 CFR parts 136, 137 &139). After the fortification, there was a critical need to update the old databases and to estimate the dietary folate intakes for nutrition monitoring and food safety evaluations (Rader et al., 2000). The validity of the estimates used to determine the potential

impact of the fortification program is dependent on the accuracy of the folate data in the national database. The weakness of the available data for the food folate found in the third report on Nutrition Monitoring in the United States (Life Sciences Research Office, 1995). Hence, the Life Sciences Research Office recommended improved methods of the folate analysis. Thus, validated analytical methods were developed after the fortification was mandated and an AOAC International official method was collaborated. The recommendations for folate intake are expressed in µg DFE.

Historical Aspect of Food Folate Analysis

During the last four decades, numerous reports have been published on food folate content and the various methods of folate determination. These methods include detection using microbiological assay (Perloff, 1977; Shin et al., 1975; DeSouza and Eitenmiller, 1990), radiobinding or radiometric assay; (Chen et al., 1983) and fluorometric, (Gregory et al.,1984; Day et al., 1981; Vahteristo et al.,1996) electrochemical (White, 1991) or spectrophotometeric methods (Selhub et al., 1988; Bagley et al., 1997), some of which are carried out in combination with gel or high-performance-liquid chromatography (HPLC). Recently, an extensive review of the interlaboratory variation of food folate analysis using various methods has been published. Among these determinations, microbiological assay appears to be most commonly used. The majority of investigators reported values of food folate content obtained by microbiological assay using *Lactobacillus casei ssp. rhamnosus* (ATCC 7469) after both heat extraction in the presence of a reducing agent(s) as well as the treatment with folate conjugase, which hydrolyzes folate polyglutamates to folates with shorter glutamyl residues such as mono or diglutamates,

which can be utilized by the assay organism. These values have been the basis of the calculations of dietary folate intake for establishment of the United States RDAs.

Methods of Folate Analysis

The most recent methods for the folate analysis include bioassays, microbiological asays, HPLC, ligand binding and radioimmuno assay. The microbiological assay using *L. casei ssp. rhamnosus* (ATCC 7469) is the most widely used along with some HPLC methods. Folate analysis is most complicated, since multiple metabolic forms are present in the biological samples. The instability of the vitamin is of primary concern.

Microbiological Methods Using Trienzyme Extractions

Microbiological assay has been considered to be one of the best and most versatile methods for the determination of food folate for the past half-century. *L. casei ssp. rhamnosus* (ATCC 7469) has been most widely used for the determination of food folate because this microorganism responds almost equally to the widest variety of folate derivatives (Rader et al., 1998).

For many years, despite its popular use, investigators had considered that microbiological assay was extremely laborious and time consuming. Also, it was difficult to establish this method as a dependable routine in each laboratory. Although numerous attempts have been made to improve the method over the years, it may be safe to say that the two most significant contributions that were made during the last 20 years changed the image of the method to less laborious, less time consuming, and more reproducible (Tamura, 2000). These contributions

include the use of cryoprotected, *L. casei ssp. rhamnosus (*ATCC 7469) and the use of a 96-well plate and a microplate reader with a computer for data reduction.

Trienzyme Extractions

During the last decade, the use of a trienzyme treatment method has been developed for more efficient extraction of folates from certain foods than the conventional methods (DeSouza and Eitenmiller, 1990; Aiso & Tamura, 1998; Martin et al., 1990; Pfeiffer et al., 1997; Rader et al., 1998, 2000; Tamura, 1998; Tamura et al., 1997). The assay of folates from foods generally involves three steps:

1) Liberation of the folates from the cellular matrix.

2) Deconjugation from the polyglutamate to the monoglutamate.

3) Detection of the biological activity of the monoglutamate forms.

Eitenmiller and DeSouza (1990) published a method for determination of food folate. In addition to the traditional treatment with conjugase, the method included treatments with a-amylase and a broad specificity protease (Pronase^{R}). They coined the term "trienzyme extraction." This method includes the sequential treatments of food homogenates with a -amylase and Pronase ^{R} in combination with the traditional folate conjugase (pteroylpoly-?-glutamyl hydrolase) treatment. This method significantly increased the measured levels of folate in many foods. Martin et al. (1990) demonstrated the broad applicability of trienzyme methodology to a variety of foods. Folate conjugase treatment is used to hydrolyze polyglutamyl folate, the primary food folate form, to monoglutamyl and diglutamyl forms, which can be utilized by *L.casei ssp. rhamnosus* (ATCC 7469) for folate determination (Tamura,1990). The

a-amylase and protease treatments digest carbohydrate and protein matrices of foods where food folates are possibly trapped. Thus, they observed a substantial increase in the in folate content of certain food items using the trienzyme treatment when compared to the traditional method alone. Numerous studies were carried out using this method in a slightly modified way. In the past decade, Tamura et al. (1998) determined the food folate values in human milk by using the heat treatment prior to the trienzyme treatment and obtained higher values than those determined by Martin et al. (1990). The order of the enzyme treatments was slightly modified wherein; the samples were treated with a- amylase and conjugase simultaneously and then digested with protease. Finally, Rader et al. (1998) found that digesting the test portion with protease and then deactivating the enzyme prior to addition of the other enzymes yielded the highest folate level from digested samples.

Rader et al. (2000) measured the total folate content of cereal- grain products after fortification using the trienzyme treatment and the microbiological assay. They reported a significant excess in some groups of the fortified products. Hence, concerns were expressed on over fortification. Shresta et al. (2000) extracted spinach and fortified bread and ready to eat cereal and reported the trienzyme treatment was a significant improvement over the single enzyme treatment only in fortified bread. Deconjugation with chicken pancreas gave slightly higher folate value than did the human plasma conjugase in all the foods except spinach. Folate assay by cyroprotected *L. casei* took shorter time, and gave better results and was more economical. Johnston et al. (2001) measured the folate concentrations in fast foods containing beef with the trienzyme folate extraction method and compared to the values using the traditional folate conjugase. One serving of hamburger, sandwich pizza and Mexican foods contained a mean of 314 ±98, 401 ±115, 221 ±45 and 282 ±126 µg of folate, respectively. They reported that

the breakfast items (165±89 µg) provided the lowest folate per serving among the other foods. The values obtained for the trienzyme treatment were markedly higher when compared to the values in the literature (extracted by the traditional method). The higher values obtained were most likely due to the fortification of folic acid in the wheat flour of the products and also due to the use of trienzyme extraction.

Iwatani et al. (2003) determined the folate content in some common Australian vegetables and reported that there was no significant difference in folate content of vegetable samples deconjugated with chicken pancreas and human plasma conjugase. The application of trienzyme extraction did not increase measurable folate values of the analyzed vegetables. The ratio of undeconjugated (free folate) to the total folate in vegetables varied from 0.19 to 0.89 average value being 0.6. The total folate content of 22 vegetables ranged from 68 to 425 µg/100g .Green leafy vegetables were very good sources of folate in the human diet. Folate content of foods commonly consumed in Korea was measured after trienzyme extraction (Yeon et al., 2003). They reported that folate values by trienzyme treatment were higher in all foods with exception of a few vegetables such as cucumber (improved strain), eggplant, and banana than those by conjugase treatment alone. Some of the values were comparable to the data using trienzyme treatment and microbiological assay in the literature and their results support the idea that food folate should be measured using the trienzyme treatment method.

Tamura et al. (1997) measured the folate content of 210 dairy products by microbiological assay and trienzyme treatment. There was an average increase of approximately 45% in folate contents in the 210 samples after trienzyme treatment over the value obtained after folate conjugase treatment alone, indicating that trienzyme treatment provided higher values in dairy products. In fact, Konings (1999) and Konings et al. (2001) reported about 20% increase in

total folate contents of dairy products after the trienzyme treatment compared to folate conjugase treatment alone. They used a buffer with pH 7.85 that is known to provide much more efficient extraction of food folate than that with a lower pH (Tamura et al., 1997). Folates in foods have been traditionally analyzed with microbiological assays, such as AOAC Official Methods 944.12, 992.05, and 960.46. AOAC Official Method 992.05 uses the conjugase enzyme treatment. The most recently published AOAC method for the analysis of the folate by the tri-enzyme procedure was a collaborative study by Devries et al., 2005. Thirteen laboratories participated in the collaborative study of 10 required and 10 optional cereal grain products, including flour, bread, cookies, baking mixes and ready to eat breakfast cereals. The standard test tube method and the microtitre plate methods were used. The relative standard deviation between the laboratories ranged from 7.4-21.6% for eight fortified products compared with values of 11-20%. Two unfortified cereal grain products showed much higher value than expected. Based on the results of this collaborative study, the microbiological assay with trienzyme extraction is recommended for adoption as Official first action study. An international inter-laboratory performance of food folate assay was evaluated using soybean four, fish powder and breakfast cereal test materials. (Puwastien et al., 2005). These materials were sent to 34 laboratories, which were asked to use their routine methods of food folate analysis. Of these, 20 used microbiological assay (17 used *Lactobacillus casei*), four used an HPLC-UV detection method, one LC-MS and one radiobinding assay for folate analysis, indicating a wide variety of folate detection methods. Among 17 laboratories where L. casei microbiological assay was performed, the inter-laboratory coefficient of variations of these test materials was 24%, 35% and 24% for soybean flour, fish powder and breakfast cereal, respectively. These observations suggest that for

food folate analysis, it is important to standardize the methods of folate extraction and detection, and the use of reliable reference materials should be encouraged.

Liquid Chromatography Methods

Folate is a general term to include closely related compounds with similar chemical structures (A pterin ring conjugated to the amine of *p*-amino benzoic acid which forms an amide bond with glutamic acid). The structural and chemical diversity of the naturally occurring folates prompted the development of several analytical procedures for their analysis. The assays encompass a fairly wide range of analytical procedures. Differentiation is crucial in folate analysis, since the vitamins show different bioavailability and stabilities. The primary advantage of the HPLC analysis is the ability to quantify the specific folate forms such as the gamma – glutamyl folate polymers. Such specificity is not obtained by other methods (Pawlosky and Flanagan, 2001). Several excellent developments in HPLC methods have occurred in the recent years. During the past decade, HPLC separation techniques with UV and/or fluorescent detection have been developed to detect the various forms of the folates in foods (Pfeiffer et al., 1997; Konings, 1999; Seyoum et al., 1993). A stable-isotope gas chromatography-mass spectrometry (GC-MS) procedure was developed for the determination of total folates in biological samples; whereby, the folates are chemically hydrolyzed to *p*-aminobenzoic acid, derivatized, and analyzed by GC-MS (Cheruppolil,1997).

Recently, an HPLC technique with fluorescence detection was developed for determination of folic acid and 5- methyl tetrahydrofolate (Doherty and Beecher,2003). The extraction is done by the trienzyme technique using rat plasma as a source of the conjugase. A high capacity styrene-divinyl benzene based solid extraction column was identified, and the

various forms of folate were separated on C-18 HPLC column which was resistant to the degradation by low pH. The mobile phase was simplified to a low pH phosphate buffer and acetonitrile. A UV-induced photolysis system was added which was controlled by the HPLC. Folic acid can be measured as a fluorescent product. This method was applied to various foods for folic acid analysis.

The availability of $^{13}C_5$ labelled folates facilitated the development of the highly specific quantitative methods for the determination of folate using mass spectroscopy detection (MS). Pawlosky and Flanagan (2001) made significant contribution to the development of the stable isotope-LC-MS method**.** A stable-isotope LC-MS assay was developed for the quantitative determination of folic acid in fortified foods (Pawlosky and Flanagan, 2001). Folic acid was extracted from food samples into a phosphate buffer, purified on a C-18 Sep-Pak cartridge, and analyzed by LC-MS in the negative ion mode using electrospray ionization. The analyte was quantified using $^{13}C_5$ folic acid as an internal standard and the coefficient of variation for the precision of the method was 5.6% based on the analysis of four sample replicates. The accuracy of the method was assessed using a standard method of addition of folic acid to a shredded whole wheat cereal. The overall quantitative efficiency of the method was evaluated using a standard reference material (Infant Formula, SRM1846). The method was applied to the determination of folic acid in several test samples (fortified breakfast cereals), and the values were in accord with the manufacturer's claim. This method advanced a LC-MS technique for the determination of folic acid in fortified foods based on stable isotope dilution methodology. The specificity of the technique and quantitative accuracy of the method in various food substrates suggests that the method may be adapted for routine analysis in other fortified foods.

Pawlosky and Flanagan (2003) described a MS method used in the validation of an HPLC determination of food folates across a wide range of sample matrixes. A series of five food reference materials that had certified values of folate concentrations and five frozen food samples were analyzed for 5-methyltetrahydrofolic acid (5-MTHFA) and folic acid using the HPLC method with fluorescence detection that was validated using an HPLC/ MS method with electrospray ionization. Identical sample specimens were extracted and analyzed in triplicate using both instrumental methods, and a comparison was made of the mean values of 5-MTHFA and folic acid resulting from these determinations. The analytes were isolated on either a high capacity strong anion exchange solid phase extraction column (HPLC method) or a phenyl Bond Elut column (MS method) prior to analyses. For quantification of the analytes by MS, ^{13}C-labeled 5-MTHFA and folic acid were added to samples as internal standards prior to enzymatic digestion and conversion of the polyglutamate forms of 5-MTHFA to the monoglutamic acid. Quantification of folic acid and 5-MTHFA using the HPLC analysis was carried out using external standards. With the exception of one reference material (pig liver), the values established for 5-MTHFA using these methods were highly comparable. In determining the variance associated with these two procedures, it was observed that the mean relative standard error for 5-MTHFA was 12% (range, 2-27%) and 11% (range, 5-25%) for the HPLC and MS methods, respectively. Folic acid was detected in only three of the samples, and the values obtained for it by either method were similar.

Stable Isotope Dilution Assays Using High Performance Liquid Chromatography–Tandem Mass Spectrometry

Rychlik (2002) synthesized labeled vitamers of folic acid to be used as internal standards in stable isotope dilution assays. Here, folic acid was synthesized by deuterating p-aminobenzoic acid, which was then coupled with glutamic acid and 6-formylpterin. Using folic acid as the starting component enabled the preparation of labeled tetrahydrofolate, 5-formyltetrahydrofolate, 5-methyltetrahydrofolate, and 10-formylfolate which were characterized by electrospray MS and collision-induced dissociation. The MS studies confirmed that the compounds could be used as internal standards in stable isotope dilution assays. Folates and their labeled analogues were analyzed simultaneously by HPLC/MS/MS using selected reaction monitoring. The folate contents were determined in meat, cereals, and vegetables using this method. Data were in good agreement with literature data, except results for broccoli, which were much lower than reported in previous studies. Several studies were carried out and folate values of various foods were determined using this method.

Ligand Binding Assays

Various biospecific methods using ligand binding have been developed for folate analysis of clinical and food samples. Biospecific assay for the water soluble vitamins were classified into two catogories (Finglas et al., 1988). Methods based upon the specific interaction of the antibody with its antigen include radio immunoassay and enzyme linked immunosorbent assay (ELISA). Assays using naturally occurring vitamin binding proteins with isotope labels or enzyme labels include radio-labelled protein binding assay (RPBA) and enzyme protein binding assay(EPBA).

Folate binding assays have been developed in the past decade for enzyme protein-binding assays (EPBA) and enzyme linked immunosorbent assay (ELISA). Radio assays have some advantages over HPLC. They are quicker, less expensive, less subject to variation, simple to perform, and have a high sample throughput (Finglas et al., 1988; Mandella & DePaola, 1984). The major limitation of the ligand binding assays is the improper response of the individual folate forms to the folate binding protein used. Careful control of the pH of the assay buffer and a good choice of the folate calibrant is necessary for the food folate measurements (Finglas et al., 1993).

Radio-Labelled Protein Binding Assays (RPBA)

Studies conducted by The Centers for Disease Control (CDC) and Food-linked Agro Industrial Research Program reported problems in calibration of kits used for RPBA assay of folate (Van den Berg et al., 1994). Also, the values obtained were not comparable with the values obtained by HPLC and microbiological assay. A study comparing several commercial RPBA kits for folate analysis of clinical samples showed problems with recovery and variability between kits and stressed the need for further standardization and optimization of assay and extraction procedures (Van den Berg et al., 1994). Attempts to subject the RPBA to folate analyses in foods have revealed several setbacks such as low precision between assays and laboratories, varying affinity to different folate forms and poor agreement with the microbiological assay. Since, standard calibration and pH are known to be crucial for the RPBA, little attention has been given to disturbances caused by food matrix and buffers. Theobald et al. (1981) suggested that matrix effects might change the background radiation and unspecific binding in the assay, resulting in false results. However, a study on evaluation of the RPBA for the folate content in berries and

milk was conducted (Stralsjo et al., 2002) .This study aimed to optimize a commercial RPBA, routinely used for clinical samples, for folate quantification in foods containing mainly 5-CH_3-H_4 folate. The assay was modified using external calibration with 5-CH_3-H_4 folate in a lower concentration range diluted in food extraction buffer, instead of the human serum albumin (HSA) provided by the kit. The modified RPBA was applied on some selected food products; milk, whey powder, rose hips, strawberries and European certified reference materials. Adjustments did not negatively affect the assay. Performance parameters included control of selectivity, absence of matrix effects, recoveries of 94-113%, precision of 1-6 CV% Folate concentrations in berries and milk, obtained by the modified RPBA were also compared with other quantification methods such as HPLC and microbiological assay.

*Enzyme Protein Binding Assays (*EPBA)

The EPBA for folate analysis in foods is a new technique reported by Finglas et al. (1988). The EPBA is based on the degree of binding of folic acid to the surfaces of microwells. Folate extraction involves a series of lengthy enzymatic steps to release the native folates bound to the food matrices and deconjugation of polyglutamyl folates to mono or other simpler measurable forms. In fortified cereals, folic acid is the major form of folate, since cereals generally contain low levels of native folate (Pfeiffer et al., 1997). Arcot et al. (2002) analyzed the folate content in fortified foods by the EPBA method using a commercial kit. The food samples were also analyzed by the microbiological assay for comparison of methods. Results from both methods showed no significant difference $p < 0.05$ in value and showed high correlation ($r = 0:89$, $p < 0.001$). The addition of 1% ascorbic acid to the extraction medium

offered better protection of folic acid against oxidation than foods extracted without ascorbic acid.

CHAPTER 3

MATERIALS AND METHODS

Preparation of Folic Acid and Total Folate Samples

The samples used for analyzing folic acid and total folate in pizza and other fast foods were collected by the United States Department of Agriculture (USDA) through the National Food and Nutrient Analysis Program (NFNAP). These included a total of 232 samples including pizzas of various types; fast food pizza(56), cheese pizza(18), meat pizza(6), meat and vegetable pizza(7), pepperoni(8). The fast food samples consisted of breakfast pastries(16), breakfast sandwiches(63), fast food sandwiches(14), taco salads(8), tacos/burritos(32), and vegetable burgers(4). Thirty pizzas (15 baked and 15 raw) were collected from a local pizzeria to examine the effects of baking on the folate retention. These included raw cheese(5), baked cheese(5), raw vegetable pizza(5), baked vegetable(5), raw supreme(5), baked supreme(5).

The samples collected through the USDA were delivered frozen and homogenized. These samples were stored at -50° C and were transferred to the refrigerator one day before the assay. The pizzas collected from the local pizzeria were chopped in a Bowl Chopper (Kramer and Grebe K.G.S. Model 33 Wallau/ Lahn -Germany) at 7°C. The cold temperature is used to prevent the oils in the pizza from melting during the grinding process. During the chopping process**,** the blade was separated and the pizza sample sticking to the side of the wall was scraped and mixed again to ensure homogenization**.** The homogenized sample was used for the folate assay.

Preparation of the Quality Control Sample

Two bags of Pillsbury all-purpose, bleached, enriched flour were purchased at the local grocery. The two bags were mixed together to ensure homogeneity and labeled and transferred into a 4 oz leak proof nalgene bottles with a polythene screw cap and refrigerated at 4 ° C. These were used through out the study.

Preparation of the Standard Stock Solution

The standard folic acid was purchased from the USP (US Pharmacopoeia). Twenty milligrams of the folic acid was weighed into a 200mL Pyrex conical flask containing 20mL of ethanol and 50mL of the distilled water. The pH was adjusted to 10.00 with 0.1N NaOH to help dissolve the folic acid and the final pH was adjusted to 7.00 with 0.05N HCl. The volume was made up to 100mL with water and transferred to 10mL Pyrex tubes and stored in the refrigerator at 4° C. This standard can be used for 6 months from the day of preparation. The standard should be discarded and a new standard should be prepared after 6 months.

Purity of the Standard Solution and Determination of the Concentration

Checking the purity of the standard is a very important step in the folate analysis. Phosphate buffer (0.1M), pH 7.0, was prepared. Potassium phosphate monobasic (13.61 g) is dissolved in water and diluted to 100mL and the pH was adjusted to 7.0 with drop wise addition of 4N sodium or potassium hydroxide. Then, the stock solution was diluted with the buffer in 1:20 ratio. The absorbance of the diluted standard was measured at 282 nm using the phosphate buffer as a blank. After measuring the absorbance of the standard and the blank, the $E^{1\%}_{1cm}$ of the standard folic acid was calculated using the following formula:

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

Where C = concentration of diluted stock standard (mg/mL).

After calculating the Extinction coefficient ($E^{1\%}_{1cm}$), the percentage purity of the standard was measured by the following formula:

=100 (calculated $E^{1\%}_{1cm}$ /reference $E^{1\%}_{1cm}$ for folic acid) (2)

The reference $E^{1\%}_{1cm}$ for folic acid used 611.7 (Eitenmiller & Landen, 1998). Finally, the true concentration of the stock solution must be calculated from the percentage purity of the standard value calculated above and the weight /volume of the stock solution used (20 mg/100ml).

The true concentration of stock solution =

(weight /volume of the stock solution used) (Percentage purity of the standard value).

This calculation gives the true concentration of the prepared stock solution.

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

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

A_{std} = 0.672 ---- measured value

A_{blank} = 0.068 ---- measured value

By the equation (1), $E^{1\%}_{1cm}$ = 10 (0.672-0.068)/0.01 = 604

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

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

= 0.1974 mg/mL = 197.4 :g/mL

Set Up for Extraction of Folic Acid and Total Folate

In a single set, 4-6 samples were analyzed. For each sample four 125mL Erlenmeyer flasks were used to compare duplicates for total folate and the folic acid. Each set had a control, recovery (of either the total folate or the folic acid) and quality control (QC) sample for total folate and QC for folic acid.

Extraction Procedure for Total folate

The extraction procedure follows the recent published procedure by De Vries et al. (2005), Rader et al. (2000), AACC Official Method (86-47).

One gram of sample was accurately weighed into 125mL Erlenmeyer flasks along with set up of 1 control extract, 1 quality control extract for total folate and a sample for recovery determination. The set up for these extract digests are explained below. Each sample was analyzed in duplicate. Fat extraction was done for samples like pizzas that contain more than 5% of fat. Fat extraction was carried out under the hood by adding 20mL of the hexane and pippeting it out after 15 min. Freshly prepared working standard solution (0.2 µg/mL) was added to the recovery sample. The addition of the standard solution depended on the amount of folic acid or total folate present in that particular type of the sample. Then, 20mL of pH 7.8 phosphate buffer and 30mL of distilled water was added to each sample, control, QC and recovery flask. All the flasks were covered with the aluminum foil and heated at 100°C for 15 min. After cooling, 10mL of the pH 7.8 phosphate buffer was added along with 1ml of the freshly prepared (2mg/mL) Pronase^{R} solution. All the samples were covered with aluminum foil and the flasks were incubated at 37°C for 3 h. The sample mixtures were then heated at 100°C for 5 min to inactivate the Pronase^{R} enzyme. After cooling to room temperature, 1mL of the freshly prepared a-amylase

solution (20 mg/mL) was added to the sample mixtures followed by 0.5mL of toluene. The sample flasks were then covered with aluminum foil and incubated at 37°C for 2 h. The chicken pancreas conjugase solution (5 mg/mL of the buffer) was prepared by weighing the required amount of the conjugase enzyme into a 150mL beaker and adding required amount of the pH 7.8 phosphate buffer. This mixture was homogenized for 10 min by placing it on a magnetic stir plate using a stir bar. The homogenized mixture was filtered into another 150mL beaker using a funnel with glass wool. Four milliliters of the filtered conjugase solution was added to the sample mixtures and then incubated at 37°C for 16 h.

After the 16 h incubation period, the samples were removed from the incubator and heated at 100°C for 5min and cooled to room temperature and then all the samples were adjusted to pH 4.5 with HCl. The final volume was adjusted to 100mL with the distilled water for all the samples including control, QC and recovery samples. Extracts were filtered through a filter paper (Whatman, size No.1) set on the funnel in a beaker. The extracts were transferred to 10mL test tubes and capped.

Extraction of Folic Acid

The extraction procedure for folic acid was similar to the total folate extraction but, the addition of Pronase^{R} and conjugase enzymes was omitted. The extractions for the total folate and folic acid were carried out simultaneously. The important step in the extraction of the folic acid was the addition of the a-amylase followed by the addition of toluene.

Control

A control (enzyme blank) without any food sample was carried through the sample digestion procedure as a total folate sample. The control was used to determine the contribution of the enzymes to the growth response of the *L. casei ssp. rhamnosus* (ATCC 7469).

Recovery

Preparation of Working Standard Solution for Recovery (0.2 µg/mL)

The stock solution of 0.1mL (200 µg/mL) was diluted with water to 100mL in a volumetric flask, which makes the final concentration to 0.2 µg/mL. It is prepared fresh on day of use. Approximately, 100% of the folic acid and total folate should be taken into account. The required amount of working standard added depended on the level of total folate and the folic acid present in the same type of food as the sample to be assayed. Estimated values were determined from the USDA Nutrient Data Bank for like samples. For example, if the assumed level of folic acid or total folate in a sample is 18 µg/100g, about 20 µg/100g should be added to the sample for the recovery determination. Because assay sample size was 1g, 1mL of working standard (0.2 µg/mL) was added to the recovery flask. That made the final volume to 0.2 µg/1g = 20 µg/100g of the food sample.

Pretreatment of Chicken Pancreas Conjugase

A cheese cloth was finely secured on top of a beaker with the help of a rubber band. A bottle of freeze dried chicken pancreas was opened and transferred to the cheesecloth and then rinsed with acetone using a disposable pipette. The beaker with the soaked conjugase was kept in the vacuum jar with vacuum turned on until the conjugase was dried completely. The conjugase

was then transferred into a bottle and covered with the aluminum foil and kept in the refrigerator for one day to dry out completely and then it was capped tightly and stored in the freezer at -4℃**.** The chicken pancreas had to be acetone washed to achieve low folate enzyme control values.

Preparation of the 0.1M, pH 7.8 Phosphate Buffer (Assay Buffer)

Sodium phosphate dibasic anhydrous (1.42 g Na_2HPO_4,), was dissolved in 100mL of water. L-ascorbic acid (1%, w/v) was added to the phosphate buffer to prevent oxidation of folate during the extraction process. The mixture was adjusted to pH 7.8 with drops of 20% (20mg/100mL) NaOH.

Reagents used

Sodium phosphate dibasic-anhydrous-(Na2HPO4-CRS:531900)

L-Ascorbic acid (1%, w/v)

Folic acid USP-Cat No: 1286005, 500mg

Acetone

Ethanol

0.05 HCl

20%NaOH

Hexane

Toluene

Chicken pancreas – Difco, Cat: 245910, Beckton Dickinson and Co., Sparks, MD 21152, Keep in a freezer.

Pronase^{R} – Calbiochem, Cat: 53702, EMD Biosciences, Inc., San Diego, CA 92121

a-Amylase – Fluka, Cat: 10065, Sigma, St Louis, MO 63178, Tel: 1-800-325-3010

Folic Acid Casei Media (Difco, Cat: 2005-11-30)

Lactobacillus broth AOAC (Difco,CAT :2002-06-30)

Lactobacillus agar AOAC (Difco,CAT :2005-04-30)

Depletion media (Lactobacillus broth diluted with folic acid casei media 1:1)

Standard stock solution

Apparatus

Incubator

Water bath

Autoclave

pH meter

96 Well microplate reader (Bio-Rad)

Bunsen burner

Disposable culture tube – Labcor (7309 Governor Way, Frederick, MD 21704, Tel: 301-620-7400), Cat: 730-004, sterile, solution basin, 80/case.

Disposable sterile filter system – Corning (Cat: 430767, Fisher Cat: 09-761-1), 0.22µm pore size, 250 mL receiver, 12/case.

Pipette tips – CLP, 5648 Copley Drive, San Diego, CA 92111, Cat: 2032.YS or 2002 S, 200 µL, racked sterile, 10 trays of 96.

Disposable multi-channel pipetter basins – Fisher Cat: 13-681-101, sterile, 100/case.

Disposable syringe – Non-sterile, 1 to 5mL.

Syringe filter – Fisher, Cat: 09-720-3, 0.22µm pore size, 13 mm diameter, Sterile, PVDF

(membrane), 100/case.

1-channel pipette – Brinkmann, 20 to 300 µL

12-channel pipette – Brinkmann (Cat: 22-46-150-8, Fisher Cat: 21-378-95), 20 to 300 µL

96 well microplates – Falcon (Cat: 353072, Fisher Cat: 08-772-2C), 50/case.

Set up for the Microplate Assay

Preparation of the Working Standard for the Microplate Assay

First, 0.1mL of the standard stock solution (200 µg/mL) was taken and diluted to 100mL with distilled water in a volumetric flask to prepare the intermediate standard (0.2 µg/mL). The working standard (2 ng/mL) was then prepared by diluting 1mL of the intermediary solution (0.2 µg/mL) to 100mL with distilled water in a volumetric flask. This standard was prepared fresh on the day of use.

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

Lactobacillus broth powder was weighed and dissolved in water (3.8 g/100mL). The solution was heated with stirring until it started to boil. Then, it was allowed to boil for 2-3 min, cooled and (10mL) dispensed into screw cap tubes. This broth was autoclaved at 121 °C for 15 min, cooled to room temperature and stored in the refrigerator at 4°C until use.

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

Lactobacilli agar was weighed (4.8g) and diluted with 100mL distilled water. It was heated with stirring until it started to boil. It was allowed to boil for 2-3 min until it was completely dissolved and cooled slightly. The prepared agar was transferred into the screw-cap

tubes and autoclaved at 121 °C for 15 min. While cooling to room temperature, the tubes were placed at an angle to produce a slant and stored in the refrigerator at 4°C until use.

Preparation of the Depletion Media (Lactobacilli broth: media=1:1)

Lactobacillus broth (3.8g/100mL) and Folic acid casei medium (9.4 g/100mL) was weighed in the ratio of 1:1 and dispensed in the required amount of water and heated with stirring until it started to boil. After boiling for 2-3 min, it was completely dissolved and cooled to approximately 37°C. The prepared mixture was transferred into the screw-type tubes and autoclaved at 121 °C for 15 min, cooled to room temperature and stored in the refrigerator at 4°C until use. This media is stable for up to 6 months at which time it was replaced.

Maintenance and Transfer of the Culture

Since the culture transfer is an important step to maintain to maintain the activated state of the culture for consistency of the assay. The culture used in the experiment was *L casei ssp. rhamnosus* (ATCC 7469). The *L casei ssp. rhamnosus* pellet (The American Type Culture Collection) was dissolved in 10mL of the Lactobacillus broth solution. This solution (0.5 – 1.0mL) was transferred to 10mL of another Lactobacillus broth solution and incubated at 37°C for 18 h. After the incubation period, the culture from the solution was transferred to the Lactobacillus agar slant and incubated at 37°C for 24 h. Then the slant was stored in the refrigerator at 4°C.

To maintain the activated culture, the culture was transferred weekly on the same day and incubated on the new slant at 37°C for 24 h. The new slant was stored in the refrigerator at 4°C.

Preparation of the Inocula

On the day of the microplate assay, the depletion media (Lactobacillus broth:media, 1:1) was taken from the refrigerator and warmed to a room temperature. Then, the culture from the 3-4 day old slant was transferred to the depletion media and incubated at 37°C for 6 h. This depletion media was used for the microplate assay after the 6 h incubation period. At this time only faint growth is discernable in the depletion media. To maintain reproducible standard curves with similar growth times, this schedule needs to be strictly adhered to.

Microplating

The sample extractions (1g/100mL) that were filtered after the 16 h digestion period were diluted with distilled water, if necessary. Based on the estimated level of the folic acid and the total folate present in the sample, dilutions were made to obtain growth spanning the concentration range of the standard curve. The dilution factor chosen for the samples were approximately the following:

a. No dilution for samples with folate range of 0-30 µg/100g of the sample.
b. 1:3 dilutions for folate range 30-80 µg/100g of the sample.
c. 1:5 dilutions for samples with folate range 80-150 µg/100g.

If higher folate concentrations values are expected such as in supplements, then higher dilutions are required. For example, pizza has an expected folate level of 45 µg/100g. If the initial weight of the sample is 1g, the folate is 0.45 µg/1g of sample. When diluted to 100mL after the extraction process, it has 0.45 µg/100mL. Then, 1:3 dilutions are made i.e, 1mL (0.0045 µg/1mL or 4.5 ng/mL) of the extract and 2mL of distilled water. After the dilutions are made, concentration of the folate is 1.5 ng/mL. During the microplating, 150µL of this

diluted extract is taken and added to 150µL of distilled water (the folate concentration is 0.225 ng/300µL) in the highest concentration well (G3 through G12). Then 7 serial dilutions are made in each row till A3 through A12. Since the concentration of the working standard is 2 ng/mL, 150µL of this standard is added to the highest concentration well (G1 and G2) containing 150µL of the distilled water. The final folate concentration in the highest concentration is 0.3 ng/300 µL which is close to the sample concentration (0.225 ng/300µL). This calculation shows how dilutions are made of samples to coordinate with the standard curve concentration range.

After the dilutions were made, the tubes were capped loosely and autoclaved at 121°C for 5 min. Along with the sample tubes a flask, 100mL measuring cylinder, and an Erlenmeyer flask with distilled water covered with aluminum foil were autoclaved.

	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
96 Well Microplate

The bench used for the assay was first cleaned with 70% alcohol and the microplate was prepared close to a lighted Bunsen burner to decrease chances of microbial contamination. The microplates were opened from the individual wrapping near the flame and the cover was labeled with an indelible pen. The sterile water was transferred into the reservoir and using a 12-channel pipetter{Brinkmann (Cat: 22-46-150-8, Fisher Cat: 21-378-95)}, 150µL of water was pipetted into the wells A1-G12 (from row A (A1-A12) to row G (G1-G12).Three hundred microliters of water was pipetted into the wells H1-H12 (blank row). Using a syringe and a sterilized syringe filter, the working standard was filtered into the reservoir (300µL of standard solution per plate). Using a 12 –channel pipetter, 150 µL of the working standard was pipetted into the wells G1-G2. Then, 150µL of the sample dilutions, control, and recovery and QC dilutions were pipetted into wells G3-G12. Each of the samples or the unknowns is pipetted into two wells through G3-G12 as duplicates. Using the 12-channel pipette, serial dilutions of the standard and the samples were made were made by transferring 150 µL from the wells G1-G12 to F1-F12, mixing 3 times in each well (by pippeting it up and down the contour of each well with 12-channel pipette 3 times). Then, 150µL of the mixture is transferred from F1-F12 to E1-E12 by mixing it 3 times. This process is continued through A1-A12 and the final 150µL from A1-A12 is discarded. After the dilutions were made, the media with the culture was prepared. The required amount of the media was transferred into the sterilized flask (15 mL/plate) using a sterilized measuring cylinder. Ascorbic acid solution (1g/10mL) was prepared and using a syringe and a sterilized syringe filter, the necessary amount of ascorbic acid solution (100 mg/100mL) was filtered into the flask containing the media. Then, the inoculum culture (1 drop/5mL of the media) incubated at 37°C for 6 h earlier was taken with a 1mL pipette and added into the flask with the media and ascorbic acid. The media with its contents was shaken properly and transferred to the reservoir. Using a

12-channel pipette, 150µL of the media mixture was added into wells G1-G12 through A1-A12.The plates were then sealed in Ziploc bags to prevent evaporation .These plates were incubated immediately at 37°C for 24-28 h.

Reading the Microplate

After the 24-28 h incubation period, one microplate was removed from the incubator and read in the microplate reader using the microplate manager program and the 595 nm absorbance filter. The highest reading point of the standard (G1-G2) was supposed to be above 0.9 absorbance which is a check for maximal growth. When this absorbance level was reached, the other microplates were removed from the incubator and each plate was mixed with a 12 –channel pipette from low concentration (A1-A12) to high concentration (G1-G12) near the flame. The growth usually reaches this point within 24-28 h, and the incubation was not checked until the 24 h time period has elapsed. The absorbance of each microwell is read in the microplate reader. The file was saved and the standard concentrations were calculated by entering 0.2 for concentration of S7 (highest concentration for the standard), and 2 as the dilution factor. The sample dilutions were entered in a similar manner. The standard curve was assembled by regression using Logistic 4PL, Linear-Linear Transformation and Linear-Linear in Axis Transformation. The unknown concentration file is exported to the Excel program to subtract the controls from the sample data and to calculate the recovery. The recovery is calculated based on the following formula:

$$\% \text{ Recovery} = 100 \times \frac{(\text{folic acid in spiked recovery sample}^{a} - \text{folic acid in unspiked sample}^{b})}{\text{folic acid added in spiked sample}^{c}}$$

Where, [a, b, c] Unit: µg/100g of sample

The sample data was corrected with the calculated recovery values.

Corrected data = (Assay data) ×(100 / % recovery).

Usually, recovery values are above 85%. Then, the outliers were removed (according to AOAC Official Method –960.46) which states that the mean values obtained for each observation should not vary by more than 10% of the average. Means and the standard deviations were then calculated for each sample, outliers discarded and final concentrations determined.

µg Dietary Folate Equivalent Calculation

It is necessary to calculate food folate value before the µg DFE can be calculated. The food folate was calculated by the difference between the total folate and the folic acid levels. This represents the amount of the native folate present in the food product .The Food folate can be calculated by the following formula:

Food Folate (µg/100g) = [Total folate (µg/100g) -Folic acid (µg/100g)]

µg Dietary folate equivalents (µg DFE) were calculated from the amounts of the folic acid added during the enrichment and the native folate in foods containing enriched flour. The µg DFE's were calculated using the following formula:

µg DFE= [Food folate (µg/100g) +1.7* Folic acid (µg/100g?]

Moisture Content

The moisture contents for 30 pizza samples collected locally were assayed by AOAC Official Method 984.25 for moisture in frozen french fried potatoes was used. The homogenized sample (5-6g) was weighed in duplicate into aluminum dishes and incubated in a mechanical convection oven at 100°C for 16 h. The samples were cooled and weighed again. The moisture content was calculated using the formula:

$$\left(\frac{\text{weight of the sample before drying }^{(g)} - \text{weight of the sample after drying }^{(g)}}{\text{weight of the sample before drying }^{(g)}} \right) * 100$$

Statistical Analysis

Statistical analysis was performed by using the Statistical Analysis System (SAS, 1990).Means were compared by the Least Significant Difference (LSD) test at α=0.05.

CHAPTER 4

RESULTS AND DISCUSSION

Evaluation of the Folic Acid and Total Folate Assay

Analytical methods are applied in innumerable fields like analytical and bio analytical chemistry, food chemistry biochemistry, clinical biology, pharmacology and related domains. The purpose of an analytical method is the delivery of a qualitative and a quantitative result with an acceptable uncertainty level; in other words, it measures the uncertainty in the method. Validating a method is investigating whether the analytical purpose of the method is achieved; wherein the analytical results obtained are within in an acceptable uncertainty level Analytical method validation forms the first level of quality assurance in the laboratory. Analytical quality assurance is the set of measures a laboratory must undertake to ensure that it can always achieve high-quality data. To achieve this, a validated method or a standardized method is used along with effective internal quality control (IQC) procedures with the use of standard reference materials (SRMs), control charts and other approaches to ensure quality. These measures may be set forth by various international organizations including Association of Official Analytical Chemists International (AOAC), International Standard Organization (ISO), Cooperation of International Traceability in Analytical Chemistry (CITAC) Eurachem Guide, 2002 and CITAC/Eurachem Guide, 2003. There are several guidelines and requirements for the different levels of quality assurance described by the regulatory bodies, standardization agencies and working group or committees. On the European level there is Eurachem that gives guidelines for

method validation, European Committee for Normalization (CEN) that works through different technical committees and working groups on the standardization of analytical methods in all sectors. On the international level, the International Union of Pure and Applied Chemistry (IUPAC), International Standard Organization (ISO), and Association of Official Analytical Chemists (AOAC) International develop validation and standardization frame works for analytical chemistry.

Internal Quality Control

In the IUPAC guidelines for IQC, Thompson and Wood defined IQC "as a set of procedures undertaken by the laboratory staff for the continuous monitoring of operation and the results of measurements in order to decide whether the results are reliable enough to be released". IQC is the elongation of the method validation that guarantees achievement of results with a required standard of accuracy. It helps in continuously checking the accuracy of the analytical data obtained day to day in the laboratory. Thus, it measures the systematic errors that lead to bias and the random errors leading to imprecision. Maintaining constant conditions can help in monitoring these errors. The word "internal" in the IQC implicates that repeatability conditions are achieved. Hence, the objective of the IQC concerns only monitoring the repeatability or the intra-laboratory precision and not the reproducibility or the inter laboratory precision. The two important aspects of the IQC are as follows.

(1) The analysis of the "control materials" such as SRMs or the spiked samples, to monitor the trueness.

(2) Replication of analysis to monitor precision.

Both these aspects form a part of the statistical control which is a tool for monitoring the accuracy of an analytical system**.** In a control chart such as a "shewhart control chart", measured values of the repeated analyses of an SRM are plotted against the run number. Based on the data in the control chart; a method is defined either as an analytical system in control or analytical system out of control. Thus, an analytical system is under control if no more than 5% of the measured value exceeds the warning limits. In this study, accuracy and recovery studies were conducted to ensure analytical quality control.

Accuracy

Accuracy defines the closeness of agreement between the accepted reference values. Measurements include precision and trueness. Precision parameters include repeatability precision (intra-run precision) intermediate precision (inter-run precision) and reproducibility precision (inter laboratory precision). Trueness describes the closeness of agreement between the expectation of the test result and the accepted reference value (true value).

Precision

Precision was measured by repeatability or intra-run precision. Repeatability precision is the precision under conditions where independent test results are obtained with the same method on identical test items in the same laboratory by the same operator using the same equipment within short intervals of time (Taverniers et al., 2004). The IQC sample used in the experiment was the bleached and enriched all- purpose flour. The IQC sample for the folic acid and total folate assays were run over a course of this study and included 25 different analyses. The IQC samples were assayed each time the folic acid and total folate assays were run on varying

numbers of test samples. The quality control charts for folic acid and total folate in enriched flour analyzed by the microbiological assay using the *L. casei ssp. rhamnosus (*ATCC 7469) are given in the Figure 2.3 and Figure 2.4, respectively. Each data point in the figure indicates the result of each run. All the data in the control charts were close to the respective means lying between the upper and the lower control line which are set by ±10% deviation of the mean. The mean value for of folic acid in the enriched flour assayed over the study was 158 µg/100g ± 7.4 (% CV=4.72) with an upper control line (UCL) of 174 µg/100g and the lower control line (LCL) of 142 µg/100g (±10% deviation of the mean). The mean value for the quality control of total folate in the enriched flour after number of trials was 180 µg/100g ± 8.4 (% CV=4.7) with an UCL of 198 µg/100g and the LCL of 162 µg/100g. The results obtained were consistent and indicated good repeatability with each assay. The above obtained values also remain within the quality control parameters specified by USDA and set at ± 10% of the mean. All the values obtained fall within the range of those reported in the semi annual USDA reports from our laboratory where the mean value for the folic acid is 158 µg/100g ± 8.2 which has an UCL of 169 µg/100g and a LCL of 138 µg/100g. The mean value for the total folate was 182 ± 8.9 µg/100g with an UCL of 200 µg/100g and LCL of 164 µg/100g with ±10% deviation. These values indicate good precision for both the folic acid and the total folate assays.

Trueness

Trueness as a measure of accuracy is expressed in terms of bias or percentages of error. Bias is the difference between the mean value determined for the analyte of interest and the accepted true value or the known level actually present. For this Certified Reference Materials (CRM) or Standard Reference Material (SRM) are used to determine the trueness of the assay (Taverniers et al., 2004). The SRM used in this experiment was infant formula SRM 1846

(National Institute of Standards and Technology (NIST), 2004). The SRM is intended for use in validating methods for proximates, calories, vitamins and trace elements. The SRM can also be used as the quality control sample for in- house quality control studies. Figure 2.5 and figure 2.6 represent the quality control charts for the folic acid and total folate analysis of SRM. The mean values obtained for the SRM for the folic acid were 123 ± 5.4 µg/100g (% CV= 4.3) with an UCL of 135 µg/100g and the LCL of 111 µg/100g. The mean value obtained for the total folate in SRM 1846 after a several trials was 140 ± 6.4 µg/100g (% CV= 4.6) with an UCL of 126µg/100g and a LCL of 154 µg/100g. The results indicate an acceptable trueness of the analytical procedure. The above obtained values fall with in the parameters of NIST for the SRM-1846. According to the NIST, the total folate value specified is 129 ± 28 µg/100g. The values specified by the NIST are slightly lower than those obtained in our laboratory (140 ± 6.4 µg/100g). The reason behind this being that the infant formula contains high amounts of folic acid and the extraction procedure followed by the NSIT involved only the conjugase enzyme while the trienzyme extraction was followed in our laboratory**.** Folate conjugase treatment is used to hydrolyze polyglutamyl folate, the primary food folate form to monoglutamyl and diglutamyl forms, which can be utilized by *L. casei ssp. rhamnosus* (ATCC 7469) for folate determination (Tamura, 1990).The a-amylase and protease treatments allow digestion of carbohydrate and protein matrices of foods where food folates are possibly trapped. Thus, a substantial increase in the in folate content of certain food items including the infant formula was observed using the trienzyme treatment when compared to the traditional method alone. The data shows that SRM 1846 contains 17 µg/100g of the food folate. The total folate level of 140 µg/100g is well within the acceptable range of 129 ± 28 µg/100g.

Recovery

Recovery is the measure of the fraction of the analyte added (spiked) to the test sample prior to the analysis. The amount of the analyte in the spiked sample is measured and compared to the concentration in the original sample. Therefore, recovery defines the losses that occur through extraction or by degradation of the analyte. Recovery values can be used to correct analytical values to represent true values in the test sample (Taverniers et al., 2004). If standard protocols are followed and the amount of spiked analyte corresponds to the amount naturally present in a specific matrix (approximately 100% of the matrix level), the recovery value is considered a primary method validation parameter. In this study, values provided by the USDA Nutrient Databank (SR 17, 2004) were used to determine spike levels for the various foods included here.

Recovery values (%) for all matrices ranged from 95 – 100. Mean recoveries were 99 ± 0.5 and 98 ± 1.4 for folic acid and total folate assays. Mean recoveries for uncooked pizzas were 98 ± 2.6 and 98 ± 1.6 (Table 2.4) for folic acid and total folate, respectively. For baked pizzas, recoveries were similar to those for uncooked pizzas. The high recovery values obtained with the foods included in this study indicates that extraction and subsequent assay procedure was suitable for the recovery of analyte.

The Folic acid, Total Folate, Food Folate and µg *DFE Levels in Pizzas*

A total of 95 USDA pizza samples were analyzed for the folic acid, total folate, food folate and µg DFE .There were five types of pizzas namely: fast food pizza (n=56), cheese pizza(n=18), meat pizza(n=6), meat and vegetable pizza(n=7) and pepperoni pizza (n=8). A total

of 30 pizzas which included 15 uncooked and 15 cooked were also analyzed. Table 2.5 gives the values for folic acid, total folate, food folate and µg DFE levels in 5 types of pizzas. The values for folic acid range from 36-63 µg/100g. Meat and vegetable pizza showed the lowest mean folic acid level of 36 ± 4.5 µg/100 g .Pepperoni pizzas had the highest folic acid levels of 63 ± 1.8 µg/100g followed by cheese pizza 59 ± 8.6 µg/100g and meat pizza 57 ± 13.5 µg/100g. The folic acid levels in meat (57 ± 13.5 µg/100g) and cheese pizzas (59 ± 8.6 µg/100g) were not statistically different at 95 % confidence interval ($P<0.05$). The folic acid in meat and vegetable pizza (36 ± 4.5µg/100g) was statistically different from all the other pizzas ($P<0.05$). However, it is difficult to make assertive conclusions about such results since the ingredient information is lacking for all the USDA samples. But then, higher amount of folic acid levels indicate samples containing higher relative amounts of enriched flour used in their preparation.

The total folate levels in the 5 types of pizzas ranged from 86-107 µg/100g. The mean value for total folate in meat and vegetable pizza has the statistically lowest value of 69 ± 13 µg/100g and that of pepperoni was the highest 107 ± 12.5 µg/100g. Pepperoni pizza was statistically different from meat (92 ±18.5 µg/100g), fast food (86 ± 14.1 µg/100g), and meat and meat and vegetable pizza (69 ± 13.3 µg/100g) at ($P<0.05$). According to the USDA databank, meat products are quiet low in folate compared to most vegetables. Hence, the pepperoni pizza most likely contains a higher ratio of flour to other ingredients. Vegetable toppings contain the following total folate levels (µg/100g): mushroom, 16; green peppers, 23; onions, 19; tomatoes, 13. Addition of such toppings to in place of meats would increase the total folate value compared to the meat type pizzas. This further substantiates that the meat based pizzas in this study contains a higher proportion of enriched flour than some of other types of pizza types.

The food folate values for all the pizzas were statistically similar (P=0.05) with an overall mean of 37.8 ± 4.8 µg/100g. The mean µg DFE content of the meat and vegetable pizza (94 ±15.1 µg/100g) is lower compared to other pizza types. The mean µg DFE content of pepperoni pizza is the highest (151 ± 19.8 µg/100g) followed by the cheese (143 ± 16.7 µg/100g), meat pizza (131 ± 27.8 µg/100g) and the fast food pizza (121 ± 20.5 µg/100g). A definitive conclusion cannot be drawn since the information on ingredient usage is lacking for the samples. However, it can be said that consumption of pepperoni pizza provides higher intake of µg DFE. The overall mean for the 95 pizzas were 53 ± 10.6 µg/100g for folic acid, 90.8 ± 14.4 µg/100g for total folate, 37.8 ± 4.8 µg/100g for food folate and 128 ± 21.8 µg/100g for µg DFE. Figure 2.7 graphically represents the folate levels in the pizzas.

The Folic Acid, Total Folate, Food Folate and µg *DFE Levels in Baked and Uncooked Pizzas after Baking*

Table 2.6 represents the various folate contents in both the baked and uncooked pizzas. The folic acid, total folate, food folate and the DFE contents of supreme baked and supreme raw were not significantly different at 95% confidence interval (P=0.05). The folic acid levels in uncooked supreme pizza was 45 ± 4.0 µg/100g while in baked supreme pizza it was 40 ± 4.3 µg/100g ;The total folate content of the uncooked supreme pizza was 89 ± 6.8 µg/100g ,while in baked supreme pizza it was 84 ± 3.7 µg/100g. The retention percentage upon baking for the supreme pizza was 95 percent for the folic acid and 93 percent for the total folate. This value indicates minimum loss of the folic acid and the total folate content in the process of baking.

Similar to the supreme pizza, there was no significant difference between the baked and the uncooked vegetable pizza (P=0.05). The folic acid content in uncooked vegetable pizza was

50 ± 2.8 µg/100g. For the baked vegetable pizza it was 48 ± 3.0 µg/100g. The decrease in the amount of the folic acid was not statistically different at 95% confidence interval (P=0.05). The total folate content in uncooked vegetable pizza was 88 ± 8.1µg/100g compared to 83 ± 3.8 µg/100g in baked pizza. The food folate in the uncooked vegetable pizza was 38 ± 3.4 µg/100g while the baked had 35 ± 4.5 µg/100g. The µg DFE values for the uncooked vegetable pizza were 124 ± 11.2 µg/100g and 116 ± 4.6 µg/100g for baked vegetable pizza. All of the above folate content values did not show any significant difference between the baked and the uncooked vegetable pizza (P<0.05). The retention percentage upon baking for the vegetable pizza was 98 percent for the folic acid and 96 percent for the total folate. It can be concluded there was minimal loss of the folate contents in the vegetable pizza due to baking. The decrease in the folic acid content in the cheese pizza showed significant difference between the baked and the uncooked (P=0.05). The folic acid content in uncooked cheese pizza was 73 ± 6.7 µg/100g compared to 61 ± 3.1 µg/100g in baked pizza. The total folate and the food folate values in cheese pizza did not show any significant difference between uncooked and the baked at 95% confidence interval. The total folate in the uncooked cheese pizza was 109 ± 6.2 µg/100g while the cooked had 102 ± 8.8 µg/100g. The µg DFE content between the baked and uncooked cheese pizza showed a significant difference at 95% confidence interval. The difference in the retention value of the folic acid, total folate food folate and the µg DFE levels between the raw and the baked type of supreme, vegetable were not significantly different at 95%confidence interval (P=0.05). Therefore, the study indicates that the vitamin is stable even at high temperatures. This study is significant since pizza consumption in the United States is soaring every year and each person consumes about 23 pounds of pizza every year (http://www.pizzaware.com).

The Folic Acid, Total Folate, Food Folate and µg DFE Contents in Fast Foods

A total of 137 USDA fast food samples were analyzed for folic acid and the total folate. The following fast foods were studied: breakfast pastries (n=16), breakfast sandwiches (n=63), fast food sandwiches (n=14), taco salad (n=8), taco burritos (n=32), vegetable burgers (n=4). Table 2.7 gives the means of folic acid, total folate, food folate, µg DFE contents in various fast foods. The fast food sandwiches had the highest amount of the folic acid 43 ± 10.9 µg/100g followed by the breakfast sandwiches 42 ± 10.9 µg/100g. The mean folic acid content in all the fast foods was statistically different at the 95% confidence interval level. An assertive conclusion cannot be drawn since the ingredient information in the fast food sandwiches is lacking. The vegetable burger showed the lowest folic acid value of 9 ± 5.1 µg/100g followed by taco salad (10 ± 4.4 µg/100g) and taco burritos (23 ± 12.1 µg/100g). The mean of the total folate in vegetable burgers was highest 73 ± 24.7 µg/100g and that of taco burritos was the lowest 42 ± 14.5 µg/100g (P=0.05). Breakfast sandwiches (59 ± 15.6 µg/100g) and fast food sandwiches (61± 30 µg/100g) did not show much difference in the values of total folate. The food folate was the lowest in breakfast pastries (11 ± 4.3 µg/100g) and highest vegetable burgers (64 ± 23.0 µg/100g). The food folate in fast food sandwiches (18 ± 9.1 µg/100g) , taco burritos (19 ± 7.3 µg/100g) and of breakfast sandwiches (17 ± 8.9 µg/100g) were not statistically different at 95% confidence interval (P=0.05). The vegetable burgers have considerably high µg DFE value of (79 ± 6.3 µg/100g). This can be explained as the vegetable burgers contain soy protein concentrate as its main ingredient. According to the listed folate content in the NDL, the soy protein concentrate has a highest total folate of 340 µg/100g. The µg DFE content of vegetable burger 79 ± 26.3 µg/100g and breakfast pastries 76 ± 14.8 µg/100g were not significantly different at 95% confidence interval (P=0.05). The µg DFE of the fast food sandwiches was the highest 88 ± 22.4

µg/100g and that of taco salad 44 ± 16.8 µg/100g was lowest (P<0.05). The bar graph in the figure 2.8 depicts the folate levels of fast foods in detail.

Moisture Content

The moisture content in the sample matrix was evaluated to correct the folate levels of the samples to a dry weight basis. The moisture content present in six types of pizzas supreme raw, supreme baked, vegetable raw and vegetable baked is given in Table 2.8. The loss in the moisture content upon baking was significant in all types of pizzas (P=0.05). The loss of moisture content in uncooked supreme pizza was highest (54.8 ± 1.4) compared to a moisture content of 49.6 ± 1.7 in the baked supreme pizza. The mean moisture content in the uncooked vegetable was 46.8 ± 5.2 while the moisture content in the baked vegetable pizza was 46.4 ± 3.7; uncooked cheese pizza was 48.8 ± 0.7, whereas baked cheese pizza was 42.6 ±1.9. The moisture content values were used to calculate the retention of the folate levels in pizzas on dry weight basis to determine the percentage retention in the baked pizzas.

Retention

The retention of the folate contents was calculated and given in the Table 2.9. The retention values were calculated using the formula:

Retention = Baked value/Raw value*100.

The retention of folic acid was highest in vegetable pizza (98%) followed by supreme pizza (95%) and lastly the cheese pizza (75%). The retention of the total folate was highest again in the vegetable pizza (96%) and the food folate retention was highest in the cheese pizza (98%). The cheese pizza had the lowest retention value of the µg DFE and the vegetable pizza had the highest value.

The Nutrient Density

Nutrient Density (ND) is the amount of a particular nutrient (carbohydrate, protein, fat, etc.) per unit of energy in a given food. The nutrient density is calculated by dividing µg DFE /100g by the energy of the food in K cal/100 g of the food. For example the µg DFE content in the meat pizza is 131 and the energy is 287 Kcals /100g .Therefore, the nutrient density for the meat pizza is:

$$\frac{131 \text{ µg DFE}/100\text{g}}{287 \text{ Kcal}/100\text{g}} = 0.456 = 45.6*10^{-2}$$

Nutrient density of pizzas and fast foods calculated from the DFE values are given in the Table 2.10, are calculated from the µg DFE values. The ND of µg DFE content is highest in pepperoni pizza ($54.7*10^{-2}$ µg DFE/Kcal) followed by cheese pizza ($50.0*10^{-2}$ µg DFE/Kcal) and meat pizza ($45.6*10^{-2}$ µg DFE/Kcal). According to the pizza industry facts, pepperoni pizza is the most popular pizza in America since it constitutes to 36% of overall pizza orders (http://www.pizzaware.com). They also state that approximately 251,770,000 pounds of pepperoni is consumed by the US population each year and 66.66% of all Americans order pizzas for dinner. U.S. per capita consumption of mozzarella cheese was 7.93 pounds in 1994 and was predicted to reach 12.51 pounds by 2004 (Source: Pizzaware.com, Business Trend Analysts, BTA.). According to Putnam and Allhouse(1999) the mozzarella cheese consumption has increased more than seven times since the 1970's to 8.4 pounds in 1997. The above facts prove that pizza plays an important role in folate nutrition, which helps in preventing various diseases. Other popular pizza toppings are mushrooms, extra cheese, sausage, green pepper and onions. The other leading sources of folate are spinach that has the highest ND of µg DFE, $843*10^{-2}$ µg DFE/Kcal, orange juice has 66.66 $*10^{-2}$ µg DFE/Kcal; broccoli has $70*10^{-2}$ µg

DFE/Kcal. Vegetable pizza is also a good source of the folate since it delivers a ND of $42.5*10^{-2}$ µg DFE/Kcal. Thus, the pizza acts as a good delivery source of various forms of the folate and, thereby, is a highly significant source of folic acid /folate to the U.S population.

Among the fast-foods, vegetable burger has the highest ND value of $51.6 *10^{-2}$µg DFE/Kcal, followed by the fast food sandwiches ($37.4 *10^{-2}$ µg DFE/Kcal) and the taco salad (29.7 µg DFE/Kcal). The breakfast pastries have the highest energy/Kcal (412). Fast food industry sales have increased more than $125 billion each year, According to a study conducted by the (CSFII) continuing Survey of the Food intake By Individuals, the energy intake from home cooked meals decreased by 11% to 20.8% for all age groups from 1997 to 1996, and the energy intake from fast foods increased by 91.2% and 208% for all age groups. Energy intake from fast foods consumption increased from 9.6% to 23.5% between 1977 and 1996 (Neilson et al., 2002). Types of fast foods consumed in the United States ranges from fast food burgers and fries to ethnic foods like tacos, fajitas, Chinese noodles, pasta and pizza (Regmi, 2001). Enriched flour is the main source of the µg DFE in these products. The ND of the µg DFE levels in wheat bread is $79.7*10^{-2}$ µg DFE/Kcal while macaroni has $87.3*10^{-2}$ µg DFE/Kcal. Rice has a ND of about $74.61*10^{-2}$ µg DFE/Kcal and noodles have $78.1*10^{-2}$ µg DFE/Kcal (SR, 17).

4.2 CONCLUSION

Fast food consumption in the United States has been dramatically increasing since the 1970's. Pizza forms an integral part of the American diet. Pizza industry alone is a $32+ billion per year, and 94% of the population of the U.S. eats pizza. As the survey of the literature indicated, sufficient information is not available on the content of folic acid and total folate in fast foods made with enriched flour.

Therefore, the purpose of this study was to determine the folic acid and total folate in various fast foods and pizzas containing enriched flour, and also to calculate the food folate and the µg DFE from the analytical data. This study also determined the effect of baking on the retention of the folic acid and the total folate in pizzas. Conclusions derived from the study include the following:

1. Analytical quality control studies showed that the method using microbiological assay and trienzyme extraction was applicable to the assay of folic acid and total folate in pizza and fast foods.

2. Accuracy measured by intra-run precision and trueness measured by assay of Infant Formula SRM 1846 indicated excellent assay repeatability and agreement to the expected SRM value, respectively.

3. Recovery values for folic acid and total folate assays were = 95%.

4. µg DFE/100g values for pizza ranged from 94-161, showing that pizza is an important source of folate to the U.S consumer.

5. µg DFE/100g values for fast foods ranged from 60-91.While not as high as pizza,these µg DFE/100g levels are high in comparison to most other foods, except for some vegetables in the U.S diet.

6. Folate in pizza was quiet suitable for baking process. Retention (%) on a µg DFE/100 basis ranged from 80(cheese pizza) to 96 (vegetable pizza).

7. Calculation of Nutrient Density µg DFE/100 indicates that pizza and fast foods compare favorably to foods such as orange juice, broccoli and spinach which are considered excellent folate sources.

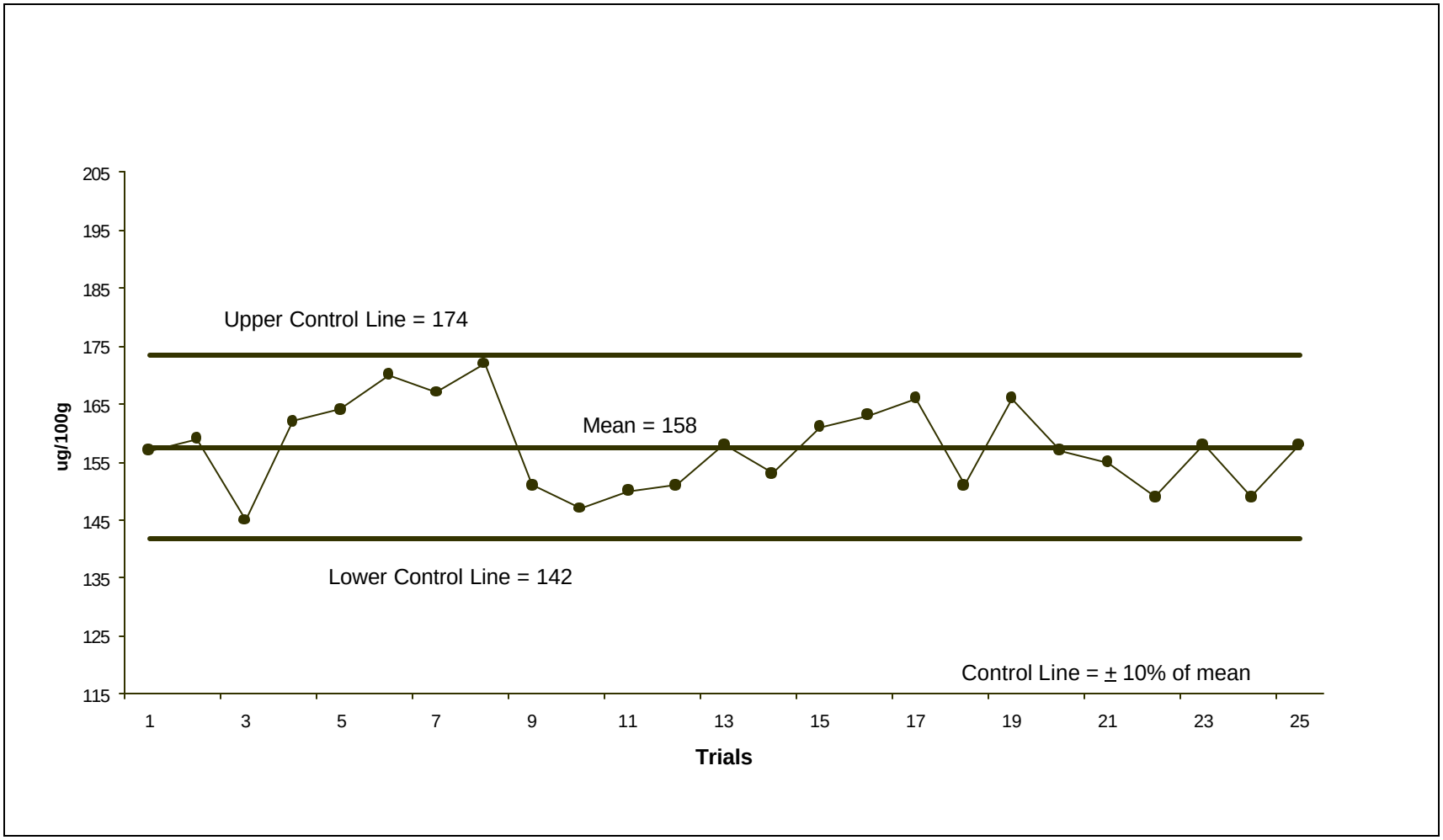


Figure 4.1

Control Chart for Folic acid in Enriched Flour.

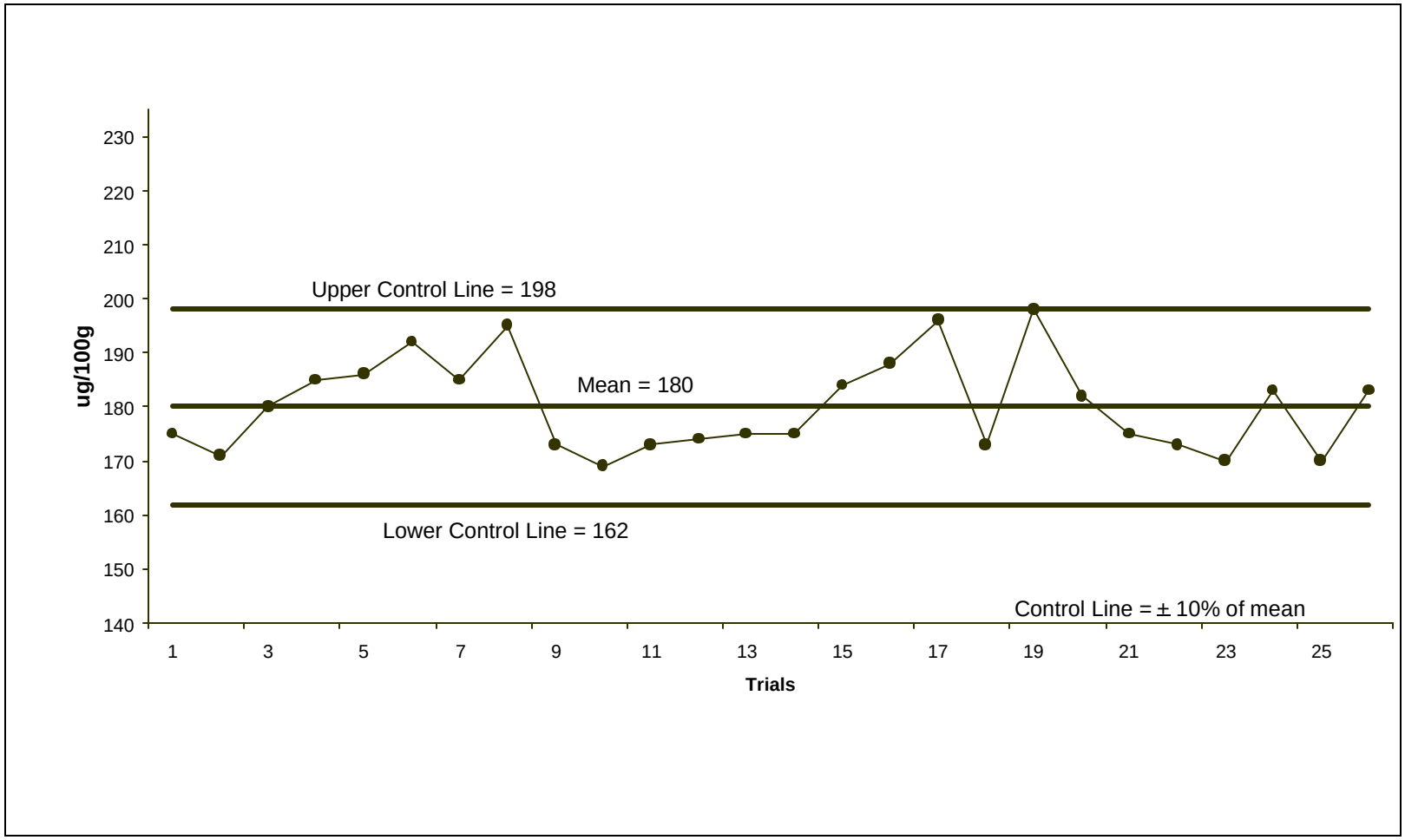


Figure 4.2

Control Chart for Total Folate in Enriched Flour.

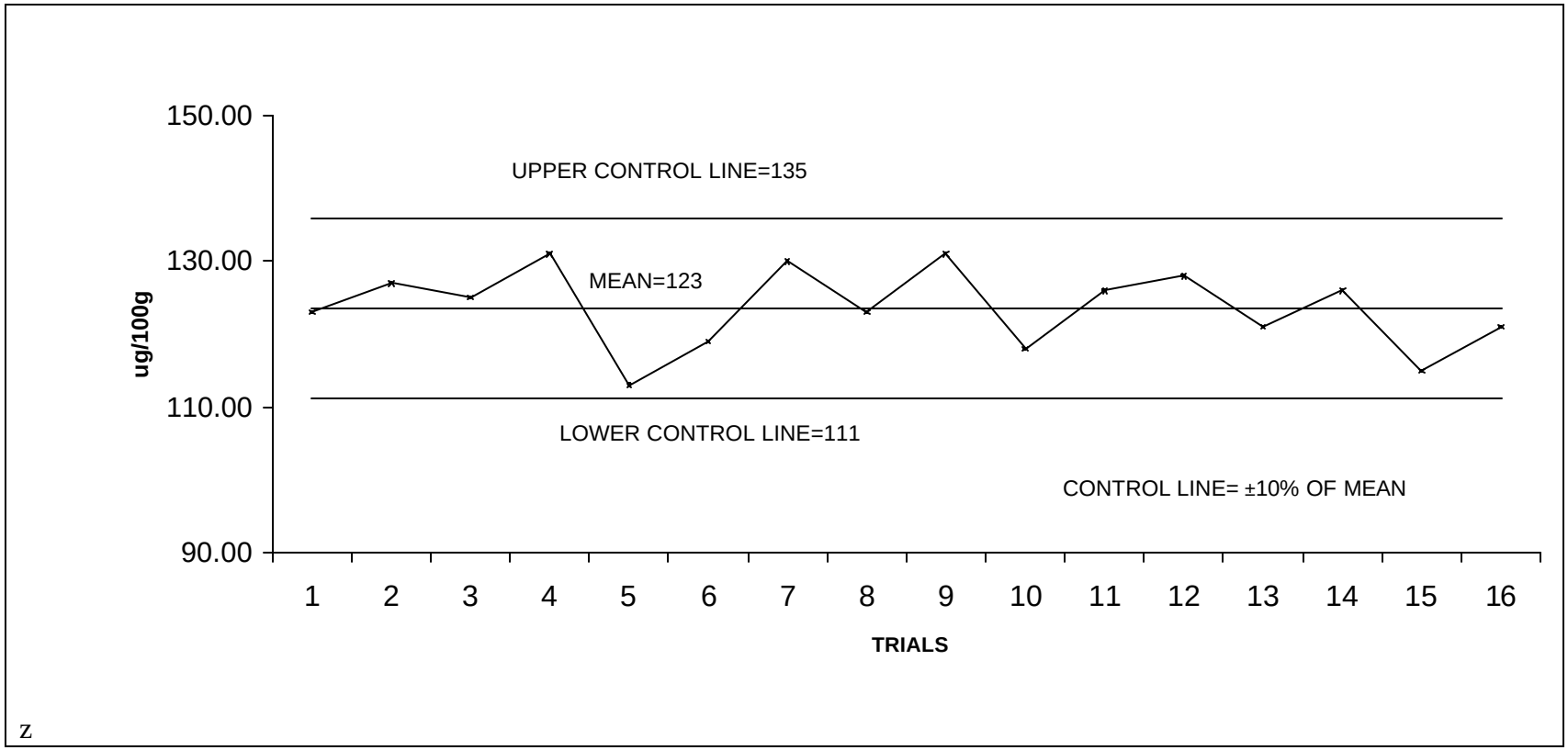


Figure 4.3

Control Chart for Folic acid in SRM 1846

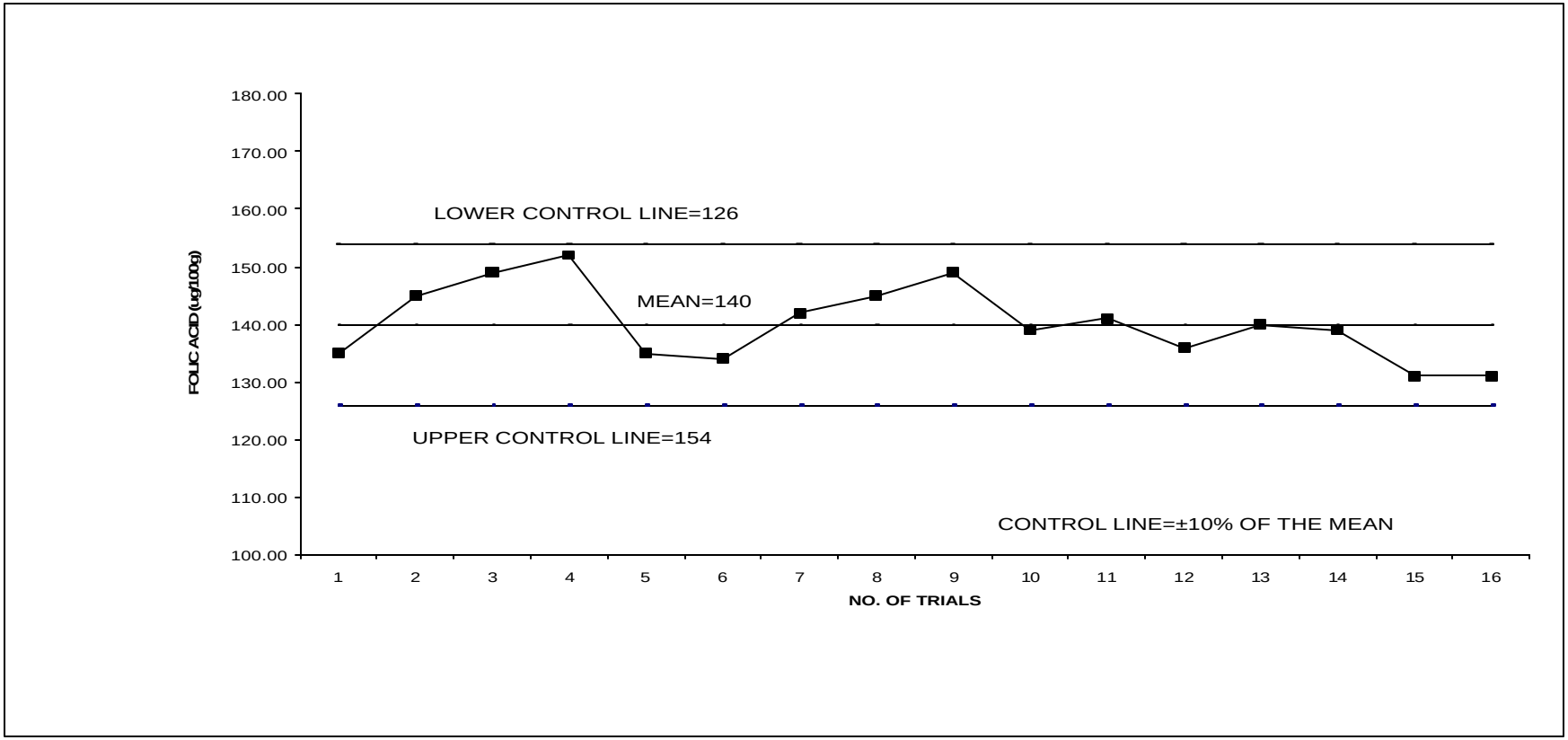
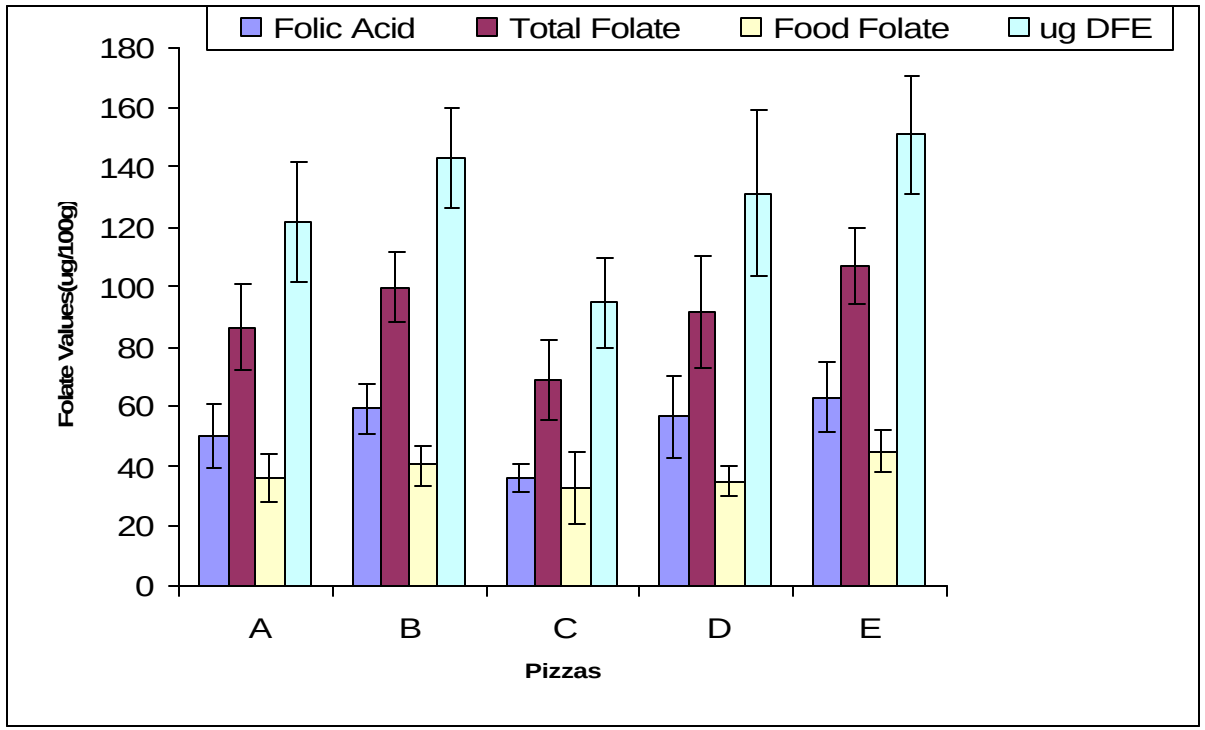


Figure 4.4

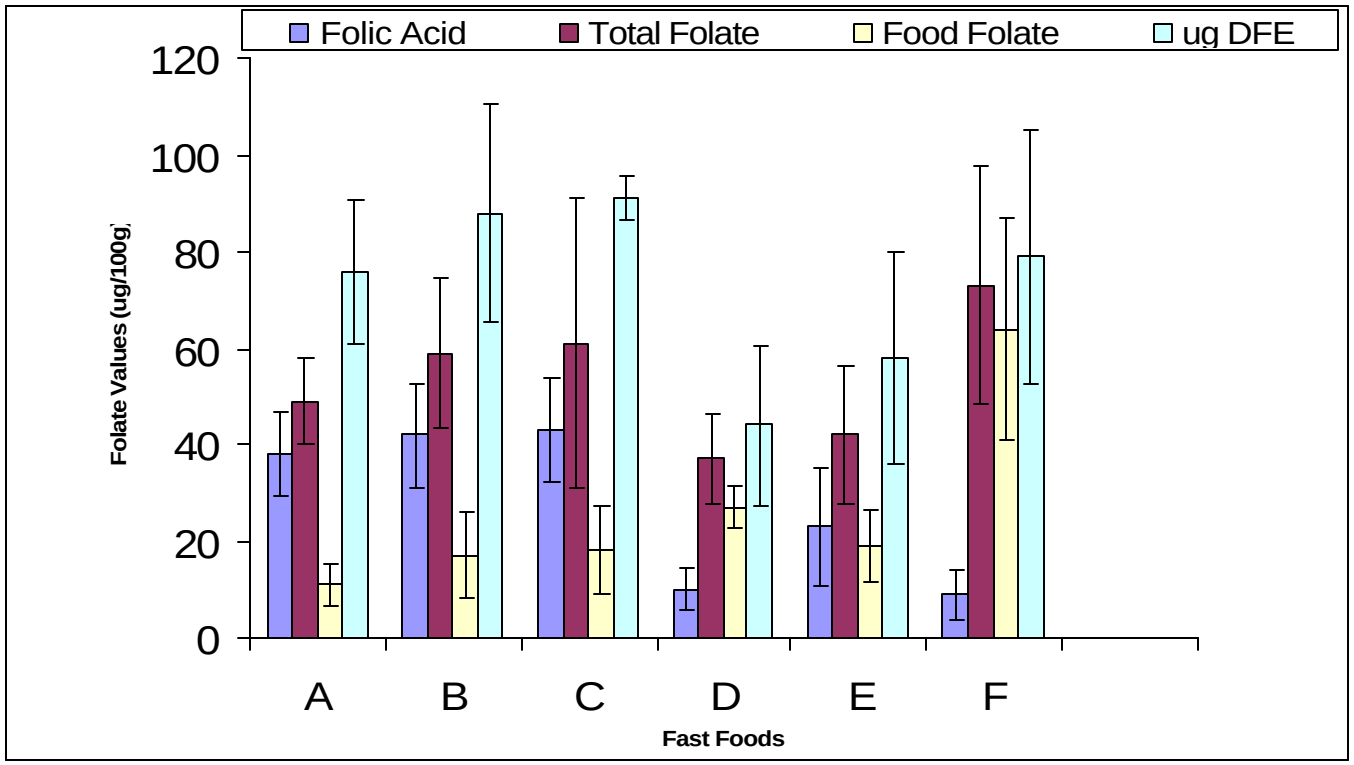
Control Chart for Total Folate in SRM 1846



A=Fast Food
B=Cheese
C=Meat and Vegetable
D=Meat
E=Pepperoni

Figure 4.5

Folic Acid, Total Folate, Food Folate and µg DFE levels in Pizzas



A=Breakfast pastries
B= Breakfast sandwiches
C=Fast Food Sandwiches
D=Taco salad
E=Taco burritos
F=vegetable burgers.

Figure 4.6

Folic Acid,Total Folate , Food Folate and µg DFE levels in Fast- Foods

Table 4.1

Analytical Recovery Values for Pizza

Pizza type(n)	Folic acid[a]	Total folate[a]
Fast food(n=12)	99 ± 0.5	98 ± 0.2
Cheese(n=5)	99 ± 3.1	99 ± 0.6
Meat and vegetable (n=2)	100 ± 2.8	99 ±1.4
Meat (n=2)	98 ± 2.2	96 ±1.3
Pepperoni(n=4)	98 ± 0.7	100 ± 0.4
Mean ± of S.D of 5 pizza types (n=25)	99 ± 0.5	98 ±1.4

[a] % ± S.D

Table 4.2

Analytical Recovery Values for Uncooked and Baked Pizzas.

	Recovery (%)			
	Uncooked		Baked	
Pizza type (n)	Folic acid	Total folate	Folic acid	Total folate
Cheese Pizza (2)	99.7	100	97.7	100
Vegetable Pizza(2)	100	96.9	97.7	95.2
Supreme Pizza(2)	95.3	98.5	100	99.9
Mean ± S.D(n=6)	98.3 ± 2.6	98.4 ± 1.6	99.1±1.3	98.4±2.8

Table 4.3

Folic Acid,Total Folate and Food Folate and µg DFE levels in Pizzas (µg/100g)[a]

Pizzas	Folic Acid[b]	Total Folate[b]	Food Folate[b]	µg DFE[b]
Fast Food (n=56)	$50^{B} \pm 10.1$	$86^{C} \pm 14.1$	$36^{B} \pm 8.1$	$121^{C} \pm 20.5$
Cheese(n=18)	$59^{AB} \pm 8.6$	$100^{AB} \pm 11.5$	$41^{AB} \pm 6.7$	$143^{AB} \pm 16.7$
Meat(n=6)	$57^{AB} \pm 13.5$	$92^{BC} \pm 18.5$	$35^{B} \pm 4.2$	$131^{BC} \pm 27.8$
Meat and vegetable(n=7)	$36^{C} \pm 4.5$	$69^{D} \pm 13.3$	$33^{B} \pm 6.9$	$94^{D} \pm 15.1$
Pepperoni(n=8)	$63^{A} \pm 11.8$	$107^{A} \pm 12.5$	$46^{A} \pm 7.2$	$151^{A} \pm 19.8$
Mean ± S.D	53 ± 10.6	90.8 ± 14.4	37.8 ± 4.8	128 ± 21.8

[a]Mean ± S.D

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[1] samples collected from fast food restaurants

[b]Means with same letters are not significantly different.

Table 4.4

Means of Folic Acid, Total Folate, Food Folate and µg DFE levels in Baked and Uncooked Pizzas (µg/100g)[a]

Sample	Folic acid[b]	Total folate[b]	Food folate[b]	µg DFE[b]
Supreme uncooked (n=5)	$45^{A} \pm 4.0$	$89^{A} \pm 3.7$	$44^{A} \pm 3.7$	$120^{A} \pm 5.8$
Supreme baked (n=5)	$40^{A} \pm 4.3$	$84^{A} \pm 6.8$	$44^{A} \pm 3.2$	$112^{A} \pm 9.8$
Vegetable uncooked (n=5)	$50^{A} \pm 2.8$	$88^{A} \pm 8.1$	$38^{A} \pm 3.4$	$124^{A} \pm 11.2$
Vegetable baked (n=5)	$48^{A} \pm 3.0$	$83^{A} \pm 3.8$	$35^{A} \pm 4.5$	$116^{A} \pm 4.6$
Cheese uncooked (n=5)	$73^{A} \pm 6.7$	$109^{A} \pm 6.2$	$36^{A} \pm 2.3$	$161^{A} \pm 4.5$
Cheese baked (n=5)	$61^{B} \pm 3.1$	$102^{A} \pm 8.8$	$41^{A} \pm 3.3$	$145^{B} \pm 13.2$

[a]Mean ± S.D
ND –Not detectable

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[b]Means for the same type of pizzas with same letters are not significantly different ($p<0.05$).

Table 4.5

Means of Folic Acid, Total Folate, Food Folate and µg DFE levels in Fast Foods (µg/100g)[a]

Type	Folic Acid[b]	Total Folate[b]	Food Folate[b]	µg DFE[b]
Breakfast Pastries(n=16)	$38^{A} \pm 8.8$	$49^{BDC} \pm 9.1$	$11^{C} \pm 4.3$	$76^{AB} \pm 4.8$
Breakfast sandwiches(n=63)	$42^{A} \pm 10.9$	$59^{ABC} \pm 15.6$	$17^{BC} \pm 8.9$	$88^{A} \pm 22.4$
Fast Food Sandwiches(n=14)	$43^{A} \pm 10.9$	$61^{AB} \pm 30$	$18^{BC} \pm 9.1$	$91^{A} \pm 4.5$
Taco Salad(n=8)	$10^{C} \pm 4.4$	$37^{D} \pm 9.3$	$27^{B} \pm 4.4$	$44^{C} \pm 16.8$
Taco Burritos(n=32)	$23^{B} \pm 12.1$	$42^{CD} \pm 14.5$	$19^{BC} \pm 7.3$	$58^{CB} \pm 22.2$
Vegetable burger(n=4)	$9^{C} \pm 5.1$	$73^{A} \pm 24.7$	$64^{A} \pm 23.0$	$79^{AB} \pm 6.3$

[a]Mean ± S.D
ND –Not detectable

---

[b]Means with same letters are not significantly different.($p<0.05$).

Table 4.6

Moisture Content in Baked and Uncooked Pizzas[a]

Pizza	Mean[a]
Vegetable raw (n=5)	46.8±5.2
Vegetable baked (n=5)	46.4±3.7
Supreme raw (n=5)	54.8±1.4
Supreme baked (n=5)	49.6±1.7
Cheese raw (n=5)	48.8±0.7
Cheese baked (n=5)	42.6±1.9

[a] % ± S.D

Table 4.7

Retention of Folate Values after Baking [a]

Type of Pizza	Folic Acid	Total Folate	Food Folate	µg DFE
Cheese	75±5.2	83±6.3	98±5.5	80±6.9
Vegetable	98±8.2	96±6.9	93±2.0	96±2.1
Supreme	95±4.1	93±1.3	92±3.2	94±2.4

[a] % ± S.D

Table 4.8

Nutrient Density of µg DFE Levels in Fast Foods and Pizzas

Sample	Kcal/100g[a]	µg DFE/100g	Nutrient Density[b] $*10^{-2}$
Fast Food	286	117	40.9
Cheese	286	143	50.0
Meat	287	131	45.6
Meat and vegetable	253	94	37.2
Pepperoni	276	151	54.7
Supreme	253	112	44.3
Vegetable	273	116	42.5
Breakfast pastries	412	76	18.4
Breakfast sandwiches	401	89	22.1
Fast-food Sandwiches	243	91	37.4
Taco salad	141	42	29.7
Taco burritos	216	59	27.3
Vegetable burger	153	79	51.6

[a] USDA National Nutrient Database for Standard Reference, SR 17, .2004. (http://www.nal.usda.gov/fnic/foodcomp)

[b] Nutrient Density = $\frac{\text{µg DFE/100g}}{\text{Kcal/100g}} = \frac{\text{µg DFE}}{\text{Kcal}}$

4.3 BIBLIOGRAPHY

Aiso, K., Tamura, T. 1998. Trienzyme treatment for food folate analysis: optimal pH and incubation time for a-amylase and protease treatments. J Nutr Sci Vitaminol 44: 361–370.

Allegra, C.J. 1990. *Cancer Chemotherapy: Principles and Practice.* ed. Chabner, B.A., Collins, J.M. p.110-153, Lippincott J.B Co, Philadelphia.

Anderson, E., Perloff, B., Ahuja, K.C., Raper, N. 2001. Tracking nutrient changes for trend analysis in the United States. J Food Comps Anal 14: 287-294.

Anderson, J.K., Kurt, R.J., Carlquist, J.F., Blair, T.L., Horne, B.D., Muhlestein, J.B. 2004. Effect of folic acid fortification of food on homocysteine-related mortality. Am J Med 116: 158-164.

Arcot, J.A., Shrestha, A.K., Gusanov, U.B. 2002. Enzyme protein binding assay for determining folic acid in fortified cereal foods and stability of folic acid under different extraction conditions. Food Control 13: 245–252.

Association of Official Analytical Chemists. Vitamin preparations, microbiological methods. In *Official Methods of Analysis*, 17th ed. Section 960.46. Arlington, VA.

Association of Official Analytical Chemists. Folic acid (pteroyl-monoglutamic acid) in infant formula, microbiological methods. In *Official Methods of Analysis,* 17th ed. Section 992.05. Arlington, VA.

Association of Official Analytical Chemists. Folic acid (pteroyl-monoglutamic acid) in vitamin preparations. In *Official Methods of Analysis,* 17th ed. Section 944.12. Arlington, VA.

Association of Official Analytical Chemists. Moisture content in frozen french fried potatoes. In *Official Methods of Analysis,* 17th ed. Section 984.25. Arlington, VA.

Bagley, P.J., Selhub, J. 1997. Analysis of folates using combined affinity and ion-pair chromatography. Methods Enzymol 281:16-25.

Bailey, L.B. 1995. Folate chemistry and metabolism. In *Folate in Health and Disease.* ed. Shane, B. p.2-3, Marcel Dekker, Inc, New York.

Bailey, L.B. 1995. Folate-cobalamin interactions. In *Folate in Health and Disease.* ed. Savage, D.G., Lindenbaum, J. p.237-286, Marcel Dekker, Inc, New York.

Bailey, L.B. 2004. Folate and vitamin B12 recommended intakes and status in the United States. Nutr Rev 62: 14-20.

Beresford, S.A.A., Boushey, C.J. 1997. Homocysteine, folic acid, and cardiovascular disease risk. In *Preventive Nutrition: The Comprehensive Guide for Health Professionals*. ed. Bendich, A., Deckelbaum, R.J. p.193–224, Humana Press, Totowa, NJ.

Bernstein, M.L, Leclerc, J.M., Bunin, G., Brisson, L., Robison, L., Shuster, J. 1992. A population-based study of neuroblastoma incidence, survival, and mortality in North America. J Clin Oncol 10: 323-329.

Boushey, C.J., Beresford, S.A., Omen, G.S., Motulsky, A.G. 1995. A quantitative assessment of plasma homocysteine as a risk factor for vascular diseases, probable benefit of increasing folic acid intakes. Am J Med Assoc 274: 1049-1057.

Bower, C., Stanley, F.J. 1989. Dietary folate as a risk factor for neural tube defects: evidence from a case-controlled study in western Australia. Med J Aust 150: 613-619.

Brattstrom, L.E., Israelsson, B., Jeppsson, J.O., Hultberg, B.L. 1988. Folic acid an innocuous means to reduce plasma homocysteine. Scand J Clin Lab Invest 48: 215-221.

Canada Gazette Part –II. 1998. Regulatory impact analysis statement, SOR/98-550,132: 3029-3033.

Chen, M.F., Hill, J.W., McIntyre, P.A. 1983. The folate in contents of foods as measured by radiometric microbiological method. J Nutr 113: 2192-2196.

Cheruppolil, R.S.K., Kolhouse, J.F. 1997. Molar quantitation of folates by gas chromatography mass spectrometry. Methods Enzymol 261: 26-34.

Czeizel, A.E. 1993. Prevention of congenital abnormalities by periconceptional multivitamin supplementation. Br Med J 306: 1645-1648.

Czeizel, A.E., Dudas, I. 1992. Prevention of the first occurrence of neural-tube defects by periconceptional vitamin supplementation. N Engl J Med 327: 1832-1835.

Day, B.P., Gregory, J.F. 1981. Determination of folacin derivatives in selected foods by high-performance liquid chromatography. J Agr Food Chem 29: 374-377.

De la Calle, M., Usandizaga, R., Sancha, M., Magdaleno, F., Herranz, A., Cabrillo, E. 2003. Homocysteine, folic acid and B-group of vitamins in obstetrics and gynecology. Eur J Obstet Gyn 107: 125-134.

DeSouza, S., Eitenmiller, R.R. 1990. Effects of different enzyme treatments on extraction of total folate from various foods prior to microbiological assay and radio assay. J Micronutr Anal 7: 37-57.

Department of Health and Human Services, Public Health Service, Food and drug administration(DHH/PHS), USA. 1993. Food standards: Amendment of standards of identity for enriched grain products to require addition of folic acid: proposed rule, (21 CFR Parts 136, 137 and 139). Federal Register, 58: 53305-53312.

Doherty, R.F., Beecher, G.R. 2003. A method for the analysis of natural and synthetic folate in foods. J Agr Food Chem 51: 354-361.

Doshi, S.N., Moat, S.J., McDowell, I.F.W., Lewis, M.J., Goodfellow, J. 2002. Lowering plasma homocysteine with folic acid in cardiovascular disease: what will the trials tell us?. Atherosclerosis 165: 1-3.

DeVries, J.W., Rader, J.I., Keagy, P.M., Hudson, C.A. 2005. Microbiological assay – trienzyme procedure for total folates in cereals and cereal foods: collaborative study. J AOAC Intl 88: 5-15.

DeVries, J.W., Rader, J.I., Keagy, P.M., Hudson, C.A. 2001. AACC collaborative study of a method for determining total folate in cereal products – microbiological assay using trienzyme extraction (AACC Method 86 – 47). Cereal Foods World 46: 216–219.

Eitenmiller, R.R., Landen Jr, W.O. 1998. *Vitamin Analysis for Health and Food Sciences.* p.411-466, CRC Press, Boca Raton, FL.

Eicher, E.R., Pierce, H.I., Hillman, R.S. 1971. The evolution of anemia in alcoholic patients. Am J Med 50: 218-232.

Feinleib, M., Beresford, S.A.A., Bowman, B.A., Mills, J.L., Rader, J.I., Selhub, J., and Yetley, E.A. 2001. Folate fortification for the prevention of birth defects: case study. Am J Epidemiol 154: 60-69.

Finglas, P.M., Faure, U., Southgate, D.A.T. 1993. First BCR intercomparision on the determination of folates in food. Food Chem 46: 199-213.

Finglas, P.M., Kwiatkowska, C., Faulks, R.M., Morgan, R. M. A. 1988. Comparison of a non-isotopic, microtitration plate folate binding protein assay and a microbiological method for the determination of folate in raw and cooked vegetables. J Micronut Anal 4: 309–322.

Flood, T., Brewster, M., Harris, J. 1992. Spina bifida incidence at birth-United States, 1983-1990. Morbid Mortal Weekly Rep 41:1.

Flood, V.M., Webb, K.L., Smith, W., Mitchell, P., Bantick, J.M., Macintyre, R., Sindhusake, D., Rubin, G.L. 2001. Folate fortification: potential impact on folate intake in an older population. Eur J Clin Nutr 55: 793-800.

Food and Drug Administration, USA .1996. Food additives permitted for direct addition to food for human consumption; folic acid (folacin); final rule, (21 CFR Part 172). Federal Register, 61: 8797-8807.

Food and Drug Administration, USA. 1993. Food standards: food labeling: health claims and label statements' folate and neural tube defects; proposed rule, (21 CFR Part 101). Federal Register, 58: 53254-53295.

Food and drug administration, USA. 1996. Food additives permitted for direct addition to food for human consumption; folic acid (folacin); final rule, (21 CFR Part 172). Federal Register, 61: 8797-8807.

Food and Drug Administration, USA. 1996. Food standards: amendment of standards of identity for enriched grain products to require addition of folic acid: final rule, (21 CFR Parts 136, 137 and 139). Federal Register, 61: 8781-8797.

Franchise Help. 2002. Top Market Share Pizza Chain Restaurant Survey.

(http://www.pizzaware.com)

Freire, W.B., Howson, C.P., Cordero, J.F. 2004. Recommended levels of folic acid and vitamin B12 fortification: A PAHO/MOD/CDC technical consultation. Nutr Rev 62: 1-2.

Gao, R.N., Levy, I.G., Woods, W.G., Coombs, B.A., Gaudette, L.A., Hill, G.B. 1997. Incidence and mortality of neuroblastoma in Canada compared with other childhood cancers. Cancer Cause Control 8: 745-754.

Glynn, S.A., Albanes, D. 1994. Folate and cancer: a review of the literature. Nutr Cancer 22: 101-l19.

Gregory, J.F. 1989. Chemical and nutritional aspects of folate research: analytical procedures, methods of folate synthesis, stability and bioavailability of dietary folates. In *Advances in Food and Nutrition Research.* p.126, Academic Press, San Diego, CA**.**

Gregory, J.F. 1997. Bioavailability of folate. Eur J Clin Nutr 51: 554-560.

Gregory, J.F., Sartain, D.B., Day, B.P.F. 1984. Fluorometric determination of folacin in biological materials using high performance liquid chromatography. J Nutr 114: 341-353.

Gucciardi, E., Pietrusiak, M.A., Reynolds, D.L., Rouleau, J. 2002. Incidence of neural tube defects in Ontario, 1986–1999. Can Med Assoc J 167: 237–240.

Gurney, J.G., Ross, J.A., Wall, D.A., Bleyer, W.A., Severson, R.K., Robison, L.L. 1997. Infant cancer in the U.S: Histology specific incidence and trends, 1973 to 1992. J Pediatr Hematol Oncol 19: 428-432.

Haytowitz, D.B., Pehrsson, P.R., Smith, J., Gebhardt, S.E., Mathews, R.H and Anderson BA. 1996. Key foods: setting priorities for nutrient analysis. J Food Comps Anal 9: 331-364.

Haytowitz, D.B., Pehrsson, P.R., Holden, J.M. 2002. The Identification of key foods for food consumption research. J Food Comps Anal 15: 183-194.

Herbert, N.N., Coleman, J.G. 1979. Hematological aspects of folate deficiency. In F*olic Acid in Neurology, Psychiatry, and Internal Medicine*. ed. Botez, M.I., Reynolds, E.H. p.63, Raven Press, New York.

Hepburn, F.N. 1987. Food consumption/food consumption interrelationships. U.S. Department of Agriculture, Human Nutrition Information Service. Administration Report No.382: 68-74.

Hoffbrand, A.V., Newcombe, B.F., Mollin, D.L. 1966. Method of assay of red cell folate activity and the value of assay as a test for folate deficiency. J Clin Pathol 19: 17-28.

Honein, M.A., Paulozzi, L.J., Matthews, T.J., Erickson, J.D., Wong, L.Y.C. 2001. Impact of folic acid fortification of the U.S food supply on the occurrence of neural tube defects. J Am Med Assoc 285: 2981–2986.

Institute of Medicine, Food and Nutrition Board. 1998. Folate. In *Dietary reference Intakes in Thiamine, Riboflavin, Niacin, Vitamin B6, Folate, Vitamin B12, Pantothenic Acid, Biotin and Choline.* p.196-305, National Academy Press, Washington, DC.

Iwatani, Y., Arcot, J., Shrestha, A.K. 2003. Determination of folate contents in some Australian vegetables. J Food Comps Anal 16: 37-48.

Johnston, K.E., Lofgren, P.A., Tamura, T. 2002. Folate concentrations of fast foods measured by trienzyme extraction method. Food Res Intl 3: 565-569.

Konings, E.J.M. 1999. A validated liquid chromatographic method for determining folates in vegetables, milk powder, liver and flour. J AOAC Intl 82: 119-127.

Konings, E.J.K., Roomans, H.H.S., Dorant, E., Golidborm, R.A., Saris, W.H.M., Van den Brandt, P.A. 2001. Folate intake of the Dutch population according to newly established liquid chromatography data for foods. Am J Clin Nutr 73: 765-766.

Laurance, K.M., James, N., Miller, M., Tennant, G.B., Campbell, H. 1981. Double-blind randomised controlled trial of folate treatment before conception to prevent recurrence of neural tube defects. Br Med J 282: 1509-1511.

Life Sciences Research Office (LSRO), Federation of American Societies for Experimental Biology, (1995). Third report on nutrition monitoring in the United States, p.92, Tables 5-12, Prepared for the Interagency Board for Nutrition Monitoring and Related Research,US Government Printing Office, Washington, DC.

Malinow, M.R., Duell, P.B., Hess, D.L., Anderson, P.H., Kruger, W.D., Phillipson, B.E., Gluckman, R.A., Block, P.C., Upson, B.M. 1998. Reduction of plasma homocystiene levels by breakfast cereal fortified with folic acid in patients with coronary heart disease. N England J Med 338: 09-15.

Mandella, R., DePaola, D.P. 1984. Measurement of tissue folates: A comparison of radioassay and microbiological methods. Nutr Research 4: 521–527.

Martin, J.I., Landen Jr, W.O., Soliman, A.G.M., Eitenmiller, R.R. 1990. Application of the tri-enzyme extraction for total folate determination in foods. J AOAC 73: 805-808.

Massaro, E.J., Rogers, J.M. 2002. *Folate and Human Development.* p.5, Preface, Humana Press, Totowa, N.J.

Milunsky, H., Jick, S.S., Jick, C.L., Bruell, D.S., MacLaughlin, K.J., Rothman, W.W. 1989. Multivitamin/folic acid supplementation in early pregnancy reduces the prevalence of neural tube defects. J Am Med Assoc 262: 2847-2852.

Mitchell, L.E., Adzick, N.S., Melchionne, J., Pasquariello, P.S., Sutton, L.N., Whitehead, A.S. 2004. Spina bifida. Lancet 364: 1885-1895.

Moore, L.L., Bradlee, M.L., Singer, M.R., Rothman, K.J., Milunsky, A. 2003. Folate intake and the risk of neural tube defects: an estimation of dose-response. Epidemiology 14**:** 200–205.

Motulsky, A.G. 1996. Nutritional ecogenetics: Homocysteine related arteriosclerotic vascular disease, neural tube defects and folic acid. Am J Hum Genet 58**:** 17–20.

Mulinare, J., Cordero, J.F., Erickson, J.D., Berry, R.J. 1988. Periconceptional use of multivitamins and the occurrence of neural tube defects. J Am Med Assoc 260: 3141-3145.

Neilson, S.J., Siega-Riz, A.M., Riz, R.D., Popkin, B.M. 2002. Trends in food locations and sources among adolescents and young Adults. Preventive Medicine 35: 107-113.

Neilson, S.J., Siega-Riz, A.M., Popkin, B.M. 2002. Trends in energy intake in US between 1977 and 1996: Similar shifts seen across age groups. Obes Res 10: 370 -378.

National Institute of Standards & Technology. 2004. Certificate of analysis. Standard Reference Material 1846. Gaithersburg, MD.

Olshan, A.F., Smith, J.C., Bondy, M.L., Neglia, J.P., Pollock, B.H. 2002. Maternal vitamin use and reduced risk of neuroblastoma. Epidemiology 13: 575-580.

Pawlosky, R.J., Flanagan, V.P. 2001. A quantitative stable-isotope LC-MS method for folic acid in fortified foods. J Agr Food Chem 49: 1282–1286.

Pawlosky, R.J., Flanagan, V.P., Doherty, R.F. 2003. A mass spectroscopic validated HPLC procedure for determination of folates in foods. J Agr Food Chem 51: 3726-3730.

Pehrsson, P.R., Haytowitz, D.B., Holden, J.M. 2003. The USDA's national food and nutrient analysis program: update. J Food Comps Anal 16: 331-341.

Perloff, B.P., Butrum, R.R. 1977. Folacin in selected foods. J Am Diet Assoc 70: 161-172.

Persad, V.L., Van den Hof, M.C., Dube, J.M., Zimmer, P. 2002. Incidence of open neural tube defects in Nova Scotia after folic acid fortification. Can Med Assoc J 167: 241–245.

Pfeiffer, C.M., Rogers, L.M., Gregory, J.F. 1997. Determination of folate in cereal-grain food products using trienzyme extraction and combined affinity and reverse phase liquid chromatography. J Agr Food Chem 45: 407-413.

Putnam, J.J and Allhouse, J.E. 1999. Food consumption, prices, and expenditures, 1970-1997. Statistical Bulletin No .965:196.

Puwastien, P., Pinprapai, N., Judprasong, K., Tamura, T. 2005. International inter-laboratory analyses of food folate. J Food Comps Anal 18: 387–397.

Rader, J.I. 2002. Folic Acid fortification folate status and plasma homocysteine. J Nutr 132: 2466-2470.

Rader, J.I., Weaver, C.M., Angyal, G. 1998. Use of Microbiological assay with tri-enzyme extraction for measurement of pre-fortification levels of folates in enriched cereal grain products the United States following fortification. Food Chem 62: 451-465.

Rader, J.I., Weaver, C.M., Angyal, G. 2000. Total folate in enriched cereal-grain products in the United States following fortification. Food Chem 70: 275–289.

Regmi, A. 2001. Market and trade economics division, E. R. S, U.S. Department of Agriculture. Changing structure of global food consumption and trade. Agriculture and Trade Report WRS-01-1.

Rychlik, M., Freisleben, A. 2002. Quantification of pantothenic acid and folates by stable isotope dilution assays. J Food Com Anal 15: 399–409.

SAS institute. 1990. *SAS user's guide*. The SAS Institute. Cary, NC.

Schorah, C.J., Devitt, H., Lucock, M.D., Dowell, A.C. 1998. The responsiveness of plasma homocysteine to small increases in dietary folic acid: A primary care study. Eur J Clin Nutr 52: 407-411.

Scott, J. 2001. Randomized trial of folic acid supplementation and serum homocysteine levels. Arch Intern Med 161: 695–700.

Seeger, D.R., Cosulich, D.B., Smith, J.M., Hultquist, M.E. 1949. Analogs of pteroyl glutamic acid. III. 4-Amino derivatives. J Am Chem Soc 71: 1753-1755.

Selhub, J., Darcy-Vrillon. B., Fell, D. 1988. Affinity chromatography of naturally occurring folate derivatives. Anal Biochem 168: 247-251.

Seyoum, E., Selhub, J. 1993. Combined affinity and ion pair column chromatographies for analysis of food folate. J Nutr Biochem 4: 448-494.

Shaw, G.M., Malley, C.D., Wasserman, C.R. 1995. Maternal periconceptional use of multivitamins and reduced risk for conotruncal heart defects and limb deficiencies among offspring. Am J Med Genet 59: 536-545.

Shin, Y.S., Kim, E.S., Watson, J.E., Stokstad, E.L.R. 1975. Studies of folic acid compounds in nature: Folic acid compounds in soybeans and cow milk. Can J Biochem 53: 338-343.

Shrestha, A.K., Arcot, J., Paterson, J. 2000. Folate Assay of foods by traditional and tri-enzyme treatments using cytoprotected *Lactobacillus casei*. Food Chem 71: 545 -552.

Smithells, R.W., Sheppard, S., Schorah, C.J. 1981. Apparent prevention of neural tube defects by periconceptual vitamin supplementation. Arch Dis Child. 56: 911-918.

Steegers, R.P., Boers, G.H., Blom, H.J., Nijhuis, J.G., Chris, M.G., Thomas Borm, G.F., Eskes, T.K. 1995. Neural tube defects and elevated homocysteine levels in amniotic fluid. Am J Obstet Gynecol 172: 1436-1441.

Stralsjo, L., Arkbage, K., Witthoft, C., Jagerstad, M. 2002. Evaluation of a radioprotein-binding assay (RPBA) for folate analysis in berries and milk. Food Chem 79: 525-534.

Thompson, M., Ellison. S., Wood, R. 2002. Pure and Appl Chem 74: 835.

Tamura, T. 1998. Determination of food folate. J Nutr Biochem 9: 285-293.

Tamura, T. 1990. Microbiological assay of folates. In *Folic Acid Metabolism in Health and Disease, Contemporary Issues in Clinical Nutrition*. ed. Picciano, M.F., Stokstad, E.L.R., Gregory, J.F.III. vol.13. p.121-137, Wiley-Liss, New York.

Tamura, T., Mizuno, Y., Johnston, K.E., Jacob, RA. 1997. Food folate assay with protease, a-amylase and folate conjugase treatments. J Agr Food Chem 45:135–139.

Taverniers, I., Bockstaele, V.E., Loose, D.M. 2004. Trends in quality in the analytical laboratory, Traceability and measurement uncertainty of analytical results. Trends Anal Chem 23: 480-552.

Theobald, R.A., Batchelder, M., Sturgeon, M.F. 1981. Evaluation of pteroylglutamic acid and N-methyltetrahydrofolic acid reference standards in a new radioassay for serum folate. Clin Chem 27: 553-555.

Thompson, J.R., Gerald, P.F., Willoughby, M.L. Armstrong, B.K. 2001. Maternal folate supplementation in pregnancy and protection against acute lymphoblastic leukaemia in childhood: A case-control study. Lancet 358: 1935-1940.

Ueland, P.M. 1995. Homocysteine species as components of redoxnthiol status. Clin Chem 41: 340-342.

US Center for Disease Control and Prevention. CDC. 1992. Recommendations for the use of folic acid to reduce the number of cases of spina bifida and other neural tube defects. Morbid Mortal Weekly Rep 41:1-7.

United States Department of Agriculture, Agricultural Research Service,USDA National Nutrient Database for Standard Reference, Release 17 .2004. Beltville Human Nutrition Research Centre, Riverdale, MD. (http://www.nal.usda.gov/fnic/foodcomp)

Vahteristo, L.T., Ollilainen, V., Koivistoinen, P.E., Varo, P. 1996. Improvements in the analysis of reduced folate monoglutamates and folic acid in food by high-performance liquid chromatography. J Agr Food Chem 44: 477-482.

Van den berg, H., Finglas, P.M., Bates, C. 1994. Flair inter comparisons on serum and red cell folate. Interl J Vit Nutr Res 64: 288-293.

Vergel, R.G., Sánchez, L.R., Heredero, B.L. 1990. Primary prevention of neural tube defects with folic acid supplementation: Cuban experience. Pregnant Diag 10: 149-152.

Wagner, C. 1996. Symposium on the subcellular compartmentation of folate metabolism. J Nutr 126: 1228-1234.

Wald, D.S., Bishop, L., Wald, N.J., Law, M., Hennessy, E., Weir, D., McPartlin, J., Scott, J. 2001. Randomized trial of folic acid supplementation and serum homocysteine levels. Arch Intern Med 161: 695–700.

Wald, N.J., Law, M.R., Morris, J.K., Wald, D.S. 2001. Quantifying the effect of folic acid. Lancet 358: 2069-2073.

Werler, M.M., Shapiro, S., Michelle, A.A. 1993. Periconceptional folic acid exposure and risk of occurrence neural tube defects. J Am Med Assoc 269: 1257-1261.

White, D.R. Jr., Lee, H.S., Kruger, R.E. 1991. Reversed-phase HPLC IEC determination of folate in citrus juice by direct injection with column switching. J Agr Food Chem 39: 714-717.

Williams, L.J., Mai, C.T., Edmonds, L.D. 2002. Prevalence of spina bifida and anencephaly during the transition to mandatory folic acid fortification in the United States. Teratology 66: 33–39.

Willis, S.L. 1931. Treatment of Pernicious anemia of pregnancy and tropical disease with special reference to yeast extract as a curative agent. Br Med J 1:1059-1064.

Yetley, E.A, Rader, J.I. 2004. Modeling the level of fortification and post-fortification assessments: U.S. experience, 2001. Nutr Rev 62: 50–59.

Yeon, M., Hyun, T.H. 2003. Folate content in commonly consumed foods in Korea measured after trienzyme extraction. Nutr Res 23: 735-746.

Young, J.L.Jr., Ries, L.G., Silverberg, E., Horm, J.W., Miller, R.W. 1986. Cancer incidence, survival, and mortality for children younger than age 15 years. Cancer 58: 598-602.

Zhang, S., Hunter, D.J., Hankinson, S.E., Giovannucci, E.L., Rosner, B.A., Coditz, G.A., Speizer, F.E., Willett, W.C. 1999. A prospective study of folate intake and the risk of breast cancer. J Am Med Assoc 281: 1632–1637.