TOTAL FOLATE IN PEANUTS AND PEANUT PRODUCTS

by

LAKSHMI KOTA

(Under the Direction of Ronald R. Eitenmiller)

ABSTRACT

Samples consisting of 222 cultivar specific samples of four different peanut types (Runner, Virginia, Spanish and Valencia) from 2 years (2005 and 2006) from 3 geographical locations (Southeast, Southwest and Virginia/Carolina) were collected for the study. No significant differences were noted among the folate levels by types for 2005 crop year peanuts ($P>0.05$). For 2006 peanuts, Spanish peanuts were statistically lower in folate than Runner and Virginia peanuts ($P<0.05$). For the Runner cultivars, significant ($P<0.05$) differences existed among cultivars with some significant year-to-year variation. Year of harvest did not have significant effect on folate content of Virginia peanuts. Folate contents among Virginia cultivars were statistically similar in 2005 and 2006. For Spanish cultivars in 2005, OLin had significantly higher folate than Tamspan 90. Overall means for both Runner and Spanish peanut types showed that high-oleic cultivars contained significantly higher total folate levels than normal cultivars ($P<0.05$). Folate levels in peanuts from Virginia/Carolina region varied significantly by production year, but peanuts from Southeast and Southwest region did not vary from 2005 to 2006.

Response surface methodology (RSM) was used to optimize the trienzyme digestion for the extraction of total folate from peanut butter. The predicted second-order polynomial model

was adequate (R^2 = 0.97) with a small coefficient of variation (3.05). Both Pronase^R and conjugase had significant effects on the extraction. Ridge analysis gave an optimum trienzyme time: Pronase^R, 1h; α-amylase, 1.5 h; conjugase, 1h. The experimental value of peanut butter (SRM 2387) was close to the predicted value from the model, confirming the validity and adequacy of the model. The optimized trienzyme digestion time when applied to peanut butter and Runner, Virginia and Spanish peanuts gave comparable values to AOAC Method 2004.05.

Mean folate levels of commercial products ranged from 66μg/100g in dry roasted peanuts to 125μg/100g in partially defatted peanut flour (28% fat). When comparing folate levels in peanuts with the levels in several tree nuts, walnuts had similar folate levels to peanuts. Folate levels in raw peanuts and roasted and blanched used to manufacture peanut butter were statistically similar (P>0.05) to the folate levels in the finished product. Retention (%) was 97.5 at the roasted and blanched stage and 95 after the milling stage.

INDEX WORDS: Total folate, peanuts, cultivars, peanut products, peanut butter, tree nuts, optimization, microbiological assay, retention.

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DEDICATION

This dissertation is dedicated

To

My husband, Praveen

For his unconditional love and encouragement

To

My lovely daughter Sanskriti,

Who is wise beyond her age.

To

My parents for giving me the best in life

And

To

Dr.Ronald R Eitenmiller and Connie S Eitenmiller

For giving me constant support through my stay in University of Georgia

Thank you all for shaping me to what I am.

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CHAPTER 1

INTRODUCTION

Consumption of peanuts and tree nuts by adult population groups has consistently shown evidence of reduced incidence of coronary heart disease (CHD). Much evidence exists from large clinical studies on nut consumption and its protective effect on cardiovascular health (Fraser and others 1992; Fraser and Shavlik 1997; Kris-Etherton and others 1999; Fraser 2000; Ellsworth and others 2001; Albert and Willet 2002; Brehme 2002; Lokko and others 2007). Regular peanut consumption lowers serum triacylglycerol concentration with reduced cardiovascular disease (CVD) risk and increases the serum magnesium concentrations (Alper and Mattes 2003). It has been established that individuals who regularly consume peanuts had higher intakes of protein with concomitant increases in arginine intake, total fat, polyunsaturated fat (PUFA), monounsaturated fat (MUFA) (Maguire others 2004), fiber (Anderson and others 2000), vitamin E (Stampfer and others 1993; Rimm and others 1993) folate, calcium, magnesium, zinc, copper (Jones and others 1997) and iron (Griel and others 2004). Nuts are also rich sources of bioactive nutrients, which indirectly have positive effects on cardiovascular health. Although nuts are not proven to reduce bodyweight, evidence from some studies shows that nuts are helpful in maintaining a healthy body weight (St-Onge 2005).

Peanuts are one of the richest sources of folate in the food supply. Folate has important roles in DNA synthesis, repair, and methylation and is inversely associated with the risk of some cancers (Lucock 2004). Interest in folate nutrition is intensified due to its role in preventing the occurrence of neural tube defects during pregnancies (Ceizel and Dudas1992) and lowering the

plasma homocysteine level which is an independent risk factor for CVD (Wald and others 2001). Reduction in CVD risk factors was primarily attributed to the beneficial fatty acid composition and folate levels in peanuts. Together with increased arginine intake from peanut protein, increased folate intake can lower homocysteine levels in the blood and prevent CVD (Feldman 2002; De Logeril 1988).

Limited nutrient composition information is available for peanuts and peanut products. In general, studies have not been done with appropriate sampling plans that ensure robust and nationally representative nutrient data. In the case of peanuts, much of the nutrient data available in the United States Department of Agriculture (USDA) National Nutrient Databank (USDA, 2008) is derived from imputed values that are estimates from analytical values obtained from similar food (Greenfield and Southgate 1992; Pehrsson and others 2003; Haytowiz and others 2002). This study aims at showing variation of total folate in peanuts due to types, cultivars, geographic locations and production years. The peanuts consisted of four basic peanut types (Runner, Virginia, Spanish and Valencia) and many cultivars within the Runner, Virginia and Spanish types from three production regions (Southeastern, Southwestern and Virginia/Carolina Region). Since the preferred cultivars rapidly change due to genetic improvement, a valid sampling plan for peanuts to cover the United States included the most significant cultivars under production. Sampling for such variables known to affect nutrient composition is necessary to ensure reliability of the data and its ultimate usefulness in nutrient composition databases (Greenfield and Southgate 1992).

Recent USDA data shows that total peanut consumption increased from 1.4 billion pounds in 1995-1996 to 1.7 billion pounds in 2006 (USDA, 2008). In United States, about 90%

of the peanut crop is processed to peanut butter, salted peanuts, confectionary products and peanut oils (American Peanut Council, 2007). Peanut butter accounts for approximately half of the edible use of peanuts. Nutrient compositional changes of peanuts associated with oil, protein, carbohydrate, mineral and water-soluble vitamin contents during roasting have been reported (Derise and others 1974; Oupadissakoon and Young 1984; Damame and others 1990). Although stability studies of other vitamins like vitamin E were established in roasted peanuts (Chun and others 2005) and in peanut butter (Chun and others 2003), folate stability has not been studied. Folate can be subjected to oxidation due to its sensitivity to heat, UV light and oxygen (Gregory 1996). Effect of processing on folate stability was studied in various food products. For example, in strawberries, total folate content was found to vary with storage, ripeness, cultivars, year of harvest, storage, and commercial processing (Stralsjo and others 2003). Similarly, free and total folate in spinach and broccoli were determined at various processing stages and steam blanching resulted in higher folate retention than water blanching (De Souza and Eitenmiller 1986). After harvesting, folate content in peanuts and peanut products can be influenced by processing and storage due to its oxidative nature. Hence, this study of folate retention in peanut butter manufacture is necessary to determine the loss of folate between different stages of processing.

DeSouza and Eitenmiller (1990) were the first to coin the term “trienzyme extraction” (Eitenmiller and others 2008). Trienzyme digestion with microbiological assay was accepted as an official method of folate analysis by AOAC International (Martin and others 1990; Eitenmiller and others 2008; DeVries and others 2001; AOAC International 2005) and was proved to be essential for the release of folate from the food matrices (Aiso and Tamura 1998). Nevertheless, studies concluded that the optimal combination of enzymes and reaction condition

varied with the food matrix. Optimization studies have been done using single enzyme or double enzyme treatments in various foods (Shreshta and others 2000; Pandarangi and LaBorde 2004; Itwani and others 2003). Aiso and Tamura (1998) demonstrated that trienzyme treatment resulted in an increase of more than 50% in the mean folate content of beef, cow's milk, white bread and spinach over conjugase treatment. Response surface methodology (RSM) has been widely used in the field of agriculture and biological research for optimizing conditions in experiments (Lee and others 2000; Mizubuti and others 2000; Madamba 2002; Kwon and others 2003; Li and Fu 2005; Liyana-Pathirana and Shahidi 2005; Tanyildizi and others 2005). Although there is ample literature on vegetable and fruit matrices (Mullin and others 1982; Desouza and Eitenmiller 1986; Lin and Lin 1999; Chen and Eitenmiller 2007). Research on optimizing conditions for trienzyme digestion in peanut products has not been completed. Therefore, the third study involves the application of RSM to optimize the trienzyme digestion for folate extraction from peanut butter as a matrix.

The objectives of this study are

- Through use of an intensive sampling plan to ensure that samples are representative of types and cultivars produced in the U.S, total folate will be determined by type, cultivar, location of growth and production years (2005 and 2006 crop years) .
- To optimize trienzyme digestion for extraction of folate in peanut butter.
- To determine the effect of peanut butter processing on folate stability.

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CHAPTER 2

LITERATURE REVIEW

History of Peanut (Arachis hypogaea L)

Peanut (*Arachis hypogaea L*) is a legume belonging to the family *Leguminosae* (Ory and Flick 1991). It is an annual herb indigenous to the Andes in South America, Mexico and Central America. Although the origin of peanuts is unknown, documented existence dates back to 950 B.C (Woodroof 1983). Peanuts were believed to be cultivated in the West Indian Islands, Mexico, Brazil, Argentina, Paraguay, and Bolivia (Hammons 1982). Historical evidence suggests that peanuts were domesticated during prehistoric times in Peru and that the European traders were responsible for disseminating the peanuts to Europe, Africa, Asia, and the Pacific Islands (Hammons 1982). The peanut gained western popularity when it reached the coast of North America from Africa via the slave trade route between 1707 and 1725. The English colonies popularized the cultivation of peanuts in North America (Woodroof 1983).

After the Civil War, when cotton crops were wiped out by the boll weevil, George Washington Carver (1864-1943), Director of the Department of Agriculture at the Tuskegee Institute, urged the farmers to plant peanuts. He led the way by finding over 300 uses for the crops. In 1890, a St. Louis physician gave peanuts another boost by making peanut butter which he prescribed to his patients as a nutritious, easily digested, high protein and low carbohydrate food (Woodroof 1983). Dr. Ambrose Straub of St. Louis patented a peanut butter-making machine in 1903 (Woodroof 1983).

Agronomy of Peanut

Peanuts are seeds of an annual legume, which grows close to the ground and produces its fruit below the soil surface. The flower of *Arachis hypogaea L* is yellow petalled and is borne above the ground (Pattee and Young 1982). After self-pollination, the flower withers and the stalks called the "pegs" develop at the base of the ovary. These pegs elongate rapidly and force the ovary into the ground for later development into a complete fruit (Pattee and Young 1982).

In the United States, peanuts are planted after the last frost in April or May when the soil temperatures reaches 65-70º F (Woodroof 1983). Peanuts may be cultivated up to 3 times, depending on the region, weed control, and grasses. A climate with approximately 200 frost-free days is ideal for a good crop (Woodroof 1983). Warm weather, adequate moisture and rich sandy soil are required for good yield. The next stage is the harvesting

Harvesting refers to the operations necessary for the removal of peanut pods from the soil. Harvesting of peanuts occurs in two stages (Woodroof 1983). The first stage includes digging that is done after 70% pods are mature. A digger loosens the plant and helps to cut the taproot while the shaker lifts the plant from the soil and shakes the soil from the roots of the plant. It also inverts the plant and exposes the plant in a windrow for sun drying (Woodroof 1983). The timing of harvest is critical since it greatly affects the yield and grade and, therefore, the value of the crop (Williams and Drexler 1981; Sanders and Bett 1995). Only a narrow window of time exists for harvest where total crop yield is high and losses are minimized (Pattee and Young 1982). Optimum harvest time ensures more mature pods, which results in higher percentages of mature seed in commercial grade sizes (McNeill and Sanders 1998).

After peanuts are harvested, the curing process begins. The term curing includes biochemical changes as well as reduction of moisture content to preserve quality (Dickens and Pattee 1973). After sun drying the peanuts for 2 or 3 days, a combine separates the pods from the plant vines. These peanuts are then placed in wagons for further curing by forcing hot air through the wagons. Freshly harvested peanuts are subjected to windrowing and mechanical curing to reduce moisture content on a wet basis, from approximately 5 to 10%. The drying rate depends on the initial moisture content, equilibrium moisture content, relative humidity, and curing temperature (Young and others 1982). Maintaining a low level of moisture in storage is important to inhibit mold growth and prevent quality deterioration during storage (Dickens and Pattee 1973). After curing, the moisture content is reduced to 8-10% for safe storage (American Peanut Council 2008).

During grading, Farmersstock peanuts that have not been shelled and cleaned, are inspected and graded to establish the quality and value of the product. This inspection determines the overall quality and farm value of the shelled products for commercial sales. Peanuts are sampled and graded by the Federal-State Inspection Service to determine their value (American Peanut Council 2008). Inspectors establish the meat content, size of pods, kernel size, moisture content, damaged kernels and foreign material. Results from these inspections determine the overall quality and value of each load. These peanuts areseparated into three classifications: segregation I, segregation II, and segregation III. These classifications are made based on the amount and type of damage apparent on the peanut kernels. Segregation I peanuts are for use in edible products and the rest are crushed for oil and meal production (Woodroof 1983).

During shelling, segregation I peanuts are cleaned to remove the stones, soil, bits of vine and other contaminants. Cleaned peanuts moved by conveyer belt through shelling machines in which the peanuts are forced through perforated grates, which separates the peanut kernels from the hulls. Shakers are used to separate the kernels and the pods (Woodroof 1983). These kernels are then sorted by size on various screens into the market grades. Then, the edible nuts are individually inspected with electronic eyes. During this process, the discolored or defective nuts as well as any remaining foreign material are eliminated, thereby decreasing the possibility of aflatoxin contamination (Woodroof 1983).

Peanut Production

The United States is a major producer of peanuts contributing approximately 10 % to the world production. Seven states account for approximately 98% of all peanuts grown in the U.S. In the year 2007, Georgia had the major proportion of peanuts which is followed by Texas, Alabama, Florida, North Carolina, South Carolina, Virginia, Mississippi, Oklahoma and New Mexico (USDA 2008a). Large numbers of peanut cultivars are grown in the United States with four types being the most popular: Runner, Virginia, Spanish, and Valencia (American Peanut Council 2008). Each type is distinctive in size and flavor. Runner peanuts are primarily used in peanut butter manufacture with medium sized kernels. Since 1940, there has been a shift to production of Runner type peanuts in the Southeast due to higher yields, good roasting flavors and wider use in peanut butter, and salted products. Runner cultivars in current production include Ap-3, Ap-4, Carver, Florida-07R, Georgia-03L, C-99R, Georgia Green, Georgia Greener, GA-02C, TamRun-96, TamRun OL01, and TamRun OL02 (The Peanut Grower 2008).

Georgia Green, the major Runner cultivar has higher yields per acre compared to other Runner cultivars, has significantly higher percentage of sound mature kernels, good grades and resistance to tomato spotted wilt virus (TSWV) and white mold. Runner peanuts are not resistant to leaf spot disease (The Peanut Grower 2008). Georgia-02C is a high oleic cultivar and can have later maturity than Georgia Green with slightly larger seed and pod size. It has a spreading runner growing habit with excellent TSWV and cylindrocladium black rot (CBR) resistance (The Peanut Grower 2008). C-99R is usually grown in the Florida region. It is a late maturing pod with excellent pod yields across a wide range of locations and good grades. The seeds are larger than the average Runner and needs more calcium for growth. These are resistant to leaf spot, white mold, rust and have small level resistance to CBR (The Peanut Grower 2008). TamRun 96 is a high yielding Runner peanut that is highly tolerant to TSWV and southern blight. It has a robust vine growth with larger vine than FlavorRunner with stronger peg attachment (The Peanut Grower 2008). TamRun OL01 is a high-oleic variety with an oleic to linoleic ratio of 15:1. It is a cross between TamRun 96 and Sun Oleic 95R. This variety has a large seed and they are usually graded as Virginia in most conditions. TamRun OL02 is also a high-oleic variety (24:1), developed from TamRun 96 and has disease resistance traits of TamRun 96 (The Peanut Grower 2008). The seed size is slightly larger than TamRun 96 and has lower sugar content than TamrRun OL01 and FlavorRunner 458 (The Peanut Grower 2008).

Virginia type peanuts are popular for their large kernel size and are particularly used for salting, confections, and roasting in shells (Woodroof 1983). They are either bunch or running in growth habit. The bunch is upright to spreading and seldom covers the ground. Cultivars of Virginia type peanuts include NC 7, NC 9, NC 10C, NC-V11, VA 93B, NC 12C, VA-C 92R,

Gregory, VA 98R, Perry, Wilson, Hull, VC-2 and Shulamit. AT-07V is a mid-oleic variety and the high-oleic varieties include Georgia-05E and Georgia HI-O/L.

NC-V11 has a spreading runner growth habit and has higher yield per acre under good growing conditions. It is less susceptible to TSWV then other Virginia cultivars (The Peanut Grower 2008).

Gregory is a large seeded Virginia cultivar with a pink seed coat, higher percentage of jumbo pods and extra large kernels. It has reduced seedling vigor and higher calcium requirements due to the large kernel size (The Peanut Grower 2008). Its growth habit is intermediate between Runner and bunch peanuts. Disease susceptibility is higher but offers higher resistance to the tomato spotted wilt virus. NC-12C is also a large seeded variety similar in maturity, plant type, seed size, shape, seed coat color, and yield to NC-7. When compared to the other Virginia cultivars, it is very susceptible to the Sclerotinia blight and has a thin hull with tendency to darkening during roasting (The Peanut Grower 2008). Perry is a high yielding Virginia cultivar, large seed CBR-resistant with pink seed coat and good pod color. Their growth habit is similar to Gregory that is between runner and bunch. VA 98R is running in growth habit and is susceptible to all peanut diseases except Sclerotinia blight. Maturity occurs a week earlier than NC-12C (The Peanut Grower 2008).

Spanish type peanuts have smaller kernel size and have higher oil content than other types of peanuts (Woodroof 1983). Spanish peanuts are grown in Southeastern and Southwestern United States. Cultivars of the Spanish group include Dixie Spanish, Improved Spanish 2B, GFA Spanish, Argentine, Spantex, Spanette, Shaffers Spanish, Natal Common (Spanish), White Kernel Varieties, Starr, Comet, Florispan, Spanhoma, Spancross, OLin,

Tamspan 90, Spanco and Wilco I (The Peanut Grower 2008). Olin is a high-oleic Spanish cultivar released from Texas A&M University in 2002 (The Peanut Grower 2008). Although it is similar to Tamspan 90 in growth habit, maturity and disease resistance, the yield potential is 5 to 10 percentage less than Tamspam 90. Tamspan 90 was released from Texas A&M University in 1990 and has excellent yield potential and disease resistance (The Peanut Grower 2008).

Valencia type peanuts are coarse, with heavy reddish stems and large foliage. Peanut pods are borne on pegs arising from the main stem and the side branches. Most of the pods are clustered around the base of the plant, and only a few are found several inches away. Valencia types are three seeded and smooth, with no constriction between the seeds. Seeds are oval and tightly crowded into the pods (Woodroof 1983). Valencias are the best flavored and preferred type for boiled peanuts. Large commercial production is primarily in Eastern New Mexico**,** especially in and around Portales, New Mexico. They are also grown on a small scale elsewhere in the South, which are comparatively tall, having a height of 125 cm (50 inches) and a spread of 75 cm (30 inches). There are two strains, one with flesh and the other with red seeds. Typical seed weight is 0.4 to 0.5 g. Cultivars of Valencias include Valencia A, Valencia C, GenTex (H & W)101, GenTex(H & W)102, GenTex(H & W)136 (The Peanut Grower 2008).

Peanut Consumption Statistics

Recent data from United States Department of Agriculture shows that total peanut consumption increased from 1.4 billion pounds in 1995-1996 to 1.7 billion pounds in 2004 (USDA 2008a). The per capita consumption increased from 5.9 pounds in 2000 to 6.6 pounds in 2004 and remained stable during 2005 (USDA 2008a). However, it decreased to 6.5 pounds in 2006.The per capita consumption of peanuts from 1970 to 2006 is given in Table-2.1. In the

United States about 90% of the peanut crop is processed to peanut butter, salted peanuts, confectionary products and peanut oils (American Peanut Council 2008). Peanut butter accounts for approximately half of the United States edible use of peanuts. Peanut butter consumption increased from less than 750 million pounds in 1995-1996 to over 800 million pounds in 2004 (USDA 2008a). Per capita consumption of peanut butter remained stable during the 1990s (average of 2.9 pounds) and increased to 3.3 pounds in 2006 which is just under half of the total peanut consumption in the U.S. The other half of the U.S edible consumption is divided between snacks, roasted nuts and confectionary (USDA 2008a). Peanuts consumed as snacks and in candy are also popular forms with per capita consumption of 1.4 and 1.2 pounds, respectively, in 2006 (USDA 2008a). Peanut consumption is important to the Georgia economy since every 1% increase in consumption can add up to $ 16.9 million to the state economy (Kearney 2007).

Production Areas

The major peanut producing areas in the United States are the Southeastern region including Georgia, Florida, Alabama, and Mississippi; the Virginia/Carolina region including Virginia, North Carolina and South Carolina; and the Southwestern region including Texas, Oklahoma, and New México. Production statistics for 2007 for these states are given in the Table 2.2 (USDA 2008a). In Southeastern region, Runner peanuts predominate with smaller production amounts of Virginia type peanuts, In Virginia/Carolina region, Virginia type predominates. In the Southwestern region, both Spanish and Valencia types are grown. Characteristics of various peanut types were given earlier in the peanut production section (USDA 2007).

Peanut Butter

Peanut butter is by far the most important product made from peanuts in the United States (USDA 2007). According to the National Agricultural Statistics Service of USDA, 901million pounds of cleaned, in shell peanuts were used for the peanut butter while 415 million pounds were used for snack, 366 million pounds for candy, and 16 million pounds for other uses. Commercial manufacture and consumption of peanut butter is an American product form (Weiss 1983). It is considered as a staple food in many American households. The popularity of peanut butter is due to its pleasing flavor, convenience for use, nutritional content and microbiological stability. The manufacture of peanut butter has advanced since its discovery. Some changes were adapted for large-scale production like addition of emulsifiers and stabilizers to prevent oil separation (Weiss 1983). The U.S Grade No.1 Runner peanuts are mostly used for the manufacture of peanut butter. Occasionally, Runner and Spanish peanuts are processed together. Since Virginias are too low in oil content, they can be only used in combination or by addition of oil prior to grinding. The manufacture of peanut butter includes the following steps (Weiss 1983).

1. Roasting

Roasting can be done in a batch or continuous process. Batch roasting allows for various roasting temperatures for various types of peanuts. In a batch method, the peanuts are roasted to 320 °F with holding time for 40-60 min. Recent Industry procedures involved multistage heating with different temperatures. Roasting at lower temperatures usually give the best flavor and longer shelf life. The next step is cooling and blanching.

2. Cooling and Blanching

The roasted peanuts are allowed to cool to room temperature before blanching. The purpose of blanching is to loosen and remove peanut skins. During blanching, the nuts are split in two causing the skin to shatter. Skin fragments are blown away leaving the heavier nuts behind.

3. Grading and Sorting

Defective peanuts are usually described as immature and underdeveloped nuts which are small and shriveled and moldy mature nuts that do not split during blanching (Weiss 1983). Graders and sorters are either revolving cages or shaker screens with specific size openings. Small screens are used before blanching to remove shrivels from large whole nuts and splits, whereas; large screens are used after blanching to hold back unsplit nuts to be discarded. Force air drafts are also used to blow lighter skins and to separate nuts from heavier stones.

4. Grinding

The roasted, blanched peanuts are ground in two stages. First, the peanuts are reduced to medium sized pieces and then ground to smooth paste with even texture. Various devices like hammer mills, homogenizers and disintegrators are used for grinding with adjustable space between the plates (Weiss 1983). The steel burr mill and multibladed cutting mill are most frequently used. Stone and steel plate mills operate by having the rotor revolve at high speed against the stator (Weiss 1983). The distance between them is adjustable and the nuts for grinding are forced between the grinding surfaces by an impeller mounted on the rotor shaft. Peanuts are poured into open top of the revolving drum. A mutibladed cutter consists of a drum

mounted on a vertical shaft rotating at 9600 rpm and the walls of the drum consist of over 200 vertically arranged tungsten carbide knives. Nuts are hurled against the knives and slashed into paste (Weiss 1983). Any remaining nuts are reduced to paste between a pair of steel plates similar to the steel plate mills. During this two-stage process, the peanut butter is heated up to 170°F. In single stage process, temperature can increase to 180°F (Weiss 1983).

Maintaining low temperature of the end product is important. The reaction of reducing sugars like dextrose, invert sugar and corn syrup with free amino groups in the peanut protein at higher temperatures causes Maillard browning**.** To prevent too much browning, sucrose can be added before milling. Stabilizer and salt can be added before the grinding process either in melted or solid form (Weiss 1983). Stabilizers like fully or partially hydrogenated vegetable oils, monoglycerides, and diglycerides of vegetable oils or combinations of these are used for peanut butter. Stabilizer prevents peanut oil from separating from the solids during storage.

5. Deaeration

Deaerators are used to remove the air from the peanut butter to decrease lipid autooxidation during storage. The hot peanut butter is pumped into the top of a closed tank under vacuum. It flows down the tank and the air is removed. Thus, the deaerated peanut butter is pumped into the chilling machine and packaged under a nitrogen blanket to minimize oxygen in the headspace of the jar (Weiss 1983).

6. Chilling and Filling

Previously, the peanut butter was filled by gravity in a thin and tall jar to maximize the heat transfer rate from the hot peanut butter in the jar. The filled peanut butter was then

circulated in chilled tunnels at 1-5 °C (Weiss 1983). Modern chilling is done with a votator A or internal scraped surface heat exchanger. The type of stabilizer used determines the filling temperatures. Peanut butter formulated with 1.8 -2.0% monoglycerides can be filled at 120-130°F.

7. Packaging

Traditional glass jars, and various polymer based jars have been used for packaging peanut butter. Shelf life of peanut butter varies from 2 yrs (glass jars) to 9months - 1 year (plastic jars).

Salted Peanuts

According to the American Peanut Council, the per capita consumption of snack peanuts was 1.4 pounds in 2006(American Peanut Council 2008). About 72% Virginias, 25% Spanish, and 3% of Runner peanuts are used for roasted nuts. Dry roasted peanuts are glazed with 1½ to 2% of oil and mixed with 2% of salt without blanching. The most common method is to blanch the peanuts first, and then oil roast (Woodroof 1983). Cooking temperatures from 280-290°F for 3-10 min are used. The typical flavor of the roasted peanuts is due to the Maillard reaction between mixtures of amino acids and carbohydrates to produce tetrahydrofuran derivatives (Woodroof 1983). The major gaseous compounds are carbon dioxide with traces of ammonia hydrogen sulfide and diacetyl. Some times when nuts are dry roasted, there is little oil on the surface of the peanuts for binding the salt. Oils, like coconut oil, are added to the oven-roasted nuts for salt binding. The most common roasting temperatures are 280 to 290°F and time is 3 to 10 min. Roasted peanuts are cooled, salted and packaged (Woodroof 1983).

Other Uses of Peanuts

The peanut industry in the United States differs from other countries since the peanuts in United States are grown mainly for food delicacies while in other countries they are grown primarily for edible oil and defatted meal that is used for animal feed. According to the USDA, the per capita consumption of peanut candies is about 1.2 pounds, 0.5 pounds for cleaned in shell peanuts, 3.3 pounds for peanut butter, 1.4 pounds for snack peanuts, giving a per capita consumption of 6.5 pounds for 2006.

Peanut Composition

In the United States, the consumption of peanuts is greater than all the other nuts combined (Table-2.3). The composition of peanuts is affected by cultivar, maturity, curing, kernel size, treatments with herbicides and fungicides, year, location, season, variety or genotype, harvest time, processing and storage, but not always by a statistically significant amount (Sanders 1980; Ahmed and Young 1982; Knauft and others 1986; Branch and others 1990; Chiou and others 1992; Sanders and others 1992; Basha 1992; Basha and Young 1992; Hashim and others1993; Grosso and Guzman1995a,1995b; Bland and Lax 2000; Pattee and others 2000). Peanuts are a rich source of many vitamins like folate (USDA 2008), vitamin E (Chun and others 2003,2005**;** Alper and Mattes 2003), minerals including copper (Jones and others 1997; Klevay 1993), magnesium (Anderson and others, 2000), zinc, calcium; fiber and the amino acid L-arginine (Alper and Mattes 2003; Palmer and others 1988). Numerous bioactive substances such as flavonoids, resveratrol and plant sterols are also present in peanuts (Sanders and others 2000). They are also a good source of oleic acid (Maguire and others 2004).

Peanuts contain relatively large quantities of protein compared to other legumes or nuts. Peanuts contain about 25% protein of which over 87% of the peanut proteins are globulins. Arachin (63%) and conarachin (33%) are the two major types of globulins (Basha and others 1976). The arachin fraction, localized in the aleurone bodies, is rich in threonine and proline but has a chemical score of only 31-38. However, conarachin found in the cytoplasm has a chemical score of 68-82. Although, peanuts have some limiting amino acids such as lysine, methionine and threonine, peanut protein can be supplemented with a complementary protein like wheat. The low ratio of lysine to arginine in plant proteins has been recognized as having an anti-atherogenic effects in animals. Peanuts are also a rich source of the amino acid arginine, the precursor of nitric oxide(NO) (Hyunh and Chin-Dusting 2006). Recently a significant cardioprotective role for L-arginine, the precursor of nitric oxide (NO) has been recognized. Dietary arginine enhances NO synthesis. NO induces smooth muscle cell relaxation (vasodilation), inhibits platelet adhesion, activation and aggregation and is antithrombotic.

Peanuts contain about 20% carbohydrate of which sucrose is the most abundant (2.9-6.4%). Other sugars like stachyose, raffinose, glucose, fructose, arabinose and galactose are also present (Ahmed and Young 1982). Peanuts are an excellent source of many minerals. Potassium, magnesium, phosphorus and sulfur are present in high amounts and are unaffected by heating (USDA 2008b). Several important vitamins such as folate, thiamin, nicotinic acid and vitamin E are present in peanuts (USDA 2008b).

Peanuts and Health Benefits

The health benefits associated with nuts (peanuts and treenuts) are thought to reflect their nutritional profile including their nutrient density, fatty acid profile and presence of bioactive compounds. Numerous reviews have consistently shown that consumption of tree nuts and peanuts have been associated with various health benefits (Dreher and Maher 1996; Turnstall-Pedoe 1998; Rainey and Nyquist 1997; Griel and Kris-Etherton 2006; Coates and Howe 2007). Much evidence exists from large clinical studies on nut consumption and its protective effect on cardiovascular health (Fraser and others 1992; Fraser and Shavlik 1997; Kris-Etherton and others 1999; Fraser 2000; Ellsworth and others 2001; Albert and others 2002; Brehme 2002; Lokko and others 2007). Further, a large number of reviews suggested an improved blood lipid profile and heart health with nut consumption (Hu and Stampfer 1999; Feldman 1999; Hu and others 2001; McKerchar 20000; Kris-Etherton and others 2001; Mukuddem-Petersen and others 2005).

Cardiovascular Heart Disease (CVD)

Five large epidemiological studies including The Adventist Health Study (Fraser and others 1992), Iowa Women's Health Study (Kushi and others 1996), Nurses' Health Study (Hu and others 1998), Physicians' Health Study (Albert and others 2002) and Cholesterol and Recurrent Events (CARE) Study have reported an inverse association between nut consumption and risk of CHD.

In the Adventists Health Study, Fraser and others (1992) first indicated the possible protective effect of nut consumption against CHD. Dietary information obtained from 31,208 non-Hispanic white California Seventh-Day Adventists showed that consumption of nuts more

than four times per week decreased fatal CHD events when compared to subjects who consumed nuts less than once per week (Fraser and others 1992). In addition, the Iowa Women's Study of 34,000 women also found that nut consumption was associated with a 40% decrease in risk of CHD (Kushi and others 1996; Ellsworth and others 2001). Dietary benefits of nut consumption further increased with the report based on the Nurses Health Study (Hu and others 1998) of 86,016 women. This study reported that substitution of the fat from one ounce of nuts for equivalent energy from carbohydrate and saturated fat reduced CHD risk 30% and 45%, respectively. In the U.S. Physicians' Health Study (Albert and others 2002), 21,454 male physicians (ages 40-84 years) with no history of heart disease at the beginning of the study where followed up for an average of 17 years. Over the period of this study, 201 sudden cardiac deaths and 566 heart disease deaths were observed. Physicians who consumed nuts two or more times per week showed a 47% lower risk of sudden cardiac death and a 30% lower risk of total coronary heart disease death **c**ompared with those who rarely or never consumed nuts. Although this benefit has been primarily attributed to the fat profile for nuts, it should also be recognized that intakes of folate and L-arginine in nuts have also been inversely associated with lowering the LDL cholesterol Levels.

Numerous clinical studies were conducted to evaluate the effects of nut consumption on lipids and lipoproteins, which are the major risk factors in heart disease. Lipid lowering property of peanuts is primarily attributed to the higher proportion of dietary mono unsaturated fatty acids (MUFA) (oleic acid) to polyunsaturated fatty acids (PUFA) (Alper and Mattes 2003; Maguire and others 2004). MUFA (oleic acid) is as effective as linoleic acid in lowering the LDL cholesterol and triglycerides without reducing the HDL cholesterol (HDL-C) (reviewed by Mukkuddem-Petersen and others 2005; Griel and Kris-Etherton 2006). These reviews showed

that inclusion of 50-100g (approximately 1.5–3.5 servings) of nuts five or more times per week in conjunction with a low fat diet would help to decrease total and LDL cholesterol and protect against CVD. Diets increasing the quantities of MUFA and PUFA supplied from almond, walnuts, hazelnuts, macadamia and pecan, lowered total and LDL cholesterol levels which are risk factors for CHD (O'Byrne and others 1997; Erario and others 2001; Rajaram and others 2001; Zibaeenezhad and others 2005; Chisholm and others 2005; Tamizifar and others 2005). The blood cholesterol lowering effects of peanuts were also examined in some studies (O'Byrne and others 1997; Kris-Etherton and others 1999; Brehme 2002). O'Byrne and others (1997) reported that a low fat diet supplemented with peanuts improved serum lipoprotein profiles when compared to a regular low fat diet for postmenopausal women with high serum cholesterol levels. Brehme (2002) reviwed 13 clinical studies lasting 3 to 24 weeks. The effect of 15 diets containing walnuts, almonds, peanuts, macadamia nuts, pecans or pistachio nuts on plasma lipids was studied. The concentration of total cholesterol and triglycerides decreased by 7 % and that of LDL cholesterol decreased by 10 %. Although the HDL cholesterol concentration increased in 6 diets (1.8 - 14.2 %), it decreased in 8 diets(0.6 and 10.2 %). Favorable fatty acid profiles (high MUFA and PUFA), folate, vitamin B-6, vitamin E, selenium, sterols, fiber and other phytochemicals in peanuts are considered as possible components to have protective effects on health. L-Arginine also plays a vital role in prevention of cardiovascular diseases. Studies have found an improvement in endothelial function after supplementation with L-arginine at doses between 8 and 21 g/day (Brown and Hu 2001). Diets containing peanuts are beneficial in patients with impaired endothelial nitric oxide synthesis and elevated cholesterol or CHD. The average daily consumption of L-arginine could be readily increased to around 8 g/day by adding approximately 70 g/day of peanuts to the average American diet (Reviewed by Coates and Howe

2007). The ability to deliver both the NO precursor and polyphenols to facilitate NO production in endothelial cells makes peanuts an ideal food for vascular health.

Diabetes type2

Nuts potentially can regulate glucose and insulin levels due to the high fat content. Garg and others (1988) reported that a high MUFA diet improved glycemia in patients with type 2 diabetes. Further evidence from the Nurses Health Study reported an inverse relation between nut consumption and type 2 diabetes (Jiang and others 2002). Consumption of peanuts and peanut butter 5 times a week reduced risk of type 2 diabetes by 27% and 21% reduction in risk of type 2 diabetes (Jiang and others 2002). Although this benefit was primarily attributed to the fatty acid profile for nuts, other components of nuts such as fiber or magnesium are inversely associated with lowering the risk of type 2 diabetes. Johnston and Buller (2005) compared the addition of either vinegar or peanuts to low and high glycemic load test meals. Both additions significantly reduced the post prandial glycemia following the high glycemic load meal. Although the reason for altering the glycemic response by peanuts is unknown, high levels of L-arginine might stimulate insulin release and glucose uptake (Ishiyama and others 2006).

Weight management

Despite having a unique nutritional profile, peanuts and some tree nuts are often avoided in diets because of their high caloric density. Although there is a misconception that inclusion of peanuts, peanut products and some tree nuts in the diet might increase the energy intake leading to weight gain and increase in Body Mass Index (BMI), evidence from a large study of 12000 participants in United States Department of Agriculture's Continuing Survey of Food Intakes by

Individuals revealed that BMI was lower in nut consumers than non-nut users (Sabate 2003). Further, clinical studies conducted by Kris-Etherton and others (1999) and Alper and Mattes (2002) proved that inclusion of nuts in an energy-restricted diet could help in limiting weight gain. There is a large body of evidence for nut consumption and maintenance of healthy body weight (Alper and Mattes 2002; Fraser and others 2002), but there is little support for nut consumption and weight loss. Recent reviews by Coates and Howe (2007) concluded that much evidence is gathered from studies showing weight maintenance where weight gain has been predicted based on energy intake data. Griel and others (2004) concluded that peanut consumption was not associated with higher BMI when included in low energy diets. Pelkman and others (2004) reported that inclusion of peanuts in low energy diets achieved the same weight loss as that of the low fat diet but was advantageous in improving CVD health. O'Byrne and others (1997) reported a 3.6 kg reduction in body weight in a 6-month dietary trial with subjects on a low fat diet without peanuts. The above studies indicate that incorporation of nuts in a low energy diet plan is an effective and safe method to boost weight reduction.

Folate and Health

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 (Willis 1931). Folate and folic acid are the two forms of folate. Folate refers to natural forms that occur in the food and folic acid is the synthetic form found in vitamin supplements and fortified foods. Folate is the general term used for this vitamin, and it exists in many chemical forms (Eitenmiller and others 2008). Synthetic folic acid is the 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). 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 2008b).

Structure of Folic Acid

Folic acid (Figure 2.1) is 2-amino –4 hydroxy-6-methylene amino benzoyl L-glutamic acid pteridine. Folic acid contains a pterin core ring structure, a para-aminobenzoic moiety and the glutamate residue. 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 α–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 (Eitenmiller and others 2008). 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 (Eitenmiller and others 2008). 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 others 2008). Although various forms exist, only the reduced forms are biologically active.

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 1998) (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 (Eitenmiller and others 2008). For example, tetrahydrofolate accepts single carbon units (methyl group) from serine or glycine and forms 5, 10 methylene tetrahydrofolate. 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 1998). 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 hemoglobin and is essential in the formation of the red blood cells (Eitenmiller and others 2008). Some folate interconversions are shown in Figure 2.2.

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 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 (Eitenmiller and others 2008). 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 the serum folate level in the blood directly leads to the decrease in the erythrocyte folate concentration (Eitenmiller and others 2008). 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 (Eitenmiller and others 2008).

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. Prior to fortification, approximately, 2500 infants were born each year with NTD'S among the 4 million births in the United States (Flood and others 1992). Additionally, 1500 fetuses with NTD'S were 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 and others 1992).

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 and others 1997; Gao and others 1997). This defect occurs in children younger than 5 years of age. The prevalence of this disease was one in 6000 to 7000 children (Bernstein and others 1992). The aggressive nature of this tumor made this disease the most common cause of cancer-related death among children 1 to 4 years old (Young and others 1986). In the 1900's, maternal folate status was been implicated in the development of fetal pathologic conditions. For example, periconceptional intake of folic acid, 0.4 to 4 mg/day, was indicated to prevent most cases of NTD (Czeizel and Dudas 1992; Wald and others 2001). Prenatal multivitamin supplementation also was associated with a lower risk of childhood brain tumors and neuroblastoma (Olshan and others 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 and others 2004). Anencephaly is the absence of the brain. It results from failure of fusion in the cranial region of the neural tube (Mitchell and others 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 and others 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 and others 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 and others 2003; Wald and others 2001).

Folate and Heart Disease

Homocysteine is an amino acid which is an intermediary in the metabolic pathway of methionine. 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. Since one of the functions of the folate involves in the conversion of homocysteine to methionine, it has a vital role in cardiovascular health (Lucock 2004; Nowack and others 2005; Eitenmiller and others 2008). A reciprocal relationship exists between blood homocysteine and water-soluble vitamins in the blood (particularly folate). Thus, understanding the interrelationships of folate, methionine, and homomocysteine and the

recognition that an elevated serum homocysteine level is an independent risk factor for cardiovascular disease has been a highly significant area of research. Folate is required for the remethylation of homocysteine to methionine, which is dependent upon sufficient levels of 5-methyltetrahydrofolate (5-CH_3-H_4 folate) as a one carbon donor (Figure 2.1 and 2.2). Numerous results from clinical studies have reported that high circulating levels of homocysteine is an independent risk factor for cardiovascular heart disease. Another consequence of disruption in methionine metabolism caused by insufficient levels of 5-CH_3-H_4 folate folate include depletion of S-adenosylmethionine(which is a methyl donor for methyltransferase reactions) (Eitenmiller and others 2008). This depletion causes decreased methylation of cytosine in DNA, resulting in increased gene transcription, DNA strand breakage, and impaired DNA repair. It also causes decreased methylation of proteins, phospholipids and neurotransmitters (Eitenmiller and others 2008). Homocysteine is metabolized by remethylation or trans-sulfuration (De la Calle and others 2003). 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 and others 2001). Recent metaanalysis showed that folic acid was the most effective homocysteine lowering agent compared to vitamin B6 and vitamin B12. In a study by Wald and others (2001), 0.8 mg/day of folic acid 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 (Schorah and others 1998; Smith and others 2008). 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 μg folic acid /100 g food (Motulsky 1996). According to Boushey

and others (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 and others 2004). The homocysteine lowering effect of folic acid was found to plateau at daily doses of 0.4 –0.5 mg (Doshi and others 2002). Evidence also suggests that these daily doses could be achieved by intake of fortified cereals (Malinow and others 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) and Smith and others (2008). 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). Folate has a dual effect on cancer, protecting against cancer initiation but facilitating progression and growth of preneo- plastic cells and subclinical cancers, which are common in the population (Smith and others 2008). Some of 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 and others 1999). Folate antagonists have been used in the cancer treatment for over 60 years, and methotrexate, which was discovered prior to 1950, remains the most widely used chemotherapeutic agent (Allegra 1990). Methotrexate is also used in treatment of rheumatoid arthritis, which is administered along with folic acid to prevent the toxicity of methotrexate.

Fortification

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 led several organizations in United States and around the world(Mexico, Canada, Chile, Hungary, UK) to recommend fortification programs (Cornel and others 2005). Also, the strong relationship between folate status and the relationship of elevated serum homocysteine levels and risk of cardiovascular heart disease (MRC 1991; Boushey and others 1995), led to fortification. 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. 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 (FDA, 1996). Since the fortification policy became mandatory**,** serum and erythrocyte folate concentrations in all sex and age groups has dramatically increased along with reduced NTD. Following the Food and Drug Administration (FDA)-mandated fortification of cereal grain products with folic acid that began in January 1998, the occurrence of NTDs declined 27%, from an annual average of 4,130 cases in 1995–1996 to 3,020 cases in 1999–2000(CDC 2004). The most common defects of NTD include neuroblastoma, spina bifida and anencephaly. Canfield and others (2005) reported a 12% reduction in cleft plate and Yazdy and others (2005) reported a decline of orofacial clefts by 85.2 to 80.2 per 1000,000 births (Yazdy and others 2005). In Chile, the rate of occurrence of spina bifida and anencephaly decreased by 51% and 46% after fortification started in 2000 (Lopez-

Camelo and others 2005). In a review, Botto and others (2006), evaluated the trends of the neural tube diseases and other malformations before and after fortification in Europe, Australia and North America. The results showed significant changes in the trends for areas with fortification but not in areas with supplementation recommendations. However, the other major birth defects did not show major trend changes after fortification. Change in prevalence of neural tube defects before and after fortification was evaluated in Australia, United States, Canada and Germany. Three studies from Australia showed a change in trend of neural tube diseases after fortification by -10 to -30% (Bower and others 2002; Botto and others 2006). Similarly, three studies in the United States were evaluated for the decrease in neural tube diseases. CDC (2004) study showed a change in neural tube diseases by -29% in all the 23 states. According to Botto and others (2006) there was a change of -29% in Atlanta and -23 % in Texas. In Canada, a change of -38% was noticed in Ontario(Ray and others 2002); 54% in Nova Scotia(Persad and others 2002); -32% in Quebec(De Wals and others 2003) and -29% in Alberta(Botto and others 2006). Similary one study in Germany showed a change of -15 %(Botto and others 2006). Although fortification in this study appeared to reduce the neural tube defects, the effects on other birth defects was unclear (Botto and others 2006).

Safe Upper Intake Level of folate

Based on the scientific evidence, 1000μg of folate was set as a safe upper limit of intake by the Institute of Medicine in 1998 (IOM 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 and Rader 2004). There is a risk of masking of pernicious anemia in patients who were

treated with 1000 μg of folic acid per day (Bailey 1995). 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 vitamin B12 (Freire and others 2004). According to a review by Smith and others (2008), the question of whether the higher folate concentrations that occur in significant sections of the population after fortification can cause harm needs much further research as does the question of whether the presence of unmetabolized folic acid in the blood (Smith and others 2008).

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 1998). Dietary reference intakes are 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 1998)

µg Dietary Folate Equivalent (µg DFE)

Numerous studies explored the differences in bioavailability of folic acid used in supplements and fortification when compared to the natural folate (Gregory 1997; Pfeiffer and others 1997a; Cuskelly and others 1996; Wei and others 1996; Sauberlich and others 1987). Based on these studies the Institute of Medicine (IOM 1998) recommended the use of a term called the µg Dietary Folate Equivalent (µg DFE) to adjust for the differences in the bioavailability of the folic acid and food folate. Folic acid when taken with food is 85 % bioavailable while food folate is 50 % bioavailable. Thus when a mixture of folate and folic acid is taken, folic acid is 85/50=1.7 times more bioavailable than food 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).

Folic acid supplements when consumed on an empty stomach are 100% bioavailable (Gregory 1997). When compared to food folate only half as much of food folate is necessary when consumed on an empty stomach. Hence,

1 µg DFE= 1 µg food folate =0.5 µg folic acid taken on an empty stomach and 0.6 µg folic acid taken with meals.

Recommended Dietary Allowances (RDA)

Folates are present in most foods such as legumes (peanuts, cowpeas, peas), leafy greens, citrus fruits (orange juice), vegetables (broccoli, cauliflower) and liver. The RDAs and the ULs for folic acid according to the Institute of Medicine are given in the following tables**.** Table 2.4 and Table 2.5

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 (DeSouza and Eitenmiller 1990), radiobinding or radiometric assay; (Chen and others 1983) and fluorometric, (Vahteristo and others 1996) or spectrophotometeric methods (Selhub and others 1988; Bagley and Selhub 1997), some of which are carried out in combination with gel or high-performance-liquid chromatography (HPLC). An extensive review of the interlaboratory variation of food folate analysis using various methods has been published (DeVries and others 2001, 2005). 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.

Microbiological Assay with Trienzyme Extraction

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 (Tamura and others 1997). AOAC Official Method 2004.05 is a microbiological method developed for folate analysis in cereal grain products (AOAC International, 2005). *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 and others 1998). For many years, despite its popular use, investigators had considered that microbiological assay was

extremely laborious and time consuming. In addition, 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 (DeSouza and Eitenmiller 1990; Tamura 1990). 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.

Principle of Microbiological Assay

Microbiological assay of the vitamin analysis is based on the nutritional requirement of a microorganism for a certain vitamin. The growth of the test microorganism is proportional to their requirement for a specific vitamin. Thus, the concentration of this specific vitamin can be estimated by comparing the growth of the test microorganism in an extract of the vitamin-containing sample with the growth of this microorganism in the presence of a known amount of the vitamin. The turbidity caused by the growth of the microorganism is measured photometrically.

Development of the 96 Well Plate Method

Newman and Tsai (1986) developed the microplate assay and contributed to the improvement of the microbiological assay for food folates. Further, Horne and Patterson (1988) established a similar plate assay which was simpler and used cryopreservation of the *Lactobacillus casei ssp. rhamnosus* (ATCC 7469) and thus shortened the incubation time. O'Broin and Kelleher (1992) developed a microassay for serum and red cell folate using a

chloramphenicol resistant strain of *L. casei*. Microplate assay for folic acid in multi vitamin formulations was done using *Streptococcus faecalis* as the test organism (Sarma and others 1995). Lastly, Horne (1997) presented an improved microplate assay using microtitre plates with opaque black walls to resolve the problem of overestimation of folate concentration of samples in two perimeter rows of 96-well plates. Further improvements were made by Tamura (1990).

Assay Organism

Test organisms like *Lactobacillus casei* ssp. *rhamnosus* ATCC 7469, *Enterococcus hirae* ATCC 8043, and *Pediococcus acidilactici* ATCC 8081 have been used for microbiological assay of folate. They have different responses to the folates available in biological matrices. Apart from the bacteria, a protozoan, *Tetrahymena pyriformis* is also used in the folate assay. *Lactobacillus casei* ssp. *rhamnosus* responds to various natural folate forms present in biologicals, and does not respond to pteroic acid—a common folate degradation product (Eitenmiller and others 2008). *Enterococcus hirae* has the limitation in that it does not respond to 5-methyl-H_4 folate, the most common folate present in milk, other foods, tissue, and serum, and it responds to pteroic acid—a common folate degradation product (Voigt and Eitenmiller 1978). *Pediococcus acidilactici* has the most limited response and can grow on only mono-, di-,and triglutamates of 5-or10-HCO-H_4 folate. It does not respond to methyl-substituted folates. None of the organisms efficiently responds to γ-glutamyl folate with greater than 3 glutamic acid residues (Eitenmiller and others 2008). Among the three bacteria, *Lactobacillus casei* ssp. *rhamnosus* has greatest capacity for response to the γ-glutamyl folate polymers. However, its response is limited to no greater than 3 glutamates with much lower response to higher

polymeric folates (Eitenmiller and others 2008). *Lactobacillus casei* ssp. *rhamnosus* is considered the best available bacteria for folate assay, because of its response to the most forms of natural folate, its greatest capacity in response to the γ-glutamyl folate polymers compared to other bacteria, and its lack of response to pteroic acid. *Lactobacillus casei* ssp. *rhamnosus* (ATCC 7469) is the test organism used in AOAC official Method 2004.05 Total Folate in Cereals and Cereal Food, Microbiological Assay –Trienzyme Procedure (45.2.09) (AOAC International 2005).

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; Martin and others 1990; Pfeiffer and others 1997b; Tamura and others 1997; Aiso & Tamura 1998; Rader and others 1998, 2000). 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.

Trienzyme assay uses the combination of Pronase^{R}, α-amylase and conjugase for extracting folate prior to microbiological assay.

1. Pronase^{R}

Pronase^{R} is a mixture of several proteolytic enzymes including endopeptidases and exopeptidases from *Streptomyces griseus.* It is still unknown how many proteinases and peptidases are present. It cleaves almost any peptide bond. Optimum pH for Pronase^{R} is 7 – 8. It is stable over wide ranges of pH and temperature. Narahashi and Yanagita (1967) described the properties of Pronase^{R}.

2. α-Amylase

α-Amylase (1,4-α-D-Glucan-glucanohydrolase) catalyzes endohydrolysis of 1,4-α-D-glucosidic linkages in polysaccharides containing three or more 1,4-α-linked D-glucose units. α-Amylase is a glycoprotein enzyme with optimum pH of 7.0.

3. Conjugase

Folate conjugase (γ-glutamyl hydrolase) catalyzes the hydrolysis of pteroylpolyglutamates to oligoglutamates or monoglutamates. There are various natural sources of folate conjugase enzyme (Keagy 1985; Goli and Vanderslice 1992). Among them, chicken pancreas and hog kidney are the most commonly used (Tamura 1990), and the enzyme from chicken pancreas was found to be more efficient for folate extraction (Pedersen 1988). The optimum pH for the conjugase from chicken pancreas is 7.8-8.5.

DeSouza and Eitenmiller (1990) were the first to develop a method for determination of food folate. In addition to the traditional treatment with conjugase, the method included treatments with α- amylase and Pronase ^{R}. They coined the term "trienzyme extraction." This

method significantly increased the measured levels of folate in wide variety of foods. Martin and others (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* subsp.*rhamnosus* (ATCC 7469), for folate determination (Tamura 1990). The α-amylase and protease treatments digest carbohydrate and protein matrices of foods where food folates are possibly trapped or bound. 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 and others (1990) 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 and others (1990). The order of the enzyme treatments was slightly modified wherein; the samples were treated with α-amylase and conjugase simultaneously and then digested with protease. Finally, Rader and others (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 sample. Rader and others (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. Shrestha and others (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 and others (2002a; 2002b) measured the folate concentrations in fast foods using trienzyme extraction proceure and obtained higher values than those in the literature. In fact, Konings (1999) and Konings and others (2001) reported about 20% increase in total folate contents of dairy products using the trienzyme extraction compared to conjugase alone.

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 collaborated AOAC method for the analysis of the folate by the tri-enzyme procedure was a collaborative study by (DeVries and others 2001, 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 was 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 and others 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 ssp rhamnosus*), 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.

Folate Analysis in Legumes

Although folate has been analyzed in the past, total folate for legumes vary from study to study by large margin. Yon and Hyun (2003) analyzed folate in some commonly consumed Korean foods by trienzyme extraction and microbiological assay. In peanuts, they reported a value of 136 μg 100^{-1} g by trienzyme treatment and 103 μg 100^{-1}g for single conjugase treatment. Recently, Rychlik and others (2007) analyzed the folate in various legumes by stable isotope dilution assays (SIDAs). These authors reported total folate levels of 94 and 61 μg $100g^{-1}$ for two samples of dry roasted peanuts. This study by Rychlik and others (2007) used an optimized enzyme extraction and a stable isotope dilution assay with detection (SIDA) liquid chromatography couple to a mass dectector (LC/MS/MS). The use of SIDA-LC/MS/MS has been developed for folate analysis in food and blood serum (Rychlik 2004; Rychlik and Mayr 2005; Rychlik and others 2007; Koehler and others 2007; Gutzeit and others 2008; Pfeiffer and others 2004; Fazili and others 2007; Fazili and others 2008). The method is considered superior to other approaches for folate analysis because the use of stable isotopically labeled analogues as the internal standards allows correction for losses of analytes during extraction and extract clean up. Further, selectivity and precision is improved compared to microbiological assay and LC methods using florescence or UV detection (Eitenmiller and others 2008).

Literature values for peanuts are highly variable ranging from range of 17 to 240µg/100g (Rychlik and others 2007). In the study by Rychlik and others (2007), the deconjugation of folate was carried out with combined treatment of rat plasma and chicken pancreas conjugase. For most of the legumes assayed by Rychlik and others (2007), SIDA-LC/MS/MS data was 2-3 times lower and in some cases many times lower than highest literature values obtained by microbiological assay. The reported total folate values in roasted peanuts were 61 and 94 µg/100g (n=2). Rychlik and others (2007) concluded that the older literature overestimates folate levels in legumes and that intake data, likewise from legumes might be overestimated. These differences in folate contents in legumes will only account for differences of less then 5 µg/day since the average daily consumption of legumes in western industrialized countries can be as low as 1 or 2 g (Rychlik and others 2007). Nevertheless, in others parts of the world such as in western Africa and a mean daily intake of about 500g peanuts which delivers 400 µg of dietary folate daily.

Optimum Conditions for Trienzyme Digestion

Trienzyme digestion with the microbiological procedure was accepted as an official method of folate analysis by the AOAC International (DeVries and others 2001, 2005); AOAC International 2005) and was proved essential for the release of folate from the food matrices (Aiso and Tamura 1998). Nevertheless, optimization studies on spinach, milk, bread, spinach, and beef concluded that the optimal combination of enzymes and reaction conditions varied with type of the food matrix. Yon and Hyun (2003) analyzed folate in some commonly consumed Korean foods by trienzyme extraction and microbiological assay and reported that most of the foods including peanuts gave higher measurable folate values by trienzyme treatment(136 µg

100^{-1}g) when compared to single conjugase treatment (103 μg 100^{-1}g). Two researchers reported that the dual enzyme treatment is sufficient to release the bound folate from the food matrices (Shrestha and others 2000; Pandarangi and LaBorde 2004). Shrestha and others (2000) used α-amylase followed by conjugase, while Pandarangi and LaBorde (2004) used protease followed by conjugase. An optimum condition for extracting folate in various foods like spinach and some Australian vegetables was also reported. Single enzyme treatment (conjugase) gave higher folate levels than trienzyme treatment for some leafy vegetables (Shrestha and others 2000; Iwatani and others 2003). Iwatani and others (2003) used spinach and Chinese broccoli as a their test sample and reported that the trienzyme treatment gave lower folate values when compared to single enzyme(conjugase) treatment. The order of enzyme addition and the digestion pH in the above studies varied from the AOAC method 2004.05. Response surface methodology (RSM) has been widely used in the field of agriculture and biological research for optimizing conditions in experiments (Lee and others 2000; Mizubuti and others 2000; Madamba 2002; Kwon and others 2003; Li and Fu 2005; Liyana-Pathirana and Shahidi 2005; Tanyildizi and others 2005). RSM simplifies the optimizing processes by evaluating the effects of several process variables and their interaction on the response variables (Box and Wilson 1951). It is advantageous over other optimizing processes since it is less laborious and requires fewer experimental trials to evaluate multiple parameters and interactions. Recently, Response Surface Methodology was applied to optimize the trienzyme digestion for the extraction of folate from vegetables (Chen and Eitenmiller 2007). The optimum trienzyme digestion time was 1.5 h for Pronase ^{R}, 1.5 h α-amylase and 3 h for conjugase. The optimized trienzyme digestion condition was applied to five vegetables and yielded higher folate levels than the trienzyme digestion (Chen and Eitenmiller 2007). Although, there is ample literature on vegetable and fruit matrices (Mullin and others

1982; Desouza and Eitenmiller 1986; Lin and Lin 1999; Chen and Eitenmiller 2007), there is a little research on the optimizing conditions for trienzyme digestion in peanut products.

Stability Studies of Folate

Peanut butter accounts for approximately half of the edible use of peanuts in the United States. Consumption of peanuts and peanut butter 5 times a week was associated with 27% and 21% reduction in risk of type 2 diabetes (Jiang and others 2002). Peanuts are one of the good sources of folate, which has been implicated in NTD and cancer. Nutrient compositional changes of peanuts associated with oil, protein, carbohydrate, mineral and water-soluble vitamin contents during roasting have been reported (Oupadissakoon and Young 1984; Damame and others 1990). Stability studies of other vitamins like vitamin E were established in roasted peanuts (Chun and others 2005) and in peanut butter (Chun and others 2003). Folate stability is affected by food processing since it is sensitive to oxidation, light, temperature, and extremes of pH. Some of the factors affecting the folate analysis are food matrices, oxygen availability, chemical environment, extent of heating and forms of folate in the food (Eitenmiller and others 2008). Heating temperature and time were found to cause increasing losses of folates during food processing (Williams and others 1995; Wigertz and others 1997; Vahteristo and others 1998). Effect of processing on folate stability was studied in various food products. Leskova and others (2006) reviewed effects of various heat treatments on folate retention in vegetables, fruit products, legume, meat, and fish products. Nearly all heat treatments showed a negative effect on folate retention. Overall, retention was 55-90% in meat and poultry, 70-100% in fish and shellfish, 40-66% in legumes and peas, and 30-100% in vegetables. The retention values depended on the extent and level of the heat treatment. Stralsjo and others (2003) studied folate

contents in strawberries and folate retention during storage and commercial processing of strawberries. The study showed that effect of cultivar, ripeness, and year of harvest is significant for folate concentration in strawberries. Folate retention was high in intact berries during storage until 3 or 9 days (4 °C). It was indicated that almost no losses of folate occurred when strawberry desserts were made from frozen berries, which is contradictory to the data reported in four European food tables indicating that only 3-30% of folate was retained in various strawberry products like jam and stewed desserts (Souci and others 1994). Stralsjo and others (2003) also suggested that fresh strawberries and processed strawberry products were good folate sources. For instance, 250 g (fresh weight) of strawberries (about 125 μg of folate) supplies around 50% of the recommended daily folate intake in various European countries (200-300 μg /day) or 30% of the U.S. recommendation (400 μg/day). Similarly, free and total folate in spinach and broccoli were determined at various processing stages and steam blanching retained more total folate than water blanching (De Souza and Eitenmiller 1986). Stea and others (2006) studied effects of various heat treatments on folate retention in vegetables caused by different processes used in modern large-scale service systems and the food industry. The main folate forms in vegetables, tetrahydrofolates and 5-methyltetrahydrofolates, were analyzed was analyzed by HPLC. Compared to raw potatoes the retention percentage was 103% for sous-vide, 72–59% for boiling (unpeeled and peeled), and 63% for oven baking. Compared to raw green peas the retention percentage was 77% for boiling, 75% for microwaving, 73% for steam boiling, and 71% for blanching. Blanching of peas, boiling of potatoes and oven baking of unpeeled potatoes caused significant reduction. Storage at various temperatures and length of times followed by reheating caused no further significant losses of total folate.

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Table 2.1 Per capita consumption of peanuts

Year	Total Consumption in Pounds
1970	5.6
1975	6.1
1980	5.0
1985	6.4
1990	6.1
1995	5.6
2000	5.8
2001	5.9
2002	5.8
2003	6.3
2004	6.6
2005	6.6
2006	6.5

Source: USDA, Economic Research Service, 2008.

Table 2.2 Peanut production by state in the United States for 2007

State	Acreage planted (Thousand acres)	Yield pounds/acre	Production (Million lbs)	Value of production (in Millions of dollars)
Alabama	160	2,600	408	73
Florida	130	2,700	321	60
Georgia	530	3,150	1,638	324
Mississippi	19	3,300	59	12
New Mexico	10	3,500	3.5	7
North Carolina	92	2,800	252	57
Oklahoma	18	3,400	58	12
South Carolina	59	3,100	174	38
Texas	190	3,950	739	167
Virginia	22	2,700	57	12
United States	1,230	3,130	3,741	753

Source: USDA, National Agricultural Statistics Service, 2007.

Table 2.3 Per capita consumption of peanuts compared to all other nuts from 2001 to 2005

Tree nuts	2001	2002	2003	2004	2005
Almonds	0.84	1.07	1.12	0.89	0.53
Filberts	0.10	0.08	0.05	0.06	0.03
Pecans	0.48	0.43	0.42	0.50	0.52
Walnuts	0.42	0.47	0.50	0.53	0.42
Macadamias	0.07	0.06	0.08	0.11	0.09
Pistachios	0.20	0.21	0.19	0.26	0.19
Other	0.73	0.83	1.01	1.08	0.89
Total	2.85	3.15	3.37	3.44	2.66
Peanuts	5.9	5.8	6.3	6.6	6.6

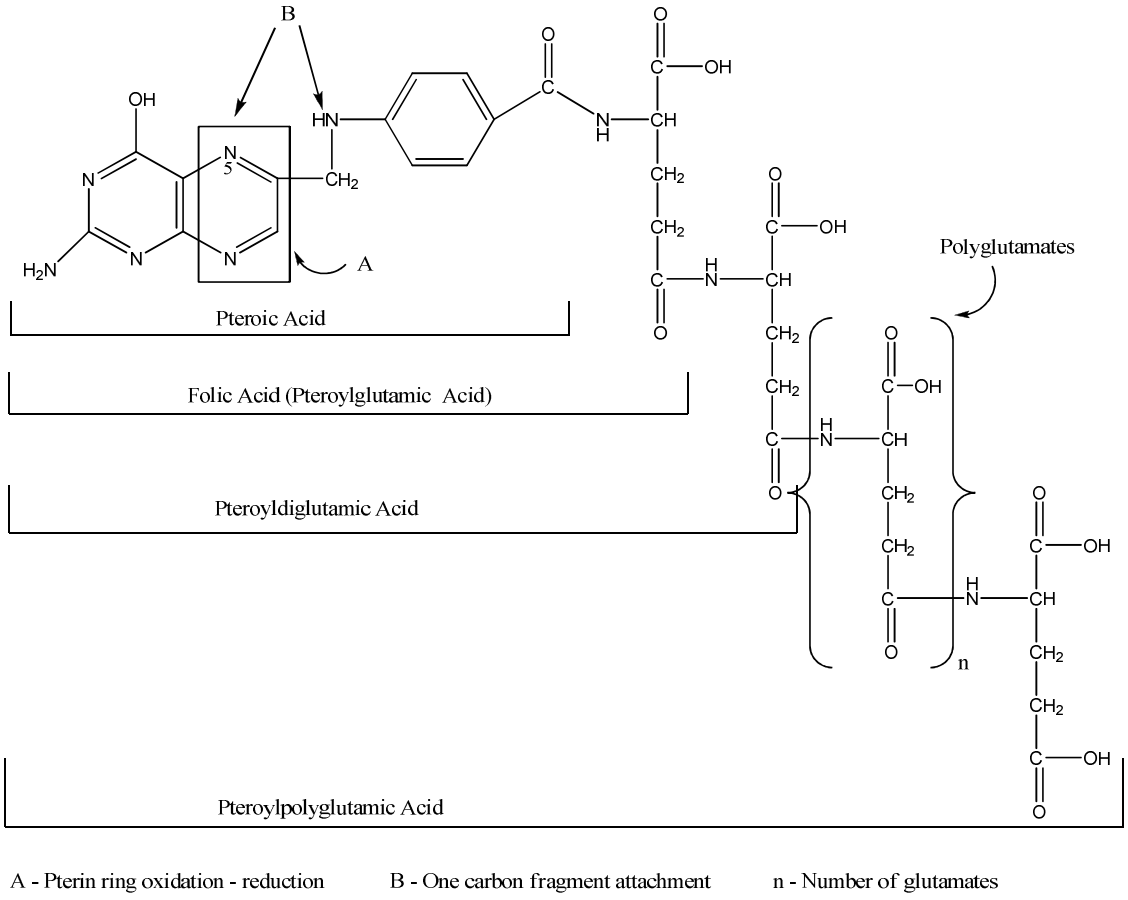
Source: USDA, Economic Research Service, 2008.

Table 2.4 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 2.5 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



A - Pterin ring oxidation - reduction B - One carbon fragment attachment n - Number of glutamates

Figure 2.1 Structure of folate and folic Acid

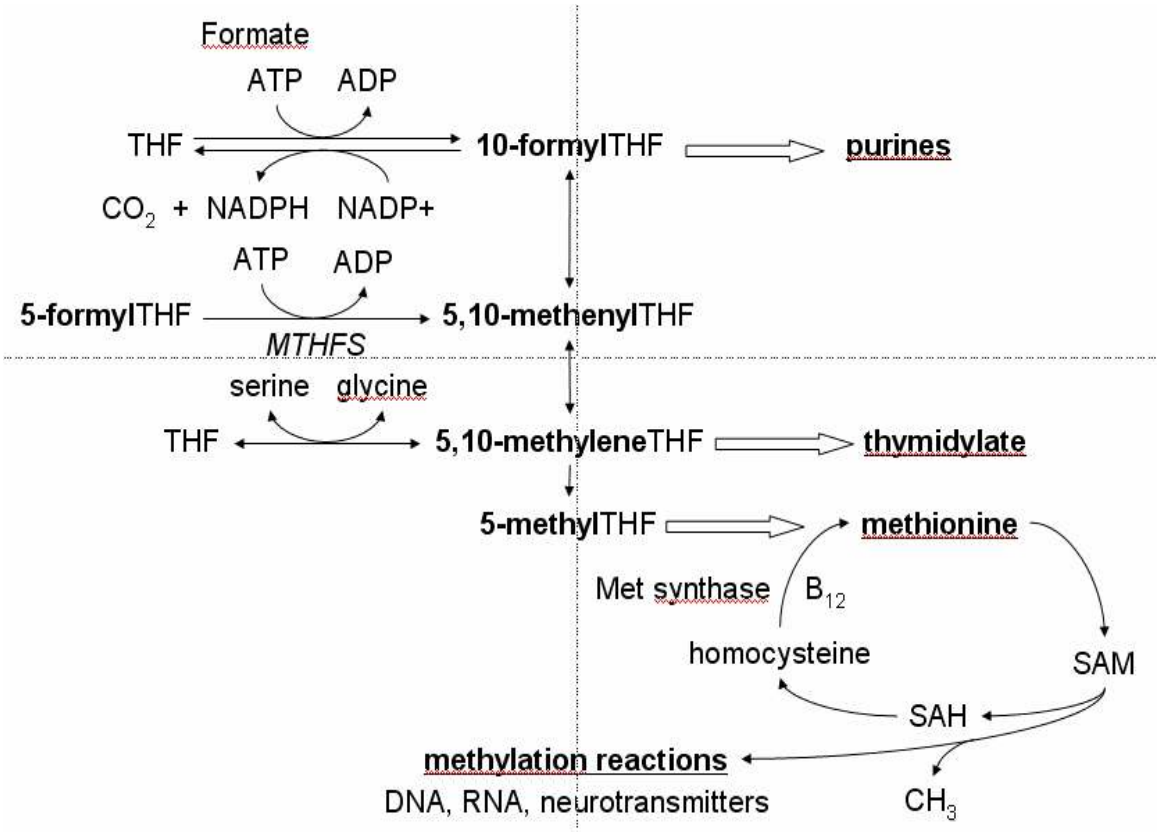


Figure 2.2 Interconversions of folate

CHAPTER 3

TOTAL FOLATE IN PEANUT TYPES AND CULTIVARS GROWN IN THE UNITED STATES[1]

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[1]Kota L, Pegg RB, Phillips RD, Eitenmiller RR to be submitted to Journal of Food Science.

ABSTRACT

The total folate content in peanuts was determined by a trienzyme extraction methodology followed by microbiological assay using *Lactobacillus casei* ssp.*rhamnosus*. Samples consisting of 222- cultivar-specific samples of four different peanut types, (Runner, Virginia, Spanish and Valencia) from 2 crop years (2005 and 2006) from 3 geographical locations (Southeast, Southwest and Virginia/Carolina) were collected by the Peanut Institute in cooperation with the American Peanut Shellers Association. The 2005 samples included 50 Runner, 26 Virginia, 9 Spanish and 1 Valencia type peanuts. The 2006 samples included 101 Runner, 24 Virginia, and 11 Spanish type peanuts. No significant differences were noted among the folate levels by types for 2005 crop year peanuts ($P>0.05$). For 2006 peanuts, Spanish peanuts were statistically lower in folate than Runner and Virginia peanuts ($P<0.05$). For the Runner cultivars, significant ($P<0.05$) differences existed among cultivars with some significant year-to-year variation. In Runner and Spanish type, high-oleic cultivars contained significantly ($P<0.05$) higher folate levels compared to normal cultivars. Folate contents among Virginia cultivars were statistically similar in 2005 (except for NC-12) and 2006 ($P>0.05$). For Spanish cultivars in 2005, OLin had significantly higher folate than Tamspan 90. Normal and high-oleic cultivars in both the years did not vary significantly ($P>0.05$) in folate content. Folate levels in peanuts from the Virginia/Carolina region varied significantly by production year, but peanuts from Southeast and Southwest region did not vary from 2005 to 2006.

Introduction

Folate, an essential water-soluble vitamin, has important roles in DNA synthesis, repair, and methylation. A deficiency of folate in the diet is associated with an increased risk of neural tube defects, cardiovascular diseases (CVD), anemia, and some cancers (Lucock 2004). Folate plays a vital role in lowering plasma homocysteine levels, which are an independent risk factor for CVD (Myles and others 2008). Numerous studies have shown that fortification of foods with folic acid or the use of folic acid as a dietary supplement leads to a dramatic decrease in neural tube defects (Botto and others 2006). This strategy, however, is expensive and hard to implement in countries worldwide. Additionally, high intakes of folic acid from fortification may increase the unmetabolized folic acid in blood, mask the symptoms of vitamin B_{12} deficiency in the elderly and may promote cancer (Smith and others 2008). Hence, consumption of natural sources of folate is the best possible way of meeting the recommended daily allowances (RDA). Most diets of the U.S population do not provide the required level to limit neural tube defects.

Peanuts are one of the richest available natural sources of folate and in one sense can be called a functional food. The United States is a major producer of peanuts contributing approximately 10 % to the world production with a per capita consumption of 6.5 pounds in 2007 (USDA 2008). Consumption of tree nuts and peanuts by adult population groups has consistently shown beneficial effects in health. Numerous studies conducted to date have reported that peanuts and tree nuts reduce LDL cholesterol concentrations and thereby the risk of CVD (Kris-Etherton and others 1999; Fraser 2000; Ellsworth and others 2001; Albert and others 2002; Brehme 2002; Lokko and others 2007). According to Alper and Mattes

(2003), regular peanut consumption lowers serum triacylglycerol concentration and increases the serum magnesium concentrations with concomitant reduction of the risk of CVD. Individuals who regularly consume peanuts have higher intakes of protein with increases in arginine, total fat, polyunsaturated fat (PUFA), monounsaturated fat (MUFA) (Maguire and others 2004), fiber (Anderson and others 2000), vitamin E (Chun and others 2005a; Lee and others 2000) folate, calcium, magnesium, zinc, copper (Jones and others 1997) and iron intake (Griel and others 2004). Reduction in CVD risk factors was primarily attributed to the beneficial fatty acid composition and L-arginine and folate levels in peanuts.

Although the folate content in various in legumes has been analyzed in the past, (Augustin and Klein 1989; Konings and others 2001; Yon and Hyun 2003), there is lack of consistent data on the folate content in peanuts. Most recently, Rychlik and others (2007) analyzed the folate in various legumes by stable isotope dilution assays (SIDAs) by the use of an optimized enzyme extraction and a stable isotope dilution assay with detection by liquid chromatography coupled to a mass dectector (LC-MS/MS). However, the possible variation of folate in peanuts due to types, cultivars, geographic production area, or years of harvest has been largely ignored. Large numbers of peanut cultivars are grown in the United States with four common types: Runner, Virginia, Spanish, and Valencia (American Peanut Council 2008). This study aims at showing variation in folate content due to type, cultivar, geographic location of growth and production year. It uses a large sample set to obtain a true representation of the variations.

Materials and Methods

Sample Collection

Samples were obtained through an intensive sampling program designed and conducted by The Peanut Institute in cooperation with the American Peanut Shellers Association. In order to meet the goals of the research, a sample set that provided the representative, cultivar identified samples covering the different peanut types and geographic regions of growth from two years was collected. This sample set represents a well defined collection for delineating peanut composition. Darlene Cowart (J.W.Leek), in cooperation with John Powell (The Peanut Institute) and Pat Kearney (PMK Associates) designed and carried out the physical aspects of sampling. In brief, the sampling effort involved:

1. Development of a uniform sampling plan that accurately determined major cultivars grown by the U.S growers in the Southeast, Southwest and Virginia/Carolina regions.
2. Based on sheller input and seed sales, cultivars for each market type were chosen.
3. Once the varieties were chosen, the shellers agreed to pull samples from wagons during the grading process. Seed growers were identified in each region and samples were taken from the seed wagons after drying the peanuts down to 10.49% moisture.
4. An official sample was pulled from each wagon and graded.
5. The sheller obtained the back half of the official grade sample and subdivided the sample down to 3 pounds.
6. The 3-pound sample was cleaned using the grade room Farmerstock cleaner and sent to the National Peanut Research Lab in Dawson, Georgia.
7. The samples were shelled and the entire sample was sent to University of Georgia.

8. About 200g of the raw kernels were added into a Cryovac B-620 bag and vacuum packaged using the Henkelman Vacuum Packager (Model 600). All the bags were labeled and stored at -60°C. (The samples were thawed prior to extraction).

Sample Description

A successful 2-year sampling plan provided 222 cultivar specific samples of four different peanut types (Runner, Virginia, Spanish, and Valencia) from two years (2005 & 2006) and 3 geographic locations (Southeast, Southwest, Virginia/Carolina). The 2005 samples included 50 Runner, 26 Virginia, and 9 Spanish, 1 Valencia type peanuts. The 2006 samples included 101 Runner, 24 Virginia, 11 Spanish type peanuts. In both years, samples were harvested from the Southeast (Georgia, Alabama, Florida), Southwest (Texas, Oklahoma) and Virginia/Carolina region (North Carolina, South Carolina, Virginia). Although Valencia type peanuts are also a major peanut type grown in the United States, only one sample was collected in 2005; therefore, the Valencia type was not included in the statistical comparisons. The 2005 samples included 16 high-oleic and 69 normal cultivar samples and, the 2006 samples included 62 high oleic and 74 normal cultivar samples. Table 3.1 summarizes the available samples by type, cultivar, year of production and growing region.

Method Evaluation

Performance parameters including accuracy and precision of the microplate assay with trienzyme extraction were evaluated following the AOAC guidelines (AOAC International 1998; AOAC International, 2002a; AOAC International, 2002b).

1. *Accuracy*

Accuracy was determined by analysis of European Commission Certified Reference Material, BCR 121 (wholemeal Flour) purchased from Resource Technology Corporation, Laramie, WY and by determination of recovery. To determine the recovery, a peanut butter sample was spiked with 60µg of folic acid standard. This spike level provided a total folate level approximately twice the expected folate amount. Recovery was calculated by the following equation (AOAC International, 2002a):

$$R(\%) = [(C_s - C_p)/C_a] - 100,$$

where R (%) is the percent recovery of added standard; C_s is folate concentration in the spiked sample; C_p is folate concentration in the unspiked sample; and C_a is the folic acid standard added. All recovery values were determined by duplicate analysis.

2. *Repeatability precision (%RSD$_r$)*

Measurement of precision with simultaneous and consecutive replicates within a laboratory is termed as repeatability precision (%RSD$_r$) (Horwitz 2003). Repeatability precision (%RSD$_r$) was determined by assay of the Pillsbury all-purpose, bleached, enriched flour purchased at the local grocery and also by assay of BCR 121 (wholemeal flour). Both

the quality control flour and BCR 121 were stored in 4 oz Nalgene bottles at 4 °C. The relative standard deviation for repeatability precision (% RSD_r) was calculated as follows:

% RSD_r= (SD * 100)/mean.

Analysis of Total Folate

Total folate was assayed microbiologically with *Lactobacillus casei* ssp. *rhamnosus (ATCC 7469),* according to the procedures outlined by Tamura (1990) and Chen and Eitenmiller (2007). A brief description of the procedural steps include the following:

1. Preparation of Standards

Twenty milligrams of the folic acid (US Pharmacopoeia) were added into a 200- mL conical flask containing 20 mL of (95%v/v) ethanol and 50 mL of deionized water. The initial pH was adjusted to 10.0 with 0.1N NaOH (to help dissolve the folic acid) and the final pH to 7.0 with 0.05N HCl. The final volume was made up to 100 mL with distilled water, the solution transferred to 10-mL Pyrex tubes and then stored at 4°C. A new standard should be prepared after 6 months.

Purity of the standard solution was determined by diluting the stock standard solution (0.2 mg/mL) with phosphate buffer (0.1 M, pH 7.0) to a final concentration of 0.01 mg/mL (1:20 dilution), measuring the absorbance of the diluted standard at 282 nm in a 1 cm quartz cell using phosphate buffer (0.1M,pH 7.0) as a blank. The purity was calculated using the following equation:

Purity of standard (%) = 100 (analyzed concentration C2 / known concentration C1)

Calculated concentration C2 = $(A_{std}-A_{blank})\cdot M/(\varepsilon\cdot b)$,

Where C1=0.01 mg/mL, C2=analyzed concentration of diluted stock standard (mg/mL),

A=absorbance, $\varepsilon=27.0* 10^3\ M^{-1}cm^{-1}$**,** b=1cm, M=molar mass of folic acid (441.40).

The absorptivity (ε) was presented by Ball (1994).

*2. Trienzyme Extraction Procedure for Total Folate***.**

The extraction procedure follows the Association of Official Analytical Chemists (AOAC) Official Method 2004.05 "Total Folates in Cereal Foods –Microbiological Assay-trienzyme procedure" which uses *Lactobacillus casei* ssp. *rhamnosus* and a recently collaborated procedure presented by De Vries and others (2005). Fat extraction was done on all the samples to remove lipids to avoid stimulatory growth of the *Lactobacillus casei* ssp. *rhamnosus* (ATCC 7469).

The extraction procedure includes the following steps:

1. Grind 10g of raw peanuts to a smooth powder in a coffee grinder with rapid pulses. Care must be taken not to paste the sample. If the sample pastes, it is difficult to prepare a homogenous ground sample.
2. Weigh 1 g of the ground sample into a150 mL (3.5cm O.D 15cm) glass tube.
3. Extract fat by adding 20 mL of hexane and vortexing for about 45 s. The mixture is allowed to stand for 10 min until there is phase separation.
4. Remove hexane layer with a Pasteur pipette.
5. Remove residual hexane under the stream of nitrogen.

6. To the defatted sample, add 20 mL of 0.1M phosphate buffer, pH 7.8 (anhydrous Na_2HPO_4, containing L-ascorbic acid -1%, w/v) and vortex.
7. Transfer sample to a 150 mL Erlenmeyer flask.
8. Wash the glass tube with two 15 mL aliquotes of deionized water to bring the total volume to 50 mL.
9. Cover the flasks with an aluminum foil and heat at 100° C for 15 min in a water bath.
10. Cool the contents to 35°C and add additional 10 mL of the phosphate buffer together with 1 mL of Pronase^R solution (2mg/mL in deionozed water, Calibochem, nr 53702, San Diago, Calif., U.S.A). Incubate the mixture at 37° C for 3 h.
11. Heat the extract at 100°C for 3 min and cool to 35°C.
12. Add 1 mL of α-amylase solution (20 mg/mL in deionized water, Fulka, nr 10065, St.Louis, Mo., U.S.A) and incubate at 37°C for 2 h.
13. Prepare chicken pancreas conjugase solution (5 mg/mL of 0.1 M, pH 7.8 phosphate buffer) by stirring for 10 min and filtering through glass wool. Add 4 mL of filtered solution and incubate at 37°C for 16 h.
14. Heat the extract at 100°C for 3 min and cool to 35°C.
15. Adjust the pH to 4.5 with 1N HCl, dilute to 100 mL with water and filter through ashless filter paper(Whatmann ^{R} No. 1,Cat: 1001-185,18.5cm).
16. Make further dilutions if necessary with, 0.1M phosphate buffer containing 1% (w/v) ascorbic acid. With a 100 mL volumetric flask, fill to the mark with deionized water.
17. Autoclave the tubes at 121°C for 5 min.

The microbiological assay was carried out using 96 well plate method (Tamura 1990; Chen and Eitenmiller 2007). Detailed procedure for microplating is provided in Appenix A.

3. *Preparation of Conjugase*

Fresh chicken pancreas provided by the Poultry Science Department, University of Georgia were cut into smaller pieces (1-2 cm) and was ground to a fine powder with dry ice using a mortar and pestle. The powdered chicken pancreas was transferred to a beaker with cold acetone (75mL). The chicken pancreas-acetone mixture was ground to a finer particle size by using a ultrasonic disintegrator (PRO 300A, Proscientific Inc. Oxford, CT). The acetone slurr was filtered through cheesecloth placed over a Buchner funnel containing filter paper (Fischerbrand, Cat: 09-795D, 11.0cm) and residual acetone was allowed to evaporate from the solid residue. The dry powder was transferred into a one ounze, glass bottle, capped tightly, and stored at -20°C. The conjugase activity of the chicken pancreas prepararion was checked following the hydrolysis of pteroyltetra-γ-L-glutamic acid. The measured activity was 0.00003 μmol/min (0.03nmol/min) per twenty milligrams of chicken pancreas conjugase preparation. Details of the specific activity measurement are provided in Appendix B.

4. *Control*

A control (enzyme blank) was carried out throughout the complete extraction procedure for quantification of total folate. The control including all enzymes and components other than the food sample was used to determine the contribution of the enzymes to the growth response of the *L. casei* ssp. *rhamnosus* (ATCC 7469). The control is important in an assay to detect the amount of folate contributed from the enzymes. In addition, the enzyme control constitutes a key quality control measure for the overall procedure.

Statistical Analysis

Statistical analysis (two-way analysis of variance) was performed using the Statistical Analysis System (SAS 9.1, Cary, NC, U.S.A.). Statistical significance between groups was determined by the Tukey test as a mean separation at $\alpha = 0.05$.

Results and Discussion

Quality Control Parameters

Accuracy

Accuracy is the closeness of the test result to the "true" or accepted value (AOAC International 2002a; 2002b). Fifty replicate analyses of BCR 121 (wholemeal flour) completed throughout the study gave a mean of 49 ± 3.5 μg/100g. The analyzed value compares closely to the reported certified value of 50 μg/100g ± 7, indicating an acceptable accuracy.

The accuracy expressed as recovery was excellent. The percent mean recoveries ± SD obtained by spiking with known levels of standards were 99.7 ± 2.2, 98.8 ±0.3, 100 ± 1.6 for Runner, Virginia and Spanish type peanuts, respectively. Closeness of the assay values to the reference concentration value of BCR 121 (wholemeal flour) and the recovery of the spiked folic acid from peanuts and peanut butter products indicate a high degree of accuracy.

Repeatability Precision (%RSD_r)

Replicate assays of in-house quality control sample (enriched flour) and BCR 121 (wholemeal flour) were used to determine %RSD_r for analysis of total folate. The % RSD of microbiological assay with trienzyme extraction was 2.7 and 9.1 for enriched flour and BCR 121, respectively. Total folate in the enriched flour was 181 ± 4.8 μg/100g and 49±3.5 μg/100g in BCR 121(Figure 3.1). The assay of both samples provided data consistently within the upper and the lower controls set at ± 10% of the mean, indicating good control of the assay.

Effect of Peanut Type and Year of Production.

The total folate contents of Runner, Virginia and Spanish type peanuts harvested in 2005 and 2006 are presented in Table 3.2. No significant differences were noted among the folate levels for 2005 crop year peanuts ($P>0.05$). For 2006 peanuts, Spanish peanuts were statistically lower in folate than Runner and Virginia peanuts ($P<0.05$). Overall means in μg/100g combining the data from the two sampling years were 92 ± 16.5 (Runner), 91± 19.1(Virginia) and 84± 13.0 (Spanish). As noted for the 2006 crop years, the overall mean for the Spanish type peanuts was significantly lower than the means of Runner and Virginia type peanuts($P<0.05$). For all peanut samples, 2006 crop year peanuts contained significantly high folate than 2005 crop year peanuts($P<0.05$)(93 ±.16.2 vs. 88.5±.18.1). Within types, only Virginia type peanuts varied significantly by crop year ($P<0.05$). Folate levels found in this study generally agree with lower published values or peanuts from older studies. Considering the data obtained by Rychlik and others (2007) by LC/MS/MS for roasted peanuts of 61 and 94 μg/100g, the current data is quiet similar and supports the opinion of

Rychlik and others (2007) that some microbiological data on legumes is much higher than "real" levels. The results show that the type and production year can affect folate content of raw peanuts.

Effect of Cultivar and Year of Production

Table 3.3 gives folate levels present in Runner cultivars peanuts produced in 2005 and 2006. Over the two production years, folate concentrations ranged from 81μg/100g (GA-03L) to 130 μg/100g (TamRun 96). Combining cultivar samples for 2005 and 2006 mean values ranged from 107 ± 6.5 μg/100g in AP-3 to 81μg/100g in GA-03L. Cultivar variations were statistically significant ($P<0.05$) with the Runner cultivars grouping into three groups as follows : Group 1 AP-3, TamRun 96, TamRun OL01; Group 2, GA-02C; Group 3, C-99R, GA-01R, Georgia Green, FlavorRunner 458, TamRun OL02 and GA-03L. The difference was 1.6 times from the highest folate concentration to the lowest. Cultivar variation in 13 strawberry cultivars (Stalsjo and others 2003) and several potato cultivars (Goyer and Navarre 2007) varied by 1.9 and 2.6 times, respectively, on a low to high folate basis. Significant ($P<0.05$) year-to-year variation was present in TamRun 96, Tamrun OL01 and FlavorRunner 458. However, for each of these cultivar samples size was quiet limited in one or both production years. Combining 2005 and 2006 samples, high-oleic cultivars contained significantly higher folate levels than normal cultivars (97±19 vs. 88 ±14μg/100g) ($P<0.05$). In summary for the Runner cultivars significant differences existed ($P<0.05$) among cultivars with some significant year-to-year variation being noted. High-oleic cultivars contained significantly higher folate levels compared to normal cultivars.

Table 3.4 presents folate data from Virginia cultivars. Over the two production years, folate concentrations ranged from 72 μg/100g (Gregory) to 122 μg/100g (NC-12). Combined data from 2005 and 2006 samples gave values ranging 122 μg/100g in NC-12 to 78 ± 9.6 μg/100g in Gregory. Combined mean variations were not significant among cultivars ($P>0.05$). Significant ($P<0.05$) year-to-year variation was noted for NC-7 and Gregory. However, sample size for both cultivars was small.

Data for the folate content in Spanish cultivars is given in Table 3.5. Spanish cultivars included Tamspan 90(normal) and OLin(high-oleic) OLin contained significantly higher folate levels than the Tamspan 90 in 2005 samples (98 vs. 82 μg/100g) and through comparisons, of the overall means (92 vs 81 μg/100g) ($P<0.05$). As noted for Runner cultivars the high-oleic cultivar contained higher folate levels compared to normal cultivar. Year to year variation was not significant ($P>0.05$) for Spanish cultivars.

Effect of Different Geographical Regions and Year of Production

To check the effects of growing locations, soil conditions on folate concentrations, peanuts harvested from 3 geographical locations were collected (Table 3.6). These regions included Southeast (Georgia, Alabama, Florida), Southwest (Texas, Oklahoma) and Virginia/ Carolina region (North Carolina, South Carolina, Virginia). The total folate for 2005 was highest in Southeast region (92 ±15 μg /100), followed by peanuts from Virginia/Carolina (87±14 μg/100) and the Southwest (84 ± 18 μg /100). For 2006, Virginia/Carolina samples (97 ± 18 μg/100) had the highest folate followed by Southwest (93± 20 μg/100) and

Southeast (91±17 μg/100). Overall means did not vary significantly among the three regions (P>0.05). There was statistical significance for peanuts from Virginia/Carolina regions between the two years of harvest but the peanuts from Southeast region and Southwest region did not show this difference. The results indicate that the production regions did not have a significant effect on the folate concentrations in peanuts harvested in 2005. However, for the peanuts harvested in 2006, Virginia/Carolina samples were higher at P<0.05.

For Runner peanuts, sufficient samples were available to compare folate levels in the Southeast samples to Runner samples collected in the Southwest (Table 3.7). Significant differences did not exist between the Runner peanuts, collected in the two production areas (P>0.05). Environmental conditions can influence the folate concentrations (Goyer and Navarre 2007).

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Table 3.1 Peanut samples collected from the 2005 and 2006 crop years

Runner			**Virginia**		
Cultivars	2005	2006	Cultivars	2005	2006
Georgia Green	22	22	NC-7	3	3
C-99R	9	15	Perry	7	6
TamRun 96	5	3	NC-V11	9	6
*FlavorRunner458	3	3	VA98R	5	6
* TamRun OL01	2	13	Gregory	1	3
* TamRun OL02	5	17	NC-12	1	0
*GA-02C	3	24			
AP-3	0	3			
GA- 01R	1	0			
GA- 03L	0	1			
Total	**50**	**101**	**Total**	**26**	**24**
Regions	2005	2006	Regions	2005	2006
Southeast	30	89			
Southwest	19	9	Southwest	6	3
Virginia/Carolina	1	3	Virginia/Carolina	20	21
Spanish			**High-Oleic and Normal** [a (b,c)]		
Cultivars	2005	2006	Cultivars	2005	2006
OLin	3	5	High-Oleic	16 (3, 13)	62 (5,57)
Tamspan 90	6	6	Normal	69 (6, 63)	74 (6, 68)
Southwest Region	9	11			

* High-oleic cultivars, [a] total sample numbers , [b] number of Spanish samples, [c] number of Runner samples.

Table 3.2 Total folate in peanut types harvested in 2005 and 2006

	Total Folate (µg/100g)		
Types	2005	2006	Overall Mean ±SD
Runner (52,101)[c]	91 ± 19.8[a,1]	93 ± 16.0[a,1]	92 ± 16.5[x]
Virginia (26,24)	84 ± 16.1[a,2]	97 ± 18.6[a,1]	91 ± 19.1[x]
Spanish (9,11)	87 ± 13.0[a,1]	83 ± 12.5[b,1]	84 ± 13.0[y]
Overall Mean	**86 ± 18.1[2]**	**93 ± 16.2[1]**	**91 ± 17.1**

[a-b,x-y] Any means in the same columns followed by the same letter are not significantly different (P<0.05) by Tukey's test.

[1-2] Any means in the same rows (2005, 2006) followed by the same number are not significantly different (P<0.05) by Tukey's test.

[c] Number of samples obtained from the year 2005 and 2006 are given in parenthesis, Data represents the mean ± standard deviation.

Table 3.3 Total folate in Runner peanuts harvested in 2005 and 2006

	Total Folate (µg/100g)		
Cultivars	2005	2006	**Overall Mean±SD**
AP-3 (0,3)[c]	-	$107 \pm 6.5^{a,b}$	**107 ± 6.5^{x}**
TamRun 96 (5,3)	$97 \pm 9.4^{ab,2}$	$116 \pm 12.5^{a,1}$	**104 ± 13.7^{x}**
*TamRun OL01 (2,13)	130^{a1}	$99 \pm 18.2^{ab,2}$	**103 ± 20.6^{x}**
*GA-02C (3,24)	$100 \pm 19^{ab,1}$	$95 \pm 14^{ab,1}$	**96 ± 15.7^{xy}**
C-99R (9,15)	$90 \pm 17^{b,1}$	$88 \pm 18.6^{b,1}$	**89 ± 13.33^{y}**
GA 01R (1,0)	89^{b}	-	**89^{y}**
Georgia Green (22,22)	$86 \pm 16^{b,1}$	$90 \pm 16.0^{b,1}$	**87 ± 14.44^{y}**
*FlavorRunner 458 (3,3)	$74 \pm 4.7^{b,2}$	$100 \pm 4^{ab,1}$	**87 ± 13.3^{y}**
*TamRun OL02 (5,17)	$100 \pm 21^{ab,1}$	$83 \pm 16^{b,2}$	**87 ± 19^{y}**
GA-03L (0,1)	81^{b}	-	**81^{y}**
High-Oleic(13,57)[c]	$97 \pm 16^{a,1}$	$98 \pm 22^{a,1}$	**97 ± 19^{x}**
Normal(37,44)	$87 \pm 18^{b,1}$	$93 \pm 16^{a,1}$	**88 ± 14^{y}**

[a-b,x-y] Any means in the same columns followed by the same letter are not significantly different ($P<0.05$) by Tukey's test.

[1-2] Any means in the same rows (2005, 2006) followed by the same number are not significantly different ($P<0.05$) by Tukey's test.

[c] Number of samples obtained from the year 2005 and 2006 are given in parenthesis , Data represents the mean ± standard deviation.

* High-oleic cultivars.

Table 3.4 Total folate in Virginia peanuts harvested in 2005 and 2006

	Total Folate (µg/100g)		
Cultivars	2005	2006	**Overall Mean±SD**
NC-12 (1,0)[c]	122	-	**122**
VA 98R(5,6)	98 ± 19[a,1]	96 ± 17[a,1]	**97 ± 16[x]**
NC-V11(9,6)	99 ± 17[a,1]	87 ± 14[a,1]	**94 ± 16.8[x]**
Perry (7,6)	93 ± 21[a,1]	85 ± 13[a,1]	**87 ± 18.6[x]**
NC-7(3,3)	93 ± 18[a,1]	73 ± 20[a,2]	**83 ± 20[x]**
Gregory(1,3)	93[a1]	72 ± 5.1[a,2]	**78 ± 9.6[x]**

[a-b,x-y] Any means in the same columns followed by the same letter are not significantly different ($P<0.05$) by Tukey's test.

[1-2] Any means in the same rows (2005, 2006) followed by the same number are not significantly different ($P<0.05$) by Tukey's test.

[c] Number of samples obtained from the year 2005 and 2006 are given in parenthesis, Data represents the mean ± standard deviation.

Table 3.5 Total folate in Spanish peanuts harvested in 2005 and 2006

	Total Folate (µg/100g)		
Cultivars	2005	2006	**Overall Mean±SD**
*OLin(3,5)[c]	98 ± 16[a,1]	88 ± 14[a,1]	**92 ± 15[x]**
Tamspan 90(6,6)	82 ± 15[b,1]	80 ± 14[a,1]	**81± 16[y]**

[a-b,x-y] Any means in the same columns followed by the same letter are not significantly different (P<0.05) by Tukey's test.

[1-2] Any means in the same rows (2005, 2006) followed by the same number are not significantly different (P<0.05) by Tukey's test.

[c] Number of samples obtained from the year 2005 and 2006 are given in parenthesis, Data represents the mean ± standard deviation.

***** High-oleic cultivars.

Table 3.6 Folate in peanuts from 3 geographic regions harvested in 2005 and 2006

Total Folate (µg/100g)			
Year	Southeast	Southwest	Virginia/Carolina
2006(89,23,24)[c]	91 ± 17[a,1]	93 ± 20[a,1]	97 ± 18[a,1]
2005(30,34,21)	92 ± 15[a,1]	84 ± 18[a,1]	87 ± 14[b,1]
Overall Mean±SD(119,57,45)	**90 ± 14[1]**	**91 ± 17[1]**	**85 ± 19[1]**

[a-b,x-y] Any means in the same columns followed by the same letter are not significantly different (P<0.05) by Tukey's test.

[1-2] Any means in the same rows (2005, 2006) followed by the same number are not significantly different (P<0.05) by Tukey's test.

[c] Number of samples obtained from Southeast, Southwest and Virginia/Carolina regions are given in parenthesis, Data represents the mean ± standard deviation.

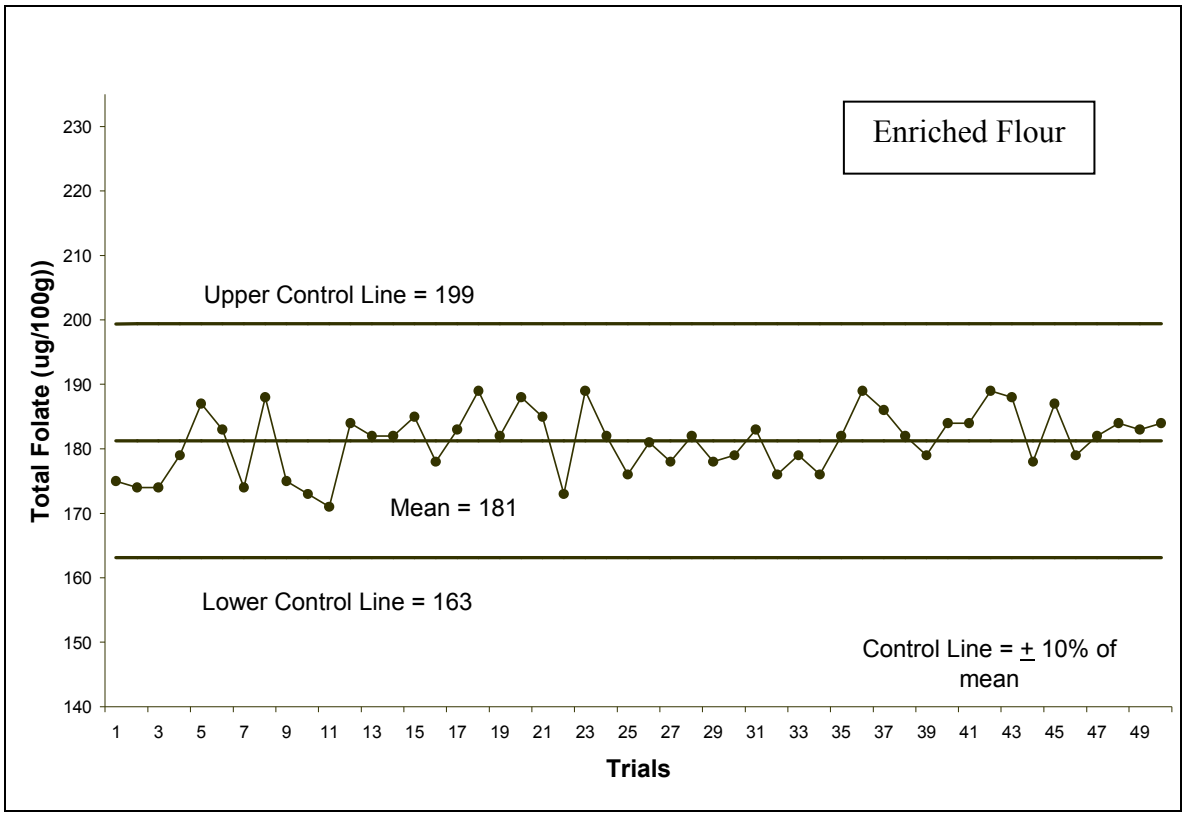
Table 3.7 Total folate in Runner peanuts from two geographical regions harvested in 2005 and 2006

	Total Folate (µg/100g)	
Year	Southeast	Southwest
2005(30,19)[c]	92 ± 17[a,1]	94 ± 21[a,1]
2006(89,9)	91 ± 15[a,1]	93 ± 17[a,1]
Mean±SD(119,28)	**90 ± 15[a]**	**92 ± 15[a]**

[a-b,x-y] Any means in the same columns followed by the same letter are not significantly different (P<0.05) by Tukey's test.

[1-2] Any means in the same rows (2005, 2006) followed by the same number are not significantly different (P<0.05) by Tukey's test.

[c] Number of samples obtained from Southeast, Southwest regions are given in parenthesis, Data represents the mean ± standard deviation.



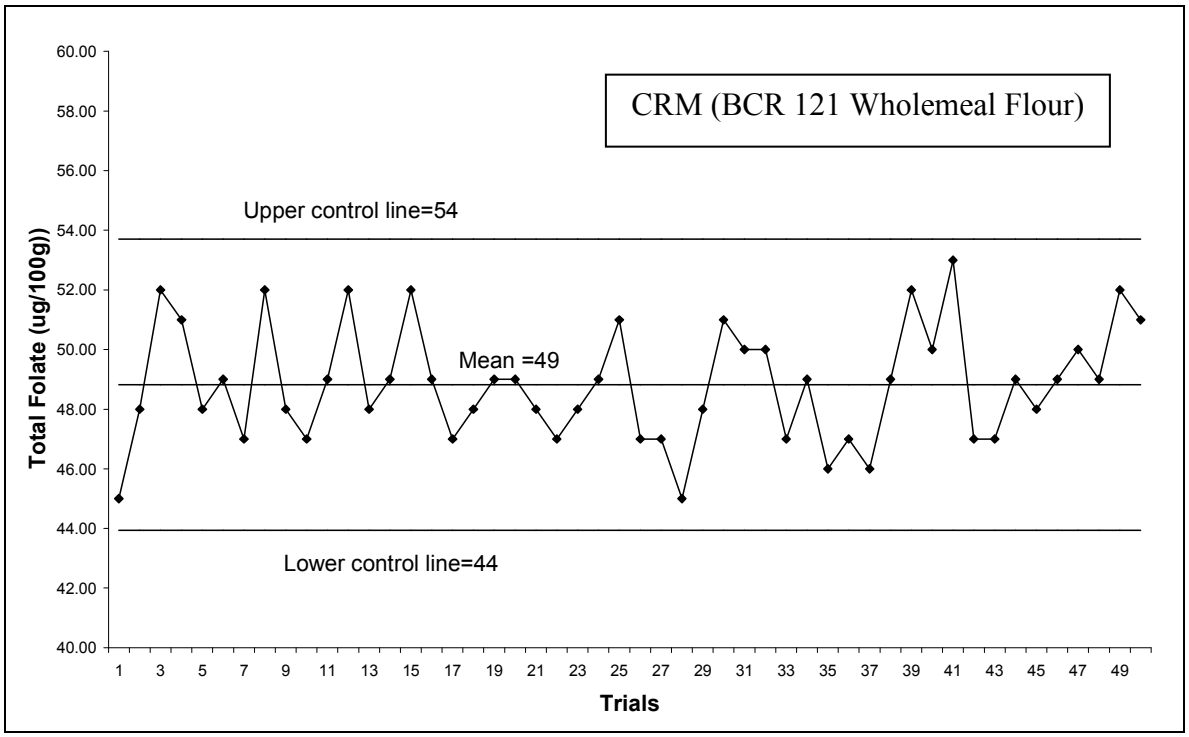


Figure 3.1 Quality control charts for total folate in enriched flour (a) and CRM (BCR 121 Wholemeal Flour) (b). Upper control line and lower control line within ± 10% of the mean. Chart repeats 50 runs.

CHAPTER 4

OPTIMIZATION OF TRIENZYME EXTRACTION FOR MICROBIOLOGICAL ASSAY OF FOLATE IN PEANUT BUTTER[1]

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[1]Kota L and Eitenmiller RR to be submitted to Journal of Food Science.

ABSTRACT

Response surface methodology (RSM) was used to optimize the trienzyme digestion for the extraction of total folate from peanut butter. Trienzyme extraction is a combined enzymatic digestion by Pronase^{R}, α-amylase and conjugase (γ-glutamyl hydrolase) to liberate the carbohydrate and protein –bound folates from food matrices for the assay of total folate. It is the extraction method used in AOAC Official Method 2004.05 for the assay of total folate in cereal grain products. The effects of various incubation times for Pronase^{R} (30 min to 1.5 h), α-amylase (30 min to 1.5 h), and conjugase (30 min to1.5h) were studied. Regression and ridge analysis were performed by the statistical analysis software. The predicted second-order polynomial model was adequate (R^2 = 0.97) with a small coefficient of variation (3.05). Both Pronase^{R} and conjugase had significant effects on the extraction. Ridge analysis gave an optimum trienzyme time: Pronase^{R}, 1h; α-amylase, 1.5 h; conjugase, 1h. The experimental value of peanut butter (SRM 2387) was close to the predicted value from the model, confirming the validity and adequacy of the model. The optimized trienzyme digestion time when applied to commercial peanut butter samples and Runner and Spanish gave comparable values to AOAC Method 2004.05.For Virginia peanuts, the optimized digestion time gave significantly higher folate than AOAC Official Method 2004.05.

Introduction

Folate measurement is routinely conducted by using trienzyme extraction followed by microbiological assay or chromatographically by high performance liquid chromatography (HPLC). Trienzyme extraction uses the combination of Pronase^{R}, α-amylase and conjugase (γ-glutamyl hydrolase) to liberate the folates from the cellular matrix and deconjugate the polyglutamate forms to monoglutamate and diglutamate forms. This method was originally introduced by DeSouza and Eitenmiller (1990) and Martin and others (1990). Trienzyme digestion with the microbiological assay was accepted as the official method of folate analysis for cereal grain products by the AOAC International (DeVries and others 2001,2005; AOAC International 2005) and was proved to be essential for the release of folate from the food matrices (Pffiefer and others 1997; Tamura and others 1997; Rader and others 1998). However, when Aiso and Tamura (1998) applied the trienzyme treatment to spinach, milk, bread, and beef, they concluded that the optimal combination of enzymes and reaction condition varied with the type of food matrix. This has been established in certain vegetables by modifying the order of enzyme addition, incubation time and pH of the buffer (Shreshtha and others 2000; Iwatani and others 2003; Pandarangi and LaBorde 2004). Two researchers reported that the dual enzyme treatment is sufficient to release the bound folate from the food matrices (Shreshta and others 2000; Pandarangi and LaBorde 2004). Shreshtha and others (2000) used α-amylase followed by conjugase, while Pandarangi and LaBorde (2004) used protease followed by conjugase. Single enzyme treatment (conjugase) also gave higher folate levels than trienzyme treatment for some leafy vegetables and breakfast cereals (Shreshtha and others 2000; Iwatani and others 2003).

Peanuts and peanut products are considered as a very good source of food folate (Rychlik and others 2007). However, definitive values for total folate are lacking in the literature. Yon and Hyun (2003) reported that most foods including peanuts gave higher measurable folate values by trienzyme treatment when compared to single conjugase treatment.

Response Surface Methodology(RSM), developed by Box and Wilson (1951), involves the use of statistical and mathematical techniques to evaluate the effects of numerous factors and their interactions on response variables. RSM reduces the experimental trials needed to evaluate multiple parameters and their interactions. It has been applied for optimizing the extracting conditions of vitamins (Lee and others 2000; Chen and Eitenmiller 2007). Recently, Chen and Eitenmiller (2007) applied this procedure to optimize trienzyme extraction of folate from vegetables. Accordingly, the optimal digestion time was 1.5h for Pronase^{R}, 1.5h for α-amylase and 3h for conjugase. In comparison to the standard procedures, the application of RSM has shortened the time of the assay, thereby, saving cost and labor for the whole experiment.

Thus, the objective of this study is to optimize the trienzyme digestion for folate extraction in peanut butter using RSM. Application to peanut butter should provide an optimized method applicable to peanuts and other peanut products.

Materials and Methods

Sample Collection

The peanut butter used in this experiment was Standard Reference Material SRM 2387

(Peanut Butter) purchased from National Institute of Standards and Technology, Gaithersburg, MD. The folate content of the SRM was not provided. A unit of SRM 2387 consisted of three jars of peanut butter containing 170 g each. Four brands of peanut butters were purchased from retail stores in Athens, Georgia. The Peanut Institute in cooperation with the American Peanut Shellers Association provided the Runner, Virginia and Spanish type raw peanuts.

Trienzyme Extraction and Microplate Assay

Total folate was measured after triezyme extraction using microbiological assay with *Lactobacillus casei ssp rhamnosus* (ATCC 7469). The trienzyme extraction followed the AOAC Official Method 2004.05(AOAC 2004 05). The microbiological assay was carried out using 96 well plate method (Tamura 1990; Chen and Eitenmiller 2007). Fat extraction of 1 g samples with 15 mL of hexane was done prior to trienzyme extraction. The detailed procedure is given in Chapter 3 under trienzyme extraction and microplate assay.

Standard Stock Solution

Twenty milligrams of the folic acid (US Pharmacopoeia) were added into a 200- mL conical flask containing 20 mL of (95%v/v) ethanol and 50 mL of deionized water. The initial pH was adjusted to 10.0 with 0.1N NaOH (to help dissolve the folic acid) and the final pH to 7.0 with 0.05N HCl. The final volume was made up to 100 mL with distilled water, the solution transferred to 10-mL Pyrex tubes and then stored at 4°C. A new standard should be prepared after 6 months.

Purity of the standard solution was determined by diluting the stock standard solution (0.2 mg/mL) with phosphate buffer (0.1 M, pH 7.0) to a final concentration of 0.01 mg/mL (1:20

dilution), measuring the absorbance of the diluted standard at 282 nm in a 1 cm quartz cell using phosphate buffer (0.1M,pH 7.0) as a blank. The purity was calculated using the following equation:

Purity of standard (%) = 100 (analyzed concentration C2 / known concentration C1)

Calculated concentration C2 = $(A_{std}-A_{blank})\cdot M/(\varepsilon\cdot b)$,

Where C1=0.01 mg/mL, C2=analyzed concentration of diluted stock standard (mg/mL),

A=absorbance, $\varepsilon=27.0*10^3\ M^{-1}cm^{-1}$, b=1cm, M=molar mass of folic acid (441.40).

The absorptivity (ε) was presented by Ball (1994).

Control

The control, including all the assay components other than the food sample was carried through the sample digestion procedure. The control was used to determine the contribution of the enzymes to the growth response of the *Lactobacillus casei ssp rhamnosus* (ATCC 7469). Although the enzyme blank values are non detectable at dilution levels of most food samples, an enzyme blank is used for each batch to show the folate in the enzymes is not a contributing factor.

Experimental Design

A fractional experimental design with 3 levels was used to observe the effect of 3 independent variables on one dependent variable. The Pronase^R digestion time (X1); α-amylase digestion time (X2); conjugase digestion time (X3) are the independent variables that have 3 coded levels (-1, 0 and 1). The dependent variable is the folate level of the SRM peanut butter. The three levels were coded based on data from the preliminary experiments with the predicted

optimized centre point. The experimental design of the 3 factors and 15 experimental points in terms of coded and uncoded levels are given in **Table 4.1** which gives total folate (μg/100g) derived from three RSM trials.

Data Analysis

The response surface regression (RSREG) of Statistical Analysis System (SAS 2002) was used to fit the experimental data to a second order polynomial to obtain the following coefficients of the equation(1):

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} + \beta_{ij} X_i X_j$$

where Y is the response for total folate (μg 100^{-1}g) ; X_i is the uncoded independent variable; β_0, β_i, β_{ii}, and β_{ij} are constant coefficients. Analysis of variance (ANOVA) and regression analysis were applied for model prediction. Estimated ridge maximum response was derived from RSREG SAS output of RIDGE MAX. Response surface and contour plots of responses were created using Sigma Plot software (version 9.0) by holding one variable constant in the estimated second-order polynomial equation.

The model verification was done by comparing the optimized time to the standard AOAC trienzyme method similar to the study by Chen and Eitenmiller (2007)**.** The folate contents of commercial peanut butter samples were assayed with the optimized extraction and compared to data determined by AOAC Official Method 2004.05. The folate valued of SRM 2387 obtained by the optimized extraction was compared to the predicted value.

Results and Discussion

Fitting the Models

Optimization of extraction parameters for the determination of folate in peanut butter was done using RSM. The experimental data for each set of variable combinations are given in **Table 4.1**. After the RSREG procedure, the data obtained from each variable set combinations were fitted to the second order polynomial equation (eq1), the regression coefficients of eq 1 are given in **Table 4.2**. The estimated values of the constant coefficients were used to predict the regression model for Y as:

$$Y = 38.62 + 32.87X_1 + 24.29X_2 + 37.91X_3 - 18.18X_1^2 - 21.70X_2^2 - 2.9X_3^2 + 26.00X_1X_2 + 10.63X_1X_3 - 37.29X_2X_3 \quad \text{(eq 2)}$$

In the above equation, Y is the response and X_1, X_2 and X_3 represent the digestion times for the enzymes Pronase^R, α-amylase and conjugase, respectively. The overall model (**Table 4.3**) when tested with ANOVA appeared adequate with an R^2 of 97%, a small coefficient of variation (3.05) and a very low probability value (<0.01). Thus, the model is highly significant and can represent the relationship between the independent variables and the dependent variable (folate response).

Folate measurement in foods is routinely conducted by using trienzyme extraction followed by microbiological assay or HPLC. As proven in the literature, trienzyme digestion is an essential step for obtaining the maximum folate values in foods. Hence, the effects of various incubation times for Pronase^R (30 min to1.5 h), α-amylase (30 min to 1.5 h) and conjugase (30 min to 1h) were studied. The overall results (**Table 4.4**) demonstrated that the Pronase^R ($p<0.05$) and conjugase ($p<0.01$) enzymes had statistical significance for the measured folate levels.

However, α-amylase did not show significance at $p<0.05$. As expected conjugase digestion was highly significant since it is necessary for deconjugation of the polyglutamates to monoglutamates.

The effects of linear, quadratic and interaction were tested for adequacy by Analysis of Variance (**Table 4.2**) for each of the independent variable. The ANOVA for linear and quadratic effects demonstrates that the model is highly significant, indicated by the Fisher's F-test with a low p value (<0.01). Thus, both the linear and quadratic effects are primary determining factors. Further, the constant coefficients of incubation time for enzymes Pronase^R (β_1), α-amylase (β_2) and conjugase (β_3) are positive, indicating the linear effects to increase the folate content (Y). However, the coefficient for α-amylase (β_2) did not show significance ($p<0.05$) indicating least importance of this variable. β_{11} β_{22} β_{33} indicate the quadratic effects, β_{12} β_{13} β_{23} show the interaction effects between the variables.

Analysis of Response Surfaces

Three dimensional response surface graphs were constructed to depict the relationship between the independent variables and the response. The response surface indicated that for conjugase and α-amylase digestions, folate levels increased at 1 h for conjugase but the α-amylase did not show a significant rise in the peak (**Figure 4.1**). This effect was observed for the α-amylase as previously shown from **Table 4.4.** For conjugase and Pronase^R treatments, the measurable folate levels increased with the incubation time and reached a maximum level in 1h and then declined (**Figure 4.2**). Similarly, (**Figure 4.3**), incubation for α-amylase increased slightly at linearly rate. However this effect was not very significant. The results indicated that the Pronase^R and conjugase were necessary to release the bound folate in peanut butter.

Optimization and Model Verification

The model was verified by the use of ridge analysis. The ridge analysis showed an optimum incubation time of 1 h for Pronase^{R}, 1 h for conjugase and 1.4 h for α-amylase. Even though α-amylase digestion did not produce a significant increase in the measurable folate, ridge analysis indicated that it is still an integral part of the extraction procedure. Contour graphs for α-amylase and conjugase (**Figure 4.1**) showed a center point at 1.2 hr and maximum folate level observed was 85 μg/100g. Similarly, the contour graph for conjugase and Pronase^{R} (**Figure 4.2**) had a center point at 1 h and the maximum folate measured was 90 μg/100g, which is close to the USDA nutrient database value of 75-90 μg/ 100g. This confirms the validity of the predicted model. However, α-amylase did not show a significant center point (**Figure 4.3**). The optimum response of the ridge analysis gave a highest value of 93 μg/100g for SRM 2387.

Model verification was done by applying the optimized digestion time to commercial peanut butter samples and three types of peanuts (Runner, Virginia and Spanish). In each case of peanut butters, the optimized trienzyme digestion gave comparable values to the AOAC Official Method 2004.05 (**Table 4.5**). The measurable folate by optimized digestion for four commercial peanut butter was 69μg/100 g, 75μg/100g, 74 μg/100g, 90μg/100 g, respectively. Similarly, the measurable folate for the above samples by AOAC Official Method 2004.05 was 72μg/100g, 79 μg/100g, 78 μg/100g and 85 μg/100g. In the case of Runner peanuts, the optimized trienzyme digestion (98 μg/100g) gave comparable values to the AOAC Official Method 2004.05 (95μg/100g). For Spanish type peanuts, although the optimized digestion gave numerically higher measurable folate as compared to AOAC Official Method 2004.05 the effect was not significant. For Virginia peanuts, the optimized digestion time gave significantly higher folate

than AOAC Official Method 2004.05. The optimized extraction time for the peanut butter matrix was simplified, thereby, allowing the trienzyme extraction with microbiological assay to be performed the same day. The study shows that folate can be extracted from peanut butter and peanuts, using considerably shorter digestion times than those used in the AOAC Method 2004.05, which is a model for the use with cereals and foods in general. This work, together with other studies or vitamin extractions method optimization by RSM (Lee and others 2000; Chen and Eitenmiller 2007) show that time and cost savings can be achieved through the use of extractions tailored to specific food matrices.

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Table 4.1 Coded incubation times for the 3 independent variables and the mean folate value from peanut butter.

	Coded Values for Independent variables			
Treatments	Pronase[R a]	α-Amylase[b]	Conjugase[c]	Total folate[d] μg/100g
1	1.5 (1)[1]	1.5 (1)	1 (0)	82.5 ± 3.2
2	.5 (-1)	1.5(1)	1 (0)	94.1 ±2.1
3	1.5 (1)	.5(-1)	1 (0)	85.8 ±1.5
4	.5 (-1)	.5 (-1)	1 (0)	62.0 ±2.2
5	1 (0)	1.5 (1)	1.5 (1)	85.8 ±3.1
6	1 (0)	1.5 (1)	.5 (-1)	82.1 ±3.0
7	1 (0)	.5 (-1)	1.5 (1)	73.6 ±2.9
8	1 (0)	.5 (-1)	.5 (-1)	80.5 ±2.9
9	1.5(1)	1 (0)	1.5 (1)	90.3 ±4.3
10	1.5(1)	1 (0)	.5 (-1)	68.5 ±3.6
11	.5 (-1)	1 (0)	1.5 (1)	70.2 ±3.1
12	.5 (-1)	1 (0)	.5 -(-1)	81.0 ±2.7
13	1 (0)	1 (0)	1(0)	89.5 ±2.0
14	1 (0)	1 (0)	1(0)	90.1±1.2
15	1 (0)	1 (0)	1(0)	89.4 ±1.4

[1] treatments were run in random order.
[a]Pronase [R]- used in concentration of 2mg/mL.
[b]α-amylase –used in concentration of 20mg/mL.
[c]conjugase –used in concentration of 5mg/mL.
[d] means from the three trials .

Table 4.2 Regression coefficients of the predicted quadratic polynomial model

Constant Coefficient[a]	Parameter Estimate	Standard Error	Computed t-value	Pr>F
β_0	38.62	10.85	3.56	0.01
β_1	32.87	15.1	2.17	0.081
β_2	24.29	19.4	1.25	0.265
β_3	37.91	14.1	2.67	0.044
β_{11}	-18.18	5.55	-3.28	0.022
β_{22}	-21.70	7.49	-2.90	0.033
β_{33}	-2.95	6.64	-0.44	0.675
β_{12}	26.00	6.41	4.05	0.009
β_{13}	10.63	4.75	2.24	0.075
β_{23}	-37.29	6.34	-5.87	0.002

[a]β_0 represents intercept, $\beta_1\beta_2\beta_3$ represent constant coefficients of Pronase^R, α-amylase and conjugase, respectively.

Table 4.3 Analysis of variance of second order response surface model

Source	DF	Sum of Squares
Model	9	1183.37***
Linear	3	335.75***
Quadratic	3	675.11***
Cross product	3	172.51**
Lack of fit	3	31.7*
Pure error	2	0.286
Total error	5	32.0465

R^2 (Coefficient of determination) =0.97 ; Coefficient of variation(CV)=3.05; DF=degree of freedom.*** significant at 1%; ** significant at 5%;* significant at 10% level.

Table 4.4 Analysis of variance of independent response

Independent variables	df	Sum of squares	Pr>F
Pronase[R]	**4**	234.35	0.0161[a]
α-Amylase	**4**	119.89	0.0607[b]
Conjugase	**4**	470.822	0.003[c]

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

Table 4.5 Comparison of total folate measured by the optimized extraction and AOAC Method 2004.05

	µg/100g	
Commercial Peanut butters(n=3)	Optimized Digestion	AOAC Method 2004.05
1	69 ± 2.4[a]	72 ± 1.3[a]
2	75 ± 0.5[a]	79 ±2.1 [a]
3	74 ± 0.7[a]	78 ± 0.5[a]
4	90 ± 1.1[a]	85 ± 1.9[a]
Peanuts(n=3)	Optimized Digestion	AOAC Method 2004.05
Runner	98±1.4 [a]	95 ±3.1 [a]
Virginia	82±2.6 [a]	71 ±1.6 [b]
Spanish	91±4.7 [a]	85 ±3.3 [a]

[a] numbers with the same letters are not significant at $p<0.05$.

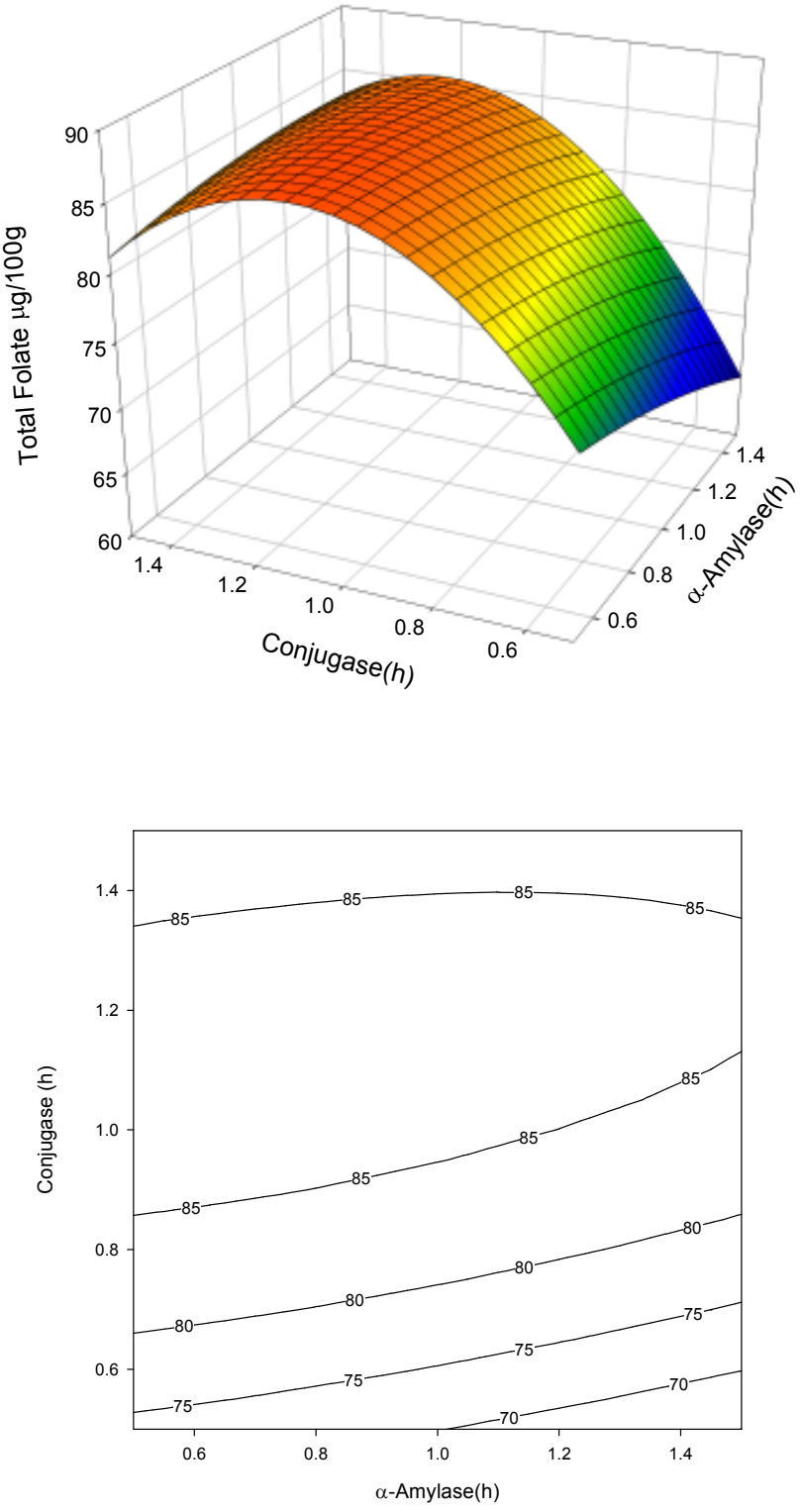

Figure 4.1 Response surface and contour plots for the effects of α-amylase and conjugase digestion times on total folate assay of peanut butter.

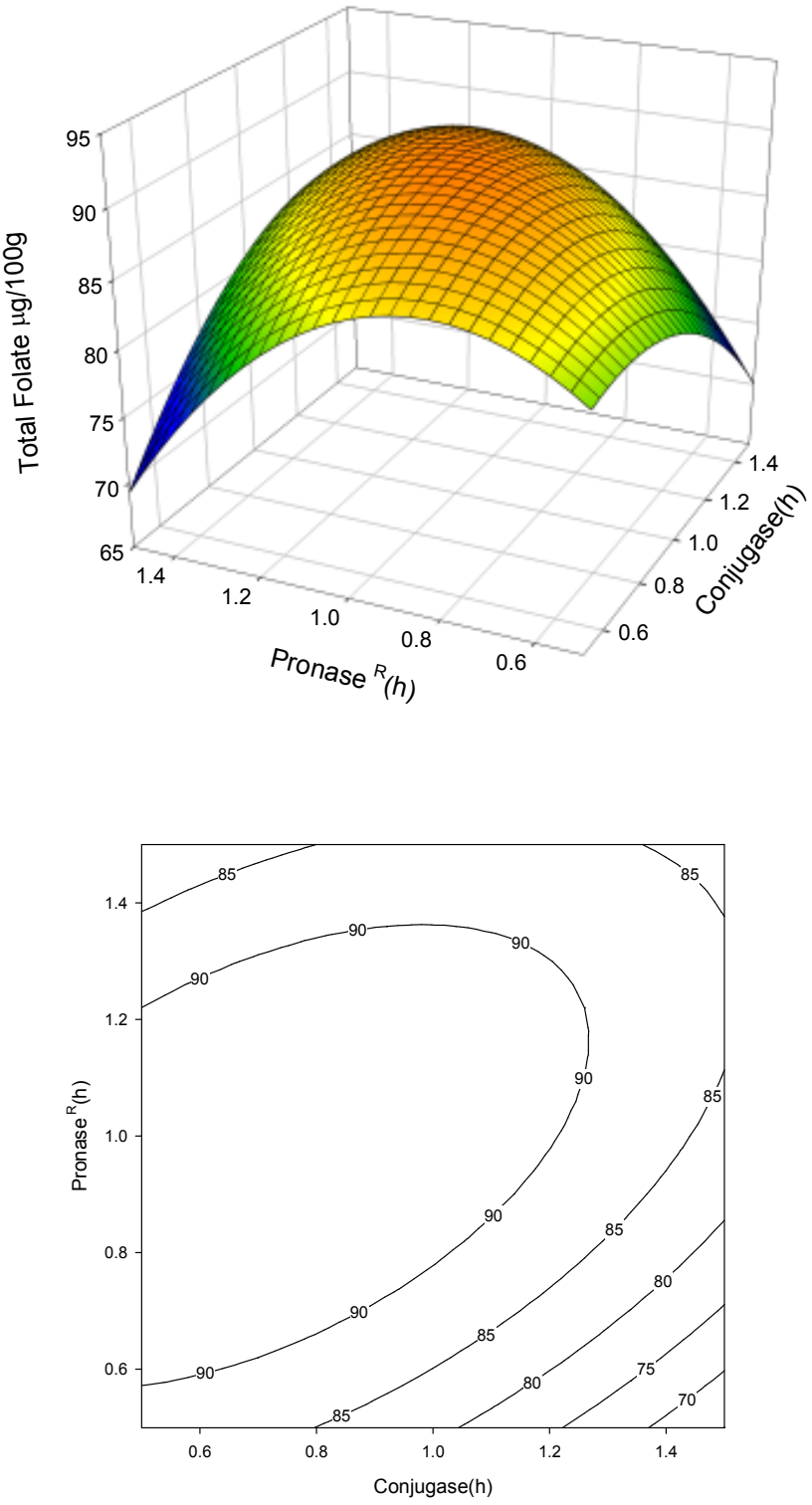

Figure 4.2 Response surface and contour plots for the effects of conjugase and Pronase^{R} digestion times on total folate assay of peanut butter.

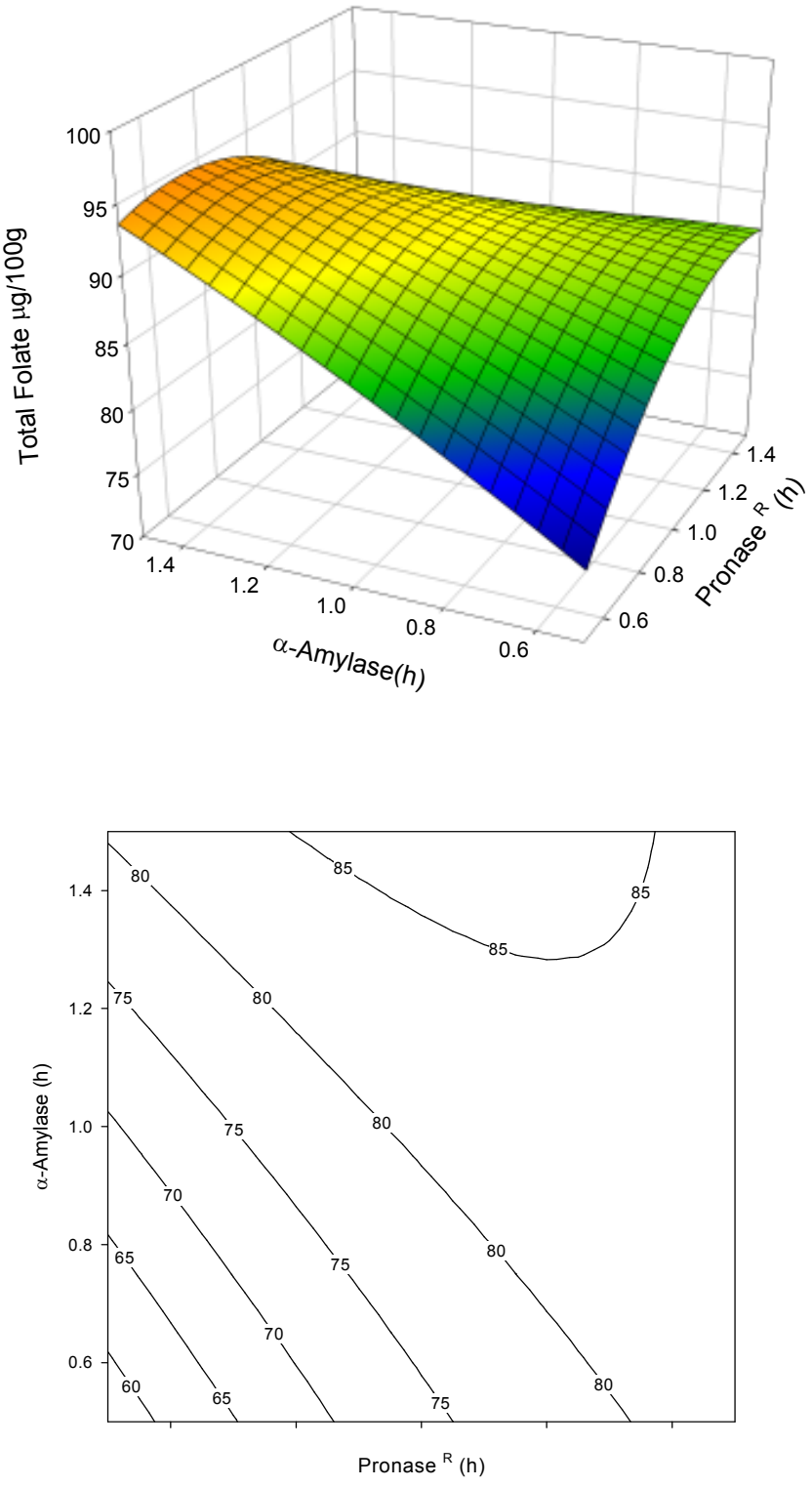

Figure 4.3. Response surface and contour plots for the effects of Pronase and α-amylase digestion times on total folate assay of peanut butter.

CHAPTER 5

TOTAL FOLATE IN PEANUT PRODUCTS, TREE NUTS AND RETENTION OF FOLATE DURING PEANUT BUTTER PROCESSING[1]

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[1]Kota L and Eitenmiller RR to be submitted to Journal of Food Science.

ABSTRACT

Retention of total folate during peanut butter processing was studied. The total folate was measured after trienzyme extraction using microbiological assay by *Lactobacillus casei spp. rhamnosus (*AOAC 2004.05*)*. Quality control studies showed that accuracy and precision of the analytical method was adequate. Mean folate levels of commercial products ranged from 66 µg/100g in dry roasted peanuts to 125 µg/100g in partially defatted peanut flour (28% fat). Comparison of folate level in peanuts with the levels in several tree nuts showed that only walnuts had similar high levels. Pecans and Brazil nuts showed comparatively lower folate levels than all the other nuts. Other tree nuts contained lower folate contents. Folate levels in raw peanuts and roasted and blanched used to manufacture peanut butter were statistically similar ($P>0.05$) to the folate levels in the finished product. Folate was stable to peanut butter processing. The folate retention based on five processing runs was 95%. The blanched and roasted peanuts had retention of 97.5% before milling.

Introduction

Peanuts are an excellent source of folate (Rychlik and others 2007). Peanut butter is by far the most important product made from peanuts in the United States. Per capita consumption of peanut butter remained stable during the 1990s (average of 2.9 pounds) and increased to 3.3 pounds in 2006 which is just under half of the total peanut consumption in the United States (USDA 2008). Much evidence exists that regular consumption of peanuts and tree nuts provides numerous health benefits. Clinical studies indicate positive effects on body weight and obesity, appetite suppression, glucose and insulin regulation, improved endothelial function and decreased risk to several diseases including coronary heart disease, cancer, and Alzheimer's disease. Many excellent reviews exist that cover the large body of literature on clinical and epidemiological studies showing the benefits of peanut and tree nut consumption (Hu and Stampfer1999; Hu and others 2001; Mukuddem-Petersen and others 2005; Griel and Kris-Etherton 2006; Segura and others 2006; Coates and Howe 2007; Smith and others 2008).

Interest in folate nutrition intensified when its role in prevention of neural tube defects during pregnancies (Cziezel and Dudas 1992) and its ability to lower plasma homocysteine levels (Wald and others 2001) were documented. An elevated homocysteine level is an independent risk factor for CVD. Reduction in CVD risk through increased consumption of peanuts and tree nuts is thought to be due to several factors including the high L- arginine and folate content (Brown and Hu 2001; Alper and Mattes 2003).

Although folate levels in peanuts and peanut products have been reported, literature values are highly variable ranging from 17 to 240 μg/100g (Rychlik and others 2007). Yon and Hyun (2003) analyzed folate in some commonly consumed Korean foods by trienzyme

extraction and microbiological assay. In peanuts, they reported a value of 136 μg/100g by trienzyme treatment and 103 μg/100g for single conjugase treatment. Recently, Rychlik and others (2007) analyzed the folate in various legumes by stable isotope dilution assays (SIDAs). Deconjugation of folate was carried out with combined treatment of rat plasma and chicken pancreas conjugase. These authors reported total folate levels of 94 μg/100g and 61 μg/100g for two samples of dry roasted peanuts.

Although a large amount of evidence exists on nut consumption and improved cardiovascular health and endothelial function, there is little information on the bioavailability of folate contained in nuts or on the effect of nut intake on serum folate (Segura and others 2006). Two short-term feeding studies assessed the effects of diets enriched with nuts on the lipid profile of hypercholesterolemic patients. They reported no change of plasma homocysteine concentrations (in patients with normal baseline homocysteine concentrations) with daily supplements of 73 g almonds or 40–65 g walnuts (Ros and others 2004). Pinto´ and others (2005) used mixed nuts as part of a folate rich diet in patients with mild hyper homocysteinemia. The results showed a modest homocysteine-lowering effect. Long-term intake of nuts as part of a healthy diet is likely to contribute to an adequate folate status (Segura and others 2006).

Folate stability is affected by food processing since it is sensitive to oxidation, light, temperature, and extremes of pH. Some of the factors affecting the folate stability are physical and chemical properties of the food matrices, oxygen availability, chemical environment, extent of heating and forms of folate in the food (Eitenmiller and others 2008). Effect of processing on folate stability was studied in various foods. Folate loss is generally related to heat processing conditions (Williams and others 1995; Wigertz and others 1997). Leaching losses occur during

boiling (Howkes and Villota 1989; Dang and others 2000). Substantial amounts of folates are normally retained during oven baking and microwaving. Stea and others (2006) studied the retention of folates in cooked, stored, and reheated peas, broccoli, and potatoes. In potatoes, sous-vide, boiling and oven baking gave retentions 103%, 78-58% and 63%, respectively. Similarly, green peas processed by boiling, micro waving, steaming and blanching retained 77%, 75%, 73% and 71%, respectively. Similar studies were also done in some legumes (Dang and others 2000; Hoppner and Lampi 1993). Folate in dried legumes showed considerable amount of loss when pretreated or presoaked before cooking (Hoppner and Lampi 1993). Each legume sample was subjected to quick soak (boiled for 2 min and held for 1 hr) and long soak (soaked for 16hr) before cooking. The mean retention for folate in legumes for 20 min cooking was 60% (without presoak), 18 % (quick soak) and 35% (long soak). When cooked for 90 min, the retention was 31% (quick soak) and 42% (long soak). Pressure cooking gave higher retention of folate than boiling in chick peas and field peas. Leaching losses was a major route of loss for folate during boiling in these legumes (Dang and others 2000).

Few studies have been published on the stability of vitamins in peanuts and the effects of processing. Chun and others (2003, 2005) studied stability of vitamin E in roasted peanuts. Vitamin E and the oxidative stability of raw and dry roasted peanuts were studied during storage at 21°C under air and vacuum. After 12 weeks, about 50% of α-tocopherol (α-T) was lost for roasted peanuts under vacuum and 90% under air. Raw peanuts retained more than 70% of each tocopherol after 38 weeks under air and vacuum (Chun and others 2005). Vitamin E retention of the total tocopherol during peanut butter manufacture was 95% (Chun and others 2003). However, effects of roasting and peanut butter processing on the stability of folate have not been reported. The objectives of this study were to determine total folate in commonly consumed

peanut products, to compare the folate levels in peanuts to commonly consumed tree nuts and to determine the effects of peanut butter manufacture on folate stability.

Material and Methods

Samples

Various of almonds (dry roasted)(n=3), cashews (oil roasted) (n=3), pine nuts(n=3), black walnuts(n=3), pecans(n=3),, brazil nuts(n=3), macadamia (dry roasted) (n=3), pistachios (dry roasted) (n=3), English walnuts(n=3), dry roasted peanuts(n=4), oil roasted peanuts(n=4), and peanut butters(n=15) , reduced fat peanut butters (n=6) were purchased from the local grocery stores in Athens, Georgia. The peanut flours, (n=12) were provided by Golden Peanut Co., Alpharetta, Georgia.

The raw peanuts used for the peanut butter manufacture included mixed cultivars but the major cultivar was Georgia Green. After harvesting, peanuts were stored in-shell in silos at an ambient temperature of 13 to 21º C until the end of the shelling season. The in-shell peanuts from silo storage were used throughout the year for shelling. After shelling, peanuts were stored at 1 to 5º C with a relative humidity of 55 to 70% (American Peanut Council 2008). Peanuts were tempered at 13º C for 48 to 72 h prior to shipping to avoid any condensation. These shelled peanuts were shipped in February 2008 in 900-kg containers to American Blanching Co. Inc., Fitzgerald, Georgia, U.S.A. No environmental controls were used during shipment. At American Blanching Co. Inc, peanuts were stored for less than 2 weeks at an ambient temperature and processed to peanut butter. Two pounds of raw and roasted and blanched

peanuts and 3 jars (530mL) of peanut butter from 5 processing runs were shipped to University of Georgia. Peanut and peanut butter samples were stored at –16 °C until assayed.

Peanut Butter Manufacture

At American Blanching Co. Inc., raw peanuts were passed over a scalping conveyor and a destoner to remove foreign materials. The raw peanuts were roasted in a Proctor-Schwartz continuous roaster at 300-310 °F in the 1st zone for 4.57 min, 355°F in the 2nd zone for 4.57 min, 365°F in zone 3 for 4.57 min and 340-355°F in zone 4 for 4.57 min. The total roasting time was approximately 23 min. Roasting conditions were adjusted to achieve Hunter color L value of 49 ± 1 (medium roasting). The peanuts were cooled by filtered ambient air in zone 5 and 6. After cooling to below 38 °C and blanching, the roasted peanuts were sorted by color to remove defective or unblanched peanuts. A two-stage milling process produced peanut butter. The first stage reduced the peanuts to a medium grind and the second stage produced a smooth, even texture. During milling, the temperature of the peanut butter can go as high as 82 °C. After milling and prior to cooling, the peanut butter was deaerated by passage over a thin-film cone deaerator (50 to 60 kPa). The deaerated peanut butter was packaged in commercial 530-mL screw top plastic jars and tempered for 48 hours at ambient temperature.

Method Performance Evaluation

Performance parameters including accuracy and precision of the microplate assay with trienzyme extraction were evaluated following the AOAC guidelines (AOAC International 1998; AOAC International, 2002a; AOAC International, 2002b).

1. Accuracy

Accuracy was determined by analysis of European Commission Certified Reference Material (BCR 121 wholemeal flour) purchased from Resource Technology Corporation, Laramie, WY and by determination of recovery. To determine the recovery, the reference peanut butter was spiked with 60µg of folic acid standard prior to extraction. This spiked level provided a total folate level approximately twice the expected folate amount in the original sample matrix (80-100 µg/100g). Recovery was calculated by the following equation (AOAC International, 2002a):

$R(\%) = [(C_s-C_p)/C_a]$ —100, where R (%) is the percent recovery of added standard; C_s is folate concentration in the spiked sample; C_p is folate concentration in the unspiked sample; and C_a is the folic acid standard added. All recovery values were determined by duplicate analyses.

2. Repeatability precision (%RSD_r)

Measurement of precision with simultaneous and consecutive replicates within a laboratory is termed as repeatability precision (%RSD_r) (Horwitz 2003). Repeatability precision (%RSD_r) was determined by assay of the Pillsbury all-purpose, bleached, enriched flour purchased at the local grocery and also by assay of BCR 121 throughout the study. Both the quality control flour and BCR 121 were stored in 4 oz Nalgene bottles at 4 °C. The relative standard deviation for repeatability (%RSD_r) was calculated as

% RSD_r= (SD * 100)/mean

Analysis of Total Folate

Total folate was assayed microbiologically with *Lactobacillus casei* ssp. *rhamnosus (ATCC 7469),* according to the procedures outlined by Tamura (1990) and Chen and Eitenmiller (2007). Detailed description of microplate assay is provided in Appendix. A

1. Preparation of Standards

Twenty milligrams of the folic acid (US Pharmacopoeia) were added into a 200- mL conical flask containing 20 mL of (95%v/v) ethanol and 50 mL of deionized water. The initial pH was adjusted to 10.0 with 0.1N NaOH (to help dissolve the folic acid) and the final pH to 7.0 with 0.05N HCl. The final volume was made up to 100 mL with distilled water, the solution transferred to 10-mL Pyrex tubes and then stored at 4 °C. A new standard should be prepared after 6 months.

Purity of the standard solution was determined by diluting the stock standard solution (0.2 mg/mL) with phosphate buffer (0.1 M, pH 7.0) to a final concentration of 0.01 mg/mL (1:20 dilution), measuring the absorbance of the diluted standard at 282 nm in a 1 cm quartz cell using phosphate buffer (0.1M,pH 7.0) as a blank. The purity was calculated using the following equation:

Purity of standard (%) = 100 (analyzed concentration C_2 / known concentration C_1)

Calculated concentration $C_2 = (A_{std}\text{-}A_{blank})\cdot M/(\varepsilon\cdot b)$,

Where C_1=0.01 mg/mL, C_2=analyzed concentration of diluted stock standard (mg/mL), A=absorbance, ε=27.0* 10^3 $M^{-1}cm^{-1,}$ b=1cm, M=molar mass of folic acid (441.40). The absorptivity (ε) was presented by Ball (1994).

2. *Preparation of Conjugase*

Fresh chicken pancreas provided by the Poultry Science Department, University of Georgia were cut into smaller pieces (1-2 cm) and was ground to a fine powder with dry ice using a mortar and pestle. The powdered chicken pancreas was transferred to a beaker with cold acetone (75mL). The chicken pancreas-acetone mixture was ground to a finer particle size by using a ultrasonic disintegrator (PRO 300A, Proscientific Inc. Oxford, CT). The acetone slurr was filtered through cheesecloth placed over a Buchner funnel containing filter paper (Fischer brand, Cat: 09-795D, 11.0cm) and residual acetone was allowed to evaporate from the solid residue. The dry powder was transferred into a one ounce, glass bottle, capped tightly, and stored at -20°C. The conjugase activity of the chicken pancreas preparartion was checked following the hydrolysis of pteroyltetra-γ-L-glutamic acid. The measured activity was 0.00003 μmol/min (0.03nmol/min) per twenty milligrams of chicken pancreas conjugase preparation. Details of the specific activity measurement are provided in Appendix B.

3. Trienzyme extraction for total folate

The extraction of folate by trienzyme digestion follows AOAC Official Method 2004.05 −Microbiological Assay with Trienzyme Procedure for Total Folates in Cereals and Cereal Foods (AOAC International 2005). The brief description of the procedure: one gram of sample (peanut butter or ground sample) weighed and fat extracted with 15 mL of hexane. Detailed steps involved in fat extraction are given below. 20 mL of pH 7.8 phosphate buffer is added and (0.1 M, containing 1% ascorbic acid) is preheated at 100°C for 15 minutes. The sample is then cooled to room temperature and 1 mL of Pronase^{R} (2 mg/mL, 107 Calbiochem, #53702, San Diego, CA) is added, followed by incubation at 37°C for 3 h. At the end of the Pronase^{R}

digestion, the sample is heated for 3 min at 100°C, cooled and digested with1 mL of α-amylase (20 mg/mL, Fluka, #10065, St Louis, MO) for 2 h at 37°C. Conjugase digestion is followed by adding 4 mL of chicken pancreas conjugase and incubating at 37°C for 16 h. At the end of the incubation, the digest is heated at 100°C for 3 min, cooled, adjusted to pH 4.5 with HCl, taken to volume of 100 mL with water and filtered through ashless filter paper (Whatman No. 2V, 12.5 cm). Although AOAC Official Method 2004.05 was collaborated for enriched cereals, the digestion was originally developed for general assay of total food folate and not limited to cereals (DeSouza and Eitenmiller 1990; Martin and others 1990).

4. Fat extraction

The fat extraction procedure includes the following steps:

1. Grind 10g of sample to a smooth powder in a coffee grinder with rapid pulses. Care must be taken not to paste the sample. If the sample pastes, it is difficult to prepare a homogenous ground sample. For peanut butters, the samples must be mixed for complete homogenization before weighing.
2. Weigh 1 g of the ground sample into a 150 mL (3.5cm O.D 15cm) glass tube.
3. Extract fat by adding 20 mL of hexane and vortexing for about 45 s. The mixture is allowed to stand for 10 min until there is phase separation.
4. Remove hexane layer with a Pasteur pipette.
5. Remove residual hexane under the stream of nitrogen.
6. To the defatted sample, add 20 mL of 0.1M phosphate buffer, pH 7.8 (anhydrous Na_2HPO_4, containing L-ascorbic acid -1%, w/v) and vortex.
7. Transfer sample to a 150 mL Erlenmeyer flask.

8. Wash the glass tube with two 15 mL aliquotes of deionized water to bring the total volume to 50 mL.
9. Cover the flasks with an aluminum foil and heat at 100^{o} C for 15 min in a water bath.
10. For samples with higher fat content (pine nuts, pecans, peanut butter) the hexane extraction is repeated 2-3 times. Further steps in the trienzyme procedure follows the AOAC method 2004 05.

5. Enzyme control

A control (enzyme blank) containing the 3 enzymes without the sample extract was included with each analytical set. The control was used to determine the contribution of the enzymes to the growth response of L*actobacillus casei ssp. rhamnosus* (ATCC 7469).

Statistical Analysis

Statistical analysis (two-way analysis of variance) was performed using the Statistical Analysis System (SAS version 9.1, Cary, NC, U.S.A.). Statistical significance between groups was determined by Tukey test as a mean separation at $\alpha = 0.05$.

Results and Discussion

Method Performance Evaluation

1. Accuracy

Accuracy is the closeness of the test result to the "true" or accepted value (AOAC

International 2002a; 2002b). Fifty replicate analysis of BCR 121, throughout the study gave a mean of 49 μg/100^{-}g ± 3.5. The analyzed value compares closely to the reported certified value of 50 μg/100g ± 7, indicating an acceptable accuracy.

The accuracy expressed as recovery was excellent. The percent mean recoveries ± SD obtained by spiking with known levels of standards were 99.8 ± 0.2, 99.32± 1.7, 98.87 ±0.87 for peanut butter, roasted peanuts and peanut flour, respectively. The percent mean recoveries for the tree nuts ranged from 99.5± 6.2 to 98.9± 4.4. Closeness of the assay values to the reference concentration value of BCR 121 and the recovery of the spiked folic acid from peanuts and peanut butter products indicate a high degree of accuracy.

2. Repeatability Precision (%RSD_r)

Replicate assays of an in-house quality control sample (enriched flour) and BCR 121 were used to determine repeatability precision (%RSD_r) for the assay of total folate throughout the study. The % RSD_r of microbiological assay with trienzyme extraction was 2.7 for enriched flour and 9.1 for BCR 121. The low % RSD_r over an extended period of time indicates excellent reproducibility of the assay. Figure 5.1 gives the quality control charts for the analysis of total folate in enriched flour and BCR 121. For each product, the data points stayed within the upper or the lower control lines set at ± 10 % of the mean. Mean values over the course of the study were 181± 4.8 μg /100g (n=50) and 49 ± 3.5 μg /100g (n=50) for enriched flour and BCR 121, respectively.

Folate Composition of Commercial Peanuts and Peanut Products

Total folate contents of commercial peanuts and peanut products are given **Table 5.1.** The total folate levels ranged from 66 μg/100 g in dry roasted peanuts to 125 μg/100 g in partially defatted peanut flour containing 12% fat. Partially defatted peanut flour containing 28% fat contained 114 μg/100 g. Total folate levels in peanut butter and reduced fat peanut butter were 70 and 68 μg/100 g, respectively.

The values reported here for commercial roasted peanuts (66 and 71 μg/100 g) and the roasted peanuts used for production of peanut butter (93 μg/100 g) are similar to values reported by Rychlik and others (2007). These authors reported total folate levels of 94 and 61 μg/100 g for two samples of dry roasted peanuts. The study by Rychlik and others (2007) used an optimized enzyme extraction and a stable isotope dilution assay (SIDA) with detection by liquid chromatography coupled to a mass detector (LC-MS/MS). The use of SIDA-LC-MS/MS has been developed for folate analysis in food and blood serum (Rychlik 2004; Rychlik and Mayr 2005; Rychlik and others 2007; Koehler and others 2007; Gutzeit and others 2008; Pfeiffer and others 2004; Fazili and others 2007; Fazili and others 2008). The method is considered superior to other approaches for folate analysis because the use of stable isotopically labeled analogues as the internal standards allows correction for losses of analytes during extraction and extract clean up. Further, selectivity and precision is improved compared to microbiological assay and LC methods using fluorescence or UV detection (Eitenmiller and others 2008).

The work by Rychlik and others (2007) showed that total folate values for most legumes were lower when measured by SIDA-LC-MS/MS analysis than literature values for similar legumes assayed by microbiological assay. Literature values for peanuts are highly variable

ranging from range of 17 to 240 μg/100 g (Rychlik and others 2007). For most of the legumes assayed by Rychlik and others (2007), SIDA-LC-MS/MS data was 2-3 times lower and in some cases many times lower than highest literature values obtained by microbiological assay. Rychlik and others (2007) concluded that the older literature overestimates folate levels in legumes and that intake data, likewise, from legumes might be overestimated.

Values obtained in this study for commercial peanut butters are similar to the USDA databank values of 92 μg/100 g for chunky style with and without salt, 74 μg/100 g for smooth style with and without salt, 60 μg/100 g smooth style, reduced fat peanut butter (USDA, 2008).

Total Folate Content of Peanuts Compared to Tree Nuts

Table 5.2 gives data comparing the total folate level in peanuts to the level in various tree nuts. Also, databank values from the USDA, German and British sources are presented for comparison. Analytical values obtained in the study and for raw peanuts assayed from 2005 and 2006 crop years (Kota and Eitenmiller 2008) show that peanuts and peanut products contain folate levels similar to that found in English walnuts(70 ± 2.4 μg/100g) and pistachios (60 ± 2.5 μg/100g). Levels of other tree nuts ranged from 9.5 ± 1.3 μg/100g in macademia nuts to 46 ± 3.6 μg/100g in pine nuts. Except for values given for dry roasted peanuts(145 μg/100g) and raw peanuts (240 μg/100g), analytical values for peanut butter and tree nuts compares closely to USDA databank values. Values of folate in pistachios and English walnuts reported in the German databank were comparable to amounts found in this study. The value for peanuts reported in the British databank (72 μg/100 g) closely compares to our analytical data.

Effect of Peanut Butter Manufacture on Folate

The folate contents measured by trienzyme extraction and microbiological assay of raw peanuts, roasted peanuts and peanut butter (n=5) from five processing runs are given in **Table 5.3**. Moisture levels of the raw peanuts (5.2%) roasted and blanched peanuts (1.17%) and peanut butter (0.99%) was used to convert the folate contents to dry weight basis.

Ingredient levels provided by The American Blanching Co, Inc. are included in **Table 5.3.** None of the ingredients other than peanuts provided folate to the formulation. Hence, the total folate in the finished peanut butter is derived from 88g of roasted and blanched peanuts per 100g product.

The retention of total folate in peanut butter is calculated based on the formula:

Retention % = (Total folate in peanut butter / Total folate in raw peanuts)*100.

The mean values for raw and roasted and blanched peanuts were 81± 1.7 µg/100 g and 79±2.2 µg/100g, respectively. These values fell in the range of those reported by Rychlik and others (2007). The total folate in the finished peanut butter was 77.2 ±3.3 µg/100 g. Although there was a slight numerical decrease in folate content from initial raw peanuts to the finished peanut butter, statistical significance did not exist($P>0.05$). The calculated folate retention for the complete process was 95%. The blanched and roasted peanuts had 97.5 % retention and a further 2.5% loss occurred during the final milling process. Thus, the study shows that losses of folates from the peanut butter manufacture are minimal.

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Table 5.1 Total folate of commercial peanut products (µg/100g)

Type of product	Total folate (µg/100g)
Dry roasted peanuts (n=4)	66 ± 5.7
Oil roasted peanuts (n=4)	71 ± 8.2
Peanut butter (n=15)	70 ± 6.4
Reduced fat peanut butter(n=6)	68 ± 3.4
Peanut flour-28%(n=6)[b]	114 ± 16.1
Peanut flour-12%(n=6)[c]	125± 11.3

[a] All samples were analyzed in duplicate

[b] Defatted peanut flour with 28% fat

[c] Defatted peanut flour with 12% fat

Table 5.2 Comparison of total folate content of peanuts to various tree nuts (µg/100g)

Nuts	µg/100g			
	Analytical values	Database		
		USDA [b]	German [c]	British[d]
Almonds(n=3) (dry roasted)	38±2.2 [a]	33	45	-
Cashews(n=3) (oil roasted)	33±1.5 [a]	25	-	-
Pine nuts(n=3)	46±3.6 [a]	35	-	-
Black walnuts(n=3)	42±3.9 [a]	31	-	-
Pecans(n=3)	27±1.1 [a]	21	-	-
Brazil nuts(n=3)	22±1.5 [a]	22	39	-
Macadamia(n=3) (dry roasted)	9.5±1.3 [a]	10	-	-
Pistachios(n=3) (dry roasted)	60±2.5 [a]	50	58	-
English walnuts(n=3)	70±2.4 [a]	98	77	-
Peanuts(Raw)(n=222)	91±17.1[a]	250	169	72
Peanut butter (n=15)	70 ± 6.4[a]	60-92	-	53
This study	77 ± 3.3 [a]		-	
Dry roasted peanuts(n=4)	66 ± 5.7 [a]	145	-	66
Oil roasted peanuts(n=4)	71 ± 8.2 [a]	120	-	52
Dry roasted peanuts(n=2)	61 ±8 [e]			
Dry roasted peanuts(n=2)	94 ±7[e] 169[f] 136[g] 17[h]			

[a] University of Georgia

[b]-United States Department of Agriculture, Agricultural Research Service, 2008, USDA Nutrient Database for Standard Reference, Release 20–1, http://www.nal.usda.gov/fnic/foodcomp USDA. 2008. [c]-Souci, SW, Fachmann, W & Kraut, H. 1994.5th Edition. Food composition and nutrition tables Stuttgart: Wissenschaftliche Verlagsgesellschaft mbH.

[d] Chan W, Brown J, Buss DH ,1994.The Composition of Foods .fourth supplement to fifth edition. The Royal Society of Chemistry and Ministry of Agriculture and Fisheries and Food.,London
[e] Rychlik and others(2007)
[f] Augustin and Klein (1989).
[g] Yon and Hyun (2003).
[h] Konings and others (2001).

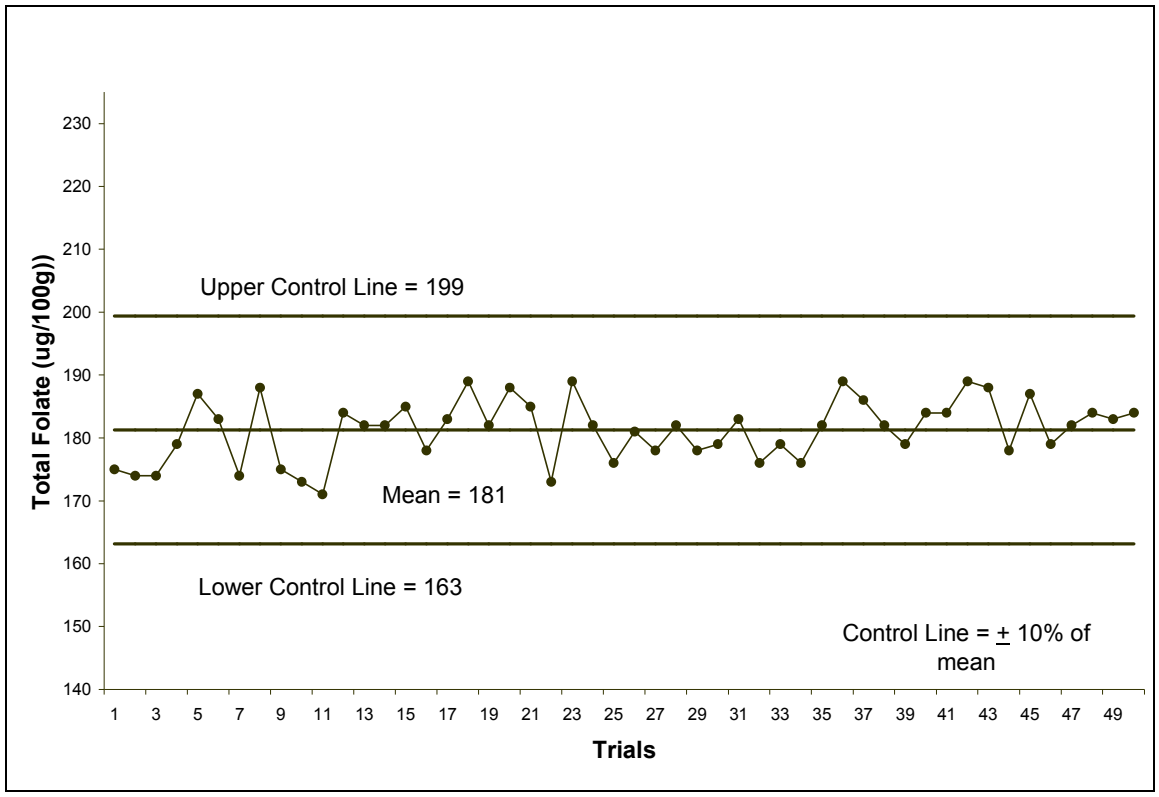
Table 5.3 Total folate and retention percentage during peanut butter manufacture (μg100g $^{-1}$)

	Grams	Total Folate[b] μg/unit (dry wt)
Ingredients in finished peanut butter(%)		
Raw Peanuts	88.0	81.0 ±1.7 [c]
Roasted and Blanched Peanuts	88.0	79.0 ± 2.2[c]
Peanut oil	3.0	-
Sugar	6.0	-
Salt	1.3	-
Stabilizer	1.7	-
Manufactured peanut butter	100	77.2± 3.3[c]
Retention through roasting and blanching		97.5
(%)		95.3
Final retention after milling(%)		

[a] Folate retention during peanut butter manufacture was based on the mean of 5 processing runs.

[b] Total folate in corresponding portion of the ingredients.

[c]Means followed by the same letter are not significantly different ($P<0.05$).



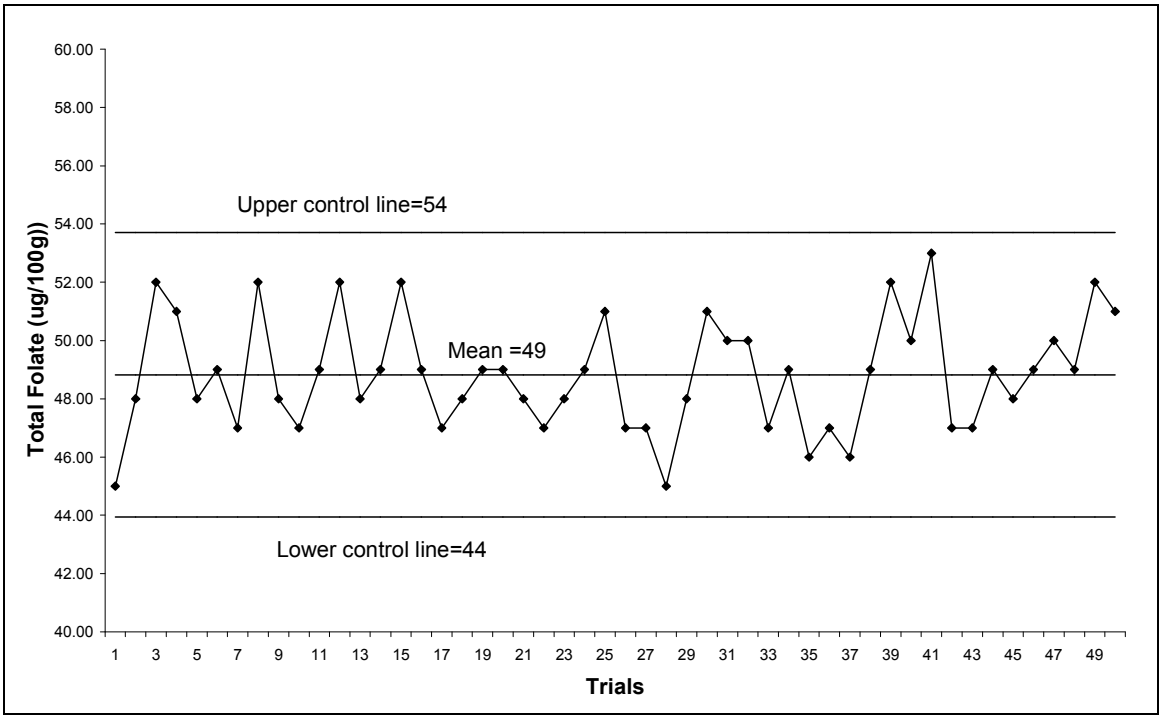


Figure 5.1 Quality control charts for total folate in enriched flour and BCR 121 (wholemeal Flour). Upper control line and lower control line are set at ± 10% of the mean.

CHAPTER 6

CONCLUSIONS

1. Statistical differences were found in the total folate content of peanuts among types, cultivars, production years and geographic region of production. Greatest variation in total folate levels were noted among cultivars within the same peanut type. Comparing the overall means by peanut type, Spanish peanuts contained lower folate levels than Runner or Virginia peanuts. Within type, high-oleic peanuts contained higher levels of total folate amounts than normal cultivars.
2. Optimal digestion times for the trienzyme extraction of total folate from peanut butter was 1h for Pronase ^{R}, 1.5 h for α- amylase and 1h for conjugase. Both Pronase ^{R} and conjugase enzymes had statistical significance for the measured folate levels ($P<0.05$). However, the α- amylase effect was not significant at $P>0.05$. Compared to AOAC Method 2004. 05, the optimized trienzyme digestion gave similar values.
3. Folate levels in commercial peanut products ranged from 66μg/100g in dry roasted peanuts to 125 μg/100g in partially defatted peanut flour (28% fat). Peanuts and peanut products contain higher folate levels than most tree nuts. English walnuts and pistachios contain folate levels similar to dry and oil roasted peanuts.
4. Peanut butter processing does not effect folate levels. A small numerical decrease in folate content occurred from the raw peanut to the roasted and blanched nut and to the finished, milled peanut butter. However, the effect was not significant ($P>0.05$).

APPENDICES

APPENDIX A

Extraction of Food by the Trienzyme Digestion

A. Extraction of Total Folate

The first day of assay

1. Turn on water bath to reach 100 ºC.
2. Weigh 1 g of the ground sample into a150 mL (3.5cm O.D 15cm) glass tube.
3. Extract fat by adding 20 mL of hexane and vortexing for about 45 s. The mixture is allowed to stand for 10 min until there is phase separation.
4. Remove hexane layer with a Pasteur pipette.
5. Remove residual hexane under the stream of nitrogen.
6. To the defatted sample, add 20 mL of 0.1M phosphate buffer, pH 7.8 (anhydrous Na_2HPO_4, containing L-ascorbic acid -1%, w/v) and vortex. Transfer sample to a 150 mL Erlenmeyer flask. Wash the glass tube with two 15 mL aliquotes of deionized water to bring the total volume to 50 mL.
7. Prepare working standard for recovery and add proper amount of standard to recovery sample
8. Add 20 mL of pH 7.8 phosphate buffer (See IV. D) and 30 mL of water to each sample.
9. Cover the flasks with aluminum foil.
10. Heat at 100ºC for 15 min.
11. Cool and add 10 mL of pH 7.8 buffer.
12. Add 1 mL Pronase^R solution (2 mg/mL in water) to each flask and mix.
13. Cover the flasks with aluminum foil and incubate at 37ºC for 3 h.

14. Heat 5 min at 100ºC and cool to room temperature (Turn on water bath before 45 min of heating).
15. Add 1 mL of α-amylase solution (20 mg/mL in water) and 0.5 mL of toluene, and mix.
16. Cover the flasks with aluminum foil and incubate at 37ºC for 2 h.
17. Prepare conjugase solution (5 mg/mL in assay buffer). Weigh conjugase, add pH 7.8 assay buffer, stir for 10 min, and filter through glass wool.
18. Add 4 mL conjugase solution and mix.
19. Cover the flasks with aluminum foil and incubate at 37ºC for 16 h.

The second day of assay

1. Heat samples at 100ºC for 5 min and cool.
2. Adjust samples to pH 4.5 with HCl, volume upto 100 mL with distilled water.
3. Filter each sample using a filter paper (Whatman, 185 mm, Cat No: 1001-185). Place a 150 mL beaker, set a funnel on the top of beaker, and place a folded filter paper on the funnel
4. Do microplate assay after any further dilutions, if necessary, using pH 6.8 phosphate buffer (See IV. E).
5. Keep original sample extracts in a –50 C freezer by transferring into disposable tubes and capping tightly until assay.

Caution 1: Enzyme preparations should be made right before each use of steps 12, 15, and 17. Do not allow enzyme solutions to stand more than 10 min. Before adding enzyme solution, temperature of extraction solution should be below 37ºC to maintain enzyme activity.

Caution 2: Prepare extra 1 mL more than the needed amount of Pronase^{R} and α-amylase solutions needed. Since glass wool holds some conjugase solution, prepare 4-5 mL more than the amount of conjugase solution needed for the assay.

Caution 3: After placing sample flasks in a boiling water bath, temperature goes down. Count heating time after temperature goes back to 100 ºC (Step 8, 12, and 18).

Caution 4: For samples high in folic acid or total folate (> 1000 μg/100 g), a high dilution factor (> 1:20) should be applied. A high dilution factor could increase standard deviation of data. → Decrease the sample weight to be assayed. Using a sample weight of 0.500 or 0.250 g is recommended for convenience in calculating data. → In case of changing the sample weight, make a note about the sample weight. Double-check that the dilution factor to be inserted to "Entering Dilutions of Unknown" is right when calculating data.

Note: Folates are light and oxygen sensitive. Use of yellow light and low actinic glassware is recommended. Samples and standard solutions should be prepared and stored under yellow light.

Purity of standard solution

1. A 0.1 M, pH 7.0 phosphate buffer was prepared by dissolving 13.61g potassium phosphate monobasic in water and diluting to 100 mL. The pH was adjusted to 7.0 with 4N potassium hydroxide (or sodium hydroxide). 4N potassium hydroxide was prepared by dissolving 22.4 g of potassium hydroxide in ~50 mL deionized water. It is cooled and diluted to 100 mL. The stock

solution is diluted with buffer by 1:20 ratio. The absorbance of diluted standard was measured at at 282 nm using 0.1M, pH 7 phosphate buffer as a blank.

2. Calculate the purity

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

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

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

(*Vitamin Analysis for the Health and Food Sciences*, R R Eitenmiller & WO Landen Jr., p 417)

- 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

  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 $E^{1\%}_{1cm}$ /table $E^{1\%}_{1cm}$) = 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

Control (Enzyme blank)

To determine contribution of enzymes to the growth response of *L. casei*, the control was assayed. A control (enzyme blank) without any food sample was carried through the sample digestion procedure as a total folate sample.

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 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.

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-(Na_2HPO_4-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 (prepared in the Laboratory, University of Georgia, Athens, Ga)

Pronase® – Calbiochem, Cat: 53702, EMD Biosciences, Inc., San Diego, CA 92121

α-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 (The Bio-Rad Benchmark Microplate Reader (Bio-Rad, Serial No. 11562, USA)

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), 0.22 □m pore size,

250 mL receiver, 12/case.

Pipette tips – CLP, 5648 Copley Drive, San Diego, CA 92111, Cat: 2032.YS or 2002 S, 200 □L,

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, 0.22□m pore size, 13 mm diameter, Sterile, PVDF (membrane), 100/case.

1-channel pipette – Brinkmann, 20 to 300 □L

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

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

A Detailed Description of the Microplate Assay

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 43 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.

Sample Dilutions

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. 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.

Autoclaving

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.

Microplating

	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	B-1	B-2	B-3	B-4	B-5	B-6	B-7	B-8	B-9	B-10	B-11	B-12	H
	1	2	3	4	5	6	7	8	9	10	11	12	

Figure A.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, the microplate was removed from the incubator and mixed three times from low concentration (row A) to the higher concentration(row G). The Bio-Rad Benchmark Microplate Reader (Bio-Rad, Serial No. 11562, USA) with Microplate Manager program (ver. 5.2) was used to read the microplates at the 595nm 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 forconcentration 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:

% Recovery = 100(folic acid in spiked recovery sample[a] – folic acid in unspiked sample [b]) / 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.

APPENDIX B

Measurement of Chicken Pancreas Conjugase Activity

The standard pteroyltetra-γ- glutamic acid (MW 828.7) was used to prepare the standard solution (0.001mg/100mL). One tenth milliliter of this standard solution (1μg/0.1mL) was added to the twelve flasks containing 30mL of deionized water and 30 mL of 0.1 M phosphate buffer (pH 7.8).The experimental conditions were similar to the folate analysis. Four milliliters of chicken pancreas acetone powder (conjugase) solution (5mg/mL of 0.1 M, pH 7.8 phosphate buffer) was added to each flask. During the first hour digestes were heated for 5 min at 100° C to inactivate the conjugase. During the second hour, digestes were removed at 15 min intervals and heated for 5 min at 100° C. The microplate assay was performed in a routine folate analysis protocol. The measurable folate values obtained are given in Table 7.1. The measurable folate values obtained are given in (Table 7.1). After the first hour, 1 μg of the measurable folates were recovered (Table 7.2). Measurable folates include pteroyl monoglutamic acid, pteroyl diglutamic acid, pteroyl polyglutamic acid with an average molecular weight 570.5 thus, 0.00003μmol/min (0.03 nmol/min) per 20 milligrams of chicken pancreas conjugase was calculated for the unit of chicken pancreas conjugase. The activity can also be measured in SI units of enzyme activity, nano katal(nkat). One katal is the amount of enzyme that converts 1 mole of substarte per second. 1 katal(kat)= 1 mol/s , 1 μmol/min=16.65nkat. The activity of twenty milligrams of chicken pancreas conjugae was 0.0005 n kat (0.5 picokatal)

Table B.1 Measurable folate released from Pteroyltetra-γ- glutamic acid (μg)

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

TableB.2 Calculation of chicken pancreas activity (μmol /min/20mg)

Time(min)	Total Folate (μg)	μmol /min
10	0.60	0.0001051710
20	0.71	0.0000622261
30	0.85	0.0000496640
40	0.97	0.0000425066
50	0.98	0.0000343558
60	1.01	0.0000295063
75	1.02	0.0000238387