WHITLEY, MADISON LISBETH. Environmental and Variety Effects on Niacin and Folate Contents in Raw and Roasted Peanuts. (Under the direction of Dr. Timothy H. Sanders and Dr. Lisa L. Dean.)

Peanuts (*Arachis hypogaea* L.) are known to be sources of several important B-vitamins, including niacin and folate. Recent research has shown that therapeutic doses of niacin are beneficial for vascular health; therefore, determination of the concentrations found in current varieties in production and potential breeding lines is needed. Folate is a term for a related group of compounds with vitamin activity important in DNA biosynthesis. Adequate levels of folate intake have been found to reduce the risk of a variety of syndromes, most notably fetal neural disorders. Current information about the concentrations and the specific types of folates present may position peanuts as an important source of this nutrient.

For the niacin assay, a series of raw and roasted samples from the 2007 and 2008 Uniform Peanut Performance Trials (UPPT) and the 2008 Core of the Core peanut germplasm collection were analyzed to compare levels of niacin across 10 growing locations. Thirty-nine total UPPT Florunner (medium grade size) and NC7 (extra large kernel {ELK} grade size) samples were analyzed using the AOAC non cereal foods method. Thirteen selected samples from the Core of the Core were also analyzed. Niacin concentration (mg/100g) of raw UPPT samples ranged from 8.20 to 25.8 with an average of 16.1 for the 2007 UPPT, 17.7 for the 2008 UPPT, and 16.8 for the Core of the Core samples. The average niacin concentration in raw Florunner and NC7 samples increased from 14.6 and 17.4 in 2007 to 16.4 and 18.9 in 2008, respectively. There was a significant difference between
niacin concentration and peanut variety, growing location and year of production. These differences are a result of slowed metabolic processes during dark respiration caused by cooler night temperatures. For the roasted UPPT samples, niacin concentration (mg/100g) ranged from 12.2 to 22.4. There was not a significant difference between raw and roasted samples, due to the stability of niacin to light and heat.

For the folate assay, a series of raw and roasted samples from the 2008 UPPT and the 2004 and 2008 Core of the Core were analyzed to compare total folate and individual folate vitamers across varying locations. Twenty-four Core of the Core and 12 UPPT samples were analyzed using a tri-enzyme method of extraction. Total folate content (µg/100g) for Core of the Core samples ranged from 207.7 to 378.3 in 2004 and 116.0 to 257.9 in 2008. The average total folate content (µg/100g) for raw Florunner and NC7 samples was 176.2 and 136.5, respectively, and for the roasted Florunner and NC7 samples was 78.9 and 72.0, respectively. Raw UPPT samples had significantly higher folate contents than roasted UPPT samples, regardless of variety. This is a result of the instability of folate at high temperatures. There was also a significant difference between Florunner and NC7 varieties, but no difference among growing locations. Of the individual folate vitamers, 5-methyl-tetrahydrofolate (THF) and 5-formyl-THF were in the highest amounts regardless of peanut collection, variety or condition. This is noteworthy since these vitamers are absorbed and used most readily by humans.

Environmental effects, including differences in light, temperature and moisture, and plant genetics play a role in the amount of vitamins produced and utilized. Environment, genetics, and the interaction between environment and genetics may be responsible for the
outcomes observed. These results suggest that germplasm/varietal differences may be sufficient for increases in niacin and folate levels through conventional breeding.
Environmental and Varietal Effects on Niacin and Folate Contents in Raw and Roasted Peanuts

by

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A thesis submitted to the Graduate Faculty of North Carolina State University in partial fulfillment of the requirements for the Degree of Master of Science in Food Science

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2010

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DEDICATION

I dedicate this work to my parents for their continued love and support and to my husband for his everlasting belief in me.
BIOGRAPHY

Madison Lisbeth (East) Whitley was born April 14, 1986 in Cedar Falls, Iowa. She is the daughter of Chip and Connie East and Robin and Stevan Jackson. She has one older sister, Katie Pirch, and two stepbrothers, Brian and Neal Cooper. Madison moved to Johnson City, Tennessee in 1987, where she went to elementary and middle school. She then moved to Radford, Virginia in 2000, where she spent the majority of her high school career as a member of the choir and as drum major of the marching band.

Madison graduated from Radford High School in May 2004 and continued her education at Virginia Polytechnic Institute and State University (Virginia Tech) in Blacksburg, Virginia. At Virginia Tech, Madison was a resident advisor and an undergraduate hall director in the Department of Residence Life. She was also involved in the nutrition and food science clubs. Madison graduated with a Bachelor of Science degree in Human Nutrition, Foods and Exercise with a concentration in Dietetics in May 2008.

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Most importantly, I owe everything to my husband, Michael. You are the reason I get up every morning. Thank you for holding my hand, coaching me towards the finish line and never giving up on me. Your smiling face reminds me how lucky I am. I love you.
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CHAPTER 1:

Literature Review
Introduction

In present-day society, most individuals tend to consume food on the basis of sensory characteristics, and not for nutritive value (Molloy 2002). Foods contain a variety of macro- and micro-nutrients that are advantageous for the human body. Two essential micronutrients are folate, the general term for a group of compounds sharing the same chemical structure, and niacin. Researchers have proven that folate, if consumed in appropriate quantities, may reduce the risk of cardiovascular disease (CVD), neural tube defects (NTDs), and cancer (Alper and Mattes 2003). It is used for one-carbon group transfers in plants and is required for several biosynthetic enzymes including *methionine synthase*, *thymidine synthase*, and *ketopantoate hydroxymethyltransferase* (Smith and others 2007). Niacin is known to be beneficial for vascular health (Mozafar 1993). It is helpful during two-electron oxidation-reduction reactions and can be used universally throughout the plant cell (Smith and others 2007). Ways to increase folate and niacin consumption and awareness of foods containing folate need to be found (Molloy 2002).

Many natural foods, including fruits, vegetables and legumes, contain folates which decrease disease risk by several mechanisms. Alper and Mattes (2003) stated that diets high in folate containing legumes, such as peanuts, may help reduce CVD and NTD risk. Although supplemental folic acid may provide adequate folate equivalents to the body, the metabolic effects may not be as beneficial as dietary folate (McCully 1998).

Research has confirmed that cooking processes deplete folate concentration in foods through oxidative and thermal losses. Peanuts, as well as other legumes and tree nuts, have significant folate contents. The specific folate compounds present in a food, the concentration
of each compound, and the overall folate content can be determined by analytical techniques. Microbiological assays were developed to determine food folate composition; however, high-performance liquid chromatography (HPLC) and mass spectrometry (MS) are methods currently used to identify and quantify folate compounds (Freisleben and others 2003). Using this methodology to verify which folate compounds and their respective quantities are present in a variety of food products, particularly various types of peanuts, will aid in determining whether or not those foods can be classified as a ‘good source of folate.’

**Peanut (Arachis hypogaea)**

Peanuts (*Arachis hypogaea*) are legumes native to South America (Hammons 1982). Early Spanish and Portuguese explorers found Indians cultivating peanuts throughout Mexico, Brazil, and Peru (Hammons 1982). From there, peanuts were dispersed throughout Europe, Africa and Asia, and were then brought to America as an inexpensive source of protein (Hammons 1982). Peanuts, belonging to the *Leguminosae* family, are considered legumes, which are comprised mainly of seeds or kernels (Hoffpauir 1953). The kernel, encased in a thin skin or testa, consists of two cotyledons and the germ (Hoffpauir 1953). The peanut kernel is acknowledged as a good source of B vitamins (Hoffpauir 1953).

There are four main market types of peanuts: runner, virginia, spanish, and valenica (Henning and others 1982). The four types of peanuts are traditionally grown in warm climates. In the United States, the major peanut-producing states are Virginia, North Carolina, South Carolina, Georgia, Alabama, Florida, Texas and Oklahoma (Henning and others 1982). These states produce 99% of the US peanut crop (Table 1) (American Peanut Council).
Table 1. U.S. peanut growing regions.

<table>
<thead>
<tr>
<th>State</th>
<th>Percent (%) Peanut Crop</th>
</tr>
</thead>
<tbody>
<tr>
<td>Georgia</td>
<td>41</td>
</tr>
<tr>
<td>Texas</td>
<td>24</td>
</tr>
<tr>
<td>Alabama</td>
<td>10</td>
</tr>
<tr>
<td>North Carolina</td>
<td>9</td>
</tr>
<tr>
<td>Florida</td>
<td>6</td>
</tr>
<tr>
<td>Virginia</td>
<td>5</td>
</tr>
<tr>
<td>Oklahoma</td>
<td>5</td>
</tr>
</tbody>
</table>

Peanuts contain a wide variety of nutrients. One ounce of peanuts provides 15% of the Reference Daily Intake (RDI) of protein, 2.4 grams (g) of dietary fiber, and nearly half of the 13 vitamins and one-third of the 20 minerals necessary for human growth and maintenance (USDA ARS 2009a). One ounce of roasted peanuts contains 29% of the RDI of Vitamin E and 10% of the RDI of folate (USDA ARS 2009a). Peanuts also contain resveratrol, which is a phytochemical associated with reducing cardiovascular disease risk (Kris-Etherton and others 2002). Despite the fact that peanuts contain high amounts of fat, they contain 81% unsaturated, or the beneficial fat and zero grams of trans fat (USDA ARS 2009a).

In plants, the biosynthetic enzymes that synthesize vitamins increase during seed germination (Smith and others 2007, Jabrin and others 2003). Mature seeds have a low rate of metabolic activity and low water content (Harvey-Gibson 1972). When harvested at an appropriate temperature with the correct amount of water and oxygen, the mature seed begins to germinate (Harvey-Gibson 1972). During seedling development, a rapid uptake of water occurs which swells the tissues, leading to increased metabolic activity and in turn, an increased rate of respiration (Harvey-Gibson 1972). This increased respiration activates
essential catalysts, called enzymes, which possess the ability to synthesize vitamins (Harvey-Gibson 1972, Jabrin and others 2003). Environmental conditions, including temperature and light, could potentially affect synthesis of these vitamins during seedling germination (Mozafar 1993). Germination and early stages of seedling growth are distinguished by major cellular events involving one-carbon metabolism, which require the presence of vitamins (Jabrin and others 2003, Mozafar 1993). Once the seedling has germinated, the root begins to penetrate the soil and a shoot extends into the surrounding environment. This allows the plant to rely on the external environment for needed nutrients (Harvey-Gibson 1972). The young plant is now dependent on the nutrients obtained through its leaves and stems. Molecules from the surrounding environment are synthesized by photosynthesis from carbon dioxide and water (Harvey-Gibson 1972, Jabrin and others 2003). The amount of vitamins synthesized must match the amount needed for one-carbon metabolism. This indicates that during phases of plant development where there are an excess of one-carbon metabolisms, high levels of vitamins, most importantly folate, must be generated (Scott and others 2000).

As previously mentioned, there are a wide variety of peanut types being cultivated around the world. One type of peanut can be grown in a specific environment and climate, while another requires the opposite environment for optimal growth. The United States maintains a germplasm collection of peanuts containing 7,432 accessions that represent the numerous types of peanuts and a range of growing conditions (Holbrook and others 1993). In order for researchers to accurately measure and assess various characteristics of the peanut plant without having to assay 7,432 accessions, a Core collection of peanuts was developed that reduced repetitiveness in the germplasm collection while maintaining a representative
sample based on growing conditions, country of origin and morphological characteristics (Holbrook and others 1993). It was established by dividing the germplasm collection into nine groups based on country of origin and other morphological characteristics and taking a random sample (Holbrook and others 1993). This Core collection represents the inherent diversity of the peanut plant (Holbrook and others 1993). About 10% of each group was selected, resulting in a Core collection of 831 accessions (Holbrook and others 1993). Since 831 accessions is still a large number for researchers to assay, the Core collection was further divided into clusters based on similar characteristics and country of origin. A random sample was selected from these clusters and a group of 112 accessions was collected, making up the Core of the Core collection. It is from this collection of peanuts that researchers are able to screen the entire germplasm collection without having to test all 7,432 samples (Holbrook and others 1993).

Uniform Peanut Performance Trials (UPPT) are a group of tests used to evaluate the potential of peanut breeding lines that have yet to be released for commercial consumption (USDA ARS 2009b). The UPPT was established in 1973 and continues to provide testing arrangements for peanut breeding programs. It provides information on the adaptability of potential new peanut cultivars over a range of diverse environmental conditions (USDA ARS 2009b). Each year, a set of advanced breeding lines is chosen, tested, and checked against the same set of cultivars from previous years. These new cultivars must meet quality and shelf-life parameters and possess chemical and sensory characteristics similar to the previous cultivars (USDA ARS 2009b). These tests provide information on shelling and post-harvest processing that is relevant to the peanut industry (USDA ARS 2009b).
Niacin Composition

Niacin, also known as nicotinic acid or vitamin B3, is a water-soluble vitamin (Figure 1) (Higdon 2010).

Figure 1. The chemical structure of niacin.

Nicotinamide, a derivative of niacin, is used to form the coenzymes nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP) in the body (Higdon 2010, Lahely and others 1999). Bioavailable niacin is comprised of free nicotinic acid and nicotinamide, NAD, and NADP (Ndaw and others 2002). Both NAD and NADP are involved in the oxidative release of energy from food (Higdon 2010).

Function of Niacin

Niacin is involved in oxidation-reduction, or redox, reactions along with other non-redox reactions. Oxidation-reduction reactions involve the transfer of electrons and as many as 200 enzymes use the niacin coenzymes, NAD and NADP, to accept and donate electrons for redox reactions (Higdon 2010). NAD is involved in energy-producing reactions including the degradation or catabolism of carbohydrates, proteins, fats, and alcohol (Higdon 2010). NADP is involved in biosynthetic or anabolic reactions including the synthesis of macromolecules, such as fatty acids and cholesterol (Higdon 2010). NAD is a reactant for two classes of enzymes that separate the niacin moiety from NAD and transfer ADP-ribose to
proteins. These enzymes play a role in cell signaling by affecting G-protein activity, which bind guanosine-5’-triphosphate (GTP) (Higdon 2010).

Along with operating in redox reactions, niacin also functions to reduce disease risk in humans. If niacin is not consumed in appropriate quantities, pellagra results (Ndaw and others 2002). Pellagra is a deficiency brought about by a lack of dietary tryptophan and niacin (Vidal-Valverde and Reche 1991). This results in symptoms such as dementia and nervous disorders. Niacin also aids in overall health maintenance, growth and development (Higdon 2010).

Sources of Niacin

Several sources of niacin include meats, fish, yeast, nuts, and legumes (Table 2) (Higdon 2010). One ounce of dry roasted, salted peanuts contains 3.8 mg of niacin, which is 20% of the Daily Values (DV). In standard nutritional labeling, foods that provide 5% or less of the DV of the particular nutrient are classified as low in that nutrient. Foods providing 10-19% of the DV are considered a good source of the nutrient, while foods providing 20% or more of the DV are considered to be high in the nutrient (Subar and others 2989). Peanuts are recognized as a good source of niacin (Higdon 2010).

Table 2. Concentration of niacin in common foods.

<table>
<thead>
<tr>
<th>Food</th>
<th>Serving Size</th>
<th>Niacin (mg)</th>
<th>% DV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salmon, cooked</td>
<td>3 ounces</td>
<td>8.5</td>
<td>57</td>
</tr>
<tr>
<td>Beef, cooked</td>
<td>3 ounces</td>
<td>3.1</td>
<td>22</td>
</tr>
<tr>
<td>Peanuts, dry roasted</td>
<td>1 ounce</td>
<td>3.8</td>
<td>20</td>
</tr>
<tr>
<td>Lentils, cooked</td>
<td>1 cup</td>
<td>2.1</td>
<td>10</td>
</tr>
<tr>
<td>Bread, whole wheat</td>
<td>1 slice</td>
<td>1.3</td>
<td>6</td>
</tr>
</tbody>
</table>
Daily Reference Intake

The Daily Reference Intake (DRI) for niacin is 16 mg niacin equivalent (NE)/day for men and 14 mg NE/day for women. One NE is equivalent to 60 mg of tryptophan (Higdon 2010). The average intake of niacin is about 30 mg NE/day for men and 20 mg NE/day for women (Higdon 2010). Despite the fact that niacin is not known to cause adverse effects in high quantities, an upper intake level (UL) has been set at 35 mg NE/day (Higdon 2010).

Niacin Biosynthesis in Plants

In plants, niacin is mainly concentrated in the aleurone layer of the kernel (Mozafar 1993). In addition, the endosperm contains material which possesses the capacity to act as a niacin precursor when translocated to the embryo (Nason 1950).

Niacin is synthesized in plants via the tryptophan degradation pathway, where tryptophan serves as a niacin precursor (Arditti and Tarr 1979). In the tryptophan degradation pathway, tryptophan is converted into kynurenine, which is then converted into 3-hydroxyanthranilic acid. 3-hydroxyanthranilic acid is a precursor for quinolinic acid, which is then decarboxylated to niacin (Figure 2) (Arditti 1967).
Despite the fact that there are several proposed pathways in which niacin is synthesized in plants, quinolinic acid has been found to be a key intermediate regardless of the pathway (Arditti and Tarr 1979). Due to the controversy over the appropriate pathway for niacin biosynthesis, additional research is needed to understand which pathway is most effective in plants (Arditti and Tarr 1979).

**Niacin Extraction and Quantification**

In order to appropriately analyze the niacin content in a food product, prior hydrolysis is necessary. Acid hydrolysis is used if biologically active niacin is being studied; however, alkaline hydrolysis is useful to release non-bioavailable niacin, providing total levels of niacin (Lahely and others 1999). Niacin can be analyzed in food products by HPLC methods, microbiological methods, and chemical assays (Van Niekerk and others 1984, Vidal-Valverde and Reche 1991). Chemical assays require the food product to react with cyanogen bromide in order to produce a pyridinium derivative (Vidal-Valverde and Reche 1991). The
chemical methods of analysis typically result in higher niacin concentrations than produced by HPLC or microbiological methods (Vidal-Valverde and Reche 1991). In the study by Lahely and others (1999), a total niacin concentration of 209 µg/g was measured in peanuts. No matter which method is used to analyze niacin concentration, niacin does not appear to be influenced by the type of extraction process utilized (Ndaw and others 2002).

**Folate Composition**

Folate, or pteroylglutamate, is a common term for a variety of compounds (Blakely and Benkovic 1984). First recognized by Lucy Wills in 1930, it is a “generic term for compounds that have vitamin activity similar to that of pteroylglutamic acid,” also referred to as folic acid (Davis 1986, Krishnaswamy and Nair 2001, Gregory 1997). Folate, water-soluble vitamin B9, exists in a variety of chemical forms, which can occur naturally in food as dietary folate or synthetically as folic acid used to fortify foods and supplements (Bailey 1998, Office of Dietary Supplements 2005, Ravanel and others 2001). It is comprised of a pterin core ring structure. This structure is conjugated to para-aminobenzoic acid (pABA) via a methylene bridge, which forms pteroic acid. The carboxyl group of pABA is bound by a peptide bond to alpha-amino groups of the glutamate molecule which connect to form folate (Figure 3) (Moat and others 2004, IUPAC-IUB 1986, Caudill 2004, Rebeille and others 2006, Basset and others 2005, Bailey 1998, Gregory 1997).
Figure 3. The chemical structure of folate.

Folate derivatives are characterized by the state of oxidation of the pteridine ring, the substituted one-carbon units at the N5 and/or N10 position, and the number of attached glutamate residues (Rebeille and others 2006, Folic Acid 2008, Seyoum and Selhub 1998). When the folate compounds are conjugated with one or more glutamate residues, they are referred to as pteroylglutamate, pteroyldiglutamate, etc (IUPAC-IUB 1986). Natural folates are polyglutamylated molecules having a short gamma-linked chain of glutamate residues attached to the first glutamate (Basset and others 2005). Folates are amphoteric and ionogenic molecules, meaning they possess the capacity to act as an acid or a base and to be ionized, respectively (Gregory 1989).

The nine most common forms, or compounds, of folate are: dihydrofolate (DHF), tetrahydrofolate (THF), folic acid (FA), 5-methyl-THF, 5-formyl-THF, 10-formyl-THF, 10-formyl-FA, 5,10-methenyl-THF, and 5,10-methylene-THF (Dang and others 2000, Molloy and Scott 2001, Basset and others 2005, De Brouwer and others 2007, Hawkes and Villota 1989). The prefixes mean that the substituent replaces one hydrogen atom in the above structure in the case of formyl- and methyl-THF, or two hydrogen atoms in the case of
methylene- and methenyl-THF (IUPAC-IUB 1986). The predominant dietary folates include DHF, THF, 5-methyl-THF, and 5-formyl-THF, with THF, 5-methyl-THF and 5-formyl-THF thought to be the most bioavailable (Phillips and others 2005). 5-methyl-THF provides the methyl group required to convert homocysteine into methionine, 5-formyl-THF is required for the biosynthesis of purines and pyrimidines in DNA and RNA, and 5,10-methylene-THF is necessary for the conversion of dUMP to dTMP and synthesis of pantothenic acid, or vitamin B5. 5,10-methenyl-THF is formed from either 5-formyl-THF or 10-formyl-THF and 5,10-methylene-THF is formed by the nonenzymatic reaction of THF with formaldehyde (Gregory 1989).

Dietary folates are naturally occurring and contain one to six additional glutamate molecules which are joined to the pteridine ring by peptide bonds (Bailey 1998). These molecules have a short gamma-linked chain of glutamyl residues that are attached to the first glutamate and are the preferred substrates for most folate-dependent enzymes (Basset and others 2005). Polyglutamate forms must be metabolized into monoglutamate form before absorption can occur in the body (Babu and Skiranria 1976). The absorption of the polyglutamate form of folate is usually 60-80% of the absorption of the monoglutamate form (Gregory 1989). Polyglutamyl folates are the main dietary folates occurring naturally in food and have a reduced pteridine ring (Gregory 1997). The compound 5-methyl-THF is the main form of folate found in the body (Verhaar and Rabelink 1999).

**Sources of Folates**

Despite the fact that folate intake in the United States is characteristically low, consuming foods that are high in folate is neither difficult nor necessarily expensive (Subar
and others 1989). Plant foods are the largest contributor of folate (Gambonnet and others 2001). Other food sources containing high levels of folate include green leafy vegetables, cooked dried beans, orange juice, milk, cabbage, white bread, fruit, cold cereal, eggs, tea, beer, corn, peas, organ meats and legumes (Table 3) (Subar and others 1989, Gregory 1997, Gregory and others 1984, Office of Dietary Supplements 2005). Legumes encompass a broad category, including beans, lentils, peas, and peanuts (Krishnaswamy and Nair 2001). Animal foods are notoriously poor sources of folate; however, trace amounts of folate are found in organ meats, such as liver (Ohr 2004).

Table 3. Concentration of folate in common foods.

<table>
<thead>
<tr>
<th>Food</th>
<th>Serving Size</th>
<th>Folate (µg)</th>
<th>% DV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beef liver, cooked</td>
<td>3 ounces</td>
<td>185</td>
<td>45</td>
</tr>
<tr>
<td>Lentils, cooked</td>
<td>½ cup</td>
<td>180</td>
<td>45</td>
</tr>
<tr>
<td>Spinach, cooked</td>
<td>½ cup</td>
<td>131</td>
<td>33</td>
</tr>
<tr>
<td>Broccoli, cooked</td>
<td>½ cup</td>
<td>50</td>
<td>15</td>
</tr>
<tr>
<td>Peanuts, dry roasted</td>
<td>1 ounce</td>
<td>40</td>
<td>10</td>
</tr>
</tbody>
</table>

Quantity of Folate in Foods

Bailey (1995) indicated that ½ cup of raw, dried peanuts contains approximately 92 micrograms (µg) of folate. Griel and others (2004) determined that 1 ounce of dry roasted, salted peanuts provides 41.1 µg of folate offering 10% of the DV. This claim was validated by the Office of Dietary Supplements (2005) which indicates that 1 ounce of dry roasted peanuts contain 40 µg and 10% of the DV of folate. As previously defined, this would be a good source of folate.

It is evident that there are a large number of dietary sources of folate. In particular, the folate concentration provided by consuming 250 kcal of peanuts is about 100 µg, which
is a quarter of the amount of folate required per day (Alper and Mattes 2003). Despite the fact that peanuts are a source of folate, Americans typically consume less than one-half ounce of peanuts per day (Dreher and Maher 1996). As a result of this low intake, the major foods sources providing the highest levels of folate in the United States are vegetables, such as spinach, green peas, and cooked beans (Subar and others 1989).

**Folate Production in Plants**

Folate synthesis in plants is closely controlled and modified in relation to the metabolic requirements which differ from one tissue to another and fluctuate during plant development (Rebeille and others 2006). Folate synthesis enzymes are increased in germinating seeds due to the continuation of active cell metabolism (Smith and others 2007). In addition, enzymes utilized during folate synthesis are produced and accumulated in proliferating tissues (Rebeille and others 2006). Highest folate contents are found in the plant cell cytosol, where various methylation reactions take place during plant photosynthesis (Periago and others 2008). Even though the cell cytosol contains the highest concentration of folates, these compounds are found in most, if not all, of the cell compartments in plants (Basset and others 2005, Rychlik and others 2007).

Folates are active in the production and maintenance of new cells and for cell division (Office of Dietary Supplements 2005, Scott and others 2000). The physiological processes taking place within the plant dictate folate requirements (Rebeille and others 2006, Rychlik and others 2007). In order to produce and maintain new plant cells, folate has the capacity to transfer single-carbon units so that one-carbon metabolism can take place (Rebeille and others 2006). The three main oxidation states in which folate can donate single carbons
include 5-methyl-THF, 5-formyl-THF, and 5,10-methylene-THF (Jabrin and others 2003). Due to the different degrees of glutamyl conjugation, plant folates function in various pathways of one-carbon metabolism (Cossins and Chen 1997). There are three main cycles that are dependent on folate and one-carbon metabolism. Those cycles include the photorespiratory cycle, the DNA biosynthesis cycle, and the methylation cycle.

The photorespiratory cycle involves the nine main folate derivates and one-carbon transfer reactions (Gambonnet and others 2001). This cycle involves the recycling of glycolate into 3-phosphoglycerate, which is required for the continuous operation of photosynthesis in plants (Gambonnet and others 2001). Photorespiration is a series of light-independent reactions which lowers the efficiency of photosynthesis by removing carbon molecules (Rebeille and others 2006). Within this process, folate is required for the interconversion of glycine to serine which takes place in the mitochondria (Basset and others 2004a, Sahr and others 2006, Rebeille and others 2006, Scott and others 2000). The folate-dependent enzymes involved in photorespiration include glycine decarboxylase and serine hydroxymethyltransferase. These enzymes tend to accumulate during greening of plants (Gambonnet and others 2001). During photorespiration, when light is present, ATP and NADPH in the leaves are exposed to oxidative cleavage. This causes reactive oxygen species to be produced by the mitochondria and plastids, which then promotes chemical breakdown and damage to the metabolic function of the cell (Basset and others 2005). Plant folates are slightly unstable and break down into pteridine and pABA-glutamyl fragments when oxidative cleavage occurs (Hanson and Gregory 2002).
Folate involvement in one-carbon metabolism is also important in two other cycles: the DNA biosynthesis cycle and the methylation cycle. These one-carbon transfers include the \textit{de novo} synthesis of certain amino acids, such as methionine, DNA, and RNA (Caudill 2004). In addition to pyrimidine and purine synthesis, amino acid interconversions contribute to cell division and proliferation (Subar and others 1989, Bailey 1998, Molloy and Scott 2001, Hoffbrand and others 1975).

Along with the one-carbon transfers required for DNA synthesis, folate is also required for the methylation cycle. These methylation reactions are necessary for a variety of biological reactions and metabolism regulation in plants and humans (Subar and others 1989, Moat and others 2004). This cycle directly involves the interconversion of homocysteine to methionine (Voutilainen and others 2001, Rebeille and others 2006, Basset and others 2004a, Krishnaswamy and Nair 2001, Folic Acid 2008, Wagner 2006, Sahr and others 2006, Scott and others 2000). Several studies have concluded that folate status is the most important determinant of homocysteine concentration since homocysteine, an amino acid, is extremely sensitive to changes in folate concentration (Office of Dietary Supplements 2005, Moat and others 2004, Molloy 2002). When there are high levels of homocysteine in the body, homocysteine is remethylated to methionine through two pathways. One of those pathways requires folate to donate a methyl group to methionine for homocysteine breakdown (Griel and others 2004, Krishnaswamy and Nair 2001, Caudill 2004, Loria and others 2000).

Folate levels vary in portions of plants. For most plants, there is a range of approximately 2 to 5 nanomoles (nmol)/g fresh weight of folate in the leaves, 0.2 to 2 nmol/g in the roots, and 0.05 to 0.5 nmol/g in fruits (Basset and others 2005). Folate biosynthesis in
plants takes place in three subcellular compartments and occurs in a multi-step process (Rebeille and others 2006, Bedhomme and others 2005). Folate synthesis and folate-mediated reactions are highly compartmentalized in plant cells between the plastids, the cytosol and the mitochondria (Diaz de la Garza and others 2007). The enzymes involved in the biogenesis of folate and pABA synthesis are found in the plastids, pterin synthesis occurs in the cytosol, and glutamate synthesis occurs in the mitochondria, the main site for folate biosynthesis (Rebeille and others 2006, Neuburger and others 1996). Folate synthesis requires several key enzymes and adequate supplies of riboflavin, niacin, pyridoxine, zinc, vitamin C, and serine (Alternative Medicine Review 2006). Folate biosynthesis is catalyzed by monomeric, monofunctional proteins (Cossins and Chen 1997).

In the first step of folate synthesis, the pterin branch of the folate molecule is catalyzed by the enzyme $GTP$ cyclohydrolase I ($GTPCHI$) to form dihydroneopterin (DHN) triphosphate (Figure 4). $GTPCHI$ is cytosolic, is a dimer with each monomer containing two $GTPCHI$ domains in tandem, and lacks targeting peptides. This step occurs outside of the cell mitochondria (Basset and others 2005, Rebeille and others 2006, Basset and others 2004b, Bedhomme and others 2005, Sahr and others 2006). In step two, dephosphorylation of DHN triphosphate occurs in two stages to produce dihydroneopterin. These two stages include the loss of pyrophosphate by a $nudix$ hydrolase and cleavage of the remaining phosphate by a nonspecific phosphatase enzyme. The lateral side chain of DHN is cleaved by dihydroneopterin aldolase ($DHNA$) to release hydroxymethyldihydropterin (Basset and others 2005, Rebeille and others 2006). In step three, chorismate is converted to pABA, which is mediated by the enzyme aminodeoxychorismate ($ADC$) synthase (Basset and others
2005, Rebeille and others 2006, Basset and others 2004b, Bedhomme and others 2005, Sahr and others 2006). pABA is then irreversibly converted to a glucose ester in step four by a cytosolic glucosyltransferase enzyme. This enzyme is esterified in the plasma (Basset and others 2005). The final three steps, steps five through seven, are localized in the mitochondria of the plant cell (Basset and others 2005). In step five, hydroxymethyldihydropterin and pABA are coupled by an ATP-dependent reaction with the enzymes dihydropteroate synthase (DHPS) and hydroxymethyldihydropterin pyrophosphokinase (HPPK) to form dihydropteroate (Basset and others 2005, Rebeille and others 2006, Basset and others 2004b, Bedhomme and others 2005, Sahr and others 2006, Scott and others 2000). Step six is a glutamylation step which is catalyzed by a dihydrogolate synthase (DHFS) enzyme and subsequent glutamations by the enzyme folylpolyglutamate synthase (FPGS) (Basset and others 2005, Rebeille and others 2006, Basset and others 2004b, Bedhomme and others 2005, Scott and others 2000). In the seventh and final step, dihydrofolate is reduced to THF. This step is mediated by a bifunctional dihydrofolate reductase (DHFR) enzyme, using NADPH as an electron donor (Figure 4) (Basset and others 2005, Rebeille and others 2006, Basset and others 2004b, Bedhomme and others 2005, Scott and others 2000).
Figure 4. Folate biosynthesis in plants.

This diagram indicates that about 50% of the folate compounds present are associated with the mitochondria, whereas only 6-7% are associated with chloroplasts (Neuburger and others 1996). THF is the dominating compound in the mitochondria (Rebeille and others 2006). Glycine and formate are potential one-carbon donors in plants which produce 5,10-methylene-THF and 10-formyl-THF (Rebeille and others 2006). Methylene-, methenyl-, and 10-formyl-THF are interconvertible and catalyzed by *methylene-THF dehydrogenase*, an NADP-dependent enzyme, and *methenyl-THF cyclohydrolase* (Rebeille and others 2006). The ability to convert 5,10-methylene-THF to 10-formyl-THF and THF shows how folate
compounds are recycled and explains the composition of the mitochondrial folate pool (Wagner 1996).

Importance of Individual Folate Compounds

Despite the fact that the term folate tends to be generalized as a combination of the various folate compounds, the individual folate compounds contribute differently to the folate pool. Separation of the folate compounds is dependent on the substitutions on the pteridine ring, yet independent of the number of glutamate residues (Clifford and Clifford 1977, Hannon-Fletcher and others 2004). Because certain folate compounds may be found more readily in plants or used more often for one-carbon metabolism in humans, it is important to distinguish between the folate compounds.

Compounds Found in Plants

The primary folate compounds found in plants are 5-methyl-THF and 5-formyl-THF, representing approximately 90% of the total folate pool (Davis 1986, Chan and Cossins 2003, Folic Acid 2008). 5-methyl-THF is typically the most bioavailable folate compound found in plants (Basset and others 2005, Hanson and Gregory 2002, Phillips and others 2005, Gambonnet and others 2001). It is produced by the enzyme \textit{5,10-methylenetetrahydrofolate reductase} (MTHFR), which is an essential enzyme in folate metabolism found in the cytosol of plant cells (De Bree and others 2002, Alternative Medicine Review 2006, Hanson and Gregory 2002, Periago and others 2008). 5-methyl-THF comprises the majority of the folate content in plant vacuoles, suggesting that 5-methyl-THF has a possible storage role in plants (Orsomando and others 2005). It may be the most appropriate folate compound used for storage since it is relatively stable at different pH levels and is easily converted to other folate
compounds through the MTHFR reactions (Orsomando and others 2005, De Brouwer and others 2007, Zhang and others 2005).

In conjunction with 5-methyl-THF, 5-formyl-THF is another stable, natural form of folate (Rebeille and others 2006, Basset and others 2005, Seyoum and Selhub 1998). It is formed when 5,10-methenyl-THF is irreversibly hydrolyzed by the enzyme serine hydroxymethyltransferase (SHMT) with glycine (Rebeille and others 2006, Basset and others 2005, Cossins and Chen 1997). 5-formyl-THF is a mitochondrial folate found predominantly in the seeds of plants (Hanson and Gregory 2002, Basset and others 2005, Orsomando and others 2005). It is relatively stable to oxidation at neural and alkaline pH levels, but less stable in acidic conditions (Hawkes and Villota 1989, Zhang and others 2005).

THF is also an important compound found in plants, but not in the same quantity as 5-methyl-THF and 5-formyl-THF. It is found in the mitochondria of plant cells and is unstable at low pH (Hanson and Gregory 2002, De Brouwer and others 2007, Zhang and others 2005). THF and 5-methyl-THF are the two major folate compounds found in the leaves of plants, with 5-formyl-THF found in smaller quantities (Chan and Cossins 2003).

In addition to these compounds, 10-formyl-THF, 5,10-methylene-THF and 5,10-methenyl-THF also contribute slightly to the folate pool found in plants (Hanson and Gregory 2002). As previously mentioned, 10-formyl-THF, 5,10-methenyl-THF and 5,10-methylene-THF are interconverted through the enzyme 5,10-methenyl-THF cyclohydrolase and the NADP-dependent enzyme 5,10-methylene-THF dehydrogenase. 10-formyl-THF is the most oxidized folate compound and is used to synthesize purines and RNA (Hanson and Roje 2001, Hawkes and Villota 1989). It is naturally occurring and easily converts to 5-
formyl-THF (Gregory 1989). 5,10-methylene-THF and 5,10-methenyl-THF are the most reduced forms of folate (Hanson and Roje 2001). 5,10-methylene-THF produces thymidylate and pantothenate, is stable to atmospheric oxygen, and is found in high quantities in the chloroplasts (Hanson and Roje 2001, Hawkes and Villota 1989, Orsomando and others 2005). 5-formyl-THF can be converted to 5,10-methenyl-THF by changing the pH level, while THF can be converted to 5,10-methylene-THF with formaldehyde or by changing the pH level (De Brouwer and others 2007).

In a study by Rychlik and others (2007), 5-methyl-THF was declared the most important folate vitamer; however, 5-formyl-THF was found in the highest quantities followed by 5-methyl-THF, 10-formyl-THF, and THF. Gregory and others (1984) found that cabbage, milk and orange juice contained high concentrations of 5-methyl-THF, reaffirming that plant foods contain high levels of 5-methyl-THF.

**Human Folate Nutrature**

Even though certain folate compounds may be more prominent in plants, the bioavailability of dietary folate depends on the quantity of folate reaching the intestine which is then absorbed by the body (Seyoum and Selhub 1998). The various folate compounds are thought to be interchangeable in human metabolism (Gregory 1997). Under normal conditions, there is little nutritional difference in the absorption and utilization of the various folate compounds (Gregory 1995). Despite this, 5-methyl-THF has been found as the predominant form of folate in the liver, plasma, intestine, kidney and red blood cells (Hawkes and Villota 1989, Diaz de la Garza and others 2007, Shane 1995, Scott and others 2000, Stokes and Webb 1999). Representing 68% of the total folate pool, 5-methyl-THF is
likely the main transport form of folate in the body (Diaz de la Garza and others 2007, Davis 1986). All other folate compounds are converted during absorption to 5-methyl-THF in the liver (Grossowicz and others 1972). The liver then secretes 5-methyl-THF in the bile, which is then absorbed in the gut (Gregory 1989, Shane 1995, Grossowicz and others 1972). 5-formyl-THF and 10-formyl-THF were found in smaller quantities compared to 5-methyl-THF (Hawkes and Villota 1989). In contradiction to 5-methyl-THF being the predominant folate compound in the human body, Hoffbrand and others (1975) found that THF was the preferred folate compound in human tissues. In addition to THF and 5-formyl-THF, 5-methyl-THF is well-absorbed by the human body for use in a variety of different functions (Bhandari and Gregory 1992). Absorption of 5-methyl-THF occurs through a carrier mediated process in which monoglutamyl forms of folate are absorbed into enterocytes of various tissues (Bhandari and Gregory 1992).

Folate Compounds in Foods

There are numerous food sources that have been analyzed by a variety of techniques to determine the levels of each folate compound. Three of those foods include spinach, sweet peppers, and wheat bread. Zhang and others (2005) found that spinach contained the highest levels of 5-methyl-THF, followed by THF, 5-formyl-THF, and 10-formyl-THF. This was confirmed by Freisleben and others (2003). Red and green sweet peppers had 5-methyl-THF in the highest concentrations, followed by 5-formyl-THF and 10-formyl-THF (Phillips and others 2006). Lastly, wheat bread was found to contain the highest levels of 5-formyl-THF, followed by THF, 5-methyl-THF, and 10-formyl-THF (Freisleben and others 2003). Even though each of these food sources contains varying levels of each folate compound, in
general, 5-methyl-THF and 5-formyl-THF are found in the highest quantities in the majority of dietary folate sources.

Stability of Folate in Foods

Folate deficiency can not only be attributed to lack of folate intake, but also to the losses of folate in the processing, preservation, and marketing of foods (McCully 1998). Certain foods contribute higher levels of folate than others, but folate content in some foods decrease when the food is harvested, processed, cooked or stored (Folic Acid 2008, Phillips and others 2005, Scott and others 2000). Raw foods, such as fruits, vegetables, and unroasted peanuts tend to be higher in folate than cooked foods due to hydrolysis of the folate compounds during heating, decreasing the overall folate content available for absorption (Moat and others 2004). These processing techniques also cause oxidative and thermal losses of folate which contribute to the decreased amount available to be taken in by the body when the food product is consumed (Gregory 1997). These processes include canning, prolonged heating, reheating, high boiling temperature, pH of cooking medium, leaching into the cooking water, and time of cooking among others (Krishnaswamy and Nair 2001, Dang and others 2000, De Brouwer and others 2007, Hawkes and Villota 1989). Since the major food sources of folate such as vegetables and beans are usually cooked before consumption, the concentration of folate is usually less than originally labeled (Subar and others 1989).

Human Folate Requirements

Humans lack the ability the synthesize folate de novo, meaning from molecules inside the body; therefore, they must obtain folate from their diet, as either plant foods, fortified foods, or supplements (Basset and others 2005). Mammals are unable to perform the first
three steps of folate biosynthesis, because they are unable to attach the initial glutamate molecule to the pterin molecule and cannot synthesize the pABA residue (Neuburger and others 1996, Folic Acid 2008). Due to this, humans need to consume large quantities of plants in order to obtain the required amount of folate needed for DNA synthesis and methylation reactions. Plant leaves tend to have the highest amount of folate, augmenting the statement that leafy green vegetables are an excellent source of dietary folate (Gambonnet and others 2001). Although the polyglutamate form of folate, which is the main form found in plants, can be taken up by humans, deglutamylation into the respective monoglutamate form provides more efficient uptake into the cells, leading to increased utilization for one-carbon metabolism (Rebeille and others 2006).

Daily Reference Intake

Based on the need and bioavailability of folate, a DRI has been established. The DRI for folate, which was increased in 1997 to provide a more appropriate intake in order to achieve cardiovascular health benefits for both healthy men and women, is 400 µg/d (Griel and others 2004, Voutilainen and others 2001, Rebeille and others 2006, Subar and others 1989). The United States Department of Agriculture specifies the DRI in µg/d for specific age categories. Children of both genders between 1 and 3 years of age should acquire 150 µg/d, while those between 9 and 13 years of age should acquire 300 µg/d. Like adults, adolescents from 14 to 18 years of age should obtain 400 µg/d (Folacin 2001, Davis 1986). In response to their increased needs due to uterine enlargement, placental and fetal development, increase in maternal red blood cell count, and possibility of NTD development, pregnant women need approximately 600 µg/d of folate (Bailey 1998). An adequate folate
supply is necessary for rapidly growing and multiplying cells (Krishnaswamy and Nair 2001). Even though the DRI is 400 µg/d, some research indicates a folate intake of 300 µg/d is thought to meet minimum folate requirements (Subar and others 1989). No adverse effects have been associated with the consumption of dietary folate in excess; however, an Upper Intake Level (UL) of 1000 µg/d of folate for all adults has still been established by the Food and Nutrition Board of the Institute of Medicine (Bailey 1998). This verifies that folate exhibits “relatively little direct toxicity” since it is flushed out of the body when in excess (Gregory 1997).

Dietary folate is biochemically measured in the body, where deficiency is identified if red blood cell folate concentrations fall below 372 nmol/liter (L) (Boushey and others 1995, Robinson and others 1998). Despite the fact that a DRI has been set at 400 µg/d of folate, most people are not obtaining the recommended dosage (Wright and others 2003). There are variations between studies regarding mean folate intake; however, intake between 250-350 µg/d appears to be representative of the adult population (Wright and others 2003, Griel and others 2004, Subar and others 1989, Dhonuske-Rutten and others 2007, Ward and others 1997, Voutilainen and others 2001). It is estimated that approximately 88% of US adults consume less than 400 µg/d of folate, indicating the need to review folate content in food products and promote foods with high quantities of folate (Boushey and others 1995).

Folate Function in Human Health and Disease Resistance

After ingestion of dietary folate, coenzyme forms of the vitamin are created in the liver. These compounds are now metabolically active and are secreted with bile into the small intestine and these compounds can be reabsorbed and distributed to tissues throughout
the body (Alternative Medicine Review 2006). When humans become folate deficient, there is an imbalance in the folate pool essential for DNA synthesis and cell repair, along with an imbalance in DNA methylation and genomic instability (Folic Acid 2008). Because cells are unable to synthesize DNA and properly regulate gene expression, altered rate of cell division and severe metabolic repercussions are likely to occur (Gambonnet and others 2001). Several of these metabolic repercussions include increased risk of NTDs, hyperhomocysteinemia, CVD, cancer, megaloblastic anemia, cervical dysplasia, periodontal disease, vitiligo, and impaired immune response (Rebeille and others 2006, Alternative Medicine Review 2006, Folic Acid 2008, Gregory 2004, De Brouwer and others 2007, Shane 1995).

Increased risk of NTDs, CVD, and cancer are the major consequences of folate deficiency. Folate has an impact on pregnancy and infant development in that if folate is deficient, NTDs, or birth defects of the brain and spinal cord, can result (Shane 1995, Bailey 1998). Folate deficiency also results in hyperhomocysteinemia, leading to CVD (Alternative Medicine Review 2006). This occurs when homocysteine is not remethylated into methionine (Rebeille and others 2006). When homocysteine levels are in excess, there is damage to the arterial wall, leading to an increased risk of heart attack, stroke, coronary artery stenosis, and venous thrombosis (Griel and others 2004, Gregory 2004, De Brouwer and others 2007). For every increase of 5 µmol/L in total plasma homocysteine, there is a 1.6 and 1.5 increase in the relative risk of CVD for men and women, respectively (Krishnaswamy and Nair 2001). If folate is consumed in proper amounts, it can improve blood flow and increase the number of signaling molecules by increasing production of nitric oxide in endothelial cells (Alternative Medicine Review 2006). Lastly, folate deficiency can lead to certain cancers, such as colon
cancer (Gregory 2004). When 5,10-methylene-THF is limited, the ratio of dUMP to dTMP increases, causing a higher inclusion of dUTP into DNA. This generates single- and double-strand DNA breaks, point mutations, and chromosomal breakage, leading to increased cancer risk (Rebeille and others 2006).

Even though these metabolic conditions are the most destructive, several of the other consequences of folate deficiency listed above are also detrimental. Megaloblastic anemia results from apoptosis, or cell death, in response to a lack of folate causing a mismatch of DNA when uracil is incorporated instead of thymidylate (Rebeille and others 2006). Folate deficiency also causes an increased resistance of the gingival to irritants, causing a reduction in inflammation and increased periodontal disease risk (Alternative Medicine Review 2006).

When analyzing these metabolic conditions as a whole, it is evident that any disease condition that increases oxidative cleavage and catabolism of folate is expected to increase folate needs (Shane 1995). However, when folate is deficient, one-carbon reactions will be disturbed and possibly compromised to some extent (Folic Acid 2008).

Decreased dietary folate intake can result in damaging metabolic conditions; yet adequate dietary folate can counteract this. For example, including peanuts in the diet increases folate, fiber, magnesium, copper, arginine, vitamin E, and niacin, all of which decrease the risk of heart disease and cancer (Ohr 2004). Griel and others (2004) confirmed that consuming peanuts five times a week was associated with a decrease in CVD risk by 39%. Consumption of other foods products such as fruits and vegetables can also increase folate levels in the body, in turn counterbalancing the metabolic implications of folate deficiency.
Folate Bioavailability

Approximately 50-67% of folate in foods is bioavailable and used for one-carbon metabolisms (Subar and others 1989). The term bioavailability includes the metabolic function of folate coenzymes, the extent of intestinal absorption, and the kinetics of folate clearance through catabolic and excretory processes (Gregory 1989). Various factors influence the bioavailability of dietary folate. Several of these include the ratio of polyglutamate to monoglutamate forms, the presence of compounds in the diet or intestine that may improve or hinder absorption, the food matrix, and the amount of folate uptake in the tissues which is contingent on the individual’s preexisting folate status (Molloy 2002, Hannon-Fletcher and others 2004). If an individual consumes foods that contain monoglutamate forms of folate and/or has preexisting low levels of folate in the body, the dietary folate consumed will be more bioavailable (Seyoum and Selhub 1998). Once dietary folate is consumed through food sources, it shows slow turnover in the body. Even though folate only has a half-life of about 100 days, the main folate compounds are interchangeable throughout human metabolism, allowing conversion of folate compounds based on need (Gregory 1997). Despite this interchangeability, the compounds are usually broken down in the manner required for their respective purpose. The dietary breakdown of folate dictates each compound’s bioavailability in the human body (Figure 5) (Molloy 2002). Folate status and bioavailability can be identified by measuring red blood cell folate (Voutilainen and others 2001). Red blood cell folate is an index of the liver and tissues in the body which are the main storage sites of folate and represent approximately a three- or four-month folate intake (Voutilainen and others 2001).
**Figure 5.** Dietary breakdown of folate in the human body.

**Bioavailability of Folate vs. Folic Acid**

Folate differs from folic acid in that folate is a natural source of the vitamin found in foods while folic acid is a manufactured, synthetic, oxidized form of folate containing one conjugated glutamate residue (Smith and others 2008). Synthetic folic acid is extremely stable and used to fortify instant breakfast drinks, breakfast cereals, pastries, diet foods, and infant formulas and can also be found in pill form (Gregory 1989, Hawkes and Villota 1989). Dietary folate is approximately 50% bioavailable (Gregory 2004, Bailey 1998, Folic Acid 2008). In comparison, folic acid is significantly more bioavailable than dietary folate (Bailey 1998, Krishnaswamy and Nair 2001). Folic acid in supplements or fortified foods is
calculated to be 1.7 times more bioavailable than dietary folate (Bailey 1998). Because folic acid is not metabolically active, it must be methylated to 5-methyl-THF before it can be used for one-carbon metabolisms (Phillips and others 2006).

The folate receptor in the body has a higher binding affinity for folic acid than for natural folate (Smith and others 2008). When folic acid is consumed in place of folate, several consequences may occur. Folic acid is more bioavailable than folate, meaning it is absorbed in the intestines, distributed through the tissues and excreted to a higher degree than folate (Bhandari and Gregory 1992). Despite this, folic acid can interfere with the metabolism, cellular transport, and regulatory functions of dietary folate by competing with the reduced forms for binding with enzymes, binding proteins, and carrier proteins (Smith and others 2008). Because of these negative effects, folic acid might not be as beneficial as once thought. Even though folic acid supplementation occurs in several food products such as bread and flour, its consumption might actually be detrimental to some individuals.

When animals consume high levels of folic acid, it may influence DNA and histone methylation, leading to phenotypic changes in subsequent generations. Increasing folic acid intake increases blood concentrations of dietary folates and unmetabolized folic acid in humans (Smith and others 2008). High levels of folic acid in the blood may be related to decreased natural killer cell cytotoxicity, which involves the cells that reject tumor cells and cells infected by viruses (Smith and others 2008). High levels of dietary folate may decrease the response of antifolate drugs to rheumatoid arthritis, cancer, malaria, and psoriasis (Smith and others 2008). Antifolate drugs cause a state of folate deficiency in the cell in order to inhibit folate-dependent enzymes along the folate metabolic pathway. DNA synthesis and
cell division are hindered by folate deficiency, both of which are involved in tumor growth (Smith and others 2008). Some individuals may also have an innate deficiency of the MTHFR enzyme which is needed to convert folic acid to 5-methyl-THF for use in the body (Alternative Medicine Review 2006). Folic acid may also worsen neurological damage related to a vitamin B12 deficiency and is hypothesized to promote tumor growth (Folic Acid/Folates 2007, Scott and others 2000). A combination of high blood folate and vitamin B12 levels in the elderly population may increase risk of cognitive impairment and anemia (Smith and others 2008). In pregnant women, an increased risk of insulin resistance and obesity in their children may occur when blood folate levels are in excess (Smith and others 2008). For these reasons, it may be more important for individuals to consume foods with higher levels of dietary folate instead of taking in synthetic folic acid.

**Folate Extraction and Quantification**

Knowing 5-methyl-THF, followed by 5-formyl-THF, is utilized most in the body and is typically found in high concentrations in plants, it is necessary to find an extraction technique to determine the overall folate content of the food, the individual folate compounds present, and the quantity of each of the folate compounds. Several obstacles are present when attempting to extract and quantify the individual folate compounds in foods. Many of the folate compounds are susceptible to interconversion and oxidative degradation (Hanson and Gregory 2002). The varying amounts and types of folate compounds, the possible destruction of the compounds by light, heat and oxygen, the lability of folate, and the existence of the compounds, especially 5-formyl-THF, at low concentrations make it extremely difficult to prepare, extract, conjugate and purify each folate compound (Rychlik and others 2007,
Gregory and others 1984, Gregory 1989, Ndaw and others 2001, Zhang and others 2005). Complete extraction may also be difficult since folates have a tendency to become trapped in the cell walls (Hanson and Gregory 2002). Most of the extraction methods utilize deconjugase enzymes to convert polyglutamates into their monoglutamate form (Rychlik and others 2007). Methods for detection, extraction, and quantification include ligand-binding methods, high-performance liquid chromatography, microbiological assays, and ultraviolet absorbance and electrochemical techniques (Hanson and Gregory 2002, Gregory 1989).

**Microbiological Assays**

For decades, microbiological assays (MAs) have been the most widely-used standard methods to determine folate content of food products (Cossins and Chen 1997, Gambonnet and others 2001, Rychlik 2004). MAs utilize *Lactobacillus casei* ATCC 7469, a lactic acid-forming bacterium, which requires the presence of folate to promote growth (Gambonnet and others 2001, Hawkes and Villota 1989). MAs do not distinguish between the single folate compounds; therefore, MAs can only be used when a total folate content for all compounds is needed (Scott and others 2000, Rychlik 2004, Freisleben and others 2003). There are several problems that arise when MAs are used to analyze folate content. First, MAs are time consuming and can cause loss of folates due to long incubation times (Zhang and others 2005). Second, ascorbic acid is used as an antioxidant to inhibit oxidative reactions of the folate compounds, which may cause high values of folate to be measured since the folate compounds exhibit greater stability when ascorbic acid is present (Hawkes and Villota 1989, Koontz and others 2005). Third, *L. casei* has a higher affinity for the monoglutamate form of folate over the diglutamate form (Hawkes and Villota 1989). Lastly, free amino acids present
may release the folate compounds by competing with the folate-binding proteins (Hawkes and Villota 1989). Even though there are several issues with MAs, the majority of the data presently known is based on MA methods (Rychlik and others 2007).

**High-Performance Liquid Chromatography**

High-performance liquid chromatography (HPLC) methods are used for analyzing folate by separating, isolating, identifying and quantifying the various folate compounds (Cossins and Chen 1997, Waters 1995). Identifying folate compounds with HPLC may be complicated due to the increased number of possible folate derivatives made by combining different “one carbon moieties, oxidation levels, and glutamate chain lengths” (Shane 1982). Despite this, the polyglutamate forms of folate in the sample must be deconjugated into monoglutamate forms which can then be coupled with fluorescence, electrochemical detection, or ultraviolet detection (Gregory 1989, Periago and others 2008, Rychlik and others 2007). Although HPLC has been used to extract and quantify individual folate compounds, it lacks specificity, is susceptible to matrix interferences, and may overestimate folate content in foods (Freisleben and others 2003, Periago and others 2008).

**HPLC-Mass Spectrometry**

HPLC is used to separate compounds while mass spectrometers (MS) generate three-dimensional data by use of an extremely fast high-repetition laser (Agilent Technologies 2001). This laser scans the injected sample and produces an image of low-molecular-weight compounds while displaying the spatial distribution of the compounds (Agilent Technologies 2001). MS produce mass spectral data which identifies the structure, quantity, molecular weight, purity, and identity of a sample (Agilent Technologies 2001). After a sample is run
through HPLC, the molecules are then ionized by MS, which then sorts and identifies the ions based on their mass-to-charge ratio (Agilent Technologies 2001). Combining HPLC and MS allows research to examine and quantify a large range of compounds.

**Environmental Conditions Influencing Vitamin Synthesis**

Plants are exposed to certain environmental conditions based on their growing location and time of harvesting. The environment fluctuates between days and seasonal cycles; however, changes in weather can cause uncertainty in the availability of nutrients. Environmental factors influence the plant’s metabolic processes (Smirnoff 1995). The primary process that powers plant metabolism is photosynthesis, which must constantly acclimate to changing environmental conditions (Keegstra and Thomashow 2002). Folate and niacin play a key role in plant photosynthesis and respiration. Because these processes must adapt, vitamin levels will fluctuate, in turn varying the rates of photosynthesis and respiration based on the present environmental factors that influence growth and development (Keegstra and Thomashow 2002). The main environmental conditions that help or hinder plant growth and contribute to the overall vitamin content of the plant include temperature, light, water stress, carbon dioxide, exposure to oxygen, and plant maturity.

**Temperature**

The temperature range compatible with plant growth is between 0°C and 45°C (Seliger and McElroy 1965). The main temperature range in which vitamins are produced in the highest quantities during plant development is between 5°C and 40°C (Ketring and others 1982). Peanut plants are sensitive to cool production environments where mild night temperatures below 20 °C occur (Maiti and others 2002), since lower temperatures cause a
decrease in the rate of photosynthesis (Keegstra and Thomashow 2002). Temperature tends to decrease when altitude is increased. Most plants produce the highest amount of vitamins at low altitude levels with the daily maximum temperature reaching 29.7°C and the minimum temperature reaching 17.3°C (Ketring and others 1982). The largest number of plant seedlings are produced at temperatures between 27.0°C and 14.5°C (Ketring and others 1982). A minimum temperature of 6.1°C and a maximum temperature of 21.7°C provide a range in which optimum plant growth, photosynthesis and development, along with vitamin synthesis can occur (Ketring and others 1982, Smirnoff 1995). Despite this, plants growing in hot climates posses a thermal defense mechanism (Maiti and others 2002). A soil temperature between 31°C and 33°C is most favorable for plant development (Ketring and others 1982). Peanut plants deteriorate quickly at high humidity levels and high temperatures because extreme temperatures can cause plant damage (Ketring and others 1982, Smirnoff 1995). Fluctuations in temperature also influence reaction rates, metabolic rates, and production of enzymes (Smirnoff 1995). Ketring and others (1982) found that vegetative growth occurs during the cool spring and early summer planting season while reproductive growth takes place during the hot summer relative to the latitude at which the crop is grown. This suggests that during vegetative growth, where the majority of folate and niacin is produced in plants, it is optimal for temperatures to be cooler.

Light

Since light is a regulator of vitamin biosynthesis, it reflects the need for higher levels of metabolic activity in photosynthetic tissues which utilize folate and niacin for one-carbon transfers (Smith and others 2007). Light intensity or irradiance, quality, and photoperiod
influence the way a plant grows and how many vitamins are produced (Ketring and others 1982). Light supplies energy for photosynthesis, which directly influences plant metabolism (Smirnoff 1995). The status of folate synthesis in leaves corresponds to the amount of light present (Jabrin and others 2003). Vitamin synthesis and accumulation are stimulated by light, due to photorespiratory activity (Jabrin and others 2003, Scott and others 2000). In order for photosynthesis to occur, irradiances must be near full sunlight for light saturation to occur (Ketring and others 1982). For example, folate synthesis is minimized when plants are shaded because it inhibits overall plant growth (Ketring and others 1982). During seedling germination, folate is synthesized more rapidly in the light than the dark (Rebeille and Douce 1999). At constant temperatures, plant growth and vitamin synthesis was increased when the photoperiod was increased from 8 to 20 hours (Ketring and others 1982). Gustafson (1953) determined that there is an increase in niacin content with an increase in day length and light, regardless of temperature. A 10-hour photoperiod is the most optimum for vitamin synthesis and accumulation (Ketring and others 1982).

Water Stress

In order for the net photosynthetic rate to be high, the range of soil water should be between 50% and 90%. Plants require a moisture level greater than 35% in order for germination to occur (Ketring and others 1982). In addition, the critical drought level should be at 50%, the moisture compensation point between 14% and 19%, and the critical wet injury level at 90% (Maiti and others 2002). The percentage of soil water corresponds to the amount of water stress on a plant. In general, water stress decreases plant growth and development, which means that plants are unable to thrive and produce high vitamin levels in
dry environments (Maiti and others 2002, Smirnoff 1995). Withholding water and water
logging both decrease net photosynthetic rate (Maiti and others 2002). In plants that are
water-stressed, the temperature in the leaves tend to be higher and transpiration tends to be
lower (Maiti and others 2002). Irrigated, rather than dryland, conditions are more appropriate
for optimum plant growth and vitamin synthesis (Ketring and others 1982).

*Carbon Dioxide*

Levels of carbon dioxide which are expected to increase over the coming decades will
influence metabolic processes that aid in plant growth and development (Keegstra and
Thomashow 2002). There is a decrease in carbon dioxide levels when night temperatures fall
below 20 °C (Maiti and others 2002). Photosynthesis increases when carbon dioxide levels
are increased, therefore increasing the amount of vitamins synthesized in the cell (Ketring
and others 1982).

*Oxygen Levels*

Folate is sensitive to chemical oxidation (Jabrin and others 2003). Oxygen stimulates
photorespiration and inhibits carbon dioxide uptake; therefore, when oxygen levels are high,
vitamins can be produced in high quantities (Ketring and others 1982).

*Plant Maturity*

Plant leaves become less efficient at synthesizing and storing vitamins with age,
which changes the bioavailability of the vitamins (Ketring and others 1982, Mozafar 1993).
Concentrations of certain vitamins may increase or decrease depending on the plant and
vitamin being referenced (Mozafar 1993). For example, folate synthesis begins to diminish
10 to 15 days after the leaf appears (Ketring and others 1982).
Other Environmental Factors

Additional environmental factors that affect vitamin synthesis include time of harvest, fertilizer, and pests. Vitamin concentration decreases after plants are harvested (Mozafar 1993). Organic and inorganic fertilizers produce different vitamin contents in plants. Organic fertilizers contain lower nitrate levels, which could possibly decrease vitamin content in plants (Mozafar 1993).

Some peanut genotypes are more stable in certain environmental conditions than others (Ketring and others 1982). Despite this, temperature range, light intensity, and water stress all relate to yield stress and vitamin production during certain growth phases (Ketring and others 1982). It is evident that there are specific environmental conditions that promote plant growth and development more than others. When plants are able to grow to their fullest potential, folate and niacin levels are thought to be the highest since more one-carbon metabolisms will be taking place. Environmental conditions featuring temperate to warm temperatures ranging from 14°C to 40°C or 57°F to 100°F, low to mid altitude that is slightly above sea level, plentiful sunlight for a minimum of 10 hours per day, irrigated soil that is 35% to 90% moist but not sopping, and high oxygen levels are the most favorable. These conditions should produce plants with high vitamin levels compared to other plants grown in colder, hotter, drier, and darker climates.

Genetically Engineering Vitamins

There are several ways in which plants can be genetically engineered to produce higher levels of vitamins so that consumption of the same quantity of a food source will produce higher levels of dietary folate and niacin intake. First, vitamins in plants can be
enhanced by over-expressing the enzymes that are the limiting steps of folate biosynthesis (Scott and others 2000). One specific enzyme that can be over-expressed is GTP cyclohydrolase-1, an important rate-limiting step during the pathway of folate synthesis (Lutz 2004, Scott and others 2000). By doing this, more folate is able to be produced by the synthesis cycle. Second, if the cofactors for vitamin biosynthesis are stabilized by binding them to vitamin-dependent proteins, more can be produced (Scott and others 2000). Third, reducing the catabolic rate of the cofactor may increase overall vitamin content (Scott and others 2000). Fourth, orientating vitamin metabolism toward a stable derivative could aid in increasing concentrations in plants (Scott and others 2000). In addition, Krishnaswamy and Nair (2001) determined that germination increases vitamin availability. If germination of a plant was able to be controlled by specific environmental conditions, vitamin availability may be increased. Lastly, it is known that folate is stored in plant vacuoles. The transport and storage processes in plants could potentially be engineered to enhance folate accumulation in the vacuoles so that more folate is stored and ready for use (Orsomando and others 2005). Even though plants can be metabolically engineered to produce higher levels of folate and niacin, if the plant is unable to grow correctly or cannot withstand certain environmental conditions, there is no advantage of having plants with extremely high vitamin contents (Smith and others 2007). Additional work needs to be done to determine whether or not genetically engineering vitamins in plants is possible and if it can produce excessive amounts of vitamins compared to what is typically produced.
Literature Cited


CHAPTER 2:
Effects of Variety, Growing Location and Year of Cultivation on Niacin Concentration in Raw and Roasted Peanuts
Abstract

Peanuts (*Arachis hypogaea* L.) are known to be sources of several important B-vitamins, including niacin (vitamin B3). For this study, a series of raw and roasted samples from the 2007 and 2008 Uniform Peanut Performance Trials (UPPT) and from the Core of the Core peanut germplasm collection grown in 2008 were analyzed to compare concentrations of niacin in peanuts from 10 U.S. growing locations. Thirty-nine total UPPT Florunner and NC7 samples and 13 selected samples from the Core of the Core were analyzed. Niacin concentration (mg/100g) of raw UPPT samples ranged from 8.20 to 25.8 with an average of 16.1 for the 2007 UPPT samples, 17.7 for the 2008 UPPT samples, and 16.8 for the Core of the Core samples. The average niacin concentration in raw Florunner and NC7 samples increased from 14.6 and 17.4 in 2007 to 16.4 and 18.9 in 2008, respectively. There was a significant difference due to peanut variety and growing location; however, there was not a significant difference due to year of production. For the roasted 2008 UPPT samples, niacin concentration (mg/100g) ranged from 12.2 to 22.4. A significant difference was not found between raw and roasted samples. The differences due to variety and location are likely the result of interactions between genetics and environment and the lack of difference between raw and roasted samples caused by the stability of niacin to light and heat. These results suggest that germplasm/varietal differences may be sufficient for increases in niacin levels through conventional breeding.
Introduction

Niacin, also known as nicotinic acid or vitamin B3, is a water-soluble vitamin (Higdon 2010). Nicotinamide, a derivative of niacin, is used to form the coenzymes nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP) in the human body (Lahely and others 1999). Bioavailable niacin is comprised of free nicotinic acid and nicotinamide, NAD, and NADP (Ndaw and others 2002). Niacin is involved in oxidation-reduction, or redox, reactions along with other non-redox reactions throughout plant cells (Smith and others 2007). Oxidation-reduction reactions involve the transfer of electrons and as many as 200 enzymes use the niacin coenzymes, NAD and NADP, to accept and donate electrons for redox reactions (Higdon 2010). NAD functions in energy-producing reactions including the degradation or catabolism of carbohydrates, proteins, fats, and alcohol (Higdon 2010). The functions of NADP are found in biosynthetic or anabolic reactions including the synthesis of macromolecules, such as fatty acids and cholesterol (Higdon 2010).

Niacin is synthesized in plants via the tryptophan degradation pathway, where tryptophan serves as a niacin precursor (Arditti and Tarr 1979). In the tryptophan degradation pathway, tryptophan is converted into kynurenine, which is then converted into 3-hydroxyanthranilic acid. 3-hydroxyanthranilic acid is a precursor for quinolinic acid which is then decarboxylated to niacin (Figure 1) (Arditti 1967, Kotah and Hashimoto 2004, Allegri and others 2003).
The Dietary Reference Intake (DRI) for niacin is 16 mg niacin equivalent (NE)/day for men and 14 mg NE/day for women. NE is equivalent to 60 mg of tryptophan (Higdon 2010). The average intake of niacin is about 30 mg/day for men and 20 mg/day for women (Higdon 2010). Niacin toxicity is rarely seen at doses typically consumed. At extremely high doses, niacin can cause the release of histamine resulting in flushing of the skin (Higdon 2010). This can be harmful to people with asthma or peptic ulcer disease. Niacin also promotes hepatic toxicity when consumed in excess, often through supplements (Higdon 2010). Because of this, an upper intake level (UL) has been set at 35 mg/day (Higdon 2010). If niacin is not consumed in appropriate quantities, nutritional deficiencies such as pellagra, a deficiency brought about by a lack of dietary tryptophan and niacin, may occur (Vidal-Valverde and Reche 1991). Common deficiency symptoms of pellagra include dermatitis, diarrhea, dementia and death, if untreated (Higdon 2010).
When niacin is consumed in appropriate quantities, it is known to be beneficial for vascular health (Mozafar 1993). Niacin favorably affects apolipoprotein (apo) B-containing lipoproteins, such as very-low-density lipoproteins (VLDL) and low-density lipoproteins (LDL), and increases apo A-I-containing lipoproteins, such as high-density lipoproteins (HDL) (Kamanna and Kashyap 2008). In addition, niacin inhibits triglyceride synthesis which causes accelerated intracellular apo B degradation, in turn decreasing secretion of VLDL and LDL particles. Niacin decreases HDL-apo A-I catabolism which increases the concentration of lipoprotein A-I HDL particles (Kamanna and Kashyap 2008). This reverses cholesterol transport and aids in decreasing inflammation (Kamanna and Kashyap 2008).

There are numerous dietary sources of niacin including yeast, poultry, meat, cereals and legumes, including peanuts (Higdon 2010). A variety of foods have been fortified with niacin, including cereals, bread and milk, thus niacin deficiency does not tend to be an issue in developed countries (Hannon and others 2007, Morris and others 2004). Despite this, peanuts are one of the most potent natural sources of niacin, containing approximately 20% of the Daily Values (DV) per serving (Daniel and Norris 1945). Peanuts (*Arachis hypogaea* L.) are a legume which belong to the *Leguminosae* family, are native to South America, and are grown underground (Hammons 1982, Hoffpauir 1953).

In order to appropriately analyze the niacin content in a food product, prior hydrolysis is necessary. Acid hydrolysis is used if biologically active niacin is being studied; however, alkaline hydrolysis is useful to release non-bioavailable niacin, thus providing total niacin content (Lahely and others 1999). Niacin can be analyzed in food products by high-performance liquid chromatography (HPLC) methods, microbiological methods, and
chemical assays (Van Niekerk and others 1984, Vidal-Valverde and Reche 1991). Chemical assays require reaction of compounds extracted from the food product with cyanogen bromide in order to produce a pyridinium derivative (Vidal-Valverde and Reche 1991). Since this reaction with cyanogen bromide is not just specific to niacin but is also common to all substituted pyridines including bound niacin, the chemical methods of analysis typically result in higher niacin concentrations than those determined by the HPLC method (Vidal-Valverde and Reche 1991). In the study by Lahely and others (1999), a total niacin concentration of 209 micrograms (µg)/gram (g) was measured in peanuts.

Using the chemical assay to verify the concentration of niacin will not only help to determine if a difference exist among peanut varieties, locations and years, but it will also aid in determining whether or not peanuts can be classified as a good source of this vitamin. The chemical assay is the official method (AOAC 961.14); therefore, the results would be acceptable to the nutrient database (USDA ARS 2009). However, research validating the HPLC methodology is needed since this method is not susceptible to interferences within the matrix (Freisleben and others 2003).

**Materials and Methods**

**Materials.** In 2007 and 2008, UPPT samples were grown in Suffolk, VA (VA); Lewiston, NC (NC); Clemson, SC (SC); Tifton, GA (GA); Headland, AL (AL); Marianna, FL (FL); Stephenville, TX (C-TX); College Station, TX (S-TX); Lubbock, TX (W-TX) and Stillwater, OK (OK). From the 2007 UPPT, Florunner (medium grade size) and NC7 (Extra Large Kernel {ELK} grade size) were obtained from all locations except C-TX. In 2008, the same varieties and sizes were obtained from all growing locations. The 2007 samples were only
analyzed as raw peanuts. The 2008 UPPT samples were analyzed as both raw and roasted peanuts, except for the GA samples which were in limited supply for roasting. Samples were roasted at 167°C for 17-20 minutes to a Hunter L value of 50 ± 1 (HunterLab 2008, Sanders and others 1989). In addition to the UPPT samples, 13 Core of the Core germplasm samples were selected from the 2008 crop year grown in Lewiston, NC. These 13 samples were a representative sampling of the germplasm available for analysis (Holbrook and others 2003).

Reagents. The reagents sulfuric acid (H₂SO₄), ammonium sulfate ((NH₄)₂SO₄), sodium hydroxide (NaOH), cyanogen bromide (CNBr), sufanilic acid, ammonium hydroxide (NH₄OH), and hydrochloric acid (HCl) were all ACS grade or better and purchased from Fisher Chemical Corporation (Fairlawn, NJ).

Standards. A niacin stock standard was prepared by weighing 0.050g of nicotinic acid (Sigma Chemical Corporation, St. Louis, MO) into a 500 milliliter (mL) volumetric flask, to which 450mL of 25% denatured alcohol had been added. The solution was then diluted to volume with 25% denatured alcohol. A niacin working standing was prepared by diluting 8mL of the niacin stock standard into 180mL of deionized (DI) water in a 200mL volumetric flask. The solution was brought to volume with DI water.

Extraction of Niacin. The AOAC non cereal food method 961.14 was utilized (Helrich 1990). In brief, whole peanuts were homogenized by grinding the seed in a Krups coffee mill (Millville, NJ) until a fine meal was obtained. Five g of each homogenized sample was weighed into an 800mL beaker. Approximately 5g of Tang (Kraft Foods, Northfield, IL) was also weighed into a 800mL beaker separately, which was used as the control sample. Two hundred mL of 1N H₃SO₄ was added to each 5g of homogenized sample and the solution
was stirred. Beakers were covered with perforated aluminum foil. The covered samples were placed in an Amsco Scientific Series 3021-S Gravity autoclave (Steris, Mentor, OH) for 45 minutes.

The remainder of the procedure was performed under low actinic lighting. After removal from the autoclave, the samples were cooled to room temperature for approximately 1 hour. The pH of each sample was adjusted to 4.5 with 10 N NaOH with an Isotemp pH meter (Fisher Scientific, Waltham, MA). The sample was transferred to a 250mL volumetric flask with water washings and diluted to volume with water (1st volume in Equation 1).

Samples were vacuum filtered through Whatman #42 filter paper (Whatman Inc., Florham Park, NJ) and 40mL of the sample (1st aliquot in Equation 1) were pipetted into a 50mL volumetric flask containing 17g of (NH₄)₂SO₄. For the standard curve, 0, 10, 20, 30, and 40mL aliquots of the working standard were pipetted into 50mL volumetric flasks containing 17g of (NH₄)₂SO₄. All volumetric flasks were diluted to volume with water (2nd volume in Equation 1) and shaken vigorously. The samples and standards were then vacuum filtered through Whatman #42 filter paper. For each sample and standard filtrate, a 1mL aliquot was pipetted into 2-50mL, glass, screw-top test tubes. One tube served as a blank for each sample and for the standards. Blank tubes were filled with 5mL of water.

The remainder of the procedure was performed under a fume hood. A Genesys 20 UV-VIS spectrophotometer (Thermo Scientific, Waltham, MA) set to 470nm was blanked with DI water. The following scheme was used to add the remaining reagents to each tube. For the blanks, 0.5mL of 0.3 N NH₄OH was added and the solution was swirled on a Fisherbrand Vortex Genie (Fisher Scientific, Waltham, MA). Two mL of 10% sulfanilic acid
was added and the solution was swirled. Last, 0.5mL of 2.4 \( \text{N} \) HCl was added, the solution was swirled and color was determined on the spectrophotometer. For the sample and standard solutions, 0.5mL of 0.3 \( \text{N} \) \( \text{NH}_4\text{OH} \) was added and the solution was swirled. Five mL of 10% CNBr was added and the solution was swirled for 30 seconds. Two mL of 10% sulfanilic acid was then added and the solution was swirled. Last, 0.5mL of DI water was added, the solution was swirled and color was determined on the spectrophotometer. The color maximum was reached 1.5 minutes after adding the sulfanilic acid solution, and began to decay after 2 minutes.

**Calculations.** The following equations were utilized to calculate niacin concentration (milligram (mg)/100g) in each sample. The standard curve of \( \Delta A_{\text{std}} \) verses niacin concentration (µg/mL) was plotted, where \( \Delta A_{\text{std}} = A_{\text{std}} - A_{\text{std blk}} \). From the standard curve, the concentration (C µg/mL) of the corresponding \( \Delta A_{\text{sample}} \) was obtained, where \( \Delta A_{\text{sample}} = \Delta A_{\text{sample blk}} \). Niacin concentration was then calculated (Equation 1).

\[
C \left( \text{from curve, } \mu\text{g/mL} \right) \times \frac{\text{1st volume (mL)}}{\text{sample weight (g)}} \times \frac{\text{2nd volume (mL)}}{\text{1st aliquot (mL)}} \times \frac{1 \text{ mg}}{100 \text{ µg}} \times 1000 \text{µg} \tag{[1]}
\]

**Statistical Analysis.** Statistics were performed using a mixed model with no interaction terms to test the significance of year (2007 versus 2008), location, variety (Florunner versus NC7), and condition (raw versus roasted). To test all pairwise comparisons among means, Tukey’s honest significant difference test and Duncan’s multiple range test were used. All statistics were performed using SAS (SAS Institute Inc. 2004).
Results and Discussion

Niacin concentration (mg/100g) for raw UPPT samples ranged from 8.20 to 25.8. The average niacin contents were compared across the 2 peanut varieties, 10 locations, and 2 years of production.

Table 1. Mean niacin concentration (mg/100g) of raw samples by variety and year.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Year 2007 Mean Content (mg/100g) ± SD</th>
<th>Year 2008 Mean Content (mg/100g) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Florunner</td>
<td>14.6 ± 3.42 a</td>
<td>16.4 ± 3.75 b</td>
</tr>
<tr>
<td>NC7</td>
<td>17.4 ± 3.41 bc</td>
<td>18.9 ± 4.62 ad</td>
</tr>
<tr>
<td>Core of the Core</td>
<td>-</td>
<td>16.8 ± 4.31</td>
</tr>
</tbody>
</table>

1 SD - standard deviation for 3 replicates per sample.
2 Numbers in columns followed by different letters are significantly different (p<0.05).

Niacin content was significantly higher in NC7 than Florunner samples for both years of production (p<0.05). Samples grown in 2007 had significantly lower niacin contents compared to those grown in 2008 (p<0.05) (Table 1). There was also a significant difference between growing locations (p<0.05) (Table 2, Figures 2-3).
Table 2. Mean niacin concentration (mg/100g) of raw UPPT samples by location.

<table>
<thead>
<tr>
<th>Location</th>
<th>Year</th>
<th>Florunner</th>
<th>NC7</th>
<th>Florunner</th>
<th>NC7</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean Content (mg/100g) ± SD</td>
<td></td>
<td>Mean Content (mg/100g) ± SD</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2007</td>
<td>(mg/100g) ± SD</td>
<td>2008</td>
<td>(mg/100g) ± SD</td>
<td></td>
</tr>
<tr>
<td>VA</td>
<td>8.15 ± 0.998 a</td>
<td>13.6 ± 0.120 a</td>
<td>16.3 ± 1.43 ab</td>
<td>15.9 ± 0.588 a</td>
<td></td>
</tr>
<tr>
<td>NC</td>
<td>12.6 ± 0.902 b</td>
<td>16.2 ± 0.883 bc</td>
<td>11.3 ± 0.141 b</td>
<td>12.8 ± 0.357 b</td>
<td></td>
</tr>
<tr>
<td>SC</td>
<td>13.6 ± 0.410 bcd</td>
<td>25.2 ± 0.764 d</td>
<td>17.9 ± 2.98 a</td>
<td>16.3 ± 1.13 a</td>
<td></td>
</tr>
<tr>
<td>GA</td>
<td>15.4 ± 0.816 cd</td>
<td>14.9 ± 1.08 ac</td>
<td>16.2 ± 1.28 ab</td>
<td>16.6 ± 1.83 a</td>
<td></td>
</tr>
<tr>
<td>AL</td>
<td>15.9 ± 3.03 d</td>
<td>17.5 ± 1.95 bf</td>
<td>15.5 ± 1.34 ab</td>
<td>16.2 ± 0.271 a</td>
<td></td>
</tr>
<tr>
<td>FL</td>
<td>13.1 ± 0.474 bc</td>
<td>18.2 ± 0.978 ef</td>
<td>12.4 ± 2.74 b</td>
<td>15.4 ± 1.26 a</td>
<td></td>
</tr>
<tr>
<td>C-TX</td>
<td>-</td>
<td>-</td>
<td>19.6 ± 0.627 a</td>
<td>24.1 ± 0.858 c</td>
<td></td>
</tr>
<tr>
<td>S-TX</td>
<td>14.6 ± 0.839 bcd</td>
<td>19.3 ± 0.516 eg</td>
<td>18.5 ± 0.624 a</td>
<td>24.5 ± 0.135 c</td>
<td></td>
</tr>
<tr>
<td>W-TX</td>
<td>18.6 ± 0.661 e</td>
<td>20.9 ± 0.462 g</td>
<td>20.3 ± 0.666 a</td>
<td>25.8 ± 0.665 c</td>
<td></td>
</tr>
<tr>
<td>OK</td>
<td>19.8 ± 2.22 e</td>
<td>14.5 ± 0.524 ac</td>
<td>20.5 ± 2.56 a</td>
<td>21.9 ± 0.895 d</td>
<td></td>
</tr>
</tbody>
</table>

1 Florunner and NC7 are peanut varieties.
2 SD - standard deviation for 3 replicates per sample.
3 Numbers in columns followed by different letters are significantly different (p<0.05).
Figure 2. Niacin concentration (mg/100g) of raw Florunner by year and location.

For the 2007 Florunner samples, OK and W-TX had significantly higher niacin contents and VA had significantly lower niacin contents than the remaining growing locations (p<0.05). In addition, AL was statistically different from FL and NC. NC was also statistically different from GA (p<0.05). Florunner samples from 2008 grown in SC, C-TX, S-TX, W-TX and OK were significantly higher in niacin content than samples grown in NC and FL (p<0.05).
For the 2007 NC7 samples, SC was statistically different from all other growing locations (p<0.05). W-TX was significantly higher than VA, NC, GA, AL, FL and OK, while S-TX was statistically higher than VA, NC, GA, AL and OK (p<0.05). FL was also statistically different from VA, NC, GA and OK; AL was statistically different from VA, GA and OK; and NC was statistically different from VA (p<0.05). For the 2008 NC7 samples, C-TX, S-TX and W-TX had significantly higher niacin contents than OK, which was significantly higher than VA, SC, GA, AL and FL, which were significantly higher than NC (p<0.05). There was a general increase in niacin content of raw peanuts in the western growing locations compared to the eastern growing locations, particularly for the 2007 and 2008 Florunner and 2008 NC7 samples. This trend was similar for the 2007 NC7 samples, with the exception of SC. The higher niacin content in these peanuts may be a result of more
favorable climate conditions that occurred during harvest, as well as the genetic variability of the plant.

Because the same varieties were grown in each location, the differences must be related to the environment. Temperature, water, and light are all important factors in vitamin synthesis (Gustafson 1953). Temperature alters reaction rates and influences the kinetic properties of enzymes within the plant (Smirnoff 1995). Optimum growth temperatures for peanut plants range between 27-30°C during daylight, which is characteristic among the 10 growing locations seen in this study (Ketring and others 1982, Gustafson 1949, Brumley and Sosebee 1978). Lower temperatures, often reached at night, tend to decrease photosynthetic rate, in turn slowing metabolic processes in the plant (Smirnoff 1995, Brumley 1974).

TX and OK have hotter days and cooler nights when compared to the other growing locations (Canty and others 2010). The higher levels of niacin content seen in peanuts grown in the western region have a similar pattern to that of sugar content in peanuts (Casini and others 2003, McMeans and others 1990). The changes in temperature from day to night influence metabolic rate, resulting in differential effects on enzymes and pathways, such as carbohydrate metabolism and respiration (Smirnoff 1995). During dark respiration, sugars, as well as other nutrients, are utilized for metabolic processes (Smirnoff 1995). Respiration is slowed when temperatures are lower, resulting in less sugar being used at night (Burke and others 2009). With less sugar being converted to ATP, it accumulates and is stored (Burke and others 2009, Rolland and others 2002). With sugar synthesis slowed, sugar stores build up, increasing the amount of sugars present in the peanut seed (Leegood 1995).
As with sugars, the significantly higher concentration of niacin in peanuts grown in TX and OK could be a result of these diurnal fluctuations. NAD and NADP, two major components of niacin, are coenzymes for several dehydrogenase enzymes, which are essential for photosynthesis and respiration (Brumley 1974, Orten and Neuhaus 1970). These coenzymes accept electrons from substrates, in which NAD then oxidizes lipids and carbohydrates present in the plant, generating ATP (Brumley 1974). NAD is essential in the mitochondrial electron transport chain and the tricarboxylic acid (TCA) cycle (Brumley 1974). During dark respiration, when temperatures are lowest, less vegetative and reproductive growth occurs in plants (Ketring and others 1982). Since the plant is not growing exponentially and does not need as much nutrients for growth, metabolic processes, such as the TCA cycle, slow (Ketring and others 1982, Smirnoff 1995). This decreases the need to use and synthesize nutrients, including niacin. Therefore, nutrients present or slowly synthesized through the night are compartmentalized and stored, which would have otherwise been utilized for nutrient synthesis and metabolism (Ketring and others 1982). Because of this, plants which are grown in environments that experience these lower night temperatures are often richer in vitamins compared to plants grown in warmer night temperatures (Gustafson 1949). Gustafson (1949, 1953) found that at decreased night temperatures, niacin tends to be more abundant.

In addition to temperature, light also plays a role in vitamin synthesis. Light supplies energy for photosynthesis and the variation of light throughout the day has an immediate effect on plant metabolism (Smirnoff 1995). Light increases niacin biosynthesis; therefore, during the night, vitamin synthesis slows (Mozafar 1993). Since vitamin synthesis and
requirement is reduced due to lack of light and cooler night temperatures, respiration and general plant metabolism slows; however, any excess niacin will accumulate in the plant (Smirnoff 1995).

In order to determine the effect of roasting on niacin content, the 2008 UPPT samples were roasted to a predetermined Hunter L value of 50 ± 1 (HunterLab 2008). Samples from each variety for all growing locations were analyzed, with the exception of GA due to lack of sample (Table 3).

Table 3. Mean niacin concentration (mg/100g) of roasted Florunner and NC7 samples for 2008 by location.

<table>
<thead>
<tr>
<th>Location</th>
<th>Florunner Mean Content (mg/100g) ± SD^1</th>
<th>NC7 Mean Content (mg/100g) ± SD^1</th>
<th>Florunner Mean Content (mg/100g) ± SD^1</th>
<th>NC7 Mean Content (mg/100g) ± SD^1</th>
</tr>
</thead>
<tbody>
<tr>
<td>VA</td>
<td>14.0 ± 0.110 ab</td>
<td>19.7 ± 1.02 a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NC</td>
<td>19.1 ± 0.739 c</td>
<td>16.3 ± 0.184 b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SC</td>
<td>16.7 ± 0.639 d</td>
<td>17.8 ± 0.497 c</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GA</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AL</td>
<td>19.6 ± 0.287 c</td>
<td>15.6 ± 0.183 b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FL</td>
<td>15.7 ± 0.462 e</td>
<td>16.3 ± 0.697 b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-TX</td>
<td>13.4 ± 0.336 b</td>
<td>21.7 ± 0.663 d</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S-TX</td>
<td>12.2 ± 0.335 f</td>
<td>22.4 ± 0.702 d</td>
<td></td>
<td></td>
</tr>
<tr>
<td>W-TX</td>
<td>14.1 ± 0.667 ab</td>
<td>21.3 ± 0.966 d</td>
<td></td>
<td></td>
</tr>
<tr>
<td>OK</td>
<td>14.7 ± 0.429 a</td>
<td>18.7 ± 0.968 ac</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

^1 SD - standard deviation for 3 replicates per sample.
^2 Numbers in columns followed by different letters are significantly different (p<0.05).

Niacin concentration (mg/100g) ranged from 12.2 to 19.6 for Florunner and from 15.6 to 22.4 for NC7 samples. There was a statistically significant difference between roasted Florunner and NC7 varieties (p<0.05). The roasted samples exhibited similar trends between
individual growing locations as their raw counterpart. Three Florunner and 4 NC7 samples increased in niacin concentration after roasting, while 6 Florunner and 5 NC7 samples decreased.

The mean niacin content (mg/100g) of raw 2008 UPPT samples were then compared to the mean niacin content (mg/100g) of roasted 2008 UPPT samples (Table 4, Figures 4-5).

**Table 4.** Mean niacin concentration (mg/100g) of 2008 raw and roasted samples by peanut variety and condition.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Condition</th>
<th>Mean Content (mg/100g) ± SD</th>
<th>Florunner</th>
<th>NC7</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Raw</td>
<td>16.4 ± 1.61 a</td>
<td>19.0 ± 0.799 b</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Roasted</td>
<td>15.5 ± 0.445 a</td>
<td>18.9 ± 0.654 b</td>
<td></td>
</tr>
</tbody>
</table>

1 SD - standard deviation for 3 replications per sample.
2 Numbers in columns rows followed by different letters are significantly different (p<0.05).

**Figure 4.** Niacin concentration (mg/100g) of raw and roasted 2008 Florunner samples across locations.
Niacin is an extremely stable vitamin that can withstand exposure to heat and light (Morris and others 2004). Significant differences were not found between 2008 raw and roasted samples of either variety (p>0.05). This is advantageous since the majority of peanuts consumed in the US are roasted (American Peanut Council).

Select samples from the Core of the Core peanut germplasm were analyzed to obtain an estimate of the range of niacin occurring in some of the germplasm collection. This range, if large, might indicate the potential to use conventional breeding to increase niacin content in new varieties. Thirteen Core of the Core samples grown in 2008 were analyzed (Figure 6).
Figure 6. Mean niacin concentration (mg/100g) of Core of the Core samples.

Niacin content (mg/100g) ranged from 13.0 to 19.3. Although this study was limited by taking three subsamples from each available sample instead of obtaining three individual replicates, the analytical variability suggests that there is a statistical difference among the samples (p<0.05). With this, there is potential for conventional breeding to increase niacin concentration in future breeding lines.

A relationship exists between the amount of niacin present in a peanut sample and the environment in which it was grown. Peanuts grown in regions with lower night temperatures tend to have increased niacin contents, since respiration and metabolic processes are slowed at cooler temperatures. With these metabolic processes not requiring the typical amount of vitamins, niacin is not used and accumulates in the plant. The significant differences between niacin concentration, peanut variety and growing location suggest that germplasm differences
may be sufficient for increases in levels of this vitamin through conventional breeding, providing consumers with a natural source of niacin.
Literature Cited


CHAPTER 3:
Comparison of Total Folate Contents and Individual Folate Vitamers in Raw and Roasted Peanuts Grown in Varying Locations and Crop Years
Abstract

Folate is the term for a related group of compounds with vitamin activity important in DNA biosynthesis. Adequate levels of folate intake have been found to reduce levels of a variety of syndromes, most notably fetal neural disorders. Peanuts (Arachis hypogaea L.) are known to provide sources of several important B-vitamins, including folate. Current information about the concentrations and the specific types of folates present may position peanuts as an important source of this nutrient. For this study, raw and roasted samples from the 2008 Uniform Peanut Performance Trials (UPPT) and several lines of the Core of the Core peanut germplasm collection grown in Tifton, GA in 2004 and in Lewiston, NC in 2008 were analyzed using high-performance liquid chromatography-mass spectrometry (HPLC-MS) to compare total folate content and individual folate vitamers across varying locations and germplasm material. Total folate content (µg/100g) in raw Core of the Core samples ranged from 207.7 to 378.3 in 2004 and 116.0 to 257.9 in 2008. The average total folate content (µg/100g) for raw Florunner and NC7 samples was 176.2 and 136.5, respectively, and 78.9 and 72.0 for roasted Florunner and NC7 samples, respectively. There was a significant difference between Florunner and NC7 varieties, but not among growing locations. Of the individual folate vitamers, 5-methyl-tetrahydrofolate (THF) and 5-formyl-THF were found in the highest concentrations. These vitamers donate single carbons for one-carbon metabolism and are the most bioavailable in plants and animals. These results suggest that germplasm differences exist which aid in increasing levels of folate through conventional breeding.
Introduction

Folate, or pteroylglutamate, is a term used to designate a variety of compounds that have vitamin activity similar to that of pteroylglutamic acid (Blakely and Benkovic 1984, Davis 1986, Krishnaswamy and Nair 2001, Gregory 1997). Folate (vitamin B9) is water soluble and exists in a variety of chemical forms, which can occur naturally in food as dietary folate or synthetically as folic acid, used to fortify foods and supplements (Bailey 1998, Office of Dietary Supplements 2005, Ravanel and others 2001). The basic structure of folate is comprised of a pterin core ring structure. This structure is conjugated to para-aminobenzoic acid (pABA) via a methylene bridge, which forms pteroic acid. The carboxyl group of pABA is linked by a peptide bond to alpha-amino groups of the glutamate molecule which connect to form folate (Moat and others 2004, IUPAC-IUB 1986, Caudill 2004, Rebeille and others 2006, Basset and others 2005, Bailey 1998).

Folate derivatives are characterized by the state of oxidation of the pteridine ring, the substituted one-carbon units, and the number of attached glutamate residues (Rebeille and others 2006, Folic Acid 2008, Seyoum and Helhub 1998). The nine most common forms, or derivatives, of folate include dihydrofolate (DHF), tetrahydrofolate (THF), folic acid (FA), 5-methyl-THF, 5-formyl-THF, 10-formyl-THF, 10-formyl-FA, 5,10-methenyl-THF, and 5,10-methylene-THF (Dang and others 2000, Molloy and Scott 2001, Basset and others 2005, De Brouwer and others 2007, Hawkes and Villota 1989). The predominant dietary folates include DHF, THF, 5-methyl-THF, and 5-formyl-THF, with the most bioavailable being THF, 5-methyl-THF and 5-formyl-THF (Phillips and others 2005). 5-methyl-THF provides the methyl group required to convert homocysteine into methionine, 5-formyl-THF is
required for the biosynthesis of purines and pyrimidines in DNA and RNA, and 5,10-
methylene-THF is necessary for the conversion of dUMP to dTMP and synthesis of pantothenic acid (vitamin B5) (Gregory 1989).

Humans are unable to synthesize folate de novo; therefore, it must be obtained from the diet. Dietary sources include green leafy vegetables, cooked dried beans, fruit, cereals, organ meats, legumes and peanut products (Subar and others 1989, Gregory 1997, Gregory and others 1984, Garratt and others 2005). Most plant products have a folate content ranging from 30-290 micrograms (µg)/100 gram (g) fresh weight (Scott and others 2000). Since this content is quite low, there is a potential to enhance folate concentration by conventional breeding techniques (Zhang and others 2005).

The Daily Reference Intake (DRI) for folate is 400µg/day (d) for men and women (Griel and others 2004, Voutilainen and others 2001, Rebeille and others 2006, Subar and others 1989). It is recommended that children of both genders between 1 and 3 years of age acquire 150µg/d, while those between 9 and 13 years of age acquire 300µg/d. Like adults, adolescents from 14 to 18 years of age should consume 400µg/d (Folacin 2001, Davis 1986). No adverse effects have been associated with the consumption of dietary folate in excess; however, an Upper Intake Level (UL) of 1000 µg/d of folate for all adults has still been established (Bailey 1998).

Folate deficiency is characterized by an imbalance in the folate pool essential for DNA synthesis and cell repair, along with an imbalance in DNA methylation and genomic instability (Folic Acid 2008). Because cells are unable to synthesize DNA and properly regulate gene expression, altered rate of cell division and severe metabolic repercussions are
likely to occur (Gambonnet and others 2001). Several metabolic consequences possibly related to increased risk include neural tube defects (NTDs), hyperhomocysteinemia, cardiovascular disease (CVD), cancer, megaloblastic anemia, cervical dysplasia, periodontal disease, vitiligo, and impaired immune response (Rebeille and others 2006, Alternative Medicine Review 2006, Folic Acid 2008, Gregory 2004, De Brouwer and others 2007, Shane 1995). Consuming adequate amounts of folate appears to be protective against some of these diseases, notably NTDs and cancer (Anonymous 2009, Kim and others 2009, Oaks and others 2010).

Although folic acid is more stable and more readily used in fortification of foods compared to dietary folate, it is thought to cause undesirable effects when consumed in high quantities. Folic acid, when consumed in amounts that exceed the rate in which human mucosa can metabolize it, can mask the diagnosis of anemia, increase the progression of neuropathy and promote tumor growth (Anonymous 2009, Hirsch and others 2009, Scott and others 2000, Sweeney and others 2009, Oaks and others 2010).

As previously mentioned, humans are unable to synthesize folate, meaning it must be consumed via plant products. Folate synthesis is closely controlled and modified in relation to the metabolic requirements which differ from one tissue to another and fluctuate during plant development (Rebeille and others 2006). Enzymes used to synthesize folate increase in germinating seeds due to the continuation of active cell metabolism (Smith and others 2007). Folates are active in the production and maintenance of new cells and for cell division (Office of Dietary Supplements 2005, Scott and others 2000). In order to produce and maintain new plant cells, folate has the capacity to transfer single-carbon units for one-
carbon metabolisms (Rebeille and others 2006). The three main oxidation states in which folate can donate single carbons include 5-methyl-THF, 5-formyl-THF, and 5,10-methylene-THF (Jabrin and others 2003).

Despite the fact that the term folate tends to be generalized as a combination of the various folate compounds, individual folate compounds contribute differently to the folate pool. Separation of the folate compounds is dependent on the substitutions on the pteridine ring (Clifford and Clifford 1977, Hannon-Fletcher and others 2004). Because certain folate compounds may be found more readily in plants or used more often for one-carbon metabolism in humans, it is important to distinguish between the folate derivatives.

There are several ways in which folate compounds can be identified and quantified. For decades, microbiological assays (MAs) have been the most widely-used standard method to determine folate content of food products (Cossins and Chen 1997, Gambonnet and others 2001, Rychlik 2004). MAs utilize Lactobacillus casei ATCC 7469, a lactic acid-forming bacterium, which requires the presence of folate to promote growth (Gambonnet and others 2001, Hawkes and Villota 1989). MAs do not distinguish between the single folate compounds; therefore, MAs can only be used when total folate concentration is desired (Scott and others 2000, Rychlik 2004, Freisleben and others 2003). High-performance liquid chromatography (HPLC) is another method used for analyzing folate by separating, isolating, identifying and quantifying the various folate derivatives (Cossins and Chen 1997, Waters 1995). Although HPLC is used to extract and quantify individual folate compounds, it is susceptible to matrix interferences and may overestimate folate content in foods (Freisleben and others 2003, Periago and others 2008). HPLC-Mass Spectrometry (MS) is useful since
the HPLC component separates individual compounds while the MS produces mass spectral data which identifies the structure, quantity, molecular weight, purity, and identity of the sample (Agilent Technologies 2001).

Because quantifying and characterizing folates is difficult because of their instability and wide array of forms, data currently available on dietary folates in food databases are often contradictory (Jastrebova and others 2003). The purpose of this research was to quantify folate levels in a variety of peanuts grown in different locations and crop years. Information about the concentrations and specific types of folates present may assist in making dietary recommendations, determining the variability among genotypes and determining if all folate forms decrease at the same rate during roasting.

**Materials and Methods**

**Materials.** Twelve lines in the Core of the Core peanut germplasm collection grown in 2004 in Tifton, GA and 2008 in Lewiston, NC, for a total of 24 samples, were available for analysis (Table 1) (Holbrook and others 1993). Data for line PI 476596 was unknown.

**Table 1.** Core of the Core peanut germplasm line characteristics.

<table>
<thead>
<tr>
<th>PI Number</th>
<th>Origin</th>
<th>Pod Type</th>
<th>Growth</th>
<th>Seed Color</th>
</tr>
</thead>
<tbody>
<tr>
<td>493581</td>
<td>Argentina</td>
<td>Valencia</td>
<td>-- (^1)</td>
<td>Red</td>
</tr>
<tr>
<td>493880</td>
<td>Argentina</td>
<td>--</td>
<td>Erect</td>
<td>Red</td>
</tr>
<tr>
<td>337406</td>
<td>Paraguay</td>
<td>Valencia</td>
<td>Spreading and Bunch</td>
<td>Purple</td>
</tr>
<tr>
<td>313129</td>
<td>Taiwan</td>
<td>Valencia</td>
<td>Spreading</td>
<td>--</td>
</tr>
<tr>
<td>356004</td>
<td>Argentina</td>
<td>Valencia</td>
<td>Bunch</td>
<td>Red</td>
</tr>
<tr>
<td>259617</td>
<td>Cuba</td>
<td>Valencia</td>
<td>Bunch</td>
<td>Red</td>
</tr>
<tr>
<td>296550</td>
<td>Israel</td>
<td>Virginia</td>
<td>--</td>
<td>Pink</td>
</tr>
<tr>
<td>325943</td>
<td>Venezuela</td>
<td>Valencia</td>
<td>Spreading and Bunch</td>
<td>Red</td>
</tr>
<tr>
<td>476636</td>
<td>Nigeria</td>
<td>Virginia</td>
<td>Spreading and Bunch</td>
<td>Pink</td>
</tr>
<tr>
<td>372305</td>
<td>Nigeria</td>
<td>Virginia</td>
<td>Spreading and Bunch</td>
<td>Mixed</td>
</tr>
<tr>
<td>407667</td>
<td>Thailand</td>
<td>Spanish</td>
<td>Bunch</td>
<td>Tan</td>
</tr>
</tbody>
</table>

\(^1\) Cells with dash (--) indicate unknown data.
Six samples, 3 Florunner (medium grade size) and 3 NC7 (Extra Large Kernel {ELK} grade size), were obtained from the 2008 UPPT collection. For each variety, the samples were grown in Lewiston, NC (NC); Marianna, FL (FL) and College Station, TX (TX). A portion of these samples was roasted at 167°C for 17-20 minutes to a Hunter L value of 50 ± 1 (HunterLab 2008, Sanders and others 1989). In all, 12 samples from the 2004 Core of the Core, 12 samples from the 2008 Core of the Core, 6 raw UPPT samples, and 6 roasted UPPT samples were analyzed.

**Folate Analysis.** The Core of the Core and UPPT samples were analyzed for total folate and individual folate derivatives (FA, 5-methyl-THF, 5-formyl-THF, THF, and 10-formyl-FA) by Medallion Laboratories of Minneapolis, MN. A tri-enzyme extraction method was utilized. The enzymes alpha-amylase, protease and chicken pancreas conjugase were used to break down total folate into the individual monomer forms.

**Statistical Analysis.** Statistics were performed using a mixed model with no interaction terms to test the significance of the growing location, variety (Florunner versus NC7), and condition (raw versus roasted) on folate content. To test all pairwise comparisons among means, Tukey’s honest significant difference test and Duncan’s multiple range test were used. All statistics were performed using SAS (SAS Institute Inc. 2004).

**Results and Discussion**

HPLC requires that the folate derivatives be de-glutamated into a monomer form by pre-treating with a conjugase enzyme (Garratt and others 2005). Coupling this with MS allows for analysis and quantification of all folate derivatives in their monomer form (Garratt and others 2005). Total folate and individual folate vitamer concentrations were obtained for
the 2004 and 2008 raw Core of the Core samples and for the 2008 raw and roasted UPPT samples via the HPLC-MS folate extraction method. Total folate content is the sum of the individual folate vitamers. Folate concentration (µg/100g) for Core of the Core samples ranged from 207.7 to 378.3 in 2004 and 116.0 to 257.9 in 2008 (Figure 1).

![Figure 1](image)

**Figure 1.** Total folate content (µg/100g) of 2004 and 2008 Core of the Core samples.

Core of the Core samples grown in 2004 have a significantly higher concentration of total folate compared to 2008 samples (p<0.05). The average total folate concentration (µg/100g) for the 2004 and 2008 Core of the Core samples is 264.8 and 195.9, respectively. The pattern seen in Figure 1 indicates that despite the significant variability among samples grown in the same year (p<0.05), the folate concentration of each sample decreased approximately the same amount from 2004 to 2008. The deviation in vitamin concentration
from one year to the next is thought to be caused by the variation in the amount of light, the magnitude and distribution of temperature, and the amount of precipitation that occurred throughout the year (Mozafar 1994). For each year, the samples were grown in one location and were exposed to one set of environmental conditions, suggesting the influence of geographic isolation on genetic variability (Moretzsohn and others 2004). The differences in the samples within the same year can be explained by distinctions in the genetic composition (Dean and others 2009). Peanut plants exhibit considerable amounts of variation in morphological, physiological and agronomic characteristics (Moretzsohn and others 2004, He and Prakash 2001). Six morphological variables that influence genetic variability include plant type, pod type, seed size, seed color, number of seeds per pod and seed weight (Upadhyaya and others 2002).

In comparison to the HPLC-MS method, Dean and others (2009) utilized the MA method to quantify folate from various Core of the Core samples. The results obtained with the HPLC-MS method were, on average, higher than the results obtained with the MA method. MAs utilize lactic acid-forming bacteria to determine total folate content (Gambonnet and others 2001). Folates are required for growth of the bacteria (Hawkes and Villota 1989). HPLC-MS is more sensitive than MA, which supports the hypothesis that HPLC-MS would produce higher folate levels (Freisleben and others 2003). This sensitivity allows for reduced susceptibility to interferences within the matrix. In addition, HPLC-MS has more selective sample clean up procedures, including a higher affinity for the folate binding protein (Freisleben and others 2003).
The average total folate content (µg/100g) for the raw Florunner and NC7 samples was 176.2 and 136.5, respectively. The total folate content (µg/100g) of roasted Florunner and NC7 samples was 78.9 and 72.0, respectively (Table 2).

**Table 2.** Total folate concentration (µg/100g) of raw and roasted UPPT samples by location.

<table>
<thead>
<tr>
<th>Location</th>
<th>Raw Florunner</th>
<th>Raw NC7</th>
<th>Roasted Florunner</th>
<th>Roasted NC7</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC</td>
<td>211.6 ± 9.66 a</td>
<td>117.9 ± 1.03 a</td>
<td>78.91 ± 3.83 a</td>
<td>78.41 ± 3.29 a</td>
</tr>
<tr>
<td>FL</td>
<td>153.2 ± 0.13 a</td>
<td>147.4 ± 2.69 a</td>
<td>77.93 ± 4.36 a</td>
<td>53.48 ± 3.77 a</td>
</tr>
<tr>
<td>TX</td>
<td>163.7 ± 5.08 a</td>
<td>144.1 ± 11.26 a</td>
<td>79.79 ± 1.69 a</td>
<td>84.10 ± 1.28 a</td>
</tr>
</tbody>
</table>

1 Florunner and NC7 are peanut varieties.
2 SD - standard deviation.
3 Numbers in columns followed by different letters are significantly different (p<0.05).

There was no statistical difference between growing locations for Florunner or NC7 samples, regardless of roasting condition (p>0.05). Raw samples had significantly higher folate concentrations than roasted samples, regardless of variety (p<0.05). Raw NC7 samples had significantly lower folate contents than raw Florunner samples (p<0.05); however, there was no difference between varieties for roasted samples (p>0.05) (Figure 2).
Figure 2. Total folate content (µg/100g) of raw and roasted UPPT samples.

Unlike other B-vitamins, folates are extremely reactive and soluble. This makes them susceptible to potential degradation during storage and heat processing, which explains why roasting caused a significant decrease in folate concentration (Scott and others 2000, Morris and others 2004).

In addition to total folate content, individual folate vitamer concentration was analyzed for both the Core of the Core and UPPT samples (Figure 3-4).
Figure 3. Concentration (µg/100g) of individual folate vitamers for 2004 and 2008 Core of the Core samples.¹

¹ Groups followed by an asterisk (*) are significantly different from groups not followed by an * (p<0.05).
Figure 4. Concentration (µg/100g) of individual folate vitamers for raw and roasted UPPT samples. Groups followed by different letters are significantly different (p<0.05).

Both figures indicate that of the individual folate vitamers, 5-methyl-THF and 5-formyl-THF were in the highest concentrations, regardless of variety or condition. For both the Core of the Core and UPPT samples, 5-methyl-THF and 5-formyl-THF had significantly higher mean contents than the remaining folate vitamers (p<0.05). Regarding the Core of the Core vitamers, samples grown in 2004 had significantly higher concentrations of FA, 5-methyl-THF and THF than samples grown in 2008 (p<0.05). In contrast, samples grown in 2004 had significantly lower concentrations of 5-formyl-THF and 10-methyl-FA than samples grown in 2008 (p<0.05). There was no difference in 10-formyl-FA content between years (p>0.05). Raw UPPT samples had statistically higher concentrations of 5-methyl-THF, 5-formyl-THF and THF compared to roasted samples (p<0.05). This was expected since folate is heat labile.
(Morris and others 2004). Although the concentration of each THF vitamer decreased after roasting, the extent of degradation differed. For example, roasting caused 5-methyl-THF to decrease 42.61 µg/100g and 27.17 µg/100g in Florunner and NC7 samples, respectively. Similarly, 5-formyl-THF was reduced by 34.61 µg/100g and 30.81 µg/100g in Florunner and NC7 samples, respectively. In contrast, THF decreased 10.13 µg/100g in Florunner samples and 12.07 µg/100g in NC7 samples. For the two vitamers with the largest concentration differences, roasting had a more significant effect on Florunner samples than NC7 samples. Despite the decrease in THF vitamers during roasting, there was not a significant decrease in the FA vitamers (p>0.05). FA, a possible breakdown product during folate extraction, is not heat labile (Scott and others 2000). In addition, there were significantly higher contents of 5-formyl-THF and 10-formyl-FA in Florunner samples than NC7 samples (p<0.05).

It is noteworthy that 5-methyl-THF and 5-formyl-THF are in the highest amount since they are the most bioavailable, compared to the other folate derivatives (Phillips and others 2006). 5-methyl-THF and 5-formyl-THF also donate single carbons for one-carbon metabolism in plants and animals (Jabrin and others 2003). Scott and others (2000) agreed that 5-methyl-THF, in its monoglutamate form, is the only significant form of folate found in the human circulation. The importance of these vitamers is evident in their function in both plants and humans.

There are some instances in which foods, particularly peanuts, are not a good source of folate since they do not contain 10-19% of the Daily Values (DV) (FDA 2009). However, since 5-methyl-THF and 5-formyl-THF are the most significant vitamers for human health, a food providing adequate amounts of these two vitamers may offer the same benefits as a food
that is designated as a good source of folate. Reevaluating the nutrition label and indicating which specific folate derivatives are present and in what quantity may be worthwhile. This would then allow foods, such as peanuts, that are not high enough in total folate to be considered a good source of this vitamin, to be recognized for its potential benefit.

The significant levels of 5-methyl-THF and 5-formyl-THF, in addition to the significant differences between folate content, peanut variety and roasting condition, suggest that germplasm differences may be sufficient for an increase in levels of folate through conventional breeding.
Literature Cited


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CHAPTER 4:

Conclusions and Future Research
This research suggests that the concentrations of niacin and folate in peanuts are influenced by the variety of peanut, the environment in which it was grown, and whether or not it was subjected to roasting temperatures. Cooler night temperatures tend to decrease photosynthetic rate, which slows metabolic processes in plants (Smirnoff 1995). This decreased need for vitamins, particularly niacin, promotes compartmentalization and storage. In addition, roasting does not affect niacin content since it is stable to heat and light. In contrast, folates are extremely sensitive to heat, causing degradation of the vitamin when subjected to high temperatures (Morris and others 2004). Because of the nutritional importance of 5-methyl-THF and 5-formyl-THF, peanuts may provide beneficial effects to humans, even if they are not considered a good source of folate.

Future work is needed to determine the bioavailability, distribution and metabolism of folate and niacin in peanut plants (King and others 2008). Researchers are still unsure how the biosynthetic pathways of folate and niacin are regulated. This limits the overall understanding of vitamin synthesis in plants (Scott and others 2000). It is also unknown what specific roles folate and one-carbon metabolism may play during plant acclimatization to environmental conditions (Scott and others 2000). It is understood that a strong relationship exists between environmental and genetic factors; however, this relationship must be further investigated to increase the concentration of vitamins in plants (Mozafar 1993). In addition, the levels of niacin and folate in plants fluctuate, which may indicate that these vitamins can accumulate in plants under particular circumstances. Determining potential strategies to increase vitamin synthesis and accumulation in plants is necessary. These include over-
expressing the enzymes that are limiting steps for synthesis, reducing the catabolic rate of the cofactor, and increasing the stability of the cofactor (Scott and others 2000).

Further refinement and optimization of the methodology used for the extraction and quantification of individual folate compounds is warranted (Phillips and others 2006). There are a wide range of extraction methods available to analyze individual folate derivatives; however, no one method has proven superior to the others (Rychlik 2004). Since folate is heat labile, the method of extracting folate cannot employ heat (Scott and others 2000). Therefore, enzymes are needed to break the matrix to leave folate in its monomer forms (Rychlik 2004). Both single-enzyme and triple-enzyme methods have been developed to extract folate from raw food material. In single-enzyme methods, a conjugase is used to break up the folate molecules into its basic, monomer forms. Several conjugases utilized include rat plasma (Konings 1999), chicken pancreas (Rychlik and others 2007), human plasma (Hyun and Tamura 2005), and hog kidney acetone powder (Jastrebova and others 2003). Triple-enzyme methods involve alpha-amylase and protease in addition to a conjugase in order to break up the starches and proteins and dissolve the food so the conjugase could then extract out the folate molecules (Konings 1999). Several triple-enzyme methods included alpha-amylase, protease, and rat plasma (Phillips and others 2006); alpha-amylase, pronase, and human plasma (Chen and Eitenmiller 2007); and alpha-amylase, protease, and chicken pancreas (Tamura and others 1997). Comparing folate concentrations after using different conjugase enzymes will aid in determining which method is the most efficient. Researchers must also keep in mind that each treatment has inherent problems and there is no procedure that utilizes the conjugase treatment of folate that is entirely satisfactory thus far.
(Scott and others 2000). Simplifying the sample preparation and shortening the time required in preparing the assay will also aid in refining the methodology (Gregory and others 1984). Folate values generated by the standard MA, which have already been assigned to nonenriched food products, should be reassessed with the HPLC method in order to update the nutrient database (USDA ARS 2009).

Comparing additional varieties of peanuts over a wider range of production years will help researchers clarify whether conventional breeding or genetic modification of peanut plants is more appropriate to increase niacin and folate concentrations. Doing so will allow peanut breeders to optimize the amount of vitamins present in their crop, in turn providing consumers with a natural and good source of niacin and folate.
Literature Cited


