SELECTED NUTRIENT ANALYSES OF FRESH, FRESH-STORED, AND FROZEN FRUITS AND VEGETABLES

by

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(Under the Direction of Ronald B. Pegg)

ABSTRACT

The objective of this two-year study was to determine and compare the status of targeted nutrients in selected fresh, fresh-stored, and frozen fruits and vegetables, while mimicking typical consumer purchasing and storage patterns of the produce. The nutrients analyzed were L-ascorbic acid (vitamin C), β-carotene (provitamin A), and folate, while the produce was blueberries, strawberries, broccoli, cauliflower, corn, green beans, spinach, and green peas. Analyses were performed in triplicate on representative samples using approved, standardized analytical methods and included a quality control plan for each nutrient; all data were analyzed by one-way ANOVA to determine the presence of significant difference in nutrient contents according to treatment (α=0.05). The findings demonstrated that fresh produce loses vitamins upon refrigerated storage over time, while their frozen counterparts retain these nutrients equally so or better. The consumers' assumption that fresh food has significantly greater nutritional value than its frozen counterpart is misplaced.

INDEX WORDS: Fresh Fruits and Vegetables, Frozen Fruits and Vegetables, Nutrients, Vitamin C, Vitamin A, Food Folate.

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DEDICATION

This thesis is dedicated to my loving parents, Xiaoming Li and Zongqiong Ma, who love me and support me in every way. In addition this work is dedicated to my loving husband, Yi Chen, who supports and shares my hopes and dreams.

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CHAPTER 1

INTRODUCTION

1.1 Differences between fresh and frozen produce

Is fresh produce significantly richer in nutrients when compared to its frozen counterparts? When selecting frozen fruits and vegetables, are consumers sacrificing nutrition for convenience and value? This study aims to address these questions by assessing the status of select key nutrients in fresh and frozen varieties of 8 types of produce comparing levels at the point of purchase and 5 days later. Nutrient analyses were performed for vitamin C, vitamin A, and folate.

Consumer habits and spending have changed significantly from previous generations. According to a 2012 report on U.S. grocery shopping trends, the economic recession of the past few years still impacts consumer spending behavior and will continue to do so in the coming years. Consumers eat at restaurants less frequently to save money and yet spend less time preparing food at home. They are seeking quality foods that are healthy, affordable, and convenient (FMI, 2012).

The majority of consumers purchase fresh fruits and vegetables, because they believe such foodstuffs are exceptionally nutritious. The term “fresh” evokes images of coming straight from the vine or field at the peak of ripeness. In reality, fresh produce is often picked well before it is ripe. It is then packaged, stored, transported, and stored again. All fruits and vegetables are

alive, even after being picked. They respire, taking in atmospheric oxygen and expelling carbon dioxide. They also transpire and lose moisture, which commonly results in shrinkage. Moisture losses and respiration cannot be prevented once the produce has been removed from its original source of water and nutrients; hence, the produce is perishable and has a limited life before it deteriorates to a point where it becomes inedible. Fruits and vegetables deemed for freezing are usually picked at peak ripeness, then steam blanched (vegetables only), and processed to preserve their nutrients. During the winter months, a variety of fresh fruits and vegetables can be more difficult to find. They are also generally more expensive, and often tasteless. Conversely, frozen produce offers the consumer convenience, quality, and variety year round. These benefits are heightened when a superior product is used, good manufacturing practices are employed in the preservation process, and the products are kept at specific temperatures.

1.2 Frozen technologies

In the early 20th century, Clarence Birdseye observed Eskimos using ice, wind, and low temperatures to freeze just-caught fish to retain the freshness of the catch during long journeys. Inspired by this, he developed a flash freezing process that revolutionized the frozen food industry. Flash freezing is a process whereby produce, subjected to cryogenic temperatures, is frozen quickly, so as to minimize the size of ice crystals formed. They are small enough to preserve cell membranes, which ensure the proper texture when thawed. Additionally, this technique preserves fruits and vegetables at their maximum flavor, texture, and color (Hilder, 1930).

Nearly a century later, another American inventor, Daniel Tippmann, took this process a step further by producing a vacuum to draw cold air through palletized food, which is called the Quick Freeze system.

1.3 Healthful benefits of fruits and vegetables

Fruits and vegetables are important sources of a number of nutrients that are under-consumed in the U.S., including folate, vitamin A, vitamin C, dietary fiber, magnesium, and potassium. According to a 2013 CDC report, 33% of American adults consume fruits and vegetables less than one time a day, despite the fact that consumption of fruits and vegetables is associated with reducing the risk of many chronic diseases. Moreover, when prepared without added fats or sugars, fruits and vegetables are relatively low in calories, and help with satiety and aid healthy weight maintenance.

The issue of healthy weight is a growing concern in the U.S. It is such a major concern that on June 18, 2013, the American Medical Association (AMA) passed Resolution 420, which recognizes obesity as a disease. According to a study from RTI International (formerly the Research Triangle Institute), by 2030, 51% of the population will be obese. It is also estimated that medical costs resulting from the obesity epidemic may be as high as $147 billion per year (Finkelstein *et al.*, 2012).

In order to combat the epidemic and promote healthfulness, the 2010 U.S.D.A. Dietary Guidelines for Americans advise individuals to increase their intake of fruits and vegetables to help control total calorie intake and manage body weight. MyPlate, the revised U.S.D.A. Food Pyramid, suggests that half of the plate should be comprised of nutrient-dense foods, like fresh

and frozen fruits and vegetables. These new guidelines also highlight the importance of variety, which is necessary to give the body the large array of vitamins and nutrients it needs.

Unfortunately, getting this variety is made difficult by the multitude of steps required to get produce to the market free of spoilage, dirt and debris, pests, and pathogenic bacteria. Spoilage and nutrient loss begin immediately post-harvest, which is problematic because transportation from farms to grocery stores can take several days or even longer. Even after transport, produce can sit in the store for a few additional days before consumers purchase them for home use. Finally, several days further may lapse between purchase and consumption. In some cases, produce originating in different parts of the world must be shipped thousands of miles, thereby increasing transportation times and storage hazards. All of these factors contribute to the diminishing of nutrients in fresh produce taken "directly" from the field and brought to the dinner table. Conversely, processing and storage times for frozen produce are significantly reduced. Freezing protects and preserves sensory and nutritional qualities over long storage periods. It is also generally regarded as superior to canning and dehydration.

These studies make it clear that dietary change should take place to make sure Americans get more nutrients from the best possible sources, but this will only occur with a more educated consumer. One of the key findings of a 2012 study suggests that the trust consumers have in food production is related to the level of knowledge consumers feel they have about where their food comes from and the related production practices. Sadly, the same study also noted that "people know more about movies, politics, history and music than they do about food production" (Sullivan, Hidgon, & Sink, 2012).

1.4 Experiment design

In this study, 8 common fruits and vegetables were analyzed for targeted nutrients. All produce was sampled both fresh and frozen, three times a year for two years, beginning in the summer of 2011 and ending in the spring of 2013. Additionally, analyses were divided seasonally by spring-to-summer, summer-to-fall, and fall-to-winter. The types of produce examined included highbush blueberries (*Vaccinium* spp., such as *V. ashei, V. corymbosum* L. and *V. corymbosum x V. darrowii*), strawberries (*Fragaria x ananassa*), broccoli (*Brassica oleracea* var. *italic*), cauliflower (*Brassica oleracea* var. *botrytis*), corn (*Zea mays*), green beans (*Phaseolus vulgaris*), spinach (*Spinacia oleracea*), and green peas (*Pisum sativum*, sometimes referred to as English peas). Acquiring fresh green peas out of season was difficult. Although it was easier to find sugar snap peas, the present study was designed for English peas to correspond with the available frozen counterpart. Therefore, one set of 2 and one set of 4 fresh and fresh-stored green peas were analyzed consecutively, as available. Produce were collected from 6 food stores within a 40-km radius of Athens, GA. The selected stores were common U.S. supermarket chains and included Walmart, Sam's Club, Kroger, Publix, Piggly-Wiggly and Ingles. Bell's served as a backup in the event that one of the products could not be found at one of the original stores. In all cases, fresh fruits and vegetables were purchased in the early morning of the day of analysis. Samples were analyzed on the day of purchase and again 5 days later, comparable to average consumer purchasing and storage habits.

Upon receipt, fruits and vegetables were respectively prepared by combining roughly 200-g portions of produce from each market in a large grey plastic tub and mixing well. Details are provided in the individual chapters when a blanching step was necessary. An equal or larger

amount of the fresh sample remained and was returned to, and stored in, the original packaging for 5 days at 4 °C. On day 5, the fresh-stored produce was combined in the same fashion as the composite prepared on day 0. Representative fresh samples from each composite were taken on day 0 and day 5, for each of the nutrient analyses. Composite samples for mineral determinations (*NB*, not part of this study) were vacuum-packed and stored at -60 °C for later analysis en masse. Frozen composites and representative samples were prepared the same as fresh; however, remaining produce was vacuum packed and stored at -60 °C.

A blanching step, prior to *trans*-β-carotene analyses, was employed for the fresh and fresh-stored produce to inactivate endogenous enzymes which can cause deterioration, notably *via* oxidation, of flavor, color, and texture, as well as cause nutrient loss. As there was no steam system to mimic an industrial blanching operation, fresh and fresh-stored vegetables were blanched intact for 1 min in boiling water, immediately chilled in an ice water bath, and then tapped onto a dry paper towel to remove excess moisture. In the case of fresh and fresh-stored corn, kernels were cut from the cob just after blanching for all nutrient analyses. Frozen vegetables did not require blanching, because they had already been subjected to it by the industry before blast freezing. Extractions of folate, vitamin C, and *trans*-β-carotene were quickly performed, as a means to prevent nutrient degradation. Analyses were completed and moisture balances were performed so that the data could be presented on either a blanched weight (b.w.), fresh weight (f.w.), or dry weight (d.w.) basis. A primary goal of this study was to provide credible and meaningful data on the nutrient status of both fresh and frozen fruits and vegetables to be used as part of a marketing strategy to better educate consumers on the healthfulness of frozen produce.

This work is divided into six chapters, including the introduction as chapter one and a brief literature review in chapter two on the present knowledge of nutrients in fresh and frozen vegetables. The third chapter reports on the L-ascorbic acid contents in the produce samples according to AOAC Official Method 967.21; Vitamin C (ascorbic acid) in vitamin preparations and juices, 2,4-dichloroindophenol titrimetric method. The fourth chapter reports on the *trans*-β-carotene content, as a representation of vitamin A, in the produce samples according to European Standard Methods EN 12823-1 and -2. The fifth chapter reports on the folate content in the produce samples according to the classical trienzyme extraction microbiological assay; AOAC Official Method 2004.05 for the assay of food folate in cereal grain products. The final chapter summarizes the conclusions drawn from the study.

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CHAPTER 2

LITERATURE REVIEW

2.1 Frozen fruits and vegetables

The 2010 U.S.D.A. Dietary Guidelines for Americans suggest that the intake of nutrient-dense foods like fruits and vegetables should be increased to meet the needs for a healthy and balanced diet. In addition to being important sources of many key nutrients, frequent consumption of a variety of colorful fruits and vegetables is associated with reduced risk of many chronic diseases, including cardiovascular disease and type-2 diabetes. A report estimated that diets high in fruits and vegetables (more than 400 g per day) could prevent at least 20% of all cancer incidences (Glade, 1999). Evidence also showed that a diet rich in fruits and vegetables reduced blood pressure (Appel *et al.*, 1997).

Freezing is one of the easiest and fastest ways to preserve the quality and texture of fresh foods. Most vegetables retain their original color, texture, and flavor better when preserved by freezing rather than canning or some other method. In order to achieve the desired results, many factors must be considered in the freezing process, including methods employed, ice crystallization size, freezer burn, freezing rate, packaging, and moisture loss (Bejarano & Venetucci, 1995).

With the technological advancement and implementation of freezing techniques and cold-chain processing, mechanical refrigeration has been both affordable and popular since the early

1950s. Frozen varieties of fruits and vegetables provide a convenient solution for transport and storage, as well as an elongated shelf-life compared to their fresh counterparts. Since the advent of commercial freezing, frozen vegetable sales have grown steadily. Total U.S. retail sales of frozen foods reached more than $25 billion in 1999, while food service manufacturing sales totaled $40.6 billion. In addition to monetary increase, the consumption of frozen vegetables increased by 20% from 1980 to 2000 (Packaged Facts, 2009). However, a more recent report (Packaged Facts, 2012), indicates sales of frozen foods have been declining. The nation's slow economic recovery has contributed to this drop as well as changes in consumer eating habits, shopping patterns, and demographics. Additionally, the lack of excitement in frozen food categories and merchandising strategies is also responsible, as is competition from fresh and prepared fresh foods, shelf-stable foods, and restaurants. The situation presents challenges and opportunities for both marketers and retailers; it impels them to look for evidence supporting the benefits of frozen foods in order to re-attract customers.

There has been a great breadth of work done to examine the nutritional quality of fruits and vegetables, especially after changes related to processing and storage. Many factors affect this quality in produce, including enzyme activity and mechanical damage. Produce that is preserved *via* freezing should be harvested at the peak of ripeness and freshness to ensure optimum quality after thawing. Before vegetables can be frozen, a critical step of deteriorative enzyme inactivation is required. This process, known as blanching, is a mild heat treatment performed by steam, hot water, hot air, or microwave radiation. In addition to inactivating damaging enzymes, blanching helps to reduce microbial loads, and decrease interstitial gases prior to further processing. It is important to note that blanching in hot or boiling water, can

result in marked losses of water-soluble vitamins and minerals by leaching. The heat treatment in blanching may also cause some losses of other thermally-labile nutrients and bioactive compounds. Nevertheless, the inactivation of oxidative and other degradative enzymes (e.g., lipoxygenase for carotenoids, polyphenol and catechol oxidases for flavonoids, and ascorbic acid oxidase for vitamin C) will help prevent further and greater losses by enzyme-catalyzed degradation during frozen storage. Without deactivation of lipoxygenase and similar enzymes, the low temperatures of frozen storage only delay the activity of these enzymes, rather than stopping them completely. In addition to preventing degradation, Baardseth (1978) reported that blanched carrots, cauliflower, and French green beans possessed better flavor and taste attributes than their unblanched counterparts.

Another major quality attribute of concern is texture. In freezing the structure of living tissue is destroyed, which can greatly affect the texture. Next to flavor, the texture of food is a critical factor for consumers. Several studies indicated that the freezing rate, which directly affects texture, influences the quality of frozen vegetables, as it changes physical properties such as density. When temperature is decreased, the water inside tissues freezes and the ice crystals expand causing cell wall destruction. Freezing as quickly as possible (*i.e.*, blast freezing) can reduce the size of the ice crystals and, therefore, reduce the magnitude of cell wall destruction, which is important because most fruits and vegetables contain over 90% water. When the product is thawed, the texture is softer than the raw product. This change of texture is especially important to note for vegetables that are then cooked before eating, because cooking further softens the food.

2.2 Specific nutrient studies

Many of the studies performed on fresh and frozen produce have been conducted to examine levels of specific nutrients and their changes over time. One such nutrient is L-ascorbic acid which degrades constantly during storage. The rate of degradation can be slowed by freezing, although a change of moisture content may also be responsible. Rickman, Barrett, & Bruhn (2007a) reported that ascorbic acid in frozen broccoli and green peas showed a similar degree of loss when compared with fresh-stored samples. However, frozen spinach and green beans only lost 20 to 30% of L-ascorbic acid after 12 months of storage, while the fresh counterparts lost over 70%. Favell (1998) found that although L-ascorbic acid levels in fresh broccoli, green beans, spinach, and carrots were generally higher, the losses of L-ascorbic acid in frozen products over a 12-month storage period were quite minimal. Phillips *et al.* (2010) investigated the retention of vitamin C in raw fruits and vegetables during the freezing process. The results showed a cumulative decrease of vitamin C in frozen oranges and potatoes at the beginning of the storage period, but noted no significant change after a prolonged period. The same findings were also reported by multiple other studies (Howard, Wong, Perry, & Klein, 1999; Patras, Tiwari, & Brunton, 2011; Hooda, & Kawatra, 2012). Martins and Silva (2004) reported that L-ascorbic acid levels in green beans stored at -18 °C decreased to 46% in the first month and decreased to 21.2% of its initial value after being stored for a 60-d period. Similarly Tosun and Yücecan (2007) found significant decrease in vitamin C content after a 6-month storage at -18 °C resulting in a total of 42.4% loss for green beans and 66.5% loss for broccoli.

Favell (1998) conducted a study that compared the vitamin C content of fresh and frozen vegetables, including peas, green beans, broccoli, carrots, and spinach. For all vegetables

studied, the vitamin C level in the commercially quick-frozen products is equal to or better than that in the 'market fresh', which is the 3-day ambient sample. The data also shows a rapid loss of L-ascorbic acid from all vegetables at ambient temperature except carrots. The variation in the rate of loss relates to surface areas, mechanical damage, sulfhydryl content, and enzyme activities. Additionally, a study by Hunter and Fetcher (2002) showed that cooked frozen green peas and frozen leaf spinach contained quantities of L-ascorbic acid greater than or equal to those in the cooked fresh products.

In a comparable study on *trans*-β-carotene and L-ascorbic acid retention in fresh and processed vegetables, broccoli, carrots, and green beans were treated and fresh-refrigerated, frozen, or canned. In the study, fresh-refrigerated vegetables were held at 4 °C for 3 weeks (broccoli and green beans) or 6 month (carrots). *Trans*-β-carotene and L-ascorbic acid were determined at specified times, before and after microwave cooking. Results showed that blanching resulted in initial vitamin C loss, but retention rates remained stable after freezing for broccoli and green beans. Fresh-refrigerated green beans lost > 90% L-ascorbic acid after 16 days of storage. *Trans*-β-carotene levels decreased slightly during freezer storage, but losses of L-ascorbic acid in blanching and cooling were greater than losses seen in freezing. Therefore in some cases, frozen vegetables may be better sources of vitamin C than their fresh counterparts. (Howard *et al.,* 1999).

Puupponen-Pimiä *et al.* (2003) studied the effect of frozen storage on the amount of folate in peas, cauliflower, broccoli, and spinach. In their study, storage at -20 °C for 18 months had minimal effects on folate levels, indicating the suppression of enzymatic reactions might effectively prevent the degradation of folate content in frozen products. Czarnowska and Gujska

(2012) suggested an initial reduction of 20% folate in frozen peas and spinach, and an 8.7 and 9.7% decrease for cauliflower and broccoli, respectively. However, the total reduction of folate was > 90% on all vegetables after 6-month storage at -20 °C.

Few recent studies were found detailing the effect of frozen storage on vitamin A levels in fruits and vegetables. Howard *et al.* (1999) analyzed the change of *trans*-β-carotene levels in frozen broccoli and green beans. The study revealed that broccoli had better retention of *trans*-β-carotene (15.7% change) than green beans (30% change) after frozen storage for 12 months.

Although mineral levels are relatively stable, they are highly dependent on the harvest processing and water used in irrigation (Rickman, Barrett, & Bruhn, 2007b). Therefore, discrepancies were found in the results previously reported.

A number of factors could have influenced the precise analysis of nutrients in these studies, including varieties and species of fruits and vegetables, differences in analytical methods, quality control, and sampling methods.

2.3 L-Ascorbic acid

Water-soluble vitamin C, or L-ascorbic acid, is a critical nutrient for human health. Vitamin C acts as an antioxidant in the human body and rapidly quenches free radicals as well as playing a vital role in regenerating oxidized vitamin E. Because humans are incapable of synthesizing vitamin C and excrete it in the urine, intake from food is necessary. The main sources of L-ascorbic acid are vegetables and fruits. Some of the best sources of L-ascorbic acid are broccoli, cauliflower, spinach, and strawberries. The Recommended Dietary Allowances (RDAs) for vitamin C are 90 mg for adult males and 75 mg for adult females (Institute of

Medicine, 2000). Failing to meet these recommendations can cause problematic deficiencies, such as scurvy, which was common historically among sailors.

Several studies reported that consumption of vitamin C is associated with many health benefits. Pauling (1995) showed that vitamin C supplementation can alleviate common cold symptoms. However, widespread belief that vitamin C has no proven effect on the common cold still remains. Although placebo-controlled trials have shown vitamin C to alleviate common cold symptoms, important questions still remain (Hemilä, 1996).

Observational studies in humans have shown an inverse relationship between vitamin C concentrations in the plasma and total serum cholesterol. A review and analysis of 51 experimental trials (McRae, 2006) strengthens the hypothesis that vitamin C supplementation may have cholesterol-lowering and cardio-protective benefits, as it elevates plasma vitamin C concentrations in patients who were previously vitamin C deficient. More well-constructed studies are needed to conclude more about the benefits of vitamin C and its role in reducing cholesterol.

Numerous studies discuss the association between ascorbic acid and cancer. Cameron and Pauling (1993) suggested that routinely high intakes of L-ascorbic acid may prevent cancer by increasing its natural existence in humans. However, studies on cancer patients showed no significant difference between vitamin C supplemented and placebo groups in their survival time (Moertel *et al*., 1985). The relation between ascorbic acid and cancer is still nebulous, as the molecular mechanism underlying anti-carcinogenic activity of L-ascorbic acid is not clearly elucidated (Naidu, 2003).

2.4 *Trans*-β-Carotene

Trans-β-carotene is the most important precursor of vitamin A for humans and is known to be very important for vision. Substantial evidence from epidemiological studies highlights the association between *trans*-β-carotene levels and decreased rates of cancer and heart disease. Mayne (1996) found that supplemental *trans*-β-carotene has been shown to reduce precancerous lesions of the oral cavity and cervix. However, some other studies suggested that β-carotene supplementation had no beneficial effect on cancer prevention; high levels were associated with increased risk of lung and gastric cancer in smokers and asbestos workers (Druesne-Pecollo *et al*., 2010). Fawzi, Chalmers, Herrera, & Mosteller (1993) showed a strong inverse association between vitamin A supplement levels and risk of mortality in children in developing countries. Recommended Dietary Allowances (RDAs) for Vitamin A are 900 μg retinol activity equivalents (RAE) for adult males under the age of 51 and 700 μg RAE for adult females under the age of 51 (Institute of Medicine, 2001). Conversely, overdosing may cause carotenodermia, a physically harmless condition that results in orange-treated skin.

High dosages of vitamin A can have toxic effects in some people. *Trans*-β-carotene capsules have been able to provide the same protection as vitamin A capsules with fewer toxic side effects. The human body converts all dietary sources of vitamin A, rich in *trans*-β-carotene, into retinol, with 1 μg of retinol being equivalent to 12 μg of *trans*-β-carotene. The conversion of *trans*-β-carotene to vitamin A has been examined in several studies (Dueker, Jones, Smith, & Clifford, 1994; Parker, 1996; Novotny, Dueker, Zech, & Clifford, 1995), but the details of its metabolism are still unclear. The major obstacle is that the metabolism of β-carotene in humans is different from most in animals. In addition, the critical enzyme in carotenoid cleavage is

unstable. Therefore, there is ongoing controversy surrounding the mechanism of β-carotene cleavage.

Trans-β-carotene also functions as an antioxidant, which quenches free radicals in the body. Therefore, it can decrease lipoprotein, which is correlated with cardiovascular disease and DHA oxidation, which is required to maintain brain function in humans.

2.5 Folate

Folate is the general term used for folic acid and poly-γ-glutamyl conjugates that exhibit the biological activity of folic acid (Eitenmiller, Landen, & Ye, 2007). Folate and folic acid are the preferred synonyms for pteroylglutamate and pteroylglutamic acid, respectively. This key nutrient is present in many natural foods, including legumes and leafy greens. Liver is also considered to be a good food source of folate. However, folic acid is not found in nature; so, it is often synthesized for fortification in both foods and pharmaceuticals.

Folate deficiency is one of the most prevalent vitamin deficiencies occurring worldwide. This deficiency can cause megaloblastic anemia (*i.e.*, the production of large immature red blood cells) as well as abdominal pain, diarrhea, mouth ulcers, skin changes, hair loss, and neurological disorders. With the recognition of the relationship between folate levels and occurrences of neural tube defects in infants, attention to folate nutrition has been raised. To reduce the number of pregnancies affected by neural tube birth defects, the United States Food and Drug Administration (U.S. F.D.A.) began to require manufacturers to add folic acid to enriched grain products (U.S. F.D.A., 1996). Since the fortification policy became mandatory in 1998, serum and erythrocyte folate concentration has dramatically increased. RDAs for folate are 400 μg

dietary folate equivalents (DFE) per day for adults and 600 μg DFE for pregnant women (Institute of Medicine, 1998).

Cooking methods strongly influence the amount of folate ingested from a food. A study in the U.K. (McKillop *et al*. 2002) of raw and cooked foods found that typical boiling times resulted in only 49% retention of folate in spinach and 44% in broccoli. Conversely, steaming spinach or broccoli resulted in no significant decrease in folate content. It is apparent that the retention of folate in various foods is highly dependent both on the food in question and the method of cooking. Therefore, health efforts to increase folate intake should incorporate practical advice on cooking.

Folate is particularly difficult to analyze and much effort has gone into developing methods for its analysis. These include bioassays, microbiological approaches, HPLC, ligand-binding, and radioimmunoassay (Eitenmiller *et al.*, 2007). The microbiological assay is one of the oldest and most applicable methods to determine total food folate content. Three bacteria and the protozoan *Tetrahymena pyriformis* were used per Eitenmiller *et al.* (2007). *Lactobacillus casei* subsp. *rhamnosus* is the most commonly employed organism for folate analysis in natural products.

Folate extraction must be performed protected from ambient light and air to minimize folate degradation. High fat samples should be defatted to prevent fatty acid stimulation of the growth response of *Lactobacillus casei* subsp. *rhamnosus*. DeSouza and Eitenmiller (1990) developed a trienzyme approach to folate extraction applicable to most foods using Pronase®, α-amylase, and chicken pancreas conjugase. The method increased measurable folate in various foods. It is the extraction method used in AOAC Official Method 2004.05 for assay of food

folate in cereal grain products. Later, Tamura, Mizuno, Johnston and Jacob (1997) used modified buffers and a specified order of enzyme addition in the trienzyme method to show significant increases in measurable folates. A study of measuring folate concentrations of fast foods by tri-enzyme extraction method (Johnston, Lofgren, & Tamura, 2002) showed that obtained folate values were markedly higher than those in the literature most likely due to the mandate of folic acid fortification in cereal-grain foods and the use of the new folate extraction technique.

However, enzymes may act differently in different samples. A study by Chen and Eitenmiller (2007) indicated that extraction with folate can be maximized using RSM techniques. It was also found that increase of the digestion time for Pronase® and α-amylase had a significant effect on folate levels. However, overnight or extended digestion with conjugase is unnecessary and possibly detrimental, because deconjugation occurs primarily within the first hour of incubation. A study on brown rice and soybeans showed that single-enzyme treatment alone, by folate conjugase from chicken pancreas may be used for these foods (Soongsongkiat, Puwastien, Jittinandana, Dee-Uam, & Sungpuag, 2010). A 2002 study found that a dual-enzyme treatment (protease and conjugase) is sufficient to determine folate content in spinach(Pandrangi, & LaBorde, 2002). In a folate study with spinach, fortified bread and ready-to-eat breakfast cereals, deconjugation with chicken pancreas gave a significant higher folate value than did human plasma in all foods except spinach (Shrestha, Arcot, & Paterson, 2000).

Traditional microbiological methods have been modified to increase speed and decrease labor intensiveness. A report by Newman and Tsia (1985) described a microbiological assay for folates adapted to use disposable 96-well plates and an automatic plate reader. The modification greatly decreased costs and made the analysis of hundreds of sample per day possible. With high

sensitivity 96-well microplates and autoplate readers have been successfully used in several laboratories. A study of measuring human milk folate with application of trienzyme treatment prior to microbiological assay indicated the treatment of protease, α-amylase and conjugase significantly increased the measurable folate in human milk by an average of 85% compared with values after folate conjugase treatment alone (Lim, Mackey, Tamura, Wong, & Picciano, 1998). In a Single Laboratory Method Performance Evaluation for the Analysis of Total Food Folate by Trienzyme Extraction and Microplate Assay (Chen, &Eitenmiller, 2007), a generated 4-parameter logistic equation was obtained. The test of parallelism demonstrated that matrix components in the food extracts did not affect the accuracy. Measured values of SRMs and CRMs were within their certified or reference values. Recoveries for all reference materials met the requirements of the AOAC guidelines for single laboratory validation. Precision measured as repeatability, including simultaneous and consecutive replicates for each SRM and CRM, met the Horwitz criterion. The results showed that trienzyme digestion using α-amylase, Pronase®, and conjugase from chicken pancreas coupled with a 96-well microplate assay provided a highly accurate, reproducible, and sensitive method for the determination of folate in a variety of foods.

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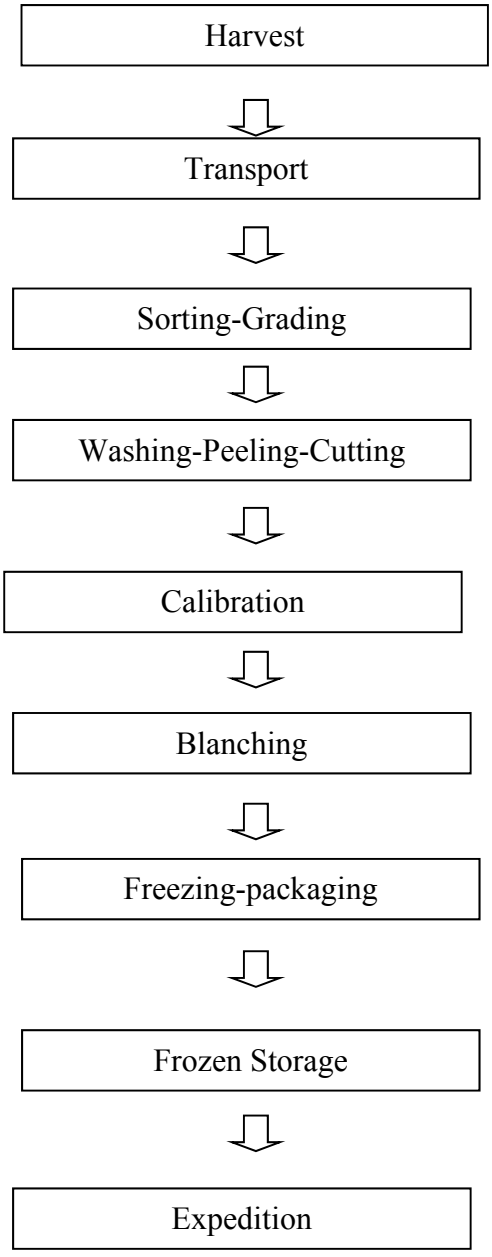

Figure 2.1 General flow diagram for the processing of frozen vegetables.

CHAPTER 3

L-ASCORBIC ACID CONTENT OF FRESH, FRESH-STORED, AND FROZEN FRUITS AND VEGETABLES

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ABSTRACT

The objectives of the study were to determine and compare the vitamin C content in selected fresh, fresh-stored, and frozen fruits and vegetables, while mimicking typical consumer purchasing and storage patterns of the produce. AOAC Official Method 967.21 was employed for the determination of L-ascorbic acid in the composite produce samples. The L-ascorbic acid contents were determined in fresh, fresh-stored, and frozen blueberries, strawberries, broccoli, cauliflower, corn, green beans, spinach, and green peas. For each of the 8 sample-types, one-way ANOVA was performed to determine the presence of significant difference in L-ascorbic acid contents according to treatment (α=0.05). The study did not find fresh-stored produce to be superior to its frozen counterpart in most cases.

3.1 Introduction

Vitamin C, also known as L-ascorbic acid, is a water-soluble vitamin that is very important in human nutrition. Specifically, vitamin C aids in the prevention of immune system deficiencies, helps in wound healing, maintenance of healthy skin, and may even play an antioxidative role in preventing the onset of certain cancers. The human body cannot synthesize vitamin C; therefore, a dietary intake of this nutrient is essential for one's health. Most fruits and vegetables are rich sources of vitamin C, but cooking can reduce the produces' vitamin C content, as it is both water-soluble and heat labile. In older times through present times, and especially in developing countries, getting a sufficient intake of vitamin C is vital to fend off deficiency diseases such as scurvy, which can lead to skin browning and bleeding from the mucous membranes.

Common analytical techniques employed to determine the vitamin C content in foodstuffs like fruits and vegetables include high-performance liquid chromatography and spectrophotometric determinations such as the enzymatic oxidation of L-ascorbic acid to dehydroascorbic acid by ascorbate oxidase. When measuring ascorbic acid iodometrically with starch, research suggests that the insolubility of the starch–iodine complex may prevent some of the iodine–titrant from reacting thereby lessening the reaction between ascorbic acid and iodine (Hossu & Magearu, 2004). Another popular technique is spectrofluoremetry based on the conversion of L-ascorbic acid to dehydroascorbic acid. The later facilitates a total vitamin C assay by *o*-phenylenediamine condensation with the formation of a fluorescent quinoxaline derivative.

For this study, AOAC Official Method 967.21 was chosen, because it has been widely used to determine the L-ascorbic acid content in fruits and vegetables and it is quicker and more cost-effective than AOAC Method 984.26. AOAC Method 967.21 is a titrimetric assay for ascorbic acid, while AOAC Method 984.26 is a semi-automated fluorometric assay for total vitamin C.

The objectives of the present study were to determine and compare the vitamin C content in selected fresh, fresh-stored, and frozen fruits and vegetables, while mimicking typical consumer purchasing and storage patterns of the produce.

3.2 Materials and Methods

AOAC Official Method 967.21 (Ascorbic acid in vitamin preparations and juices: 2,6-dichloroindophenol titrimetric method) was employed for the determination of ascorbic acid in the composite produce samples.

3.2.1 Sample handling

Fruit and vegetable samples (*i.e.*, blueberries, strawberries, broccoli, cauliflower, corn, green beans, spinach, and green peas), either fresh or frozen, were purchased from 6 supermarkets within a 40-km radius of Athens, GA. The selected stores were common U.S. supermarket chains and included Walmart, Sam's Club, Kroger, Publix, Piggly-Wiggly, and Ingles, as well as Bell's as a backup. All of the frozen produce acquired was private-label.

On the occasions when fresh fruits and vegetables were purchased, double the quantity of that of the frozen produce was acquired. Half of the purchased fresh produce was placed in a

standard kitchen refrigerator for 5 days before analysis. This procedure was intended to mimic the purchase and storage practice of fresh produce by consumers. Hence, these fruits and vegetables are referred to as fresh-stored.

A ~200-g portion of fruit or vegetable from each store was combined in a large grey plastic tub, and then mixed well to give a composite sample. In the case of fresh and fresh-stored vegetables, no samples were blanched prior to analysis except for corn-on-the-cob. Blanching was unnecessary, because each vegetable sampled was immediately placed in a nutrient-stabilizing reagent after grinding to prevent enzymatic degradation of the L-ascorbic acid. For fresh corn-on-the-cob, blanching for 1 min and then cooling the cobs in an ice bath prior to cutting off the kernels was mandatory to prevent the enzymatic degradation that would have taken place during the time it took to mix up the niblets for the composite and then sample the portions for analysis. All frozen vegetables had been blanched prior to freezing, whereas the fruits were blast frozen. Representative samples from each composite were then taken for ascorbic acid analysis.

3.2.2 Moisture analyses

A moisture analysis was performed gravimetrically in triplicate using AOAC Official Method 984.25 (Moisture {loss of mass on drying} in frozen French-fried potatoes) on all fruits and vegetables by placing a ground portion of each sample in a forced-air convection oven at 103 ± 2 °C until a constant mass was reached (AOAC International, 2006).The moisture analyses were performed to provide a uniform mass balance for all data; that is, being able to convert collected data from a fresh weight basis (f.w.) to a blanched weight basis (b.w.) to a dry weight

basis (d.w). Because the fresh and fresh-stored corn-on-the-cobs were blanched prior to preparing the composite, the moisture content of this composite was determined and compared to that of non-blanched corn. The conversion of L-ascorbic acid levels based on blanched weight (b.w.) to fresh weight (f.w.) was calculated using the following equation:

$$\text{L-ascorbic acid content (f.w., mg/100 g)} = \text{L-ascorbic acid (b.w., mg/100 g)} \times \frac{(1-M_b)}{(1-M_f)}$$

where, M_b is the moisture content of the blanched corn-on-the-cob; and M_f is the moisture content of the fresh (unblanched) corn-on-the-cob.

3.2.3 Reagent preparation

All chemicals were ACS-reagent grade, unless otherwise specified. The *meta*-phosphoric–acetic acid nutrient-stabilizing reagent and the 2,6-dichloroindophenol reagent were prepared just prior to each analysis. When not in use, the solutions were stored at 4 °C. L-Ascorbic acid (USP-grade) standard solutions for quality control determinations were prepared fresh daily.

meta-Phosphoric acid–acetic acid extractant

In a 500-mL volumetric flask, 15 g of *meta*-phosphoric acid pellets (CAS 37267-86-0; VWR International, Suwanee, GA) were dissolved in 200 mL of deionized water, to which 40 mL of glacial acetic acid (Fisher Scientific, Suwanee, GA) were added. Once the *meta*-phosphoric acid chips were fully in solution, the volume was adjusted to 500 mL with more

deionized water. The reagent was then filtered through Whatman No. 1 filter paper (Fisher) into an amber bottle.

Ascorbic acid standard

Fifty milligrams of USP-grade L-ascorbic acid (Mallinckrodt Chemical, Inc., procured from VWR International) was weighed and quantitatively transferred to a Pyrex® 50-mL Class A low-actinic (red) volumetric flask with the *meta*-phosphoric acid–acetic acid extracting reagent. The flask was then filled to mark with the *meta*-phosphoric acid–acetic acid.

2,6-Dichloroindophenol reagent

One hundred milligrams of 2,6-dichloroindophenol sodium salt, hydrate (CAS 620-45-1; Acros Organics, 98^{+} purity from Fisher) and 84 mg of $NaHCO_3$ (VWR) were dissolved in 100 mL of deionized water. Once all compounds were in solution, the final volume was adjusted to 400 mL with deionized water, then filtered through Whatman No. 1 filter paper into a glass-stoppered bottle, and wrapped in aluminum foil to prevent photo-oxidation of the vitamin C.

3.2.4 Standardization of the 2,6-dichloroindophenol reagent

Using a fixed-volume borosilicate glass pipet, 5 mL of the *meta*-phosphoric–acetic acid extracting reagent was dispensed into each of three 125-mL Erlenmeyer flasks, followed by 1 mL of the ascorbic acid standard solution *via* a Gilson Pipetman *Neo*® (Gilson, Inc., Middleton, WI). With gentle swirling, the test solutions were titrated against the indophenol solution until a light-rose color lasting longer than 5 s remained. Three blanks were also prepared; these

contained 5 mL of the *meta*-phosphoric–acetic acid extracting reagent and 1 mL of deionized water. The blanks were titrated against the indophenol reagent and the average mL reading for these was subtracted from the volume of indophenol consumed by the test solutions. The concentration of the indophenol standard solution was then calculated and expressed as mg of ascorbic acid equivalents per mL of indophenol solution.

3.2.5 Sample preparation and extraction

Under yellow lighting, in order to minimize photo-oxidation, a representative quantity (~100 g) of each composite fruit or vegetable was ground in a 70-W Black & Decker one-touch chopper (Model HC306, Applica Consumer Products, Inc., Miramar, FL) just preceding the assay, followed by stabilization of the L-ascorbic acid in a test portion with the *meta*-phosphoric acid–acetic acid reagent. Triplicate samples were stabilized in the *meta*-phosphoric–acetic acid reagent to prevent enzymatic and oxidative destruction of the vitamin C.

A 10-g portion of the ground fruit or vegetable was placed in a mortar to which a small volume of the *meta*-phosphoric acid–acetic acid reagent was added, followed by maceration with a pestle until all particulates were in suspension. The mixture was filtered through a funnel containing glass wool into a Pyrex® 100-mL Class A low-actinic (red) volumetric flask. The glass wool was rinsed, and the flask filled to mark with the *meta*-phosphoric acid–acetic acid reagent; the contents were then well mixed.

One at a time, 30-mL aliquots were pipetted into a 125-mL Erlenmeyer flask and titrated against the 2,6-dichloroindophenol solution until a light-rose color lasting longer than 5 s was

evident. The titration was repeated 2× more for each test sample. Similarly, three blanks containing just 30 mL of the *meta*-phosphoric acid–acetic acid reagent were also titrated.

3.2.6 Calculations

The ascorbic acid content in sample was calculated as follows (AOAC International, 2006),

$$\text{mg ascorbic acid/g} = (X-B) \times (F/E) \times (V/Y)$$

where, X is the average mL for test sample titration; B is the average mL for blank titration; F is the mg of ascorbic acid equivalent to 1.0-mL indophenol standard solution; E is the mass (g) of sample assayed; V is the volume of the initial test solution; and Y is the volume of the test solution titrated.

Because of the mass change as a result of blanching, L-ascorbic acid contents in fresh and fresh-stored corn-on-the-cob were corrected according to their moisture contents before and after blanching. Each sample was analyzed in triplicate. Mean and standard deviations were calculated.

3.3 Quality Control

To validate the accuracy and interday precision (*i.e.*, relative repeatability standard deviation, $\%RSD_r$) for the analysis of L-ascorbic acid by the titrimetric method in the fruit and vegetable samples, a quality control plan was established.

Accuracy

In this study, a certified reference material (CRM) from the European Commission Joint Research Center, Institute for Reference Materials and Measurements, BCR® 431 (Brussels

sprouts powder) was purchased from the Resource Technology Corporation (Laramie, WY). Analysis values of all examined produce were compared against the CRM over the course of the study. Accuracy was assessed by comparing the data value obtained from a measurement to that of the accepted value of the reference material. Bias, which is defined as the difference between the analytical value from that of the accepted value provided on the CRM's certificates of analysis, was also determined (Horwitz, 2003). The bias and percent accepted value were calculated as follows:

$$\text{Bias} = \mu - x$$

$$\text{Accepted value (\%)} = x/\mu \times 100$$

where, x is the analytical value; and μ is the accepted value provided by the certificate of analysis of the reference sample.

Recovery is defined as the fraction of the analyte measured after addition of a known quantity of the analyte to the sample. Recovery was determined on the basis of AOAC guidelines (2000). BCR® 431 (Brussels sprouts powder) was spiked with known levels of L-ascorbic acid. Final spiking levels per gram of the CRM were 2.5, 5.0, and 10.0 mg, which represent levels of 50, 100, and 200%. Each frozen fruit and vegetable was also spiked with an appropriate quantity of L-ascorbic acid based on its endogenous ascorbic acid content, covering levels of 50, 100, and 200%. All spiking experiments were performed in triplicate. The % recovery of the added USP L-ascorbic acid standard was calculated as follows:

$$\text{Recovery (\%)} = [(C_s - C_p)/C_a] \times 100$$

where, C_s is the L-ascorbic acid concentration in the spiked sample; C_p is the L-ascorbic acid concentration in the unspiked sample; and C_a is the mass of the L-ascorbic acid standard added.

Precision

Precision, or reproducibility, is the degree to which repeated measurements under unchanged conditions show the same results. The precision of the assay was determined by repeatability (intraday precision) and intermediate precision (interday precision) of the CRM. Precision was determined in three replicates of the CRM on the same day (intraday precision), and daily 3× over a period of two weeks (interday precision). Precision can be approximated as the relative standard deviation between trials and can be expressed as follows:

$$\%RSD = (SD \times 100)/\bar{x}$$

where, %RSD is the relative standard deviation; SD is the standard deviation; and $\bar{x}$ is the mean.

3.4 Data Analysis

The L-ascorbic acid contents were determined in fresh, fresh-stored, and frozen blueberries, strawberries, broccoli, cauliflower, corn, green beans, spinach, and green peas. The analyses were performed over the span of two years in 6 distinct time frames, namely Summer to Fall 2011, Fall 2011 to Winter 2012, Winter to Spring 2012, Summer to Fall 2012, Fall 2012 to Winter 2013, and Winter to Spring 2013. Within each time period, the L-ascorbic acid content was measured in triplicate for all combinations of treatments and samples. The data for each time frame, although reported individually in the results, are treated as replicates in our statistical analysis of the differences among treatments. Therefore, the ascorbic acid content of each treatment/sample combination was assessed according to 18 replications.

For each of the 8 sample-types (*i.e.*, each specific fruit or vegetable), one-way ANOVA was performed to determine the presence of significant difference in L-ascorbic acid contents

according to treatment (α=0.05). Data transformation was applied when necessary to adequately meet the assumption of normal distribution for ANOVA, and the Weighted Least Squares method was employed in the instances in which the equal variance assumption of the ANOVA was violated. All necessary statistical diagnostic checks (e.g., residuals versus predicted value plot, Q–Q plot of residuals, and histogram plot of residuals) were performed to verify acceptability of ANOVA implementation. In the cases in which a statistically significant difference was observed among the three treatments, the Tukey's Studentized Range multiple comparisons test was performed to determine which specific pairs of treatments (*i.e.*, fresh vs. frozen, fresh vs. fresh-stored, and fresh-stored vs. frozen) showed significant differences from one another (α=0.05).

All data analyses were performed using SAS software, version 9 of the SAS system for Windows (SAS Institute Inc., Cary, NC).

3.5 Results and Discussion

L-Ascorbic acid, extracted from fruits and vegetables, was measured quantitatively by titrating against a standardized solution of 2,6-dichloroindophenol in sodium bicarbonate. In this titrimetric assay, ascorbic acid reduces an oxidation-reduction indicator dye, namely 2,6-dichloroindophenol, to a colorless product. At the end-point, any excess un-reduced indicator dye will be visible as rose-pink in acidic solutions.

Vitamin C is exhaustively extracted from produce samples and the titration is performed in the presence of a *meta*-phosphoric–acetic acid reagent to maintain a suitable acidity for the reaction, as well as to avoid auto-oxidation of L-ascorbic acid at higher pH values.

Quality Control

Accuracy

Accuracy and $\%RSD_r$ measures for the ascorbic acid assay are reported in Table 3.1. Ascorbic acid was extracted from the CRM, BCR® 431 (Brussels sprouts powder), and analyzed. According to its certificate of analysis, BCR® 431 contains 483 ± 24 mg vitamin C/100 g. Comparison of the analytical value to the accepted value from the certified value of the CRM was within the accepted range reported for BCR® 431. The bias value was +10. The ratio of the analytical value to the accepted value, expressed as a percentage (% of accepted value), can be used to evaluate accuracy. For ascorbic acid, the % accepted value was 97.9 indicating a close agreement between the analytical and accepted value. Interday precision ($\%RSD_r$) was excellent at a value of 0.6. Together, the accuracy and $\%RSD_r$ measures indicated that the ascorbic acid analyses of the fruit and vegetable samples were accurate and repeatable. Noteworthy is that many nutrient profiling studies fail to report on these parameters.

The recoveries of ascorbic acid from BCR® 431 for spiked samples at levels of 50, 100, and 200% were 101 ± 4, 97 ± 3 and 105 ± 3%, respectively. The recoveries ranged from 97 to 105%. For fruit and vegetable samples, the recoveries ranged from 93 to 105%, thereby demonstrating excellent recovery of vitamin C from the test samples (see Table 3.2). Such high recoveries from the CRM and produce experiments validate the accuracy of the L-ascorbic acid assay.

Precision

Intraday precision (repeatability) was determined by running 9 replicates of the CRM over 3 days. The findings are presented in Table 3.1. The RSD of the data were calculated and

shown to be less than 5%. The daily (intraday) and day-to-day (interday) precision of the CRM gave the following results: based on n=3, the ascorbic acid analysis of the CRM showed a relative standard deviation of 0.64%. In terms of inter-day precision, the ascorbic acid analysis of BCR® 431 (Brussels sprouts powder) gave a relative standard deviation of 2.33%. In both cases, %RSD values were found well within the 5% limit, indicating that the current method is repeatable.

Statistics

The question of interest is whether or not the L-ascorbic acid contents in frozen fruits and vegetables are significantly ($p < 0.05$) different from those of their fresh and/or fresh-stored counterparts. Consumers generally assume that the nutrient content in fresh produce is greater than that which is frozen. Because the range of ascorbic acid contents in the selected produce varied quite markedly, it was more reasonable to analyze each fruit and vegetable separately. To illustrate, the L-ascorbic acid content in fresh blueberries was only ~13 mg/100 g, f.w. whereas in fresh strawberries it was ~55 mg/100 g, f.w. Consequently, it is not appropriate to fit just one statistical model to the L-ascorbic acid data. Instead, 8 different models were fitted based on 54 observations (*i.e.*, 6 trials per fruit or vegetable × 3 replicates per sample ×3 treatments {fresh, fresh-stored, and frozen}). Seasonality was not considered in this study because of lack of knowledge of the source and processing of the fresh and frozen fruits and vegetables.

The mean L-ascorbic acid contents determined in fresh, fresh-stored, and frozen blueberries and strawberries as well as the corresponding fresh and frozen values from the U.S.D.A. National Nutrient Database for Standard Reference (R26), herein after referred to simply as Database, are presented in Table 3.3. There was no statistical ($p > 0.05$) difference in

the levels of vitamin C for all three berry types. For frozen blueberries, the mean value determined was 9.9 ± 5.7 mg/100 g, f.w., which is much higher than the Database value of 2.5 ± 2.4 mg/100 g, f.w., based on only 3 observations. With reference to both values, there were large standard errors of the means. Possible explanation for this variation may stem from the employment of different cultivars, but the preparation of the composite each time prior to vitamin C analysis should have decreased this variability. For fresh and fresh-stored blueberries, the standard errors of the means were much less than that of the frozen berry composites. One might have expected less variance in the content of L-ascorbic acid in frozen blueberries; the range was from 3.0 mg/100 g to 19.8 mg/100 g, possibly being due to the impact of non-uniform blast freezing.

Not just blueberries but for all produce analyzed in this study, the mean value for L-ascorbic acid in the fresh samples was always higher than that of the fresh-stored product. This is expected, as the nutrient content would change with the respiration/lifespan of the fruit. What this study lacks is uniform control over the age, handling, and treatment of the fruit before it was analyzed. As stated in the objective, this investigation was designed to mimic the purchasing and storage habits of produce by consumers before consumption. The findings show that the vitamin C content of fresh blueberries stored at 4 °C for 5 days decreased by 22%. A study by Kalt, Forney, Martin, & Prior (1999) found that ascorbate level in low-bush blueberries dropped by 27% after 8 days held at 20 °C. These authors postulated that the losses may be from extra vacuolar vitamin C.

For strawberries, the ascorbic acid contents in the fresh and fresh-stored strawberries were 55.2 ± 9.8 and 48.3 ± 12.2 mg/100 g, f.w., respectively, and were consistent with the

Database value of 58.8 ± 2.5, based on 9 observations. However for frozen strawberries, the vitamin C content of 54.7 ± 9.6 mg/100 g, f.w. was higher than the value of 41.2 ± 9.1 reported by the U.S.D.A. It is interesting that greater variation in the vitamin C content of the analyzed strawberries from values reported in the Database was not found. Olsson *et al.* (2004) reported a 2 to 5 fold variation in the ascorbic acid level among 4 different cultivars. These authors also reported that the concentration of bioactive compounds in strawberries varied according to the type of cultivar, ripening stage, and length of storage. Just like blueberries, the fresh strawberries had a greater mean content of vitamin C compared with its fresh-stored counterpart. The data showed a 12.5% decrease of L-ascorbic acid in fresh strawberries stored at 4 °C for 5 days after purchase. The loss was minor compared to that of blueberries. Kalt *et al.* (1999) found no ascorbate losses in strawberries during 8 days of storage at 0, 10, 20, and 30 °C. What is noteworthy is that the mean content of the frozen berries were greater than that of the fresh-stored strawberries. Yet, there is no statistical ($p > 0.05$) difference in the level of vitamin C between the fresh, fresh-stored, and frozen berries.

The mean L-ascorbic acid contents determined in fresh, fresh-stored, and frozen broccoli, cauliflower, corn, green beans, and spinach, as well as the corresponding fresh and frozen values from the Database are presented in Table 3.4. The mean values for fresh, fresh-stored, and frozen broccoli were 50.2 ± 8.5, 46.7 ± 10.8, and 50.6 ± 3.3 mg/100 g, f.w., respectively, and there were no statistical ($p > 0.05$) differences. The vitamin C content in frozen broccoli was similar to the 56.4 ± 2.9 mg/100 g, f.w. value of the Database; however, this was not the case for fresh broccoli, whose reported value is 89.2 ± 4.0 mg/100 g, f.w. It is possible that lack of a blanching step before taking a representative sample from the broccoli composite was responsible for some

degradation of vitamin C, but unlike corn-on-the-cob, there was no significant tissue damage to the broccoli to release oxidases until the grinding step in the chopper, followed immediately by sampling and addition of the *meta*-phosphoric acid–acetic acid nutrient-stabilizing reagent. A decrease of only 7% L-ascorbic acid was noted in fresh broccoli stored at 4 °C for 5 days after purchase. Consistent with this work, Favell (1998) found no significant decrease of vitamin C in fresh broccoli stored at 4 °C for 7 days. A study by Schonhof, Kläring, Krumbein, Claußen, & Schreiner (2007) found that temperature had a strong influence on the L-ascorbic acid content. These authors found at 15 to 20 °C the L-ascorbic acid content decreased by up to 38% as compared to broccoli grown at 7 to 12 °C. Although seasonal variability was not examined in this study, the observation by Schonhof *et al.* (2007) reinforces the point that horticultural practices and growing conditions also markedly impact nutrient levels, not just cultivar selection, handling, and storage conditions.

The mean values for fresh, fresh-stored, and frozen cauliflower were 45.1 ± 6.1, 42.0 ± 6.4, and 40.4 ± 7.5 mg/100 g, f.w., respectively, and there were no statistical ($p > 0.05$) differences. The vitamin C content in fresh cauliflower was similar to the 48.2 ± 3.8 mg/100 g, f.w. value reported in the Database, while the frozen cauliflower analyzed in this study yielded a slightly lower value than the Database's 48.8 ± 1.7 mg/100 g, f.w. A 7% decrease in vitamin C was observed in fresh cauliflower stored at 4 °C for 5 days after purchase. Unlike the frozen, neither the fresh nor fresh-stored cauliflower samples were blanched. It is possible that the lower ascorbic acid mean in frozen cauliflower, albeit not statistically so, might be a result of blanching. Volden, Bengtsson, & Wicklund (2009) found an average 19% loss of L-ascorbic acid in cauliflower as a result of blanching. Lisiewska and Kmiecik (1996) also reported that 28 to 32%

losses in the vitamin C content had occurred due to blanching. Yet, further losses in the nutrient during frozen storage were slight. After 12 months of frozen storage, Lisiewska and Kmiecik (1996) found a further reduction of ascorbic acid in cauliflower of only 6 to 13%.This finding clearly illustrates the stability of L-ascorbic acid in frozen storage.

The mean values for fresh, fresh-stored, and frozen corn were 6.2 ± 0.9, 5.7 ± 0.9, and 6.2 ± 1.3 mg/100 g, f.w., respectively, and there were no statistical ($p > 0.05$) differences. The vitamin C contents in fresh and frozen corn were similar to the 6.8 ± 0.6 and 6.4 ± 1.6 mg/100 g, f.w. values reported in the Database, respectively. The L-ascorbic acid content in corn was lowest of all produce analyzed. Because the fresh and fresh-stored corn cobs were blanched prior to preparing the composite, the moisture content of the composite was determined gravimetrically by oven drying and compared to that of non-blanched corn. A correction factor, as described in the experimental section, was employed to be able to report the results on a f.w. basis. An interesting experiment would have been not to blanch the corn before stripping it off the cob to see if the enzymatic degradation of vitamin C was pronounced. This was not done, as it was a logical step to blanch the corn. The mean ascorbic acid contents in fresh corn and frozen corn were similar and slightly higher than that of the fresh-stored produce. A 5% loss in L-ascorbic acid was noted in fresh corn-on-the-cob stored at 4 °C for 5 days after purchase. The similar nutrition value in frozen corn ought to be a major attraction to consumers, because the frozen product needs much less handling before consumption compared with fresh corn.

The mean values for fresh, fresh-stored, and frozen green beans were 7.7 ± 3.7, 6.7 ± 3.6, and 11.0 ± 2.3 mg/100 g, f.w., respectively. The frozen green beans possessed a mean L-ascorbic acid level significantly ($p < 0.05$) greater than both the fresh and fresh-stored samples. The

vitamin C contents in the frozen green beans were similar to the 12.9 ± 1.5 mg/100 g, f.w. value reported in the Database, but the fresh sample was lower than the Database's 12.2 ± 0.8 mg/100 g, f.w. value. It is interesting to note that the Database's mean content of vitamin C for fresh green beans is lower than that of its frozen counterpart; our findings match this observation. Makhlouf *et al.* (1995) also found vitamin C in the raw beans had an average of 8.2 mg/100 g, which corresponds with our finding. In this study, a loss of 13% ascorbic acid was noted for fresh green beans stored at 4 °C for 5 days after purchase. Although the loss was not significant compared to the fresh sample, other researchers have reported marked ascorbic acid loss with extended storage. For instance, Howard, Wong, Perry, & Klein (2008) found a rapid loss of L-ascorbic acid in fresh green beans during refrigeration for 3 weeks. Although blanching prior to freezing slightly decreased the L-ascorbic acid content, the authors reported that its retention remained stable after freezing. Martin and Silva (2004) found that the nutritional and sensory parameters of green beans were retained at storage temperatures of 5, -6, and -12 °C, thereby indicating that the beans' quality held well during frozen storage. Favell (1998) noted a 77% decrease in L-ascorbic acid in fresh green beans stored at 4 °C for 7 days. Because the handling practices and age of the picked green beans reaching the supermarkets are unknown, it is not surprising based the previous studies mentioned above, that frozen green beans can contain more vitamin C than those which are "fresh." This is something of which the consumer is not aware.

The most interesting finding in the L-ascorbic acid assay was for spinach. The mean values for fresh, fresh-stored, and frozen spinach were 25.2 ± 3.5, 19.6 ± 5.5, and 14.5 ± 5.7 mg/100 g, f.w., respectively. All means were statistically ($p < 0.05$) different from one another! The vitamin C content in fresh spinach was similar to the 28.1 ± 4.1 mg/100 g, f.w. value

reported in the Database, based on 7 observations. However for frozen spinach, the value of 5.5 ± 0.7 mg/100 g, f.w. was markedly lower than that determined in this study. In fact, the mean ascorbic acid content in frozen spinach was ~1.6× greater than the Database value. What can account for the variation? Spinach possesses a large surface area relative to the other fruits and vegetables examined in this study; so, blanching may have had a detrimental effect on its vitamin C content. Note that only the frozen sample had been blanched before it was analyzed, while the fresh and fresh-stored test samples were placed in the nutrient-stabilizing reagent after grinding. This may possibly account for why the vitamin C content in frozen spinach is significantly ($p < 0.05$) less than the fresh, non-blanched, counterpart. It is also very apparent that refrigerated storage results in a significant ($p < 0.05$) loss of vitamin C: a 22% decrease of L-ascorbic acid was noted for fresh spinach that had been stored at 4 °C for 5 days after purchase. On the other hand, Giannakourou and Taoukis (2003) found higher levels of L-ascorbic acid in frozen spinach: the contents ranged from 25 to 34 mg/100 g, f.w. The difference might have been due to cultivar selection or how blanching was performed. Other studies have also noted poor retention of vitamin C in spinach. Favell (1998) reported a 75% decrease in L-ascorbic acid in fresh spinach stored at 4 °C for 7 days. This loss is markedly greater than the ~30% loss of L-ascorbic acid these authors observed in frozen spinach stored at -20 °C for 12 months. Hunter and Fletcher (2002) reported a total loss of L-ascorbic acid in fresh spinach stored for 4 days at 20°C. He partially attributed the loss to permeation of oxygen through the spinach leaves. Consequently, in consideration of rapid L-ascorbic acid losses post-harvest, freezing would have an obvious advantage compared to refrigeration.

The mean L-ascorbic acid contents determined in fresh, fresh-stored, and frozen green peas as well as the corresponding fresh and frozen values from the Database are presented in Table 3.5. Unlike the other produce, fresh green peas, also referred to as English peas, could only be procured in the spring of both years of the study; hence, Table 3.5 lists sampling times as opposed to seasons. The mean values for fresh, fresh-stored, and frozen green peas were 19.0 ± 5.1, 15.4 ± 3.4, and 21.1 ± 3.3 mg/100 g, f.w., respectively. Fresh and frozen green peas were not statistically ($p > 0.05$) different from one another, even though the absolute mean value of L-ascorbic acid in frozen green peas was greater than that of fresh peas. For fresh-stored green peas, however, the L-ascorbic acid content was reduced and statistically ($p < 0.05$) different from the latter two types. With reference to the Database, the vitamin C content of frozen green peas listed was consistent with that found in the present study; the former being 18.0 ± 0.7 mg/100 g, f.w., based on 7 observations. For fresh green peas only one observation is given in the Database at a value of 40 mg/100 g, f.w., which cannot be deemed as reliable for comparative purposes. A study by Makhlouf *et al.* (1995) also found raw peas to possess vitamin C levels 45% below the Database value. These authors also noted that there was actually no marked difference between the content of L-ascorbic acid in frozen and fresh green peas. In other studies, frozen peas were found to have a good retention of L-ascorbic acid. For instance, Sistrunk, Gonzalez, & Moore (1989) reported that frozen green peas retained the highest nutritional quality compared with other processing methods. With no processing, a 19% loss of L-ascorbic acid was noted for fresh green peas stored at 4 °C for 5 days. This observation is consistent with the findings of Favell (1998), who reported a 15% loss of L-ascorbic acid in fresh green peas during 7 days at 4 °C compared to a 10% decrease in frozen green peas stored at -20 °C for 12 months. Hunter and

Fletcher (2002) observed that fresh green peas stored at ambient temperatures lost ~50% of their L-ascorbic acid content after 7 days, indicating a dramatic loss in the nutrient if fresh green peas were not properly stored. Finally, an older study by Fellers and Stepat (1935) reported a 51.5% ascorbic acid loss in fresh green peas during 24-48 h after picking on a fresh/wet weight basis.

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Table 3.1 Accuracy and interday precision (%RSD_r) determined by analysis of the L-ascorbic acid extracted from BCR® 431 (Brussels sprouts powder).

Analyte	Mass fraction (mg/100 g)			% of accepted value[4]	%RSD_r[5]
	Accepted value[1]	Analytical value[2]	Bias[3]		
Vitamin C (total ascorbate)	483 ±24	473 ± 11	10	97.9	0.6

[1]The accepted value of L-ascorbic acid was from the certified value of BCR® 431.

[2]Values (mean ± SD) are based on 9 replicate analyses.

[3]Bias = Accepted value − Analytical value.

[4]The ratio of the analytical value to accepted value expressed as a percentage.

[5]RSD_r, interday relative standard deviation (SD/mean × 100)

Table 3.2 L-Ascorbic acid recoveries in frozen fruits and vegetables.

Frozen Produce	Recovery (%)
Blueberry	93 ± 2
Strawberry	104 ± 2
Broccoli	105 ± 3
Cauliflower	94 ± 3
Corn	99 ± 5
Green Beans	95 ± 7
Spinach	103 ± 1
Green Peas	98 ± 4

[1]Values (mean % ± SD) are based on 3 replicate analyses using USP-grade L-ascorbic acid.

Table 3.3 L-Ascorbic acid content (mg AA/100 g, f.w.) in selected fresh, fresh-stored, and frozen fruits.[1]

Fruits	Factors	Year 2011			Year 2012			Year 2013	Mean±SD[2]	U.S.D.A. Mean±SE[3]
		Summer	Fall	W/S[4]	Summer	Fall	W/S			
Blueberry	Fresh	13.3±0.06	6.3±0.11	13.5±0.54	19.0±2.92	11.6±1.34	15.2±3.04	13.1±4.23A	]	9.7±0.89 (*n*=4)
	Fresh-Stored	12.6±0.05	5.0±0.11	11.5±0.75	10.3±1.40	11.0±1.54	11.1±1.27	10.2±2.66A	]	
	Frozen	4.3±0.05	3.0±0.02	10.2±0.40	9.7±0.25	19.8±1.86	12.2±0.17	9.9±5.71A		2.5±2.37 (*n*=3)
Strawberry	Fresh	62.2±0.01	61.6±0.25	68.6±0.43	46.4±4.71	48.5±4.92	44.1±0.90	55.2±9.83A	]	58.8±2.47 (*n*=9)
	Fresh-Stored	45.9±0.29	60.3±0.81	68.2±0.26	37.8±1.19	38.5±1.24	38.9±1.55	48.3±12.21A	]	
	Frozen	59.1±0.34	60.2±0.02	67.7±0.51	55.8±2.50	44.6±1.49	40.8±0.57	54.7±9.61A		41.2±9.06 (*n*=6)

[1]Abbreviations: AA – ascorbic acid; f.w. – fresh weight.

[2]Mean ± SD values calculated based on 18 individual observations. Those followed by a different letter within a column (for each sample) reflect significant differences ($p \leqslant 0.05$) according to ANOVA and means separation with Tukey's Studentized Range *via* SAS software.

[3]U.S.D.A. National Nutrient Database for Standard Reference (R26) sample means ± standard error {SE} (*n* = number of data points) for: Broccoli, raw; Broccoli, frozen, chopped, unprepared; Cauliflower, raw; Cauliflower, frozen, unprepared; Corn, sweet, yellow, raw; Corn, sweet, yellow, frozen, kernels cut off cob, unprepared; Beans, snap, green, raw; Beans, snap, green, frozen, all styles, unprepared; Peas, green, raw; Peas, green, frozen, unprepared; Spinach, raw; Spinach, frozen, chopped or leaf, unprepared; Blueberries, raw; Blueberries, frozen, unsweetened; Strawberries, raw and Strawberries, frozen, unsweetened.

[4]W/S –Winter/Spring.

Table 3.4 L-Ascorbic acid content (mg AA/100 g, f.w.) in selected fresh, fresh-stored, and frozen vegetables.[1]

Vegetables	Factors	Year 2011			Year 2012			Year 2013	Mean±SD[2]	U.S.D.A.[3] Mean±SE
		Summer	Fall	W/S[4]	Summer	Fall	W/S			
Broccoli	Fresh	66.0±0.01	55.2±0.28	42.0±0.26	45.0±0.49	48.9±0.51	44.3±0.32	50.2±8.47A	89.2±3.98 (*n*=19)	
	Fresh-Stored	65.5±0.04	53.5±0.24	32.3±1.43	44.0±0.78	42.6±1.35	42.2±1.30	46.7±10.75A		
	Frozen	55.3±0.65	48.9±0.32	49.0±0.33	47.9±0.70	54.8±0.93	47.7±0.75	50.6±3.32A	56.4±2.85 (*n*=32)	
Cauliflower	Fresh	46.8±0.42	39.8±0.22	54.6±0.40	36.9±0.27	49.0±1.04	43.5±1.38	45.1±6.07A	48.2±3.77 (*n*=28)	
	Fresh-Stored	45.5±0.35	32.8±0.40	50.8±0.20	36.0±0.27	46.0±0.58	41.1±0.54	42.0±6.35A		
	Frozen	44.2±0.33	52.2±0.33	32.4±0.78	36.6±0.03	44.6±0.46	32.1±0.75	40.4±7.50A	48.8±1.67 (*n*=34)	
Corn	Fresh	5.5±0.001	6.3±0.31	7.1±0.11	4.8±0.13	7.1±0.56	6.0±0.38	6.2±0.90A	6.8±0.57 (*n*=7)	
	Fresh-Stored	5.2±0.19	5.0±0.18	7.0±0.27	4.7±0.13	6.7±0.19	5.7±0.18	5.7±0.90A		
	Frozen	6.0±0.08	5.0±0.19	6.5±0.50	4.5±0.25	7.1±0.35	8.2±0.18	6.2±1.29A	6.4±1.55 (*n*=68)	
Green Beans	Fresh	12.6±0.001	12.3±0.50	6.5±0.27	3.5±0.70	4.5±0.17	6.8±0.13	7.7±3.66A	12.2±0.75 (*n*=9)	
	Fresh-Stored	11.4±0.01	11.4±0.15	5.5±0.28	3.4±0.20	2.7±0.17	6.0±0.13	6.7±3.60A		
	Frozen	12.9±0.18	12.3±0.18	8.2±0.25	14.2±0.18	9.5±0.46	8.7±0.22	11.0±2.34B	12.9±1.48 (*n*=80)	
Spinach	Fresh	26.5±0.44	29.1±0.14	20.0±0.11	21.3±0.11	27.8±0.65	26.7±0.62	25.2±3.49A	28.1±4.13 (*n*=7)	
	Fresh-Stored	16.1±0.37	11.4±0.11	19.7±0.15	19.2±0.15	26.0±0.74	25.0±0.72	19.6±5.51B		
	Frozen	13.8±0.18	12.2±0.05	26.6±0.37	12.5±0.19	11.5±0.27	10.2±0.21	14.5±5.70C	5.5±0.69 (*n*=11)	

[1,2,3,4]See footnotes of Table 3.3 for description of the samples reported from the U.S.D.A. National Nutrient Database for Standard Reference (R26).

Table 3.5 L-Ascorbic acid content (mg AA/100 g, f.w.) in fresh, fresh-stored, and frozen green peas.[1]

Vegetable	Factors	Sampling Period						Mean±SD[2]	U.S.D.A.[3] Mean±SE
		1	2	3	4	5	6		
Green Peas	Fresh	13.0±0.20	27.2±5.89	16.8±1.68	17.2±2.01	20.2±2.65	19.8±1.84	19.0±5.11A	40.0 (*n*=1)
	Fresh-Stored	12.3±0.73	12.3±0.40	14.6±2.35	14.8±1.65	18.0±1.58	20.6±2.35	15.4±3.42B	
	Frozen	19.5±0.06	23.7±0.19	19.5±0.11	17.2±0.25	19.8±1.8	26.6±0.18	21.1±3.27A	18.0±0.66(*n*=7)

[1,2,3]See footnotes of Table 3.3 for description of the samples reported from the U.S.D.A. National Nutrient Database for Standard Reference (R26).

CHAPTER 4

TRANS-β-CAROTENE CONTENT, AS A REPRESENTATION OF VITAMIN A, OF FRESH, FRESH-STORED, AND FROZEN FRUITS AND VEGETABLES

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ABSTRACT:

The objectives of the study were to determine and compare the *trans*-β-carotene content in selected fresh, fresh-stored, and frozen fruits and vegetables, while mimicking typical consumer purchasing and storage patterns of the produce. European Standard Methods EN 12823-1 and -2 (2000) were employed to analyze the *trans*-β-carotene content in the selected fruit and vegetable samples. The *trans*-β-carotene contents were determined in fresh, fresh-stored, and frozen blueberries, strawberries, broccoli, cauliflower, corn, green beans, spinach, and green peas. For each of the 8 sample-types, one-way ANOVA was performed to determine the presence of significant difference in *trans*-β-carotene contents according to treatment (α=0.05). The study did not find *trans*-β-carotene (*i.e.,* representation of vitamin A) contents in fresh-stored produce to be superior to its frozen counterpart in most cases.

4.1 Introduction

Vitamin A is a fat-soluble vitamin found widely in plants. It is responsible for giving carrots, sweet potatoes, squash, and other vegetables their orange color. Vitamin A refers to all isoprenoid compounds that possess the biological activity of all-*trans*-retinol. As an accepted term, "provitamin A" is used to differentiate carotenoid precursors of vitamin A from carotenoids without vitamin A activity. Vegetables, including carrots, sweet potatoes, and dark-green leafy vegetables like spinach, are great sources of provitamin A carotenoids for humans. Dietary vitamin A is designated as "performed vitamin A" when consumed as a dietary constituent of animal products. *Trans*-β-carotene ($C_{40}H_{56}$, Figure 4.1) is the most important precursor of vitamin A in humans; it also acts as an antioxidant to deactivate reactive lipophilic free radicals.

Deficiency of vitamin A in one's diet can lead to night blindness, and even complete blindness in severe cases. Conversely, an overdose may cause carotenodermia, a physically harmless condition resulting in an orange skin tint. There are some controversial opinions regarding β-carotene and cancer. Several studies indicate that β-carotene may prevent certain types of cancer due to its antioxidative properties, while others suggest an increased risk of lung cancer with β-carotene supplementation. A re-examination of the literature and segregation of supplemented β-carotene with natural sources of β-carotene from food may be warranted.

There are many methods available in the literature on how to analyze vitamin A, including colorimetric, spectrophotometric, spectrofluorometric, classical column as well as thin-layer chromatography, and high-performance liquid chromatography (HPLC; both normal- and reversed-phase with either ultraviolet-visible or fluorescent detection). Generally, HPLC analysis

with UV-visible detection is the recommended assay for carotenoids in fruits or vegetables. This is because many of the other methods overestimate vitamin A activity. Biologically-inactive carotenoids are more difficult to completely extract from the provitamin A carotenoids (Eitenmiller, Landen, & Ye, 2007).

Reversed-phase high-performance liquid chromatography (RP-HPLC) is a separation technique whereby compounds interact with both a stationary and a mobile phase. In RP-HPLC, the stationary phase is less polar relative to the mobile phase. Of course, polarity is a relative term. Molecules interact with the modified silica of the stationary phase, which typically comprises spherical silica beads (of a 3 to 5-μm particle size) with either octyl, octadecyl, or C_{30} groups bound to the silica *via* covalent bonds. A mixture being separated by RP-HPLC elutes from the column in order of decreasing polarity. Often the mobile phase composition needs to be gradually changed from a polar one to a less polar one over time in gradient RP-HPLC work in order to efficiently separate all of the bioactives in a mixture. An isocratic method was preferred and achieved in this work using a C_{30} stationary phase, which was chosen for its superior selectivity for a variety of carotenoid isomers (Emenhiser, Sander, & Schwartz, 1995; Sharpless, Thomas, Sander, & Wise, 1996; Bell, Sander, & Wise, 1997), for which the details are provided in the experimental section.

The objectives of the present study were to determine and compare the *trans*-β-carotene content (also calculated as vitamin A) in selected fresh, fresh-stored, and frozen fruits and vegetables, while mimicking typical consumer purchasing and storage patterns of the produce.

4.2 Materials and Methods

European Standard Methods EN 12823-1 and -2 (2000) were employed to analyze the *trans*-β-carotene content (provitamin A) in the selected fruit and vegetable samples. The analysis required a saponification step followed by extraction of the unsaponifiable fraction and HPLC analysis. HPLC analysis was accomplished *via* an unpublished method by Sanders, described in a technical note obtained from YMC™ Separation Technologies, entitled "Separation of carotenoids found in algae (YMC™ Separation Technology, 2007)".

4.2.1 Sample handling

Fruit and vegetable samples (*i.e.*, blueberries, strawberries, broccoli, cauliflower, corn, green beans, spinach, and green peas), either fresh or frozen, were purchased from 6 supermarkets within a 40-km radius of Athens, GA. The selected stores were common U.S. supermarket chains and included Walmart, Sam's Club, Kroger, Publix, Piggly-Wiggly, and Ingles, with Bell's as a back-up. All of the frozen produce acquired was private-label.

On the occasions when fresh fruits and vegetables were purchased, double the quantity of that of the frozen produce was acquired. Half of the purchased fresh produce was placed in a standard kitchen refrigerator for 5 days before analysis. This procedure was intended to mimic the purchase and storage practice of fresh produce by consumers. Hence, these fruits and vegetables are referred to as fresh-stored.

A ~200-g portion of vegetable or fruit from each store was combined in a large grey plastic tub, and then mixed well to give a composite sample. A blanching step was employed, for the fresh and fresh-stored produce to inactivate endogenous enzymes, which can cause

deterioration and nutrient loss. All frozen vegetables had been blanched prior to freezing, whereas the fruits were blast frozen. Representative samples from each composite were then taken for β-carotene analysis. A set of fresh samples were stored at 4 °C and the experiment was repeated 5 days later.

4.2.2 Blanching and saponification

Each fresh or fresh-stored composite sample of fruit or vegetable was heat-treated. The samples were blanched for 1 min in a pot filled with boiling water (100 °C) to minimize enzymatic degradation of the β-carotene from lipoxygenase prior to grinding and saponification. In the case of fresh and fresh-stored corn, kernels were cut from the cob post blanching and prior to grinding and saponification. A blanching step was not necessary for the frozen vegetables, as they had already been steam blanched prior to freezing. After blanching, the composite sample was chilled in an ice water bath and tapped onto dry paper towels to remove excess water. The sample was ground in a 70-W Black & Decker one-touch chopper (Model HC306, Applica Consumer Products, Inc., Miramar, FL) just preceding the assay.

Saponification was accomplished by weighing and combining 10 g of the finely ground sample and 1 g of pyrogallol (CAS 87-66-1; Acros Organics, 99% purity from Fisher) in a 125-mL Erlenmeyer flask with a 24/40 joint. Pyrogallol was added to serve as an antioxidant during saponification. One hundred milliliters of 95% (v/v) ethanol and 20 mL of 60% (w/v) KOH were added to the flask followed by swirling of the mixture. Blanketed under nitrogen and with a Snyder column attached, the flasks containing the test samples were placed in an 81 ± 2 °C water

bath. The samples were saponified under reflux for 45 min, then removed and placed in an ice water bath to cool.

4.2.3 Moisture analyses

A moisture analysis was performed gravimetrically in triplicate on all fruits and vegetables by placing a ground portion of each sample in a forced-air convection oven at 103 °C until a constant mass was reached (usually 24 h). The moisture analyses were performed to provide a uniform mass balance for all data; that is, being able to convert collected data from a fresh weight basis (f.w.) to a blanched weight basis (b.w.) to a dry weight basis (d.w). Because the composite of all fresh and fresh-stored produce was blanched prior to saponification, the moisture contents of the composites were determined and compared to their non-blanched counterparts. The conversion of *trans*-β-carotene levels based on blanched weight (b.w.) to fresh weight (f.w.) was calculated using the following equation:

$$\textit{trans}\text{-}\beta\text{-carotene (f.w., } \mu g/100\ g) = \textit{trans}\text{-}\beta\text{-carotene (b.w., } \mu g/100\ g) \times \frac{(1-M_b)}{(1-M_f)}$$

where, M_b is the moisture content of the blanched produce; and M_f is the moisture content of the fresh or fresh-stored (unblanched) produce.

4.2.4 Extraction of the unsaponifable fraction

The contents of the cooled saponified sample were quantitatively transferred to a 1-L separatory funnel, fixed with a funnel and glass wool. Hexanes (ACS grade, Fisher) were used to extract the *trans*-β-carotene. The rinsing/transfer step included rinsing the saponification flask with 80 mL of deionized water followed by ~125 mL of hexanes. The combined layers were

vigorously shaken for 60 s and *in situ* separation of the aqueous and organic layers was achieved. The lower aqueous phase was removed *via* the funnel's PTFE stopcock into the original 125-mL Erlenmeyer flask. The upper organic layer was quantitatively transferred to a 500-mL Erlenmeyer flask with a small portion of hexanes. The aqueous layer was then returned to the 1-L separatory funnel and the extraction was repeated 2× more, pooling the organic layers. The pooled extract was quantitatively returned to the separatory funnel and washed 3× with 150 mL of deionized water, or until the pH was neutral (7.0), as determined by pH paper (Fisher). The extract was then filtered over anhydrous sodium sulfate (Fisher) through a glass funnel filled with a glass wool plug into a 1-L round-bottom flask. The extract was then concentrated to near dryness with a Büchi Rotavapor R-210 using a V-700 vacuum pump connected to a V-850 vacuum controller (Büchi Corporation, New Castle, DE) at 45 °C. Final traces of solvent were removed using a nitrogen evaporator (N-EVAP™ 111, Organomation Associates, Inc., Berlin, MA). The dried extract was resuspended in a known volume of the HPLC mobile phase methanol: methyl tertiary-butyl ether (MeOH: MTBE, 75:25, v/v). An aliquot of the resuspended extract was transferred to a 1.5-mL polyethylene microcentrifuge tube and spun for 2 min at 10,000×g. The supernate was transferred to a 2-mL HPLC amber vial (Agilent Technologies), capped and subjected to HPLC analysis. All samples were analyzed in triplicate.

4.2.5 HPLC separation of all *trans*-β-carotene

An Agilent 1200 series quaternary pump with degasser, autosampler, diode array detector and ChemStation software were employed and fitted with a YMC™ C_{30} carotenoid HPLC column (4.6 × 250 mm, 3-µm particle size; Waters Corporation, Milford, MA), YMC™

carotenoid S-3 DC guard cartridge (4.0 × 200 mm) and YMC™ direct connect end fitting. Elution parameters entailed an isocratic system at a flow rate of 2.0 mL/min with 75:25 (v/v) CH_3OH:MTBE as the mobile phase according to unpublished research, courtesy of Sander (YMC™ Separation Technology, 2007). Injection volumes were sample dependent and typically 20 or 100 μL. Visible detection was monitored at a $\lambda = 450$ nm and the column temperature was maintained at 35 °C.

A range of volumes (20 to 100 μL) of working solutions of a commercial source of *trans*-β-carotene (CAS 7235-40-7, type I, synthetic, ≥ 93%, Sigma-Aldrich Co., St. Louis, MO) were injected into the HPLC-system. Retention time mapping of the separated peaks of the sample against the β-carotene standard solution was performed to identify the *trans*-β-carotene in the sample. Further confirmation of the retention time was achieved by the standard additions method of spiking the fruit and vegetable test samples with the commercial *trans*-β-carotene standard. Quantitative analysis was then performed with an external standard; the peak areas pertaining to *trans*-β-carotene were integrated and the results compared against the corresponding values for the standard calibration curve.

4.2.6 Calculations

The mass concentration, ρ, of total *trans*-β-carotene in mg/100 g of the sample (triplicate samples, averaged) was calculated using the following equation:

$$\rho = \frac{A_s \times C \times V_s \times V_{st} \times 100}{A_{st} \times M \times V_{is} \times 1{,}000}$$

where, A_s is the peak areas for the β-carotene isomers obtained with the sample test solution; C is the corrected purity of the standard solution (μg/mL) {see details in the quality control plan}; V_s

is the total volume of sample test solution (mL); V_{st} is the injection volume of the standard solution (μL); A_{st} is the peak area for *trans*-β-carotene obtained with the standard solution in units of area; M is the sample mass (g); V_{is} is the injection volume of the sample test solution (μL); 1,000 is the conversion factor for μg to mg; and 100 is the conversion factor for the content to be reported per 100-g sample.

Because the fresh and fresh-stored composite samples were blanched before the *trans*-β-carotene analysis, their masses changed slightly. As described above, the *trans*-β-carotene contents in fresh and fresh-stored samples were therefore corrected according to their moisture contents before and after blanching. Each sample was analyzed in triplicate; means and standard deviations were calculated.

4.3 Quality control plan

To validate the accuracy and interday precision (*i.e.*, relative repeatability standard deviation, $\%RSD_r$) for the analysis of *trans*-β-carotene in the fruit and vegetable samples, a quality control plan was established.

Concentration and purity of the purchased trans-β-carotene

The *trans*-β-carotene commercial standard (20 mg) was weighed and transferred to a Pyrex® 100-mL Class A low-actinic (red) volumetric flask with ~20 mL of *n*-hexane, sonicated for 60 s using a 104X Ultrasonik™ cleaner (Dentsply International, York, PA) to fully dissolve the crystals, and the volume brought to mark with additional *n*-hexane; the final concentration was 200 μg/mL. A 3.0 μg/mL working solution was prepared by transferring 150 μL of the stock solution *via* a Gilson Pipetman *Neo*® into a 10-mL Class A low-actinic volumetric flask and

filling to mark with *n*-hexane. To determine the concentration and purity of the commercial *trans*-β-carotene standard, the absorbance of the *trans*-β-carotene stock solution in *n*-hexane was measured spectrophotometrically in a quartz cuvette at the wavelength maximum of 453 nm using an Agilent 8453 UV-visible diode array spectrophotometer (Agilent Technologies, Inc., Wilmington, DE). The mass concentration, ρ (in mg/mL), was calculated based on the following equation:

$$\rho = \frac{A_{\lambda 453} \times 10^4}{2{,}592}$$

where, $A_{\lambda 453}$ is the absorbance of the stock *trans*-β-carotene solution at the wavelength maximum of 453 nm; and 2,592 is the $E^{1\%}_{1cm}$ value of *trans*-β-carotene in *n*-hexane (*NB* that it may change considerably with the composition of the solvent).

Preparation of the trans-β-carotene standard and calibration solutions (for HPLC)

Twenty milligrams of the *trans*-β-carotene commercial standard and 1 g of pyrogallol were weighed and quantitatively transferred to a Pyrex® 100-mL Class A low-actinic volumetric flask with ~20 mL of 75:25 (v/v) CH_3OH:MTBE (*i.e.*, the mobile phase). The solution was sonicated for 60 s using the Ultrasonik™ cleaner (Dentsply) to fully dissolve the crystals, and brought to volume with additional mobile phase; the final stock concentration was 200 μg/mL. The flask was stoppered, sealed with Teflon tape, and stored at -30 °C for up to 3 weeks. Working *trans*-β-carotene standard solutions were prepared from the stock solution at concentrations of 1, 3, 75, 150 and 200 μg/mL mobile phase. Before HPLC analysis, an aliquot of each solution was transferred to 2-mL HPLC amber vials, capped, and then placed in the autosampler magazine.

Linearity

The linearity of the calibration curves from the absorption responses of the diode array detector was analyzed over concentrations ranging from 1 to 200 μg/mL using the *trans*-β-carotene working solutions; all working solutions were prepared in triplicate. The linearity was evaluated by linear regression analysis, which was calculated by least-squares regression analysis.

Accuracy

In this study, a CRM from the European Commission Joint Research Center, Institute for Reference Materials and Measurements, BCR® 485 (mixed vegetables), was purchased from the Resource Technology Corporation (Laramie, WY). Accuracy was assessed by comparing the data value obtained for a measurement to that of the accepted value of the CRM. Bias, which is defined as the difference between the analytical value from that of the accepted value provided on the CRM's certificates of analysis, was also determined (Horwitz, 2003). The bias and % accepted value were calculated as follows:

$$\text{Bias} = \mu - x$$

$$\text{Accepted value (\%)} = x/\mu \times 100$$

where, x is the analytical value; and μ is the accepted value provided by the certificate of analysis of the reference sample.

Recovery is defined as the fraction of the analyte measured after addition of a known quantity of the analyte to the sample. Recovery was determined on the basis of AOAC guidelines (2000). Ground fruit and vegetable samples as well as the BCR® 485 (mixed vegetables) CRM were spiked with known levels of *trans*-β-carotene. Final spiking levels per gram of sample were

1, 50, and 100 μg. Each spiking experiment was performed in triplicate. The % recovery of the added *trans*-β-carotene standard was calculated as follows:

$$\text{Recovery (\%)} = [(C_s - C_p)/C_a] \times 100$$

where, C_s is the *trans*-β-carotene concentration in the spiked sample; C_p is the *trans*-β-carotene concentration in the unspiked sample; and C_a is the mass of the *trans*-β-carotene standard added.

Precision

Precision, or reproducibility, is the degree to which repeated measurements under unchanged conditions show the same results. The precision of the assay was determined by repeatability (intraday precision) and intermediate precision (interday precision) of both the *trans*-β-carotene standard and test sample solutions. Precision was determined in 5 replicates of both the working *trans*-β-carotene solution (3 μg/mL) and the test samples on the same day (intraday precision) and daily for 5 times over a period of 4 weeks (interday precision). Precision can be approximated as the relative standard deviation between trials and can be expressed as:

$$\%RSD = (SD \times 100)/\bar{x}$$

where, %RSD is the relative standard deviation; SD is the standard deviation; and $\bar{x}$ is the mean.

Sensitivity

The limit of detection (LOD) and limit of quantitation (LOQ) were the concentrations of *trans*-β-carotene that yielded a measure peak with a signal-to-noise ratio of 3:1 and 10:1, respectively.

4.4 Data Analysis

The *trans*-β-carotene contents, as a representation of vitamin A in the produce, were determined in fresh, fresh-stored, and frozen blueberries, strawberries, broccoli, cauliflower, corn, green beans, spinach, and green peas. The analyses were performed over the span of two years in 6 distinct time frames, namely Summer to Fall 2011, Fall 2011 to Winter 2012, Winter to Spring 2012, Summer to Fall 2012, Fall 2012 to Winter 2013, and Winter to Spring 2013 (see format of Table 4.3.1). Within each time period, the *trans*-β-carotene content was measured in triplicate for all combinations of treatments and samples. The data for each time frame, although reported individually in the results, are treated as replicates in our statistical analysis of the differences among treatments. Therefore, the *trans*-β-carotene content of each treatment/sample combination was assessed according to 18 replications.

For each of the 8 sample-types (*i.e.*, each specific fruit or vegetable), one-way ANOVA was performed to determine the presence of significant difference in *trans*-β-carotene contents according to treatment (α=0.05). Data transformation was applied when necessary to adequately meet the assumption of normal distribution for ANOVA, and the Weighted Least Squares method was employed in the instances in which the equal variance assumption of the ANOVA was violated. All necessary statistical diagnostic checks (e.g., residuals versus predicted value plot, Q–Q plot of residuals, and histogram plot of residuals) were performed to verify acceptability of ANOVA implementation. In the cases in which a statistically significant difference was observed among the three treatments, the Tukey's Studentized Range multiple comparisons test was performed to determine which specific pairs of treatments (*i.e.*, fresh vs.

frozen, fresh vs. fresh-stored, and fresh-stored vs. frozen) showed significant differences from one another (α=0.05).

All data analyses were performed using SAS software, version 9 of the SAS system for Windows (SAS Institute Inc., Cary, NC).

4.5 Results and Discussion

In this study, a YMC™ polymeric C_{30} reversed-phase carotenoid HPLC column was employed to separate and identify the content of all *trans*-β-carotene in the samples of fruits and vegetables. The C_{30} phase provided a high number of interaction sites for more complete partitioning and resolution of positional carotenoid isomers. Advantages of this approach included isocratic elution capabilities, greater selectivity, and moderate solvent toxicity as well as cost.

Quality Control

The purity of the *trans*-β-carotene commercial standard was 94.0%. On the basis of the purity check, the concentration in the stock standard solution was 188 μg/mL. For the daily working standard, the stock solution was diluted with an appropriate volume of the mobile phase to give concentrations of 0.94, 2.82, 9.4, 70.5, 141, and 188 μg *trans*-β-carotene/mL. Concentrations of *trans*-β-carotene in the test samples were calculated from the peak area determined by the UV-visible diode array detector of the HPLC at λ = 450 nm in injection volumes ranging between 20 to 100 μL.

Linearity

The regression equation for *trans*-β-carotene was $y = 15.579x + 26.848$ when plotting the peak area (y) versus concentration (x); the correlation coefficient ($R^2 = 0.9977$) was highly significant, thereby indicating that injection volumes between 20 to 100 μL into the HPLC showed good linearity between peak area and injection volume.

Specificity

All composite samples were analyzed by HPLC for *trans*-β-carotene content according to a method described in a technical note (YMC™ Separation Technology, 2007), utilizing a YMC™ C_{30} carotenoid column. Figure 4.2 depicts an example chromatogram of a broccoli sample, demonstrating the excellent resolution of the carotene isomers using this particular column and isocratic elution. Based on the conditions of the assay, *trans*-β-carotene eluted from the column at 7.15 min with no interfering compounds around this retention time. The ChemStation software reported a peak purity of > 99%.

Accuracy

Accuracy and $\%RSD_r$ measures for the *trans*-β-carotene analysis by the HPLC protocol are given in Table 4.1. *Trans*-β-carotene was extracted from BCR® 485 and analyzed. According to its certificate of analysis, BCR® 485 (mixed vegetables) contains 23.7 ± 1.5 mg *trans*-β-carotene/kg (mass fraction of dry matter). Comparison of the analytical value to the accepted value from the certified value of the CRM was within the accepted range reported for BCR® 485. The bias value was small and negative (-0.06). The ratio of the analytical value to the accepted value expressed as a percentage (% of accepted value) can be used to evaluate accuracy. For *trans*-β-carotene the % of accepted value was 100.25 indicating an extremely close agreement

between the analytical and accepted value. Interday precision (%RSD_r) was 1.35. Together, the accuracy and %RSD_r measures indicate that the *trans*-β-carotene analyses of the fruit and vegetable samples by the HPLC assay were accurate and repeatable. Noteworthy is that many nutrient profiling studies fail to report on these parameters.

The recovery of *trans*-β-carotene from BCR® 485 (mixed vegetables) for spiked samples was 94.5 ± 2.0. For frozen fruit and vegetable samples, the recoveries ranged from 90.7 to 99.8%, thereby demonstrating excellent recovery of *trans*-β-carotene from the test samples (see Table 4.2). Such high recoveries from the CRM and produce experiments validate the accuracy of the *trans*-β-carotene assay.

Precision

Intraday precision (repeatability) was determined by running control samples that had been spiked with a concentration of the analyte, which was ~25% higher than the threshold value. The RSD of these data were calculated and shown to be less than ± 25.8%. The daily (intraday) and day-to-day (interday) precision of the *trans*-β-carotene working solution gave the following results: based on n=5, the HPLC analysis of a 3-µg *trans*-β-carotene/mL working solution showed a relative standard deviation of 1.4%. In terms of interday precision, 3-µg *trans*-β-carotene/mL interday HPLC analysis gave a relative standard deviation of 13% (over 4 weeks). In both cases, the %RSD values were found well within the 15% limit, indicating that the current method is repeatable.

Sensitivity

The LOD and LOQ of *trans*-β-carotene by the proposed method were found to be 0.067 µg/mL and 0.22 µg/mL, respectively.

Tables 4.3.1, 4.4.1, and 4.5.1 report the *trans*-β-carotene contents in fresh, fresh-stored, and frozen fruits and vegetables based on their fresh weight (f.w.). The conversion of *trans*-β-carotene levels based on their blanched weight (b.w.) to fresh weight (f.w.) is described in the experimental section above. Tables 4.3.2, 4.4.2, and 4.5.2 give the *trans*-β-carotene contents, based on the b.w. for all of the fruits and vegetables examined, while Tables 4.3.3, 4.4.3, 4.5.3 report the vitamin A (*i.e.*, retinol activity equivalents, RAE) levels based on the fresh weights, which were calculated from the determined *trans*-β-carotene values by RP-HPLC. The equation employed to convert from *trans*-β-carotene to vitamin A (RAE) is as follows:

$$\text{vitamin A (RAE) in } \mu g/100\ g = 1/12 \times \mu g\ \textit{trans}\text{-}\beta\text{-carotene}/100\ g\ \text{sample}$$

For the purposes of this discussion, only the *trans*-β-carotene results based on their f.w. will be commented upon, as the results of the other bases are merely numerical derivatives. Table 4.3.1 gives the mean *trans*-β-carotene contents determined in fresh, fresh-stored, and frozen blueberries and strawberries as well as the corresponding fresh and frozen values from the U.S.D.A. National Nutrient Database for Standard Reference (R26), herein after referred to simply as Database. In most of the cases, the L-ascorbic acid values measured in this study were consistent relative to the values reported in the Database. However, this was not the case for *trans*-β-carotene values. The mean values for fresh, fresh-stored, and frozen blueberries were 196 ± 44, 136 ± 26, and 74 ± 24 μg of *trans*-β-carotene/100 g, f.w., respectively. All means were statistically ($p < 0.05$) different from one another! Furthermore, the *trans*-β-carotene content in fresh blueberries was markedly different, roughly 5× more than the 32 ± 3 μg/100 g, f.w. value reported in the Database, based on 18 observations. Why this is so, is uncertain. Even though the range of the *trans*-β-carotene content goes up to 89 μg/100 g, f.w. in the Database, our mean of

196 ± 44 μg *trans*-β-carotene/100 g, f.w. was surprising. For this reason, the validity of the *trans*-β-carotene commercial standard and calibration curves were rechecked, each time a blueberry sample was analyzed by the RP-HPLC methodology; no anomalies were found. It is also difficult to postulate that variation in cultivars, climatic conditions, status of berry maturities, and growing regions were solely responsible for the difference in the *trans*-β-carotene values of fresh blueberries. Unfortunately, it is impossible to compare our individual observations with the values reported in the Database. A 31% loss in *trans*-β-carotene content was noted in fresh blueberries stored at 4 °C for 5 days after purchase. Although a mean value of 74 μg *trans*-β-carotene/100 g, f.w. was found in frozen berries, the Database lists an imputed value of 28; so, no legitimate comparison is possible.

The mean values for fresh, fresh-stored, and frozen strawberries were 21.2 ± 6.5, 13.2 ± 4.0, and 20.7 ± 10.5 μg *trans*-β-carotene/100 g, f.w., respectively. Only the fresh-stored sample was significantly different ($p < 0.05$) from the other berry types. In other words, the vitamin A content in fresh and frozen strawberries were not statistically ($p > 0.05$) different. Just like the blueberries, the *trans*-β-carotene content of fresh strawberries was markedly greater than the value of 7 ± 0.2, based on 8 observations, reported in the Database. Surprisingly, the Database lists a greater imputed value of 27 μg *trans*-β-carotene/100 g, f.w. for frozen strawberries. A ~42% loss in *trans*-β-carotene was noted in fresh strawberries stored at 4 °C for 5 days after purchase. Although only two fruits were investigated, a trend suggests that *trans*-β-carotene degradation is quite pronounced after harvesting of berry fruits.

Table 4.4.1 summarizes the *trans*-β-carotene content of fresh, fresh-stored, and frozen produce based on the f.w. for broccoli, cauliflower, corn, green beans, and spinach. The mean

values for fresh, fresh-stored, and frozen broccoli were 2,020 ± 390, 1,710 ± 300, and 940 ± 200 μg/100 g, f.w., respectively, and there were no statistical ($p > 0.05$) differences between the fresh and fresh-stored test samples. Frozen broccoli was lower and statistically ($p < 0.05$) different from its fresh counterparts. Again, the *trans*-β-carotene content of fresh broccoli was magnitudes higher than the 360 μg/100 g, f.w. value listed in the Database, based on 124 observations. It is difficult to believe this value, especially when the Database lists that frozen broccoli possesses almost double the *trans*-β-carotene content (*i.e.*, 610 μg/100 g, f.w.) than that of its fresh counterpart. The only conclusion reachable is that the fresh samples were ground without blanching and then sat for a period before analysis allowing time for the enzymatic degradation of *trans*-β-carotene. In this study, both the fresh and frozen broccoli were found to possess markedly greater contents of *trans*-β-carotene concentrations than the values reported in the Database. Different methods can also lead to the variance in *trans*-β-carotene levels. For instance, Biehler, Mayer, Hoffmann, Krause, & Bohn (2010) reported carotenoid content means in fresh broccoli ranging from 2,300 to 3,500 μg/100 g using different determination methods. These authors also reported that the *trans*-β-carotene content in frozen broccoli was lower than its fresh analog. Even though not statistically different, roughly a 15% loss in *trans*-β-carotene content was noted in fresh broccoli stored at 4 °C for 5 days after purchase. Howard, Wong, Perry, & Klein (1999) reported that the *trans*-β-carotene content in broccoli increased by 19% in 1994 and decreased by 64% in 1995 after 3 weeks of storage at 4 °C; no pattern of retention was evident. Hussein *et al.* (2000) found a decrease of only 10% in *trans*-β-carotene content in fresh broccoli stored at 4 °C for 10 days. The discrepancy in this study from ours might possibly be because of post-harvest variance in sampling of a representative sample. Different packaging can also result

in a marked difference on the retention of total carotenoids during transport after processing and storage. Specifically, Barth and Zhuang (1996) found that modified atmosphere packaging prevented any loss in carotenoids of broccoli florets stored at 5 °C for 6 days, while unwrapped florets or florets wrapped in perforated film lost ~50% of their carotenoid content under the same conditions. Other studies reported better stability of carotenes in frozen broccoli. For example, Martin, Sweeney, Gilpin, & Chapman (1960) found no decrease in carotene contents of broccoli stored at 0 °C for 61 weeks. Wu, Perry, & Klein (1992) too suggested no difference in β-carotene content of frozen broccoli during 16 weeks of storage.

For cauliflower, the mean *trans*-β-carotene content in fresh products was found to be 24.2 ± 11.4 μg/100 g, f.w., whereas in the Database it is listed as zero based on 4 observations. Other studies also found detectable levels of *trans*-β-carotene in fresh cauliflower: Kurilich *et al.* (1999) reported 70 μg *trans*-β-carotene/100 g, f.w. in fresh white cauliflower. More recently, a study by Gebczynski and Kmiecik (2006) found 130 μg *trans*-β-carotene/100 g in fresh cauliflower on a dry matter basis. Our findings showed that the *trans*-β-carotene contents in fresh, fresh-stored, and frozen cauliflower were not statistically ($p > 0.05$) different. Very interesting is an investigation by Selman (1994), who reported higher *trans*-β-carotene contents in boiled cauliflower compared with raw cauliflower, which might de due to better release of the carotenoid from the food matrix for extraction. In this study, a 26% decrease in *trans*-β-carotene was noted in fresh cauliflower stored at 4 °C for 5 days after purchase.

The mean *trans*-β-carotene contents for fresh, fresh-stored, and frozen corn were 65.4 ± 34.1, 35.7 ± 20, and 77.2 ± 24 μg/100 g, f.w., respectively. Even though the absolute mean for frozen corn was higher than its fresh counterpart, the values were not statistically ($p > 0.05$)

different. On the other hand, the *trans*-β-carotene in frozen corn was significantly ($p < 0.05$) greater than that of the fresh-stored produce. Both the contents of *trans*-β-carotene in the fresh and frozen corn were found to be higher than the Database values. A study by Scott and Eldridge (2005) also reported a higher *trans*-β-carotene content in frozen golden whole kernel corn and white shoepeg compared to that of their fresh counterparts. The increase in *trans*-β-carotene observed in frozen corn may be a result of water loss from the kernels, because the blanching and freezing process can dehydrate vegetables slightly. In fresh corn, the *trans*-β-carotene contents ranged from 20.2 to 111 μg/100 g, f.w. A possible explanation for the wide range observed is variation in what parts of the world the corn-on-the-cob comes from throughout a calendar year as well as the different cultivars sold in supermarkets. In this study, a 45% decrease in *trans*-β-carotene was noted in fresh corn-on-the-cob stored at 4 °C for 5 days after purchase. Forty-five is the highest percentage of *trans*-β-carotene loss amongst the fresh fruits and vegetables examined in this study. This implies that there is a great nutritional advantage of frozen corn when it comes to vitamin A compared to fresh corn.

The mean *trans*-β-carotene contents for fresh, fresh-stored, and frozen green beans were 501 ± 71, 360 ± 114, and 359 ± 117 μg/100 g, f.w., respectively. The fresh green beans possessed a statistically ($p < 0.05$) greater content of *trans*-β-carotene than the fresh-stored and frozen produce, which themselves were not statistically ($p > 0.05$) different. The contents of *trans*-β-carotene in fresh and frozen green beans determined by this study were higher than their respective Database values of 379 (n=77) and 292 (n=5). A 23% decrease in *trans*-β-carotene was noted in fresh green beans stored at 4 °C for 5 days after purchase. A similar study by

Howard *et al.* (1999) reported an average loss of 10% for *trans*-β-carotene in green beans refrigerated for 16 days.

Of all of the fruits and vegetables examined, spinach contained the highest content of *trans*-β-carotene. The mean values for the fresh, fresh-stored, and frozen produce were 11,100 ± 2,220, 8,900 ± 1,720, and 8,500 ± 2,280 μg/100 g, f.w., respectively. Just like green beans, the fresh spinach possessed a statistically ($p < 0.05$) greater content of *trans*-β-carotene than the fresh-stored and frozen produce, which themselves were not statistically ($p > 0.05$) different. The spinach samples analyzed in this study had levels of *trans*-β-carotene markedly higher than that reported in the Database. In fact, the fresh spinach in this study possessed ~2× the 5,630 ± 770 μg *trans*-β-carotene/100 g, f.w. level given in the Database. Very interesting is that the imputed value of 7,040 μg/100 g, f.w. for frozen spinach is higher than that of fresh spinach, which was based on 5 observations. Pandrangi and LaBorde (2004) also found *trans*-β-carotene contents in fresh spinach ranging from 5,400 to 12,700 μg/100 g, f.w. A 20% loss in *trans*-β-carotene was noted in fresh spinach stored at 4 °C for 5 days after purchase. Simonetti, Porrini, & Testolin (1991) reported a 10% decrease in *trans*-β-carotene content of spinach based on wet weight over 3 weeks of storage. On the other hand, Pandrangi and LaBorde (2004) reported an 84.3% retention of carotenoids in fresh spinach stored at 4 °C for 8 days.

Unlike the other produce, fresh green peas, also referred to as English peas, could only be procured in the spring of both years of the study; hence, Table 4.5.1 reports sampling times as opposed to seasons and gives the *trans*-β-carotene content of fresh, fresh-stored, and frozen green peas based on their fresh weight; the mean values were 957 ± 343, 610 ± 150, and 1,084 ± 224 μg/100 g, f.w., respectively. Even though the absolute mean for frozen green peas was

higher than its fresh counterpart (1,084 vs. 957 μg *trans*-β-carotene/100 g, f.w.), the values were not statistically ($p > 0.05$) different. On the other hand, the *trans*-β-carotene in frozen green peas was significantly ($p < 0.05$) greater than that of the fresh-stored produce. The content of *trans*-β-carotene determined from this research for fresh green peas was found to be greater than the Database value of 449 ± 109, based on 40 observations. In the case of frozen green peas, the Database gives an imputed value of 1,225 μg *trans*-β-carotene/100 g, f.w. Guerrant *et al.* (1947) reported that blanching had little effect on the carotene content in green peas. A 36% loss in *trans*-β-carotene was noted in fresh green peas stored at 4 °C for 5 days after purchase. This conclusion was supported by Simonetti *et al*. (1991), who also found a significant decrease of *trans*-β-carotene in peas after 3 weeks of refrigerated storage. The observation seems to suggest that there might be a nutritional advantage for consuming frozen green peas when it comes to vitamin A compared to fresh green peas of unknown age.

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Table 4.1 Accuracy and interday precision (%RSD_r) determined by analysis of the *trans*-β-carotene extracted from BCR® 485.

Analyte	Mass fraction (mg/kg)			% of accepted value[4]	%RSD_r[5]
	Accepted value[1]	Analytical value[2]	Bias[3]		
trans-β-carotene	23.7 ± 1.5	23.76 ± 0.32	-0.06	100.25	1.35

[1]The accepted value of *trans*-β-carotene is calculated from the certified value of BCR® 485 based on the % mass fraction.

[2]Values (mean ± SD) are based on 5 replicate analyses.

[3]Bias = Accepted value – Analytical value.

[4]The ratio of the analytical value to accepted value expressed as a percentage.

[5]RSD_r, interday relative standard deviation (SD/mean × 100)

Table 4.2 *Trans*-β-carotene recoveries in frozen fruits and vegetables.

Frozen Produce	Recovery (%)[1]	RSD (%)[2]
Blueberry	99.8 ± 2.7	2.7
Strawberry	98.9 ± 2.9	2.9
Broccoli	96.1 ± 3.8	4.0
Cauliflower	96.2 ± 2.1	2.2
Corn	94.2 ± 3.2	3.4
Green Beans	94.5 ± 3.1	3.3
Spinach	91.8 ± 4.1	4.5
Green Peas	90.7 ± 3.6	4.0

[1]Values (mean % ± SD) are based on 3 replicate analyses using the commercial *trans*-β-carotene.

[2]RSD = relative standard deviation.

Table 4.3.1 *Trans*-β-carotene content (μg/100 g, f.w.) in selected fresh, fresh-stored, and frozen fruits.[1]

Fruits	Factors	Year 2011			Year 2012			Year 2013	Mean±SD[2]	U.S.D.A. Mean±SE[3]
		Summer	Fall	W/S[4]	Summer	Fall	W/S			
Blueberry	Fresh	175±34.5	239±38.2	252±17.9	203±74.5	164±43.6	141±23.1	196±43.6A	]	32±3.3 (*n*=16)
	Fresh-Stored	146±2.3	174±7.5	111±25.6	108±50.2	150±21.8	124±6.7	136±25.7B		
	Frozen	116±30.7	87±18.0	75±6.0	59±6.5	58±6.3	51±7.0	74±24.3C		28 (Imputed)
Strawberry	Fresh	20.6±4.4	18.0±6.2	31.0±2.1	14.1±1.8	16.7±0.3	26.9±1.9	21.2±6.5A	]	7±0.2 (*n*=8)
	Fresh-Stored	15.0±1.6	17.6±7.6	11.4±5.9	6.0±2.3	16.3±1.2	12.9±6.6	13.2±4.0B		
	Frozen	15.2±8.9	9.8±3.4	10.8±5.7	35.0±5.0	31.0±2.3	22.1±8.5	20.7±10.5A		27 (Imputed)

[1]f.w. – fresh weight.

[2]Mean±SD values calculated based on 18 individual observations. Those followed by a different letter within a column (for each sample) reflect significant differences ($p \leqslant 0.05$) according to ANOVA and means separation with Tukey's Studentized Range *via* SAS software.

[3]U.S.D.A. National Nutrient Database for Standard Reference (R26) sample means ± standard error {SE} (*n* = number of data points) for: Broccoli, raw; Broccoli, frozen, chopped, unprepared; Cauliflower, raw; Cauliflower, frozen, unprepared; Corn, sweet, yellow, raw; Corn, sweet, yellow, frozen, kernels cut off cob, unprepared; Beans, snap, green, raw; Beans, snap, green, frozen, all styles, unprepared; Peas, green, raw; Peas, green, frozen, unprepared; Spinach, raw; Spinach, frozen, chopped or leaf, unprepared; Blueberries, raw; Blueberries, frozen, unsweetened; Strawberries, raw and Strawberries, frozen, unsweetened.

[4]W/S –Winter/Spring.

Table 4.3.2 *Trans*-β-carotene content (μg/100 g, b.w.) in selected fresh, fresh-stored, and frozen fruits.[1]

Fruits	Factors	Year 2011			Year 2012			Year 2013	Mean±SD[2]	U.S.D.A. Mean±SE[3]
		Summer	Fall	W/S[4]	Summer	Fall	W/S			
Blueberry	Fresh	158±31.4	241±38.6	222±16.9	196±53.9	157±41.9	136±22.3	185±49.5A	]	32±3.3 (*n*=16)
	Fresh-Stored	121±1.9	170±7.3	112±25.8	103±47.6	139±19.9	121±6.5	128±26.6B		
	Frozen	104±1.8	84±17.4	78±6.2	55±6.0	57±5.8	50±6.8	71±21.0C		28 (Imputed)
Strawberry	Fresh	20.6±4.4	15.0±5.1	25.7±1.5	13.3±4.2	14.8±0.3	25.0±2.2	19.1±5.9A	]	7±0.2 (*n*=8)
	Fresh-Stored	13.3±4.2	14.6±5.8	10.4±5.4	4.7±1.5	14.2±0.8	10.0±5.3	11.1±4.5B		
	Frozen	11.7±6.7	7.8±2.7	9.8±5.2	32.0±4.6	27.3±1.5	19.1±7.3	18.0±10.3A		27 (Imputed)

[1]b.w. – blanched weight.

[2]Mean±SD values calculated based on 18 individual observations. Those followed by a different letter within a column (for each sample) reflect significant differences ($p \leqslant 0.05$) according to ANOVA and means separation with Tukey's Studentized Range *via* SAS software.

[3]U.S.D.A. National Nutrient Database for Standard Reference (R26) sample means ± standard error {SE} (*n* = number of data points) for: Broccoli, raw; Broccoli, frozen, chopped, unprepared; Cauliflower, raw; Cauliflower, frozen, unprepared; Corn, sweet, yellow, raw; Corn, sweet, yellow, frozen, kernels cut off cob, unprepared; Beans, snap, green, raw; Beans, snap, green, frozen, all styles, unprepared; Peas, green, raw; Peas, green, frozen, unprepared; Spinach, raw; Spinach, frozen, chopped or leaf, unprepared; Blueberries, raw; Blueberries, frozen, unsweetened; Strawberries, raw and Strawberries, frozen, unsweetened.

[4]W/S –Winter/Spring.

Table 4.3.3 Vitamin A, RAE (μg/100 g, f.w.) in selected fresh, fresh-stored, and frozen fruits.[1]

Fruits	Factors	Year 2011			Year 2012			Year 2013	Mean±SD[2]	U.S.D.A. Mean±SE[3]
		Summer	Fall	W/S[4]	Summer	Fall	W/S			
Blueberry	Fresh	14.6±2.93	19.9±3.17	21.0±1.60	17.4±5.15	13.6±3.64	11.8±1.90	16.4±4.41A	]	3 (Imputed)
	Fresh-Stored	12.2±0.17	14.5±0.64	9.2±2.14	9.0±4.16	12.5±1.78	10.3±0.57	11.1±2.64B		
	Frozen	9.7±0.16	7.2±1.49	6.3±0.49	4.9±0.55	4.8±0.49	4.2±0.59	6.2±2.00C		2(Imputed)
Strawberry	Fresh	1.7±0.37	1.5±0.52	2.6±0.16	1.2±0.37	1.4±0.02	2.2±0.18	1.8±0.57A	]	1±0.031(*n*=6)
	Fresh-Stored	1.3±0.14	1.5±0.57	1.0±0.49	0.5±0.17	1.4±0.07	1.1±0.57	1.0±0.47B		
	Frozen	1.2±0.70	0.8±0.28	0.9±0.47	2.9±0.42	2.6±0.14	1.8±0.70	1.7±0.93A		2±0.51 (*n*=5)

[1]f.w. – fresh weight.

[2]Mean±SD values calculated based on 18 individual observations. Those followed by a different letter within a column (for each sample) reflect significant differences ($p \leqslant 0.05$) according to ANOVA and means separation with Tukey's Studentized Range *via* SAS software.

[3]U.S.D.A. National Nutrient Database for Standard Reference (R26) sample means ± standard error {SE} (*n* = number of data points) for: Broccoli, raw; Broccoli, frozen, chopped, unprepared; Cauliflower, raw; Cauliflower, frozen, unprepared; Corn, sweet, yellow, raw; Corn, sweet, yellow, frozen, kernels cut off cob, unprepared; Beans, snap, green, raw; Beans, snap, green, frozen, all styles, unprepared; Peas, green, raw; Peas, green, frozen, unprepared; Spinach, raw; Spinach, frozen, chopped or leaf, unprepared; Blueberries, raw; Blueberries, frozen, unsweetened; Strawberries, raw and Strawberries, frozen, unsweetened.

[4]W/S –Winter/Spring.

Table 4.4.1 *Trans*-β-carotene content (μg/100 g, f.w.) in selected fresh, fresh-stored, and frozen vegetables.[1]

Vegetables	Factors	Year 2011			Year 2012			Year 2013	Mean±SD[2]	U.S.D.A.[3] Mean±SE
		Summer	Fall	W/S[4]	Summer	Fall	W/S			
Broccoli	Fresh	1.55±0.36	1.72±0.18	2.30±0.14	2.11±0.13	2.22±0.25	2.25±0.43	2020±390A	]	360±10
	Fresh-Stored	1.46±0.34	1.33±0.23	2.05±0.23	1.70±0.21	1.63±0.20	2.07±0.17	1710±300A	]	(*n*=124)
	Frozen	1.10±0.16	1.35±0.24	0.85±0.07	0.73±0.04	0.75±0.06	0.85±0.07	940±200B		610(*n*=1)
Cauliflower	Fresh	14.1±1.5	23.8±1.3	44.2±1.4	12.8±0.5	28.7±4.3	21.7±6.6	24.2±11.4A	]	0±0.01(*n*=4)
	Fresh-Stored	8.1±0.1	22.6±8.3	40.0±6.2	9.2±3.0	18.7±3.0	8.4±0.3	17.8±12.6A	]	
	Frozen	14.8±2.4	31.3±6.0	10.0±2.9	18.6±9.0	12.0±3.7	12.5±2.5	16.5±7.7A		7(Imputed)
Corn	Fresh	111.4±2.9	45.7±16.6	20.2±1.4	78.7±17.1	92.0±0.1	44.5±2.7	65.4±34.1AB	]	47(Imputed)
	Fresh-Stored	20.0±6.6	38.4±3.8	16.6±2.9	34.1±6.9	72.9±4.6	32.0±0.1	35.7±20.0B	]	
	Frozen	127.1±20.7	33.3±7.8	53.0±1.2	71.9±18.0	74.0±20.9	103.8±13.8	77.2±34.0A		49(Imputed)
Green Beans	Fresh	430±182	670±70.7	373±94.6	657±48.1	347±71.0	476±49.0	501±70.5A	]	379±49.0
	Fresh-Stored	595±351	412±51.9	397±34.7	189±37.7	322±62.4	244±45.1	360±144B	]	(*n*=77)
	Frozen	276±25.1	288±92.4	483±40.7	391±18.9	408±32.3	295±46.1	359±117B		292(*n*=5)
Spinach	Fresh	9.41±0.82	11.3±0.23	9.37±0.11	9.73±0.39	11.4±0.51	15.1±0.11	11100±2200A	]	5630±770
	Fresh-Stored	8.30±0.11	10.7±0.36	8.27±0.85	6.07±0.19	9.58±0.20	10.4±0.90	8900±1720B	]	(*n*=5)
	Frozen	7.46±1.57	8.17±2.45	6.07±0.23	11.2±0.60	6.64±0.94	11.3±0.56	8500±2280B		7040(Imputed)

[1,2,3,4]See footnotes of Table 4.3.1 for description of the samples reported from the U.S.D.A. National Nutrient Database for Standard Reference (R26).

Table 4.4.2 *Trans*-β-carotene content (μg/100 g, b.w.) in selected fresh, fresh-stored, and frozen vegetables.[1]

Vegetables	Factors	Year 2011			Year 2012			Year 2013	Mean±SD[2]	U.S.D.A.[3] Mean±SE
		Summer	Fall	W/S[4]	Summer	Fall	W/S			
Broccoli	Fresh	1.17±0.27	1.18±0.12	1.56±0.09	1.48±0.09	1.45±0.16	1.61±0.31	1410±240A	]	360±10
	Fresh-Stored	1.15±0.27	0.91±0.15	1.44±0.15	1.16±0.14	1.18±0.15	1.45±0.12	1220±300A	]	(*n*=124)
	Frozen	0.75±0.05	1.11±0.03	0.69±0.06	0.61±0.04	0.60±0.05	0.69±0.06	740±200B		610(*n*=1)
Cauliflower	Fresh	12.1±1.3	20.8±1.1	37.8±1.1	12.1±0.9	25.7±3.8	17.0±7.5	20.0±9.7A	]	0±0.01(*n*=4)
	Fresh-Stored	7.1±0.1	19.6±7.2	36.0±5.6	8.3±2.1	15.7±2.5	7.0±0.1	15.6±12.0A	]	
	Frozen	11.8±1.9	26.3±5.0	7.7±2.1	15.6±6.7	10.0±2.6	9.5±1.9	13.5±7.2A		7(Imputed)
Corn	Fresh	92.6±2.2	37.6±13.7	22.2±1.6	83.2±36.5	89.0±0.1	42.5±2.6	61.2±31.7A	]	47(Imputed)
	Fresh-Stored	19.0±6.3	35.4±3.5	17.6±3.1	37.1±7.5	69.7±4.5	30.0±0.1	34.8±20.1B	]	
	Frozen	107.1±17.4	30.3±7.1	46.0±1.1	61.9±15.5	63.7±17.5	88.7±11.0	66.3±28.4A		49(Imputed)
Green Beans	Fresh	490±166	459±67.6	373±81.4	453±52.3	347±70.3	476±51.6	433±93.8A	]	379±49.0
	Fresh-Stored	451±266	419±53.0	362±8.6	206±40.6	331±64.1	266±48.7	339±120.5B	]	(*n*=77)
	Frozen	209±20.9	293±76.8	440±35.1	426±16.2	419±31.0	321±42.1	338±96.7B		292(*n*=5)
Spinach	Fresh	7.60±0.66	8.56±0.18	7.64±0.09	7.56±0.09	8.53±0.38	10.5±0.07	8400±1100A	]	5630±770
	Fresh-Stored	5.89±0.78	8.08±0.68	6.21±0.64	5.12±0.16	6.96±0.15	7.43±0.64	6610±1210B	]	(*n*=5)
	Frozen	5.93±1.24	5.87±1.76	5.16±0.19	9.08±0.49	2.56±0.66	7.60±0.37	6030±2240B		7040(Imputed)

[1,2,3,4]See footnotes of Table 4.3.2 for description of the samples reported from the U.S.D.A. National Nutrient Database for Standard Reference (R26).

Table 4.4.3 Vitamin A, RAE (μg/100 g, f.w.) in selected fresh, fresh-stored, and frozen vegetables.[1]

Vegetables	Factors	Year 2011			Year 2012			Year 2013	Mean±SD[2]	U.S.D.A.[3] Mean±SE
		Summer	Fall	W/S[4]	Summer	Fall	W/S			
Broccoli	Fresh	129±30.2	143±14.6	191±11.3	176±10.6	185±20.6	187±35.9	169±31.0A	]	31(Imputed)
	Fresh-Stored	122±22.2	111±18.9	171±17.8	142±17.1	136±16.8	172±13.7	143±29.4A		
	Frozen	91.6±5.8	112±3.1	70.6±5.7	60.7±3.6	62.5±5.1	70.4±5.7	78.0±29.3B		52(Imputed)
Cauliflower	Fresh	1.2±0.13	2.0±0.11	3.7±0.10	1.1±0.08	2.4±0.35	1.8±0.80	2.0±0.95A	]	0 (Imputed)
	Fresh-Stored	0.7±0.01	1.9±0.69	3.3±0.52	0.8±0.19	1.6±0.25	0.8±0.10	1.5±1.03A		
	Frozen	1.2±0.20	2.6±0.50	0.8±0.23	1.5±0.66	1.0±0.26	1.0±0.21	1.4±0.69A		1(Imputed)
Corn	Fresh	9.3±0.21	3.8±1.39	1.7±0.12	6.6±2.88	7.1±0.01	3.7±0.22	5.5±2.90A	]	9(Imputed)
	Fresh-Stored	1.7±0.55	3.2±0.32	1.4±0.25	2.8±0.58	6.1±0.39	2.7±0.01	2.9±1.53B		
	Frozen	10.6±1.72	2.8±0.65	4.4±0.10	6.0±1.51	6.2±1.69	8.6±1.07	6.4±2.86A		10(Imputed)
Green Beans	Fresh	35.8±15.3	55.9±5.8	31.1±7.9	54.6±4.1	28.7±5.9	39.0±4.1	42.7±8.4A	]	35(Imputed)
	Fresh-Stored	49.6±29.2	34.4±4.3	33.1±0.8	15.7±3.1	26.8±5.2	20.3±3.7	30.7±14.9B		
	Frozen	15.9±2.1	23.1±7.8	19.1±3.3	55.4±1.5	25.7±2.7	29.0±3.9	30.6±9.8B		27(Imputed)
Spinach	Fresh	784±68.5	943±19.2	806±9.5	811±9.6	949±42.4	1261±8.8	926±171A	]	469(Imputed)
	Fresh-Stored	691±91.2	894±75.6	689±70.9	506±16.2	798±16.8	868±74.8	745±154A		
	Frozen	622±130	681±204	505±18.8	934±50.2	554±142.4	946±46.6	707±204A		586(Imputed)

[1,2,3,4]See footnotes of Table 4.3.3 for description of the samples reported from the U.S.D.A. National Nutrient Database for Standard Reference (R26).

Table 4.5.1 *Trans*-β-carotene content (μg/100 g, f.w.) in fresh, fresh-stored, and frozen green peas.[1]

Vegetable	Factors	Sampling Period						Mean±SD[2]	U.S.D.A.[3] Mean±SE
		1	2	3	4	5	6		
Green Peas	Fresh	639±172	856±39.0	1462±125	1261±67.6	598±3.6	924±34.4	957±343A	449±109 (*n*=40)
	Fresh-Stored	531±43.6	455±35.3	693±19.3	970±76.8	453±26.6	560±103	610±150B	
	Frozen	1323±48.4	1206±41.3	1024±76.5	938±44.5	1124±53.3	833±18.4	1084±224A	1225(Imputed)

[1,2,3]See footnotes of Table 4.3.1 for description of the samples reported from the U.S.D.A. National Nutrient Database for Standard Reference (R26).

Table 4.5.2 *Trans*-β-carotene content (μg/100 g, b.w.) in fresh, fresh-stored, and frozen green peas.[1]

Vegetable	Factors	Sampling Period						Mean±SD[2]	U.S.D.A.[3] Mean±SE
		1	2	3	4	5	6		
Green Peas	Fresh	601±162	744±33.8	1273±66.7	1050±55.7	554±3.0	813±30.0	859±290A	449±109 (*n*=40)
	Fresh-Stored	487±40.0	395±31.0	630±18.0	923±73.0	431±25.3	514±42.3	597±185B	
	Frozen	1070±35.6	1058±32.9	927±62.6	897±38.7	1033±66.8	797±16.0	973±122A	1225(Imputed)

[1,2,3]See footnotes of Table 4.3.2 for description of the samples reported from the U.S.D.A. National Nutrient Database for Standard Reference (R26).

Table 4.5.3 Vitamin A, RAE (µg/100 g, f.w.) in fresh, fresh-stored, and frozen green peas.[1]

Vegetable	Factors	Sampling Period						Mean±SD[2]	U.S.D.A.[3] Mean±SE
		1	2	3	4	5	6		
Green Peas	Fresh	53.2±14.3	71.3±3.2	122±6.4	105±5.6	49.9±0.2	77.0±2.8	79.7±27.5A	38(Imputed)
	Fresh-Stored	44.3±3.7	37.9±3.0	57.8±1.6	80.8±6.4	37.8±2.2	46.6±3.8	50.9±15.7B	
	Frozen	110±4.0	100±3.4	85.5±6.4	78.1±3.7	93.8±6.7	69.5±1.5	90.3±18.0A	103(Imputed)

[1,2,3]See footnotes of Table 4.3.3 for description of the samples reported from the U.S.D.A. National Nutrient Database for Standard Reference (R26).

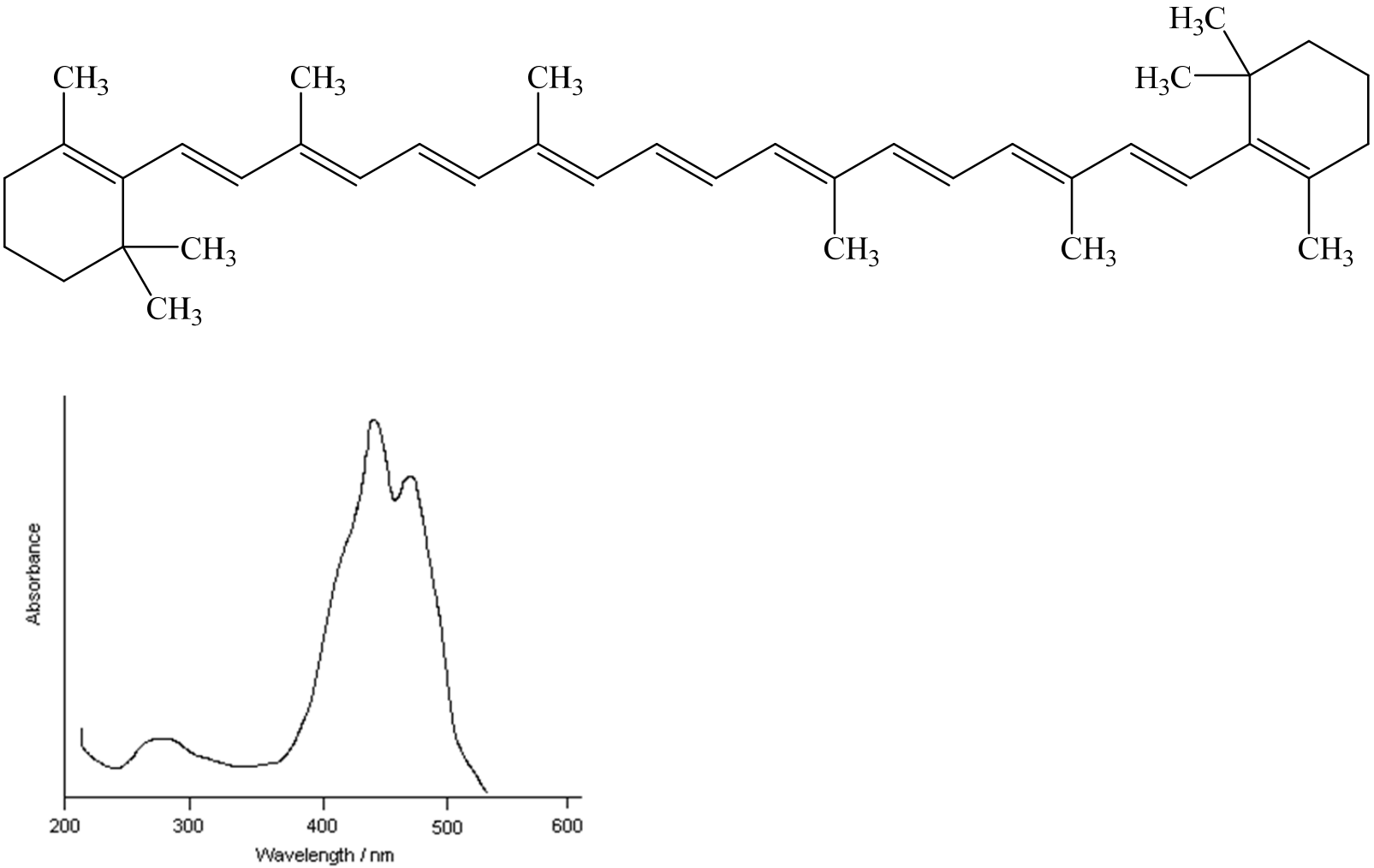

Figure 4.1 Chemical structure of *trans*-β-carotene and its absorption spectrum in *n*-hexane.

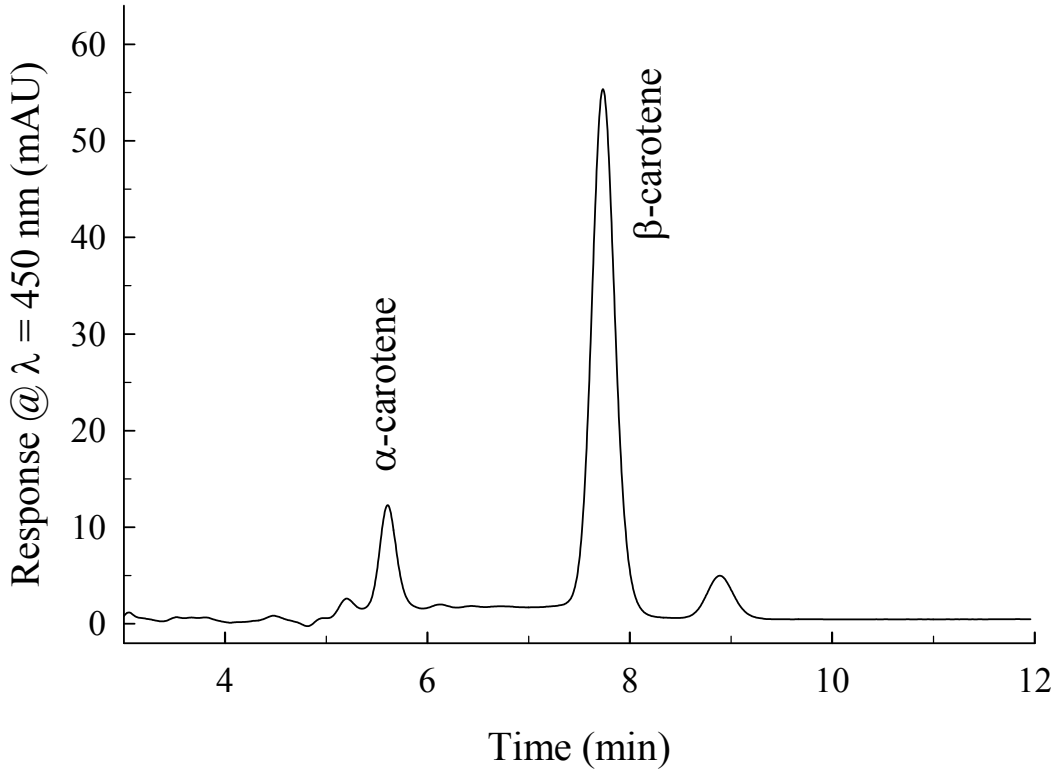

Figure 4.2 Chemical separation of *trans*-β-carotene from α-carotene and other carotenoids in broccoli.

CHAPTER 5

FOLATE CONTENT OF FRESH, FRESH-STORED, AND FROZEN FRUITS AND VEGETABLES

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ABSTRACT:

The objectives of the study were to determine and compare the folate content in selected fresh, fresh-stored, and frozen fruits and vegetables, while mimicking typical consumer purchasing and storage patterns of the produce. AOAC Official Method, 2004.05, was employed to analyze the folate content in the selected fruit and vegetable samples. The folate contents were determined in fresh, fresh-stored, and frozen blueberries, strawberries, broccoli, cauliflower, corn, green beans, spinach, and green peas. For each of the 8 sample-types, one-way ANOVA was performed to determine the presence of significant difference in folate contents according to treatment (α=0.05). The study did not find fresh produce to be superior to its frozen counterpart in most cases.

5.1 Introduction

Folate is a generic term for a family of structurally-related heterocyclic compounds based on the structure of pteroic acid, conjugated with one or more L-glutamic acid residues (*i.e.,* glutamates) linked through the γ-carboxyl of the amino acid. These naturally-occurring folates are typically in a reduced form and have multiple L-glutamyl residues. Though folic acid, pteroyl-L-monoglutamic acid (Figure 5.1, $C_{19}H_{19}N_9O_6$), cannot be found in nature, it is a common and stable form used in food fortification and available in dietary supplements. Its fully oxidized conjugated pterin ring makes this compound more stable than natural folates, often referred to as food folate, and therefore increases its shelf life (Young *et al.*, 2011).

The biosynthesis of folate and its various metabolites play a vital role in human nutrition. Folate functions as a coenzyme in the oxidation, reduction and transfer of one-carbon groups in the metabolism of some amino acids (e.g., glycine, methionine, serine) and homocysteine, in the synthesis of thymidylate and purines, in the methylation of DNA and histones, and in the initiation of protein synthesis (Lucock, 2000; de Brouwer, Zhang, Storozhenko, van der Straeten, & Lambert, 2007). As a consequence of the biochemical role folate plays in the body, important health benefits are associated with adequate folate intake. In particular, the role of folate in reducing the risk of cardiovascular disease and neural tube defects (e.g., spina bifida and anencephaly) in pregnant women is well recognized (Honein, Paulozzi, Mathews, Drickson, & Wong, 2001). In 1992, the U.S. Public Health Service recommended that all women of childbearing years consume 400 μg of folic acid daily; however, a survey indicated that only 29% of such women were following this recommendation (CDC, 1999). To combat this problem, the U.S. F.D.A. authorized the addition of folic acid to enriched grain products in March of 1996 and

made compliance mandatory by January 1998, with the goal of this fortification to increase the daily intake of folic acid by at least 100 μg. This authorization is still in force.

Naturally-occurring folate comprises a group of mono- and poly-glutamate derivatives of pteroic acid (4-[pteridin-6-methyl)amino] benzoic acid). In foods the predominant forms are tetrahydro-, dihydro-, formyl-, and methyl-tetrahydrofolate (Phillips *et al.*, 2005). Though wheat-based products fortified with folic acid are dietary sources of this vitamin, fruits and vegetables serve as a good source of naturally-occurring folate, primarily 5-methyltetrahydrofolate, which is highly bioavailable (Konings *et al.*, 2001).

Folates are vulnerable to destructive oxidation. Because the vitamin occurs in multiple chemical forms, its sensitivity to the analytical technique of choice will differ based on the forms. The classical approach for analyzing the total folate content in foods is complex. It comprises two key steps: first an extraction of the nutrient in its various forms from a food using three enzymes, and second, a quantification of the nutrient by a microbiological assay. The trienzyme extraction method for folate analysis was developed in the mid-1990s, and is now widely employed. In fact, it is recognized as an official method by AOAC International. This method involves the use of α-amylase, Pronase® protease, and a chicken pancreas conjugase. Pronase® protease and α-amylase help to liberate folate bound to proteins and from the food matrix in general. Once liberated, conjugase is added in order to permit the hydrolysis of polyglutamyl folate moieties to mono- or di-glutamyl folate. These latter forms of folate are important, because they are utilized for bacterial growth in the microbiological assay.

For the microbiological assay itself, *Lactobacillus casei* subsp. *rhamnosus* (ATCC® 7469™) culture is plated in a 96-well microtiter plate along with liberated folate samples. The

folate content is determined by quantitative comparisons between the turbidity of the *L. casei* growth of extracts (*i.e.*, test samples) against that of known concentrations of a folic acid standard. The extent of turbidity developed over a specified time is measured spectrophotometrically and then related back to the quantity of folate in the extract prepared by the trienzyme methodology. Different vitamers of folate are not distinguished by this assay; hence, the total folate content is reported. Because folate is sensitive to light, action must be taken to prevent light damage during analyses.

5.2 Materials and Methods

Folate analysis was per the operating procedure for microplate assay of folic acid and total folate with calculation of dietary folate equivalents as summarized by Chun, Chen, Kota, and Eitenmiller, an unpublished report, based on AOAC Official Method 2004.05.

5.2.1 Sample handling

Fruit and vegetable samples (*i.e.*, blueberries, strawberries, broccoli, cauliflower, corn, green beans, spinach, and green peas), either fresh or frozen, were purchased from 6 supermarkets within a 40-km radius of Athens, GA. The selected stores were common U.S. supermarket chains and included Walmart, Sam's Club, Kroger, Publix, Piggly-Wiggly, and Ingles, as well as Bell's as a backup. All of the frozen produce acquired was private-label.

On the occasions when fresh fruits and vegetables were purchased, double the quantity of that of the frozen produce was acquired. Half of the purchased fresh produce was placed in a standard kitchen refrigerator for 5 days before analysis. This procedure was intended to mimic

the purchase and storage practice of fresh produce by consumers. Hence, these fruits and vegetables are referred to as fresh-stored.

A ~200-g portion of fruit or vegetable from each store was combined in a large grey plastic tub, and then mixed well to give a composite sample. In the case of fresh and fresh-stored vegetables, no samples were blanched prior to analysis except for corn-on-the-cob. Blanching was unnecessary because each vegetable sampled was immediately placed in a phosphate buffer after grinding to prevent enzymatic degradation of the folate. For fresh corn-on-the-cob, blanching for 1 min and then cooling the cobs in an ice bath prior to cutting off the kernels was mandatory to prevent the enzymatic degradation that would have taken place during the time it took to mix up the niblets for the composite and then sample the portions for analysis. All frozen vegetables had been blanched prior to freezing, whereas the fruits were blast frozen. Representative samples from each composite were then taken. Because folate is light sensitive, all assays were conducted under yellow lights.

5.2.2 Moisture analyses

A moisture analysis was performed gravimetrically in triplicate on all fruits and vegetables by placing a ground portion of each sample in a forced-air convection oven at 103 °C until a constant mass was reached (usually 24 h). The moisture analyses were performed to provide a uniform mass balance for all data; that is, being able to convert collected data from a fresh weight basis (f.w.) to a blanched weight basis (b.w.) to a dry weight basis (d.w). Because the fresh and fresh-stored corn-on-the-cobs were blanched prior to preparing the composite, the moisture content of this composite was determined and compared to that of non-blanched corn.

The conversion of folate levels based on blanched weight (b.w.) to fresh weight (f.w.) was calculated using the following equation:

$$\text{folate content (f.w., µg/100 g)} = \text{folate (b.w., µg/100 g)} \times \frac{(1-M_b)}{(1-M_f)}$$

where, M_b is the moisture content of the blanched corn-on-the-cob; and M_f is the moisture content of the fresh (unblanched) corn-on-the-cob.

5.3.1 Preparation of reagents for the trienzyme extraction assay

Phosphate buffer

A 0.1 M phosphate buffer was prepared by dissolving 1.42 g of Na_2HPO_4 and 1 g of L-ascorbic in 100 mL of deionized water and then adjusting the pH to 7.8 with 4 N NaOH and a pH meter. Each sample requires ~35 mL of buffer; so, the quantity prepared fresh each day was dependent on the number of samples processed in a given day.

Protease reagent

Pronase® protease, a non-specific protease from *Streptomyces griseus*, which can digest proteins to free amino acids, was purchased from VWR International (Suwanee, GA). The product (Cat No. 537002-50KU) was the 50 KU size, where 1 KU = 1,000 units, and possessed an activity of ≥ 45,000 proteolytic units/g dry weight. Pronase® (20 mg) was weighed out, transferred to a 10-mL volumetric flask, and dissolved in deionized water. The solution was prepared fresh each day and stored in a refrigerator at 4 °C when not in use.

α-Amalyse reagent

1,4-α-D-Glucan glucanohydrolase (*i.e.*, α-amylase) from *Aspergillus oryzae* was acquired from the Sigma-Aldrich Chemical Company (St. Louis, MO). The product (Cat No. 10065-50G) had

an activity of 35.7 U/mg, where 1 U corresponds to the amount of enzyme which liberates 1 μmol of maltose per min at pH 6.0 and 25 °C. α-Amylase (200 mg) was weighed out, transferred to a 10-mL volumetric flask, and dissolved in deionized water. The solution was prepared fresh each day and stored in a refrigerator at 4 °C when not in use.

Conjugase reagent

A crude preparation of acetone-washed chicken pancreata was used (see details below for its preparation). The lyophilized powder (100 mg) was weighed out, dissolved in 20 mL of the phosphate buffer; and sonicated for 5 min. The solution was then filtered through glass wool to remove any particulates. This reagent was prepared fresh each day and stored in a refrigerator at 4 °C when not in use.

5.3.2 Isolating the conjugase enzyme from chicken pancreata

Chicken pancreata (~60) were harvested from freshly-slaughtered chickens at the University of Georgia's Department of Poultry Science farm (Athens, GA). The pancreata were packaged in zip lock pouches and frozen at -80 °C overnight. Then ~15 g of frozen pancreata were combined with ~300 mL of cold acetone (-78 °C, prepared by dry ice addition to the organic solvent) in a 500-mL Erlenmeyer flask. The contents were blended at 25 × 1,000 rpm using a PT 3100 Polytron homogenizer with a Kinematica AG PT-DA 3012/2TM generator (Fisher Scientific Co., Suwanee, GA). The slurry was poured into a Büchner funnel, lined with Whatman No. 1 filter paper, connected to a suction flask and a vacuum system. After suction filtration, the filter cake was rinsed with 3 × ~100 mL portions of cold acetone. The crude preparation containing the chicken pancreas conjugase enzyme was scrapped from the filter

paper, allowed to dry in a fume hood at room temperature for 1 h, and then lyophilized in a FreeZone® 2.5-L bench-top freeze dryer (Labconco Corporation, Kansas City, MO) to ensure that all traces of acetone and moisture were removed. The product (referred to as the conjugase powder) was transferred to an amber-glass bottle and stored in a refrigerator at 4 °C until used.

5.3.3 The trienzyme extraction assay

For each fruit and vegetable, 100-g subsamples were taken from the homogeneous composite and ground in a 70-W Black & Decker one-touch chopper (Model HC306, Applica Consumer Products, Inc., Miramar, FL) just preceding the assay. Triplicate samples were subjected to the trienzyme assay using the following method (*NB*, the description forthwith is made for a single tube): the ground sample (1.0 g) was weighed into a borosilicate glass folate tube (36 *i.d.* × 155 mm), to which 20 mL of the phosphate buffer were added and a sufficient quantity of deionized water to bring the volume up to 50 mL. The sample was homogenized with the Polytron homogenizer using a 3012/2TM generator at 25 × 1,000 rpm for 60 s. After homogenization, two drops of toluene were added to retard bacterial activity. The tube was then wrapped with aluminum foil and placed in a Thelco boiling water bath (Model 83, Precision Scientific Co.) for 15 min. Using an ice water bath, the tube and its contents were cooled to room temperature. One milliliter of the Pronase® solution was added and the tube was then placed in a 5.0-ft^3 Isotemp™ standard lab incubator (Model 650D, Fisher Scientific) at 37 °C for 3 h. The tube was returned to the boiling water bath for 3 min to inactivate the protease, followed by a cooling step. Afterwards, 1 mL of the α-amylase solution was added followed by a 2-h period in the incubator at 37 °C. Finally, 4 mL of the chicken pancreas conjugase solution were added

followed by an additional incubation at 37 °C for 16 h. The folate tube was returned to the boiling water bath for 3 min to inactivate the conjugase. Using an ice water bath, the tube was cooled to room temperature and the pH of the solution was adjusted to 4.5 with (1+1, v/v) HCl and a pH meter. The acidified solution was then quantitatively transferred to a 100-mL volumetric flask. The flask was filled to mark with deionized water and the solution filtered through Whatman No. 1 filter paper into an Erlenmeyer flask. The extract was dispensed into 1.5-mL disposable/conical microcentrifuge tubes (Cat. No. 20170-038, VWR International), wrapped with aluminum foil, and stored at -40 °C until analyzed.

As an internal quality control measure, the folic acid fortified Gold Medal, enriched, all-purpose flour (Kroger, Athens, GA) was extracted by the trienzyme assay described above. From the nutrition label, 30 g of this flour contains 10% of the recommended daily value of 400 µg of folic acid. Therefore, the folic acid concentration in flour is roughly 133 µg/100 g flour.

5.3.4 Microplate assay

5.3.4.1 Reagent preparation

Lactobacilli broth

15.2 g of BD Difco™ *Lactobacilli* broth, AOAC powder (Cat No. 290110, procured from VWR International) were weighed, transferred to a 500-mL Erlenmeyer flask and dissolved in 400 mL of deionized water. Using a hot plate/stirrer, the broth was brought to a boil, held there for 2-3 min, cooled to room temperature using an ice water bath, and then dispensed (10 mL aliquots) *via* a Gilson Pipetman P10 mL into 20 × 150-mm screw-cap culture tubes (Cat No. 14-932D, Fisher Scientific). The tubes were loosely capped and autoclaved with a steam sterilizer

autoclave (AMSCO, Erie, PA) at 121 °C for 15 min. After the tubes had cooled, they were tightly capped and stored in a 4 °C refrigerator until used.

Lactobacilli agar

19.2 g of BD Difco™ *Lactobacilli* agar, AOAC powder (Cat No. 290010, procured from VWR International) were weighed, transferred to a 500-mL Erlenmeyer flask and dissolved in 400 mL of deionized water. Using a hot plate/stirrer, the agar was brought to a boil, held there for 2-3 min, cooled to ~40 °C using an ice water bath, and then dispensed (10 mL aliquots) *via* a Gilson Pipetman P10 mL into 20 × 150-mm screw-cap culture tubes (Cat No. 14-932D, Fisher Scientific). The tubes were loosely capped and autoclaved at 121 °C for 15 min. During cooling, the tubes went positioned on their side to create a slanted angle (*i.e.*, the product referred to as slants). After the agar had solidified, the tubes were tightly capped and stored at 4 °C refrigerator until used.

Lactobacilli culture activation and maintenance

Freeze-dried *Lactobacillus casei* subsp. *rhamnosus* (ATCC® 7469™) was acquired from the American Type Culture Collection (Manassas, VA). The lyophilized culture was dispersed in 10 mL of the *Lactobacilli* broth (solution A). Between 0.5 to 1 mL of solution A was diluted with another 10 mL portion of *Lactobacilli* broth (solution B). Solutions A and B were placed in the incubator at 37 °C for 18 h. If the culture grew well in solution B, it was transferred and streaked onto one of the *Lactobacilli* agar slants, but if not, then solution A was streaked onto the agar slant. The freshly inoculated slant was incubated at 37 °C for 24 h and then placed in the refrigerator. To maintain viability of the culture, a portion of the culture was taken every 7 d

from the stored slant and transferred to a new slant. The new slant was incubated at 37 °C for 24 h and then placed in the refrigerator.

Depletion medium

7.6 g of *Lactobacilli* broth, AOAC, and 18.8 g of BD Difco™ folic acid *casei* medium powder (Cat No. 282210, procured from VWR International) were weighed, transferred to a 500-mL Erlenmeyer flask and dissolved in 400 mL of deionized water. Using a hot plate/stirrer, the media was brought to a boil, held there for 2-3 min, cooled to room temperature using an ice water bath, and then dispensed (10 mL aliquots) *via* a Gilson Pipetman P10 mL into 20 × 150-mm screw-cap culture tubes (Cat No. 14-932D, Fisher Scientific). The tubes were loosely capped and autoclaved at 121 °C for 15 min. After the tubes had cooled, they were tightly capped and stored in a 4 °C refrigerator until used.

Folic acid casei medium

28.2 g of BD Difco™ folic acid *casei* medium powder were weighed, transferred to a 500-mL Erlenmeyer flask and dissolved in 300 mL of deionized water. Using a hot plate/stirrer, the media was brought to a boil, held there for 1-2 min, and cooled to room temperature using an ice water bath. Prior to filtering, the workbench was wiped with 70% (v/v) ethanol. The medium was then filtered, near a flame to help prevent bacterial contamination, using a 250-mL Corning™ disposable sterile filter system (Cat No. 09-761-140, Fisher Scientific). Briefly, the filter system was removed from its aseptic packaging and the spout connected to a vacuum hose. Media was poured into the upper chamber and a vacuum was applied. After all of the solution had been filtered through the 0.22-µm cellulose acetate membrane, the upper unit of the filter

was removed and the bottle then capped tightly. The sterile media was stored in a 4 °C refrigerator, but warmed to room temperature before use in the microplate assay.

Folic acid stock solution

Under yellow lighting, 20 mg of USP-grade folic acid (CAS 59-30-3; Sigma-Aldrich Chemical Co., St. Louis, MO) were accurately weighed and transferred to an Erlenmeyer flask with ~20 mL of 95% (v/v) ethanol (ACS grade, Fisher Scientific). The solution was then diluted with ~30 mL of deionized water. To facilitate the dissolution of folic acid, the pH was raised to 10.0 with 0.1 N NaOH and then adjusted to 7.0 with 0.05 N HCl. The 50-mL solution was quantitatively transferred to a Pyrex® 100-mL Class A low-actinic (red) volumetric flask and then filled to mark with deionized water. Aliquots (10 mL) of this stock solution were transferred to borosilicate glass culture tubes, which were capped, wrapped with aluminum foil, and stored in a 4 °C refrigerator until used.

5.3.4.2 Microplate assay

The preparation for the microplate assay was as follows: A tube containing the depletion media was warmed to room temperature. The culture from a 3-5 day old slant was transferred to the depletion media. The tube was then incubated at 37 °C for 6 h before further use. To ensure sterility, all supplies were autoclaved at 121 °C for 15 min and were cooled, capped and/or sealed. These included a 250-mL Erlenmeyer flask filled with 100 mL of deionized water, a 50-mL graduated cylinder, a 125-mL Erlenmeyer flask, Gilson diamond pipette tips, and a jar of 1.5-mL disposable/conical microcentrifuge tubes.

Sample extracts (from the trienzyme extraction) and those of the wheat flour for quality control (QC) determinations were thawed to room temperature, then transferred to 20-mL Wheaton amber vials. The vials were diluted to the appropriate ratio (determined by trial and error; details to follow) with deionized water, and were capped. The dilution factor employed was based on the estimated folate concentration for the fruit or vegetable in question. Turbidity was determined for each test sample and was compared to that of the folic acid standard. If the measurements were similar, then the appropriate dilution had been employed in the microbiological assay. If not then a more dilute or concentrated test sample was used. A ratio of 1:10 (v/v) was determined for the QC sample, while a 1:1 ratio was chosen for test samples with a folate range of 0 to 30 µg/100 g fruit or vegetable; 1:3 for test samples with a folate range of 30 to 80 µg/100 g fruit or vegetable; 1:5 for test samples with a folate range of 80 to 150 µg/100 g fruit or vegetable; and 1:10 for test samples with a folate content of 150 to 200 µg/100 g fruit or vegetable. The amber vials were autoclaved at 121 °C for 5 min, capped loosely, and the vials were capped tightly after cooling to room temperature using an ice water bath.

Several solutions were prepared fresh before each microplate assay. A working solution of folic acid (~2 ng/mL) was prepared. Briefly, a tube of the folic acid stock solution (~200 µg/mL) was removed from the refrigerator and tempered to room temperature. A 100-µL aliquot was transferred *via* a Gilson Pipetman *Neo*® to a Pyrex® 100-mL Class A low-actinic (red) volumetric flask, diluted to mark with deionized water and mixed well. Once mixed, a 1,000-µL aliquot of the first diluted standard was transferred *via* a Gilson Pipetman *Neo*® to a second Pyrex® 100-mL Class A low-actinic (red) volumetric flask; the flask was diluted to mark and mixed well.

An ascorbic acid reagent was then prepared. Briefly, 1 g of USP-grade ascorbic acid (L-ascorbic acid, Mallinckrodt Chemical, Inc. supplied by VWR International) was weighed and quantitatively transferred into a 10-mL volumetric flask using deionized water. The flask was then diluted to mark and warmed under running water to fully dissolve the crystals. Once dissolved, the solution was re-cooled to room temperature.

Before the microplate assay, the CCI biosafety laminar-flow hood (Contamination Control, Inc., Kulpsville) was wiped down with 70% (v/v) ethanol and run for > 60 min to ensure a sterilized environment in the hood before plating. Under the hood, the folic acid working solution and L-ascorbic acid reagent were syringe filtered using BD 3-mL Luer-Lok™ syringes (Cat No. BD309657, VWR International) and 0.22-µm polyvinylidene fluoride (PVDF) sterile filters (Cat No. 09-720-3, Fisher Scientific) into sterilized 1.5-mL disposable/conical microcentrifuge tubes.

All steps of the microplate assay were executed in a biosafety cabinet. All reagents and solvents transferred to the wells were done using sterilized Gilson diamond tips (Gilson Inc., Middelton, WI). A sterile, 96-well, polystyrene Falcon™ tissue culture plate with a flat-bottom and lid (Cat No. 08-772-2C, Fisher Scientific) was removed from its aseptic package. Using a 12-channel Gilson Pipetman M Multichannel, 300 µL of sterilized water were taken up from a 50-mL Costar™ sterile disposable reagent reservoir (Cat No. 4871, procured from VAR International) and dispensed along row H (*i.e.*, the bottom row, see Fig 5.2 for plate set up) of the microplate as a "blank" row; 150 µL were added to all other rows. Then, 150 µL of the sterile-filtered folic acid working solution (~2 ng/mL), sterilized flour extract, and sterilized test sample extracts were added to wells G1-G3, G4-G6, and G7-G12, respectively, *via* a 200-µL single

channel Gilson Pipetman. Using the 12-channel Pipetman with sterilized tips, the solutions in row G were mixed 3×. After mixing, 150 µL of the solutions (be it folic acid standard, flour extract, or two test sample extracts) were transferred from wells G1-G12 to F1-F12. The solutions in wells F1-F12 were mixed 3× and 150 µL of the diluted solutions were then transferred to each subsequent row of wells (E thru A), until the last row (A) on the microplate was filled. In row A (wells A1-A12), 150 µL were removed from the microplate and disposed. Growth media for each plate was prepared by adding 15 mL of the folic acid *casei* medium reagent, 150 µL of the sterilized ascorbic acid reagent, and 45 µL of the depletion medium incubated with the culture to a 125-mL sterilized flask, and mixing well. This solution was poured into a sterile reagent reservoir; 150 µL were pipetted into every row, except for the blank row (H). Each prepared microplate was individually placed inside a zip-lock sandwich bag and transferred to the incubator for 18 h at 37 °C.

After 18 h the microplates were removed from the incubator and the contents in the wells of rows A thru G were mixed well with the 12-channel Pipetman. Absorbance readings for the microplate were taken at λ = 650 nm using a FLUOstar Omega microplate reader (BMG LABTECH Inc., Cary, NC). The assay was carried out using bottom scanning and at an incubation temperature of 25 °C. The concentrations of folate (*i.e.*, folic acid equivalents) in the trienzyme extracts of the fruits and vegetables were calculated by MARS Data Analysis software.

5.3.5 Calculation of folate levels in fruits and vegetables

Turbidity data on the folate level in the samples was collected and calculated by the FLUOstar Omega microplate reader. Polynomial regression (a form of linear regression) was

employed to calculate a standard curve based on the absorbance readings of the folic acid standard at different concentrations. For each microplate analyzed, a new standard curve was created; folic acid concentration versus absorbance was fitted with a third-order polynomial. In other words, the curve is sigmoidal, but has a linear portion associated it where concentration can be determined from absorbance readings according to Beer's Law (see the box in Figure 5.3). Using this region of the curve and taking into account appropriate dilution factors and sample masses, the folate contents in the fruits and vegetables were determined and reported as μg folic acid equivalents/100 g, f.w. Because the mass of fresh and fresh-stored corn samples changed during blanching, the folate content in these was corrected according to the moisture content before and after blanching. Each sample was analyzed in triplicate. Mean and standard deviations were calculated.

5.4 Quality control

To validate the accuracy and interday precision (*i.e.*, relative repeatability standard deviation, $\%RSD_r$) for the analysis of folate in the fruit and vegetable samples, a quality control plan was established.

Concentration and purity of the folic acid standard solution

A 0.1 M phosphate buffer (pH = 7.0) was prepared by dissolving 13.61 g of KH_2PO_4 in 100 mL of deionized water and then adjusting the pH to 7.0 with 4 M NaOH. A 10-mL aliquot of the stock solution was diluted with the phosphate buffer at a ratio of 1:20 (v/v). The absorbance of the working folic acid standard was measured at λ = 282 nm using an Agilent 8453 UV-visible diode array spectrophotometer (Agilent Technologies, Inc., Wilmington, DE). A mixture

of the prepared phosphate buffer and water at a ratio of 1:20 (v/v) was used for background correction. The purity of the folic acid in the test sample was calculated as follows:

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

where, A_{std} is the absorbance of the test solution; A_{blank} is the absorbance of the reagent blank; and C is the concentration of the diluted folic acid stock solution (mg/mL).

The reported $E^{1\%}_{1cm}$ for folic acid at $\lambda = 282$ nm in a 0.1 M phosphate buffer at pH 7.0 is 611.7 (Eitenmiller, Landen, & Ye, 2007); thus, the purity of the standard prepared for the experiment and its true concentration are calculated as follows:

$$\text{Purity} = \text{the calculated } E^{1\%}_{1cm}/611.7 \times 100$$

$$\text{Concentration (mg/mL)} = (\text{exact mass of folic acid weighed in mg/100 mL}) \times \text{purity}/100$$

Preparation of the folic acid standard and calibration solutions

A working solution of folic acid standard (~2 ng/mL) was prepared freshly before each microplate assay. Briefly, a tube of the folic acid stock solution (~200 µg/mL) was removed from the refrigerator and tempered to room temperature. A 100-µL aliquot was transferred *via* a Gilson Pipetman *Neo*® to a Pyrex® 100-mL Class A low-actinic (red) volumetric flask, diluted to mark with deionized water and mixed well. Once mixed, a 1,000-µL aliquot of the first diluted standard was transferred *via* a Gilson Pipetman *Neo*® to a second Pyrex® 100-mL Class A low-actinic (red) volumetric flask; the flask was diluted to mark and mixed well. Working standards and sample extracts were assayed under identical conditions for each microplate. Standard curves were checked every day to ensure consistency in the microbiological assay.

Accuracy

In this study, a CRM from the European Commission Joint Research Center, Institute for Reference Materials and Measurements, BCR® 485 (mixed vegetables), was purchased from the Resource Technology Corporation (Laramie, WY). Accuracy was assessed by comparing the data value obtained for a measurement to that of the accepted value of the CRM. Bias, which is defined as the difference between the analytical value from that of the accepted value provided on the CRM's certificates of analysis, was also determined (Horwitz, 2003). The bias and % accepted value were calculated as follows:

$$\text{Bias} = \mu - x$$

$$\text{Accepted value (\%)} = x/\mu \times 100$$

where, x is the analytical value; and μ is the accepted value provided by the certificate of analysis of the reference sample.

Recovery is defined as the fraction of the analyte measured after addition of a known quantity of the analyte to the sample. Recovery was determined on the basis of AOAC guidelines (2000). Ground fruit and vegetable samples as well as the BCR® 485 (mixed vegetables) CRM were spiked with known levels of folic acid. Final spiking levels per gram of sample were 1.57, 3.15, and 4.72 μg, which represent levels of 50, 100, and 150%. Each spiking experiment was performed in triplicate. The % recovery of the added folic acid standard was calculated as follows:

$$\text{Recovery (\%)} = [(C_s - C_p)/C_a] \times 100$$

where, C_s is the folic acid concentration in the spiked sample; C_p is the folic acid concentration in the unspiked sample; and C_a is the mass of the folic acid standard added.

Precision

Precision, or reproducibility, is the degree to which repeated measurements under unchanged conditions show the same results. BCR® 485 was employed as the CRM for validation of folic acid analyses, as noted above. The precision of the assay was determined by repeatability (intraday precision) and intermediate precision (interday precision) of the CRM. Precision was determined from 22 replicates of the CRM over 4 days.

Precision can be approximated as the relative standard deviation between trials and can be expressed as:

$$\%RSD = (SD \times 100)/\bar{x}$$

where, %RSD is the relative standard deviation; SD is the standard deviation; and $\bar{x}$ is the mean.

Quality control chart

As an internal quality control measure, the folic acid-fortified Gold Medal, enriched, all-purpose flour (Kroger, Athens, GA) was extracted by the trienzyme assay described above. From the nutrition label, 30 g of this flour contains 10% folic acid, based on the recommended daily value of 400 μg. Therefore, the folic acid concentration in flour is ~133 μg/100 g flour. For QC analysis, a mean line, upper control limit (UCL = mean + 20% of the mean), and lower control limit (LCL = mean - 20% of the mean) were established. Each data point was plotted on the QC chart with a corresponding number. When a value fell outside of either the UCL or LCL, the assay was considered invalid and the sample analysis from the run discarded. Routine monitoring of the QC chart was performed to ensure that there was no contaimination, excessive bacterial growth, or other similar problems with the microplate.

5.5 Data Analysis

The folate contents in fresh, fresh-stored, and frozen samples were assessed within blueberries, strawberries, broccoli, cauliflower, corn, green beans, spinach, and green peas. The analyses were performed over the span of two years in 6 distinct time frames, namely Summer to Fall 2011, Fall 2011 to Winter 2012, Winter to Spring 2012, Summer to Fall 2012, Fall 2012 to Winter 2013, and Winter to Spring 2013 (see format of Table 5.1). Within each time period, the folate content was measured in triplicate for all combinations of treatments and samples. The data for each time frame, although reported individually in the results, are treated as replicates in our statistical analysis of the differences among treatments. Therefore, the folate content of each treatment/sample combination was assessed according to 18 replications.

For each of the 8 sample-types (*i.e.*, each specific fruit or vegetable), one-way ANOVA was performed to determine the presence of significant difference in folate contents according to treatment (α=0.05). Data transformation was applied when necessary to adequately meet the assumption of normal distribution for ANOVA, and the Weighted Least Squares method was employed in the instances in which the equal variance assumption of the ANOVA was violated. All necessary statistical diagnostic checks (e.g., residuals versus predicted value plot, Q–Q plot of residuals, and histogram plot of residuals) were performed to verify acceptability of ANOVA implementation. In the cases in which a statistically significant difference was observed among the three treatments, the Tukey's Studentized Range multiple comparisons test was performed to determine which specific pairs of treatments (*i.e.*, fresh vs. frozen, fresh vs. fresh-stored, and fresh-stored vs. frozen) showed significant differences from one another (α=0.05).

All data analyses were performed using SAS software, version 9 of the SAS system for Windows (SAS Institute Inc., Cary, NC).

5.6 Results and Discussion

Quality Control

The purity of the folic acid stock standard was 98.0%. On the basis of the purity check, the concentration in the stock standard solution was 196 µg/mL. For the daily working standard, the stock solution was diluted with deionized water to give concentrations of 1.96 ng folic acid/mL. The levels of folic acid equivalents in the fruit and vegetable samples were then determined from the calibration curve. A typical calibration curve of folic acid concentration versus absorbance is depicted in Figure 5.3. Non-linear regression gave a third-degree polynomial equation of $y = 2189.7x^3 - 524.48x^2 + 440.66x + 0.0263$ with a correlation coefficient (R^2) of 0.9987. The microplate reader was programmed to calculate a polynomial equation for the folic acid standard of each plate. In other words, a new standard curve was generated for each plate assayed.

Accuracy

Accuracy and $\%RSD_r$ measures for the folic acid analysis by the microplate protocol are given in Table 5.4. Folic acid was extracted from BCR® 485 (mixed vegetables) and analyzed. According to its certificate of analysis, the CRM contains 315 ± 28 µg of folic acid/100 g. Comparison of the analytical value to the accepted value from the certified value of the CRM was within the accepted range reported for BCR® 485. The bias value was negative. The ratio of the analytical value to the accepted valued expressed as a percentage (% of accepted value) can

be used to evaluate accuracy. The percentage of analytical value to acceptable value was 95%. Together, the accuracy and $\%RSD_r$ measures indicated that the folic acid analyses of the fruit and vegetable samples by the microplate assay were accurate and repeatable. The recoveries of folic acid from BCR® 485 at spike levels of 50, 100, and 150% were 95 ± 4, 100 ± 9, and 107 ± 9%, respectively. The recoveries ranged from 92 to 108%. The high recoveries validate the accuracy of the folic acid analysis performed. Noteworthy is that many nutrient profiling studies fail to report on these parameters.

Precision

Intraday precision (repeatability) was determined by running 22 replicates of the CRM over 4 days. The RSD of these data was calculated and shown to be less than 5%. The daily (intraday) and day-to-day (interday) precision of the CRM gave the following results: based on n=4, the folic acid analysis of the CRM showed a daily RSD of 5.62%. In terms of interday precision, the folic acid analysis of BCR® 485 gave a RSD_r of 4.47%. In both cases, the %RSD values were determined to be well within the ±10% limit, indicating that the current method is repeatable. A QC chart was also used to monitor the status of the assay. An example is depicted in Figure 5.4; here, 16 folic acid concentrations from the QC flour sample were collected on 4 days over a two week period. All folic acid concentrations fell within the UCL and LCL, thereby indicating that the experimental conditions were constant during this period.

Table 5.1 reports the folate contents (as folic acid equivalents) in fresh, fresh-stored, and frozen blueberries and strawberries; the mean values for blueberries were 11.5 ± 4.5, 9.6 ± 3.8, and 13.8 ± 3.9 μg/100 g, f.w., respectively. For blueberries, the folate contents determined in this study over a two year period were almost double of the corresponding values reported in the

U.S.D.A. National Nutrient Database for Standard Reference (R26), hereinafter referred to simply as Database. It seems that the folate content of fresh blueberries in 2011 was greater than that in 2012. Many different factors including the source of the blueberries, environmental factors, and age of the "fresh" product at the time of purchase can cause such variation. Roughly a 17% decrease of folate in fresh blueberries stored at 4 °C for 5 days after purchase was noted. The mean folate content in frozen blueberries was greater than that of fresh and fresh-stored blueberries. While the difference was not significant ($p > 0.05$) for fresh blueberries, it was, however, for the fresh-stored samples. Moreover with the frozen blueberries, there was less variation in the range of values over the time periods examined (*i.e.*, 10.2 to 19.6 μg/100 g). This conclusion is different from that reported in the Database.

The mean folate contents of fresh, fresh-stored, and frozen strawberries, reported in Table 5.1, were 43 ± 12.7, 38.5 ± 4.5, and 39.7 ± 8.5 μg/100 g, f.w., respectively. For all strawberries, no significant difference ($p > 0.05$) was found in the folate contents. Roughly a 10% decrease in the folate content of the fresh-bought strawberries was observed after 5 days of storage at 4 °C. Our results indicate that the folate contents in both fresh and frozen products were 1.5 to 2× greater than the corresponding Database values. Moreover, the folate data from this study was found to be in line with those of other studies. For instance, Strålsjö, Witthöft, Sjöholm, & Jägerstad (2003a) reported that the average folate concentration in 13 different strawberry cultivars was 47 ± 9 μg/100 g, whereas the Database average in fresh strawberries was only 24 ± 5.5 μg/100 g; we report a mean of 43 ± 12.7 μg/100 g. Strålsjö, Åhlin, Witthöft, & Jastrevova (2003) noted that the folate content in the best 8 cultivars (in terms of folate levels) varied from 73 (*Lina*) to 99 (*Melody*) μg/100 g. In a more recent study, Tulipani *et al.* (2008) reported that

the folate levels in 9 different strawberry cultivars ranged quite markedly from 12.8 to 96 µg/100 g. This study also listed greater folate contents compared to the Database. Using an HPLC assay, Vahteristo, Lehikoinen, Ollilainen, & Varo (1997) reported a content of 36 µg folate/100 g fresh strawberries.

Table 5.2 summarizes the mean folate contents of fresh, fresh-stored, and frozen produce based on the f.w. for broccoli, cauliflower, corn, green beans, and spinach. The mean values for fresh, fresh-stored, and frozen broccoli were 72.6 ± 7.7, 67.4 ± 8.7, and 61.7 ± 9.3 µg folic acid equivalents/100 g, f.w., respectively. There were no statistical ($p > 0.05$) differences between the fresh and fresh-stored test samples, but frozen broccoli was lower in folate and statistically ($p < 0.05$) different from its fresh counterparts. Studies have indicated a loss of folate resulting from blanching of fresh broccoli. Specifically, DeSouza and Eitenmiller (1986) observed losses of 60 and 9% after water and steam blanching, respectively, compared with fresh broccoli. It should be noted that neither the fresh nor fresh-stored broccoli analyzed in this study was blanched, as samples from the composite were immediately placed in a phosphate buffer. The frozen broccoli, however, had been blanched prior to freezing and this may account for why its mean folate content is statistically ($p < 0.05$) different from its fresh counterparts. For fresh broccoli, the values determined in this study were slightly greater than the value of 63 ± 12.7 µg folic acid equivalents/100 g, f.w. listed in the Database, based on 27 observations. Even though not statistically different, roughly a 7.2% loss in folate content was noted in fresh broccoli stored at 4 °C for 5 days after purchase.

The mean folate contents for fresh, fresh-stored, and frozen cauliflower were 68.9 ± 4.4, 65.0 ± 5.5, and 67.7 ± 8.5 µg folic acid equivalents/100 g, f.w., respectively, and there were no

statistical ($p > 0.05$) differences. The folate content in fresh cauliflower was slightly greater than the 57 ± 5 μg/100 g, f.w. value reported in the Database based on 27 observations. For the frozen cauliflower samples analyzed in this study, the folate level was similar to the imputed value of 64 μg/100 g, f.w. listed in the Database. Fresh cauliflower stored at 4 °C for 5 days after purchase showed only a 6% decrease in its folate. Other studies have reported higher folate contents in fresh cauliflower than those in this work. For instance, Puupponen-Pimiä *et al.* (2003) reported 122 μg folate/100 g fresh cauliflower, while Vahteristo *et al.* (1997) found 85 μg/100 g fresh cauliflower. It was suggested that the difference in folate contents was due to variability in the cultivars tested. It should be pointed out, however, that the microbiological assay for folate can lead to erroneous data if the protocol is not followed carefully and a quality control plan is not in place to monitor the repeatability and precision of the assay.

The mean folate contents for fresh, fresh-stored, and frozen corn were 50.2 ± 10.5, 37.2 ± 10.1, and 50.6 ± 6.7 μg folic acid equivalents/100 g, f.w., respectively. Even though the absolute mean for frozen corn was higher than its fresh counterpart, the values were not statistically ($p > 0.05$) different. On the other hand, the folate in frozen corn was significantly ($p < 0.05$) greater than that of the fresh-stored produce. Both the contents of folate in the fresh and frozen corn were found to be higher than the Database values of 42 ± 3.4 and 36 ± 5.5 μg folic acid equivalents/100 g, f.w., respectively. In this study, a 26% decrease in folate was noted in fresh corn-on-the-cob stored at 4 °C for 5 days after purchase. Twenty-six was the highest percentage of folate loss amongst the fresh fruits and vegetables examined in this study. Just as it was for vitamin A, the findings suggest that there is a great nutritional advantage of frozen corn when it comes to folate compared to fresh corn.

The mean folate contents for fresh, fresh-stored, and frozen green beans were 36.8 ± 10.0, 30.2 ± 6.9, and 28.5 ± 12.0 μg folic acid equivalents/100 g, f.w., respectively. The fresh green beans possessed a statistically ($p < 0.05$) greater content of folate than the frozen produce, which was not statistically ($p > 0.05$) different from that of the fresh-stored green beans. Again it should be noted that neither the fresh nor fresh-stored green beans analyzed in this study was blanched, as samples from the composite were immediately placed in a phosphate buffer. The frozen green beans, however, were blanched prior to freezing and this may account for why its mean folate content is statistically ($p < 0.05$) different from its fresh counterpart. The content of folate in fresh green beans determined by this study was similar to the Database value of 33 ± 2.7 μg folic acid equivalents/100 g, f.w. (n=8), but 2× that of the frozen value of 15 ± 2.6 (n=3). An 18% decrease in folate was noted in fresh green beans stored at 4 °C for 5 days after purchase.

Of all of the fruits and vegetables examined, spinach contained the highest content of folate. The mean values for the fresh, fresh-stored, and frozen produce were 134 ± 20.1, 129 ± 16.5, and 141 ± 13.8 μg folic acid equivalents/100 g, f.w., respectively. No statistical ($p > 0.05$) differences were noted in the folate content of the spinach types. The spinach samples analyzed in this study had levels of folate lower than that reported in the Database (194 ± 35.6, based on 6 observations). However, the result was consistent with the study by Rychlik (2004), who found the folate in fresh spinach varied from 96.1 to 159.2 μg/100 g. Pandrangi and LaBorde (2004) reported total folate in commercially-packaged spinach ranged between 84 and 225 μg/100 g with a mean value of 160 ± 42 μg/100 g. The folate level for the frozen spinach was not statistically ($p > 0.05$) different from the Database value of 145 ± 8.3, based on 6 observations. De Souza and Eitenmiller (1986) reported a 60% loss in folic acid for blanched spinach, whereas

Cooper, Chen, & King (1983) found that blanching in boiling water for 3 min resulted in a 33% loss compared with fresh spinach and microwave blanching without water, which only had a 14% loss relative to fresh spinach. It indicated that blanching can result in great loss of folate, but varying times and methods can lead to different quantities of folate being lost due to blanching. It is interesting to note that the frozen spinach sample possessed the highest mean folate content, albeit it not statistically ($p > 0.05$) different from the fresh samples; yet, it was the only blanched sample. Roughly a 4% loss was found in fresh spinach on average after being stored at 4 °C for 5 days after purchase. On the other hand, Chen, Song, & Kirsch (1983) found a 26% decrease in folate content of spinach stored at 4 °C for 7 days. These authors suggested that blanching and immediate freezing of spinach can retain more than 80% of the folacin in the fresh spinach when the air was excluded from the packaging.

Unlike the other produce, fresh green peas (also referred to as English peas) could only be procured in the spring of both years of the study; hence, Table 5.3 reports sampling times as opposed to seasons and gives the folate content of fresh, fresh-stored, and frozen green peas based on their fresh weight; the mean values were 54.2 ± 10.3, 47.2 ± 4.5, and 58.1 ± 13.1 μg/100 g, f.w., respectively. Even though the absolute mean for frozen green peas was higher than its fresh counterpart (58.1 vs. 54.2 μg folic acid equivalents/100 g, f.w.), the values were not statistically ($p > 0.05$) different. On the other hand, the folate in frozen green peas was significantly ($p < 0.05$) greater than that of the fresh-stored produce. The content of folate determined from this research for fresh green peas was found to be less than the Database value of 65 μg folic acid equivalents/100 g, f.w. based on a single observation. Stea, Johansson, Jägerstad, & Frølich (2006) found 47.9 μg/100 g of folate in raw green peas. In the case of

frozen green peas, the Database lists an imputed value of 53 μg folic acid equivalents/100 g, f.w. Roughly a 13% loss was observed in fresh green peas after storage at 4 °C for 5 days. Selman (1994) noted that the folate content in boiled green peas was much lower than raw green peas. Although there might be some loss in blanching because of the water solubility of folate, the high retention of folate in frozen storage compensated for this.

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Table 5.1 Food/total folate content (μg/100 g, f.w.) in selected fresh, fresh-stored, and frozen fruits.[1]

Fruits	Factors	Year 2011			Year 2012			Year 2013	Mean±SD[2]	U.S.D.A. Mean±SE[3]
		Summer	Fall	W/S[4]	Summer	Fall	W/S			
Blueberry	Fresh	12.0±2.40	18.8±0.86	14.1±1.24	6.0±0.20	10.9±0.75	7.1±1.14	11.5±4.52AB	]	6±0.1(*n*=12)
	Fresh-Stored	9.0±5.00	15.2±0.71	10.3±1.19	4.4±0.51	10.8±0.38	7.8±0.35	9.6±3.79B		
	Frozen	13.0±1.03	15.8±2.81	19.6±4.61	12.2±2.43	12.0±3.12	10.2±0.42	13.8±3.93A		7±1.3(*n*=3)
Strawberry	Fresh	45.7±8.42	47.4±5.23	39.8±5.98	33.4±7.69	51.4±7.10	40.1±3.83	43.0±12.7A	]	24±5.5(*n*=3)
	Fresh-Stored	39.6±4.10	43.6±1.38	35.0±2.82	32.7±4.88	41.7±2.31	39.3±1.62	38.5±4.54A		
	Frozen	44.7±1.96	45.5±1.57	35.8±6.97	30.2±2.24	47.6±13.05	34.7±2.52	39.7±8.50A		17±5.4(*n*=3)

[1]f.w. – fresh weight.

[2]Mean ± SD values calculated based on 18 individual observations. Those followed by a different letter within a column (for each sample) reflect significant differences ($p \leqslant 0.05$) according to ANOVA and means separation with Tukey Studentized Range *via* SAS software.

[3]U.S.D.A. National Nutrient Database for Standard Reference (R26) sample means ± standard error {SE} (*n* = number of data points) for: Broccoli, raw; Broccoli, frozen, chopped, unprepared; Cauliflower, raw; Cauliflower, frozen, unprepared; Corn, sweet, yellow, raw; Corn, sweet, yellow, frozen, kernels cut off cob, unprepared; Beans, snap, green, raw; Beans, snap, green, frozen, all styles, unprepared; Peas, green, raw; Peas, green, frozen, unprepared; Spinach, raw; Spinach, frozen, chopped or leaf, unprepared; Blueberries, raw; Blueberries, frozen, unsweetened; Strawberries, raw and Strawberries, frozen, unsweetened.

[4]W/S –Winter/Spring.

Table 5.2 Food/total folate content (μg/100 g, f.w.) in selected fresh, fresh-stored, and frozen vegetables.[1]

Vegetables	Factors	Year 2011			Year 2012			Year 2013	Mean±SD[2]	U.S.D.A.[3] Mean±SE
		Summer	Fall	W/S[4]	Summer	Fall	W/S			
Broccoli	Fresh	74.7±8.80	75.5±6.07	69.9±1.09	79.6±8.85	68.1±11.1	67.7±3.67	72.6±7.68A	]	63±12.7(*n*=27)
	Fresh-Stored	65.6±2.46	65.4±10.9	62.2±2.90	80.3±8.76	65.7±10.4	65.4±2.95	67.4±8.68A	]	
	Frozen	61.5±19.1	57.7±5.82	61.4±2.01	66.3±4.97	59.5±2.33	63.9±10.8	61.7±9.28B		67±1.8(*n*=3)
Cauliflower	Fresh	68.9±16.9	64.6±15.8	65.4±5.54	72.2±3.49	71.6±1.59	62.8±10.46	68.9±4.40A	]	57±5.3(*n*=27)
	Fresh-Stored	66.4±2.18	64.7±7.58	65.2±7.10	70.1±2.82	71.0±9.47	61.3±3.12	65.0±5.49A	]	
	Frozen	72.5±13.0	67.3±0.96	66.9±12.9	76.0±13.2	70.8±2.06	68.1±2.96	67.7±8.47A		64(Imputed)
Corn	Fresh	60.5±12.1	42.9±8.25	61.3±6.89	48.6±3.07	49.1±6.17	38.8±1.36	50.2±10.5A	]	42±3.4(*n*=7)
	Fresh-Stored	30.3±6.59	38.2±8.67	24.8±3.52	47.6±0.78	49.4±3.16	33.2±1.96	37.2±10.1B	]	
	Frozen	45.1±1.96	56.0±9.90	48.9±10.5	52.9±2.21	53.8±4.98	46.9±2.21	50.6±6.73A		36±5.5(*n*=6)
Green Beans	Fresh	31.9±4.18	25.6±3.23	31.6±5.20	34.3±4.98	46.1±0.42	51.4±7.10	36.8±10.0A	]	33±2.7(*n*=8)
	Fresh-Stored	25.9±6.27	21.7±2.23	28.0±3.65	31.2±0.98	33.8±2.99	40.7±2.31	30.2±6.90AB	]	
	Frozen	27.6±1.15	18.2±4.06	15.4±2.74	27.0±1.44	35.3±0.94	47.6±13.1	28.5±12.0B		15±2.6(*n*=3)
Spinach	Fresh	119±5.0	160±5.7	140±1.6	147±4.6	119±8.7	121±31.6	134±20.1A	]	194±35.6(*n*=6)
	Fresh-Stored	122±6.3	146±10.9	136±6.0	147±2.1	106±7.4	119±4.1	129±16.5A	]	
	Frozen	154±7.8	144±4.8	136±13.5	147±11.1	145±12.6	121±6.6	141±13.8A		145±8.3(*n*=11)

[1,2,3,4]See footnotes of Table 5.1 for description of the samples reported from the U.S.D.A. National Nutrient Database for Standard Reference (R26).

Table 5.3 Food/total folate content (μg/100 g, f.w.) in fresh, fresh-stored, and frozen green peas.[1]

Vegetable	Factors	Sampling Period						Mean±SD[2]	U.S.D.A.[3] Mean±SE
		1	2	3	4	5	6		
Green Peas	Fresh	64.8±5.19	52.0±7.62	63.3±4.51	45.6±2.46	48.1±9.78	51.7±14.70	54.2±10.3AB	65(*n*=1)
	Fresh-Stored	43.4±5.81	49.8±2.49	49.7±3.86	45.8±2.39	48.0±2.91	46.3±7.33	47.2±4.46B	
	Frozen	41.2±7.52	64.9±6.59	52.7±6.48	76.9±10.21	55.7±9.36	57.2±5.83	58.1±13.1A	53(Imputed)

[1,2,3]See footnotes of Table 5.1 for description of samples reported from the U.S.D.A. National Nutrient Database for Standard Reference (R26).

Table 5.4 Accuracy and interday precision (%RSD_r) determined by analysis of the folic acid extracted from BCR® 485.

Analyte	Mass fraction (μg/100 g)			% of accepted value[4]	%RSD_r[5]
	Accepted value[1]	Analytical value[2]	Bias[3]		
Folic acid	315 ± 28	299 ± 13	-16	95	4.47

[1]The accepted value of folic acid was from the certified value of BCR® 485

[2]Values (mean ± SD) are based on twenty-two replicate analyses.

[3]Bias = Accepted value – Analytical value.

[4]The ratio of the analytical value to accepted value expressed as a percentage.

[5]RSD_r, interday relative standard deviation (SD/mean × 100)

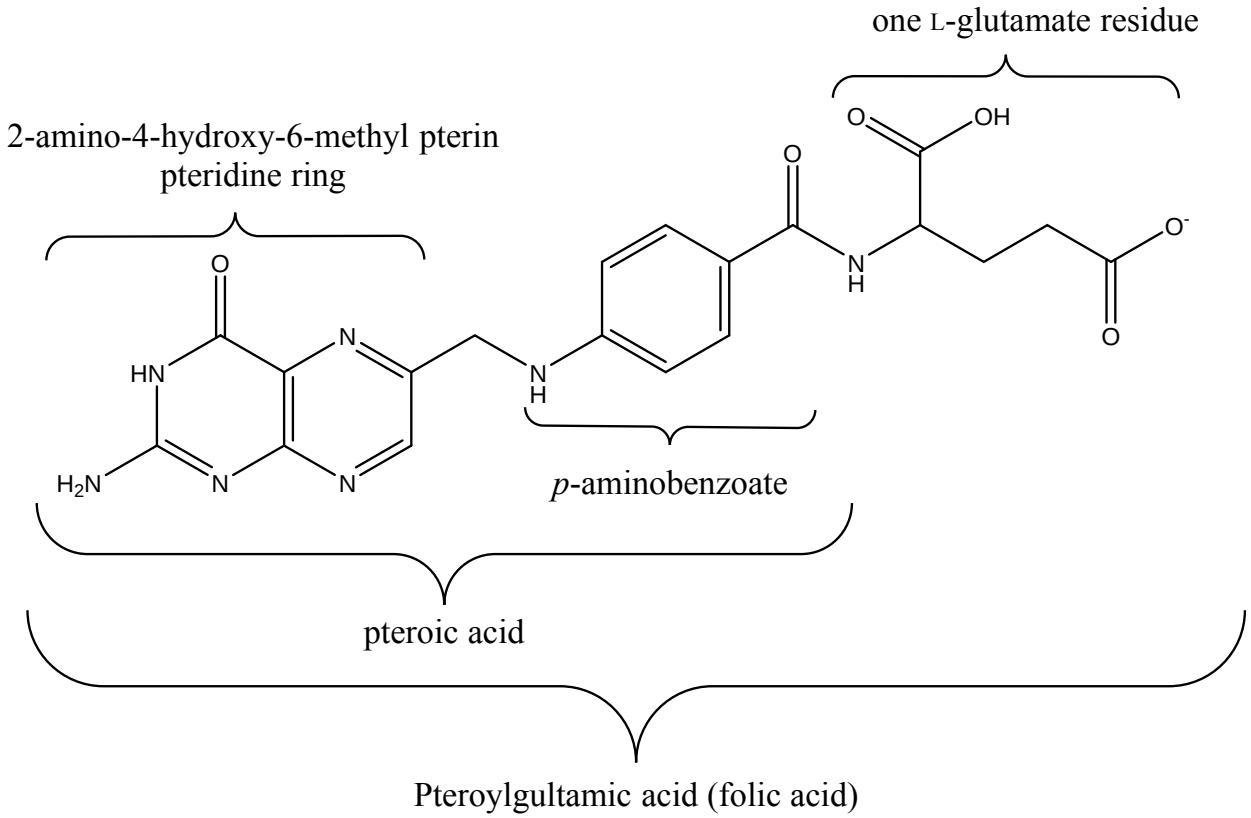

Figure 5.1 Chemical structure of folic acid.

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

Folic acid standard | Flour sample for QC | Test sample 1 | Test sample 2

Figure 5.2 Layout of the microplate for the folate assay.

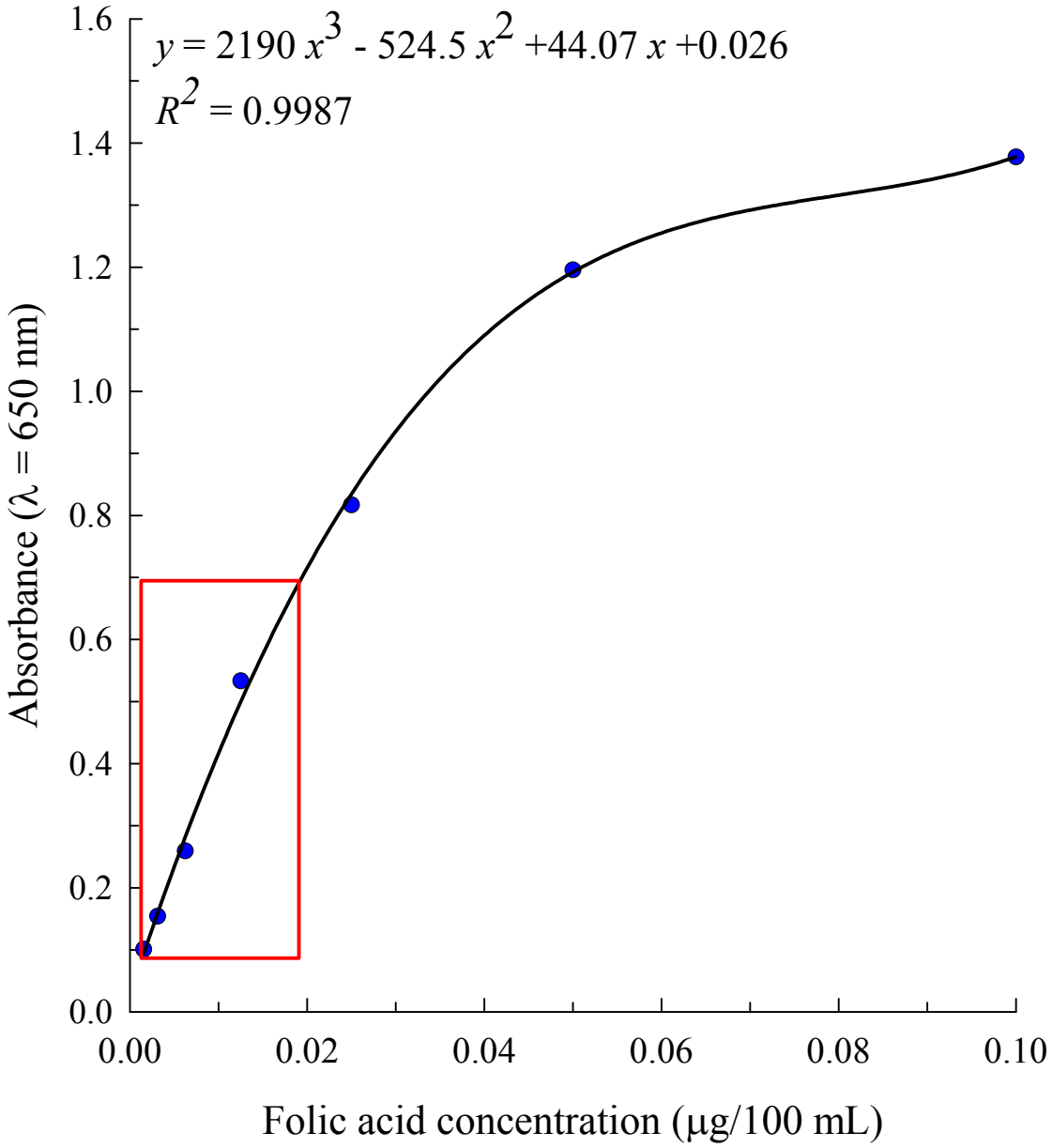

Figure 5.3 An example of a typical standard curve generated in the microbiological assay of folate concentration versus absorbance[1].

[1]The inserted box depicts the linear region of this polynomial curve, which can be used for folate quantification in the fruit and vegetable samples.

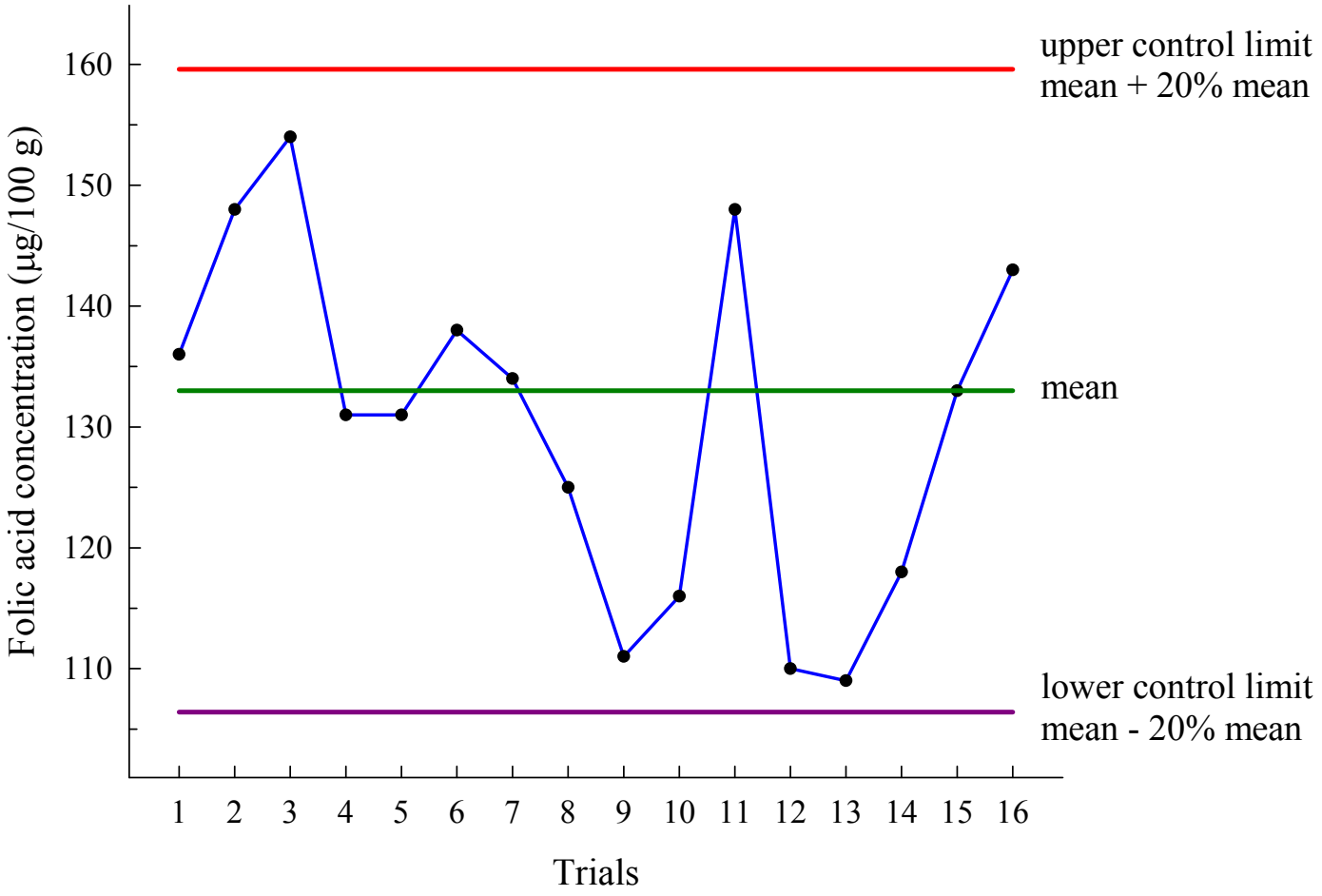

Figure 5.4 The quality control assessment of folic acid in enriched, all purpose-flour based on 16 measurements.

CHAPTER 6

CONCLUSIONS

Variables, such as product origin, time of harvest, storage period and conditions, pretreatment of the produce, as well as processing time and conditions are important inherent factors affecting the results of the analytical assays performed in this work and the compilation of the data. This study did not attempt to track cultivar types, country of origin (or U.S. state) of the produce, or environmental/weather patterns, the primary goal was to compare nutrient levels in different produce based on grocery patterns. Therefore, targeted fruits and vegetables were purchased either fresh or frozen from local grocery chains and then stored per typical customer habits.

The 8 fruits and vegetables selected for nutrient analyses in this study were blueberries, strawberries, broccoli, cauliflower, corn, green beans, spinach, and green peas. The nutrients measured were vitamin C (*i.e.*, ascorbic acid), vitamin A (*i.e.*, reported as *trans*-β-carotene content) and folate. When comparing these nutrient levels in fresh, fresh-stored, and frozen fruits and vegetables, some interesting and valuable findings were found. In the majority of situations, no significant difference was determined between the selected nutrient contents in fresh and frozen products as well as fresh samples that had been stored in a refrigerator for 5 days after purchase. This observation is consistent with some other research studies. Yet, future studies with a strong experimental design are warranted to consolidate the conclusion.

For broccoli, no significant difference was found between the vitamin C contents in fresh, fresh-stored, and frozen products. However, the vitamin A (reported as *trans*-β-carotene) levels in fresh and fresh-stored broccoli were both higher than that of the frozen counterpart; no significant difference existed between the fresh and fresh-stored samples. This finding indicated a reasonable stability of *trans*-β-carotene in fresh, uncut broccoli during refrigerated storage. The stability may be due to protection of this nutrient from the relatively hard cell structure of its surface and the fact that the fresh broccoli, unlike the frozen sample, was not cut up into pieces until only just before the vitamin A analysis was performed. In terms of folate, the mean value of fresh-stored broccoli based on n=18 was slightly greater than that of frozen broccoli, but statistically there was no significant difference between the two.

For corn, no significant difference was found in the vitamin C and *trans*-β-carotene contents for the fresh, fresh-stored, and frozen samples. Yet, the folate contents in both fresh and frozen corn were found to be significantly greater than that of fresh-stored corn, and no significant difference existed in the folate levels of the fresh and frozen corn. The finding suggests that regular consumption of frozen corn as opposed to fresh corn of indeterminate age might provide a superior and consistent level of folate intake. This could be especially true in the U.S. during the winter season when fresh corn-on-the-cob is imported to the country. Moreover, if fresh corn is stored in a refrigerator for a period of 5 days after purchase, or even longer before consumption, it will contain markedly less folate than its frozen counterpart.

No significant difference was found between the nutrients in fresh and frozen green peas, but there was a marked decrease in L-ascorbic acid and *trans*-β-carotene contents during storage; frozen green peas were found to contain higher L-ascorbic acid and *trans*-β-carotene than fresh-

stored green peas. This may attributable to the vulnerability properties of green peas. Because the surface area for green peas is large and delicate, vitamins may be easily lost during storage. Additionally, no significant difference was found in folate contents between fresh, fresh-stored and frozen green peas. Therefore, frozen green peas can be a better choice compared with fresh counterpart for customers, because vitamin C and vitamin A contents were more stable in the frozen products. By the time when fresh green peas were actually consumed, which can be even longer than 5 days, the vitamin content in them may be lower than the frozen green peas.

In spinach, L-ascorbic acid and *trans*-β-carotene contents in fresh produce were found higher than in frozen spinach, while no difference was found between fresh-stored and frozen spinach for *trans*-β-carotene. No significant difference was noted in the folate content between fresh, fresh-stored, and frozen spinach. The finding indicated the possibility that frozen contains more nutrients when customers actually consume them. Firstly, customers are uncertain about how long fresh spinach has been out on displayed in the grocery store before it is purchased and brought to the household. Secondly, nutrients are lost in the refrigerator before consumption. Although fresh spinach may contain more nutrients initially, frozen spinach may possess the same level at the point of consumption.

For blueberries, while no significant difference was found in L-ascorbic acid contents in the three types of products, *trans*-β-carotene contents in both fresh and fresh-stored blueberries were found to be significantly higher than in frozen blueberries.

In conclusion, most of the findings of this study support the hypothesis that frozen fruits and vegetables possess equal or even higher levels of nutrients value compared with fresh counterparts, particularly fresh-stored ones. Future studies are warranted to consolidate the

conclusions reached in this work. Season and cultivar variations should be considered. To promote consumers to consider frozen fruits and vegetables as a healthful option, more studies which demonstrate that the level of nutrients in frozen produce is equal to or superior to fresh counterparts is needed.