MICROPLATE ASSAY WITH TRIENZYME EXTRACTION FOR TOTAL FOLATE ANALYSIS IN FOODS

by

LIWEN CHEN

(Under the direction of Ronald R. Eitenmiller)

ABSTRACT

In order to verify the trienzyme digestion coupled with a 96-well microplate assay is suitable for folate analysis in a variety of foods, single laboratory method evaluation was conducted using reference materials representing a broad selection of food matrices. A four parameter logistic equation of the calibration curve was generated using nonlinear regression. Accuracy, recovery and precision of the method met acceptable criteria as presented by AOAC International guidelines. Limit of detection and limit of quantitation were 0.3 and 0.6 μg/100g, respectively. The results showed that trienzyme digestion 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.

The trienzyme digestion was optimized for food folate extraction from vegetables using response surface methodology (RSM). The predicted second-order polynomial model was adequate ($R^2 = 0.947$), without significant lack of fit ($p > 0.1$). Both Pronase^R and α-amylase have significant effects on the extraction. Ridge analysis gave an optimum trienzyme digestion times: Pronase^R, 1.5 h; α-amylase, 1.5 h; conjugase, 3 h. The experimental value for CRM 485 (mixed vegetables) under this optimum digestion was close to the predicted value from the

model, confirming the validity and adequacy of the model. The optimized trienzyme digestion condition was applied to 5 vegetables and yielded higher folate levels than AOAC Official Method 2004.05.

In order to provide reliable folate data for fruits, vegetables and their commercial products, 250 fruit samples and 171 vegetables samples were collected through the National Food and Nutrient Analysis Program (NFNAP) or other United States Department of Agriculture (USDA) food sampling programs. The assay procedure followed AOAC Official Method 2004.05. Folate in most of the fruits and fruit products ranged from 10~80 μg/100g. Most tropical fruits (30-77) are high or concentrated natural sources of folate. Folate contents in vegetables and their products varied from 3 to 307 μg/100g. Edamame showed the highest folate content (307, μg/100g), followed by leafy vegetables (137-249), green beans (28), broccoli (67), corn (38), green peas (31), carrots (26), orange juice (25).

INDEX WORDS: food, folate, contents, trienzyme extraction, method validation, optimization, microbiological assay, microplate

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DEDICATION

This dissertation is dedicated

to

my father Yuhua Chen,

who rooted it in my heart that

knowledge is the most important thing to seek after.

To

my mother Yinghui Li,

who loves me and let me be myself, and

encouraged me to go for my career goal.

To

my brother Guohao Chen,

who takes care of the family.

AND

To

my Lord and Savior Jesus Christ,

who guides my way to fulfill His purpose.

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CHAPTER 1

INTRODUCTION

This study validated the microplate assay with trienzyme digestion for total folate analysis in a broad selection of food matrices. Further investigation was conducted to optimize the trienzyme digestion for food folate extraction from vegetables. Finally, using microplate assay with trienzyme extraction, this study gave an overview of folate content in commercial vegetable and fruit products; thereby, providing useful information for healthy diet.

Microbiological assay, using *Lactobacillus casei subsp. rhamnosus* (ATCC 7469), is one of the best and most versatile methods for the determination of food folate (Tamura, et al., 1997). A 96-well microtiter plate assay with microcomputer analysis was one of the modifications to improve microbiological assay, making it less laborious, less time consuming, and more reproducible (Newman and Tsai, 1986; Horne and Patterson, 1988; Tamura, 1990; Horne, 1997).

Trienzyme extraction, introduced by Eitenmiller and his colleagues (DeSouza and Eitenmiller, 1990; Martin, et al., 1990), is a combined enzymatic extraction for folate analysis with Pronase^{R}, α-amylase and conjugase (γ-glutamyl hydrolase). Food folate is liberated from non-specific binding by carbohydrates and proteins with α-amylase and Pronase^{R} digestion. Conjugase removes glutamic acid residues from γ-glutamyl folates with three or more glutamic acid residues. Deconjugation of folate is necessary because *Lactobacillus casei subsp. rhamnosus* (ATCC 7469) can not respond to poly γ-glutamyl folates with three or more glutamic acid residues (Goli and Vanderslice, 1992).

Although the microplate assay (Tamura, 1990) and trienzyme digestion (DeSouza and Eitenmiller, 1990; Martin, et al., 1990) for food folate analysis were introduced in 1990, the combination of microplate assay and trienzyme digestion for folate analysis in food was still at the early stage. The purpose of the combination was to extract folate from the food matrix completely for accurate measurement, save time, materials and labor. In order to evaluate if this purpose was fulfilled, it was necessary to evaluate the method performance parameters of the microplate assay combined with trienzyme extraction. Few papers have been published in this area.

During the period of this study, microbiological assay with trienzyme extraction was accepted as an official method for folate analysis in cereal grain products by AOAC International (DeVries, et al., 2005; AOAC International, 2005). However, the official recommendation of this method was limited to cereal grain products. Evidence was still needed in order to extend its application to other food matrices. Evaluation of method performance parameters was needed in order to verify that the microbiological assay with trienzyme digestion was suitable for folate analysis in a broad selection of food matrices with an acceptable uncertainty level.

Method performance evaluation included single-laboratory (in-house) and inter-laboratory (collaborative) validation. Our study was single-laboratory method performance evaluation, in which calibration curve and matrix effect (parallism), accuracy, recovery, precision, limit of detection and limit of quantitation were evaluated.

Most plant-derived foods, especially fruits and vegetables, are main source of dietary folate. Koehler et al (1997) reported that fruits and vegetable provided 20.8 % and 23.2 % of folate intake of older adults, respectively. They also found that fruits and vegetables provided 23 of the top 50 folate sources. Increasing consumptions of fruits and vegetables were shown to

improve dietary folate status, preventing cardiovascular disease and neural tube defects in the general population (Brouwer, et al., 1999; Koebnick, et al., 2001; Chen, et al., 2005). A high fruit and vegetable diet was also recommended to potentially reduce cancer risk (Bailey, 2003).

Optimum conditions for extracting food folate from vegetables were recently under investigation. Working on spinach, Pandrangi et al (Pandrangi and LaBorde, 2004) found an optimum incubation time of 8 h for protease digestion, while α-amylase digestion did not appreciably affect measurable folate. Australian researchers reported that single enzyme extraction with conjugase alone gave higher measurable folate levels than trienzyme extraction for leafy vegetables (Shrestha, et al., 2000; Iwatani, et al., 2003). However, digestion pH and order of enzyme addition in these literatures were varied from AOAC Official Method 2004.05.

Response Surface Methodology (RSM) is a collection of statistical and mathematical techniques useful for developing, improving, and optimizing processes (Myers and Montgomery, 1995). Originally described by Box and Wilson (1951), RSM evaluates the effects of several process variables and their interactions on response variables. RSM is less laborious and time-consuming than other optimization approaches, requires fewer experimental trials to evaluate multiple parameters and their interactions. RSM has been widely used for optimizing conditions in agricultural and biological research (Lee, et al., 2000; Mizubuti, et al., 2000; Madamba, 2002; Kwon, et al., 2003; Li and Fu, 2005; Liyana-Pathirana and Shahidi, 2005; Tanyildizi, et al., 2005). Our study applied RSM to optimize the trienzyme digestion for folate extraction from vegetables, using Certified Reference Material (CRM) 485 mixed vegetables to represent the vegetable matrix.

Several reports have been published on folate contents of some vegetables (Miyamoto, et al., 1973; Malin, 1977; Leichter, et al., 1978; Mullin, et al., 1982; Desouza and Eitenmiller, 1986;

Lin and Lin, 1999; Melse-Boonstra, et al., 2002). Iwatani et al (2003) reported folate contents of Australian vegetables. This study provides a thorough overview of folate contents in a broad selection of fruit and vegetable commercial products, providing more helpful information for establishing a healthy diet with adequate folate intake.

The objectives of this study were:

1. To establish and validate that microplate assay with trienzyme digestion was suitable for folate analysis in foods.
2. To optimize trienzyme digestion for extracting folate from vegetables.
3. To provide a thorough overview of folate contents in a broad selection of fruit and vegetable commercial products for establishing a healthy diet with adequate folate intake using samples collected by the United States Department of Agriculture for analysis at the University of Georgia.

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CHAPTER 2

LITERATURE REVIEW

Folate and Folic Acid

Folate is the general term inclusive of folic acid (pteroylglutamate) and poly-γ-glutamyl conjugates that exhibit the biological activity of folic acid. Folate was first isolated in the early 1940s from green leaves, liver, and yeast. Folates include natural forms occurring in the food, while folic acid is the synthetic form commonly used for food fortification and formulation of supplements and pharmaceuticals. Folates are labile, sensitive to oxidation, light, heat and extremes of pH. The level of instability differs with the individual vitamers. Folic acid is more stable than folate under most conditions (Reed and Archer, 1980; Gregory, 1989; Hawkes and Villota, 1989; Ball, 1994b).

Chemical Structure of Folate and Folic Acid

Folic acid has three main components: pterin (2-amino-4-oxopteridine), a para-aminobenzoic acid moiety, and a glutamate residue. The structures of folic acid and folate are shown in Figure 2.1. Pteroic acid is the parent compound. It contains a pterin core ring structure which is conjugated to para-aminobenzoic acid via a methylene bridge to form pteroic acid (Bailey, 1995). Folic acid is formed by binding the carboxy group of the para-aminobenzoic acid to the amino group of the glutamate. Natural folates comprise a large group of heterocyclic compounds that are based on the pteroic acid structure conjugated with two or more L-glutamates through γ-glutamyl amide linkages. Folic acid and folate are the preferred synonyms for pteroylglutamic acid (single glutamic acid) and polypteroylglutamate, respectively (Bailey,

1995). The pteroylglutamates and their corresponding acids are named after the number of glutamate residues attached: for instance, pteroyldiglutamic acid has two glutamates and pteroylpolyglutamate contains more than two glutamates. The pterin ring in pteroylglutamic acid may be in the oxidized state or may be reduced to dihydrofolate (DHF) or tetrahydrofolate (THFA). The active co-enzyme forms are N-5 methyl, N-5 or N-10 formyl, N-5 formimino, N-5, 10 methylene, and N-5,10 methenyl folate, all of which have one carbon unit at N-5 or N-10 or between N-5 or N-10 of the pterin ring (Eitenmiller and Landen, 1998). The coenzyme forms of folate, differ from the vitamin in two respects: they are reduced compounds (5,6,7,8-tetrahydropterins), and they are modified by the addition of glutamate residues bound to one another through γ-glutamyl amide linkages. Only the reduced forms of folate are biologically active. Various folate coenzyme forms are given in Table 2.1. The anionic polyglutamyl moiety, usually five to six residues long, participates in the binding of coenzyme to enzymes and helps keep the coenzymes inside cells since lipid membranes are impermeable to charged molecules.

Function of Folate

Folate functions in one carbon metabolism. Various forms of tetrahydrofolate, including N-5 methyl, N-5 or N-10 formyl, N-5 formimino, N-5, 10 methylene, and N-5,10 methenyl tetrahydrofolate, act as acceptors or donors of single carbon units and play an important role in various metabolic reactions by transferring single carbon units (methyl, methelene, methenyl, formyl, formimino groups). For instance, tetrahydrofolate accepts single carbon units (methyl group) from serine, converts serine to glycine, and forms 5,10-methylenetetrahydrofolate. Folate coenzymes also involved in other amino acid interconversions such as conversion of histidine to glutamic acid, and homocysteine to methionine. In cell division, folate coenzyme are involved in the synthesis, repairing, and functioning of the DNA (Wagner, 1996). Folate plays a vital role in

the reproduction of cells in the fetus and is highly essential whenever there is a rapid proliferation of cell growth, especially during pregnancy, infancy and child growth. Folate helps to maintain the nervous system and functions of the intestinal tract (Wagner, 1996; Institute of Medicine, 1998). It also acts as the carbon carrier in the formation of the "heme" and is essential in the formation of the red blood cells.

Folate and Health

Megaloblastic Anemia

Megaloblastic anemia is the major clinical manifestation of folate deficiency resulting in slowed DNA synthesis (Herbert and Coleman, 1979) Megaloblastic anemia is the production of immature red blood cells. Clinical symptoms of megaloblastic anemia are weakness, fatique, difficulty concentrating, irritability, headache, palpitations and even shortness of breath in the advanced stage(Gibson, 2005). Inadequate folate intake causes a decrease in serum folate concentration and erythrocyte folate concentration decreases accordingly. The concentration of homocysteine increases and leads to megaloblastic changes with rapidly dividing cells characterized by impaired DNA synthesis (Ball, 2004). Then, macrocytic anemia develops because of reduced erythrocyte count. Finally, all three measures of anemia (hematocrit hemoglobin concentration and erythrocyte concentration) are depressed. Studies indicated that the serum folate level in the blood directly leads to the decrease in the erythrocyte folate concentration (Hoffbran, et al., 1966; Eichner and Hillman, 1971).

Neural Tube Defects (NTD)

Neural tube defects (NTD) are birth defects resulting from the failure of the neural tube to

close during the first four weeks of gestation. The most common defects of NTD include neuroblastoma, spina bifida and anencephaly. Neuroblastoma is a malignant (cancerous) tumor that develops from nerve tissue, which occurs in infants and children. Spina bifida is a birth defect that involves the incomplete development of the spinal cord or its coverings. The term spina bifida comes from Latin and literally means "split" or "open" spine. Spina bifida occurs at the end of the first month of pregnancy when the two sides of the embryo's spine fail to join together, leaving an open area. Anencephaly is absence of the fetal cranial vault. Exposure of cerebral tissue to amniotic fluid precludes brain development. Anencephaly is a form of neural tube defect that is typically an isolated birth defect that is not related to chromosomal abnormalities. The defect may occur before the recognition of conception because the closure of the neural tube occurs between 18 and 27 days after conception. Among the 4 million births in the United States, there were approximately 2500 infants born each year with NTD (Flood, et al., 1992). Various factors may play a role in the occurrence of NTD, including genetics, geography, socioeconomic status, month of conception, race, nutrition, maternal health, maternal age and reproductive history.

Folic acid status associates with the reduction of pregnancies with NTD (Czeizel and Dudas, 1992; Wald, et al., 2001). Several clinical studies revealed a decrease in occurrence of NTD after the periconceptional supplementation of folic acid (Laurence, et al., 1981; Smithells, et al., 1981; Vergel, et al., 1990; Wald, 1991; Czeizel, 1993; Werler, et al., 1993). Consumption of multivitamins with folic acid would also reduce the occurrence of NTD (Bower and Stanley, 1989; Milunsky, et al., 1989; Czeizel and Dudas, 1992; Werler, et al., 1993; Shaw, et al., 1995). In 1992, the US Public Health Service (PHS) issued a recommendation that “all women of child bearing age in the United States consume 0.4 mg of folic acid per day to reduce their risk of a

neural tube defect in pregnancy."

Folate plays a vital role in the reproduction of cells in the fetus and is highly essential whenever there is a rapid proliferation of cell growth, especially during pregnancy, infancy and child growth. However, the mechanism of action of folate associated with NTD is still poorly understood.

Cardiovascular Disease

During the past decade or so, interest in the role of adequate folate nutrition was also intensified for its association to cardiovascular disease (Boushey, et al., 1995; Moat, et al., 2004) Elevated plasma homocysteine is a risk factor for cardiovascular disease (Anderson, et al., 2000; Clarke and Stansbie, 2001). For every 10% elevation of homocysteine, there was nearly the same rise in the risk of developing severe coronary heart disease (Verhoef, et al., 1997). Vitamin supplementation with folic acid substantially reduced blood homocysteine (Brattstrom, et al., 1998), which resulted in the reduction of cardiovascular disease. Homocysteine is an amino acid which is an intermediary in the metabolic pathway of methionine. Methionine and homocysteine can interconvert into one another. Homocysteine can be methylated by 5-methltetrahydrofolate to form methionine. Thus, lack of folate intake will interrupt the conversion from homocysteine to methionine, lead to the accumulation of homocysteine and increase the risk of cardiovascular disease. Scientific evidence showed that 0.8 mg/day of folic acid could produce maximum reduction in plasma homocysteine levels (Wald, et al., 2001). Vitamin B6 and B12 are also significant to the metabolic pathway of methionine. Hence, it was suggested that folic acid supplements combined with B6 and B12 may offer a preventative measure against cardiovascular diseases (Brattstrom, et al., 1988; Schorah, et al., 1998). Research showed that

9% of male and 54% of female coronary artery deaths in the United States out of around 50,000 deaths each year could be prevented by mandatory fortification of grain products with 350 mg folic acid / 100 g food (Motulsky, 1996). It was found that daily dose of folic acid to lower homocysteine was 0.4-0.5 mg (Doshi, et al., 2002), which could be achieved by intake of fortified grain products (Malinow, et al., 1998).

Cancer

Diminished folate status is associated with higher risk of carcinogenesis (Lucock, 2004) . Low levels of folate were indicated to be a risk factor for breast cancer when associated with high alcohol intake (Zhang, et al., 1999). The association of folate with cancer may result from the involvement of folate in DNA synthesis, especially in methylation reactions as well as in DNA repair. Hypomethylation of DNA was observed in colorectal cancers, and possible protective role of folate in carcinogenesis was reviewed (Glynn and Albanes, 1994).
Folate is also recognized for its role in reducing macular degeneration and neurodegenerative disorders including stroke, Alzheimer's disease and Parkinson's disease (Mattson and Haberman, 2003; Mattson and Shea, 2003; Lucock, 2004; Nowak, et al., 2005).

Folic Acid Fortification

The notable benefits of folic acid for prevention of NTD has led several organizations in the United States and around the world to recommend folic acid fortification programs (Centers for Disease Control, Food and Drug Administration, Bio Med Central, Canada). The fortification of folic acid was first proposed in the United States (US) in 1973. After careful review, the health claim for dietary supplements in relation to the neural tube defects was authorized in 1994. On March 17, 1996, the FDA issued a final rule effective from January 1st of 1998 that all flour

and enriched cereal grain products are required to be fortified with folic acid at levels ranging from 0.43 mg to 1.4 mg per pound of the product (Food and Drug Administration, 1996). Amendments of standards of identity were recognized for enriched grain products for addition of folic acid . Folic acid fortification was also mandated in Canada in 1998 (Canada Gazette Part-II, 1998), and Australia.

Prevalence of NTD has greatly decreased since fortification. Honein showed that the birth prevalence of NTD decreased by 19% after mandatory fortification when compared to those before fortification (Honein, et al., 2001). Williams et al (2002) revealed that prevalence of spina bifida and anencephaly decreased by 31% after fortification. In Canada, Persad et al (2002) reported that the incidence of NTD decreased by 54% after the mandatory fortification. Decreased incidence of the NTD was attributed to folic acid fortification. Homocysteine level was also found to decreased significantly after the implementation of fortification (Anderson, et al., 2004; Tucker, et al., 2004). Two recent papers compared the National Health and Nutrition Examination Surveys before fortification and after fortification. Prevalence of low serum folate levels decreased after fortification and prevalence of high serum folate levels increased in the elderly (Dietrich, et al., 2005; Pfeiffer, et al., 2005).

Apart from US, Canada, Mexico, Chili and Hungary also initiate folic acid fortification program (Cornel, et al., 2005).

DFE

The Institute of Medicine Panel on Folate, Other B Vitamins, and Choline (Institute of Medicine, 1998) established the μg Dietary Folate Equivalent (μg DFE) to compensate for differences in bioavailability between synthetic folic acid and native food folate. It was indicated

that folic acid taken with food is 85 percent bioavailable while native food folate is only 50 percent bioavailable (Melse-Boonstra, et al., 2004a; Melse-Boonstra, et al., 2004b). Thus, folic acid taken with food is 1.7 (85/50) times more bioavailable than folate. When a mixture of folic acid and food folate is taken, μg DFE is calculated by the following formula:

$$\mu g \text{ of DFE provided} = [\mu g \text{ of food folate} + (1.7 \times \mu g \text{ of synthetic folic acid})]$$

Supplements of folic acid when consumed in an empty stomach are nearly 100 percent bioavailable (Gregory, 1997). If compared with food folate, only half as much of folic acid is needed if taken on an empty stomach. Hence, 1 μg of DFE provided = 1 μg of food folate = 0.5 μg of folic acid taken on an empty stomach = 0.6 μg of folic acid taken with meals.

Dietary Reference Intake (DRI)

Dietary Reference Intake (DRI) is a general term for a set of reference values used for planning and accessing nutrient intake for healthy people. It includes the Recommended Dietary Allowance (RDA), Adequate Intake (AI), Estimated Average Intake (EAR) and Tolerable Upper Intake Level (UL). The RDA recommends the average daily intake that is sufficient to meet the nutrient requirements of nearly all (97-98%) healthy individuals in each age and gender group. An AI is set when there is insufficient scientific data available to establish a RDA. The AIs meet or exceed the amount needed to maintain a nutritional state of adequacy in nearly all members of a specific age and gender group. The EAR is the daily intake value that is estimated to meet the requirement of half of the healthy individuals. The UL is the maximum daily intake unlikely to result in adverse health effects (Institute of Medicine, 1998).

DRI of Folate

Recommendations for intake of folate are expressed in μg DFE according to the Institute of Medicine of the National Academy of Sciences (Institute of Medicine, 1998). The RDA and UL for folate in μg DFE / day are given in Table 2.2 and Table 2.3.

Upper Intake Levels of Folate

According to the scientific evidence, 1000 μg DFE /day was set as a safe upper limit of folate intake by the Institute of Medicine in 1998 (Institute of Medicine, 1998). Studies showed that intake of folate exceeding this level masks the symptoms of pernicious anmia caused by vitamin B12 deficiency (Bailey, 1995; Yetley and Rader, 2004). After peer reviw of the availale data, FDA concluded that the risk was minimal if the folate intakes were less than 1000 μg DFE /day in patients with vitamin B12 deficiency. In countries with prevalent vitamin B12 deficiency, it was recommended that the food should be fortified with both folic acid and vitamin B12 (Freire, et al., 2004).

Stability of Folate and Folic Acid

Folic acid is a stable synthetic form. Most naturally occurring folate derivatives in foods are sensitive to oxidation, light, temperature and extremes of pH. Thus, folate stability is affected by food processing. Affecting factors include variation in food matrices, oxygen availability, chemical environment, extent of heating and forms of folate in the food (Eitenmiiler and Landen, 1998). Increasing severity of heating temperature and time were found to cause increasing losses of folates during food processing (Williams, et al., 1995; Wigertz, et al., 1997; Vahteristo, et al., 1998). Ultra high temperature (UHT), which sterilizes foods at 135-150 °C for a few seconds, is

the common thermal process for liquid foods. Generally, less than 20% loss of folate was found in UHT, but up to 43% was also observed (Andersson, 1993). A low oxygen concentration was shown to decrease the degradation of 5 –methyltetrahydrofolic acid during UHT, although unable to completely prevent it (Viberg, et al., 1997). Nguyen et al. (2003) studied the stability of folic acid and 5-methyltetrahydrofolic acid during thermal treatment combined with high hydrostatic pressure using reverse phase liquid chromatography. They found that folic acid is quite stable under the high pressure treatments, while degradation of 5-methyltetrahydrofolic acid was enhanced by increasing pressure, with a remarkable degradation occurred at temperature above 40 °C. A spectroscopic study of the ultraviolet photodegradation of aqueous folic acid under aerobic condition was provided by Off et al. (2005). They found that folic acid was cleaved into ρ-aminobenzoyl-L-glutamic acid and 6-formyl pterin when exposed to ultraviolet radiation. When the irradiation continued, 6-formyl pterin was degraded to pterin-6-carboxylic acid.

Gujska and Majewska (2005) studied the effect of baking on added folic acid and endogenous folates in rye breads using HPLC combined with fluorescence and UV detection. They found that added folic acid decreased from flour to the bread stage. The total losses depended on baking process and ranged from 12~21%. The total endogenous folate content showed almost no changes, which may confirm that folates were produced during leaving of the dough during fermentation. Osseyi et al. (2001) studied the stability of added folic acid and some endogenous folates including trtrahydrofolate (THF), 5-formyl-THF, 10-formylfolate, and 5-methyl-THF during breadmaking. They found a good stability of added folic acid and native folates during the baking process. Compared with the flour from which they are made, increased amounts of endogenous folate were found in dough and bread.

Leskova et al. (2006) reviewed effects of various heat treatments on folate retention in vegetables, fruit products, legume, meat and fish products. Nearly all heat treatments showed a negative effect on folate retention. Overall, retention was 55-90% in meat and poultry, 70-100% in fish and shellfish, 40-66% in legumes and peas, and 30-100% in vegetables. The retention values depended on the extent and level of the heat treatment.

Fruits and vegetables are important sources of natural dietary folate. Koehler et al. (1997) reported that vegetable and fruit provided 23.2 % and 20.8 % of folate intake of older adults, respectively. They also found that fruits and vegetables provided 23 of the top 50 folate sources. Increasing the consumption of vegetables and fruits improves dietary folate status, preventing cardiovascular disease and NTD in the general population (Brouwer, et al., 1999; Koebnick, et al., 2001; Chen, et al., 2005). Folate-dense fruits and vegetables diet was also recommended to potentially reduce cancer risk (Bailey, 2003).

The stability of folate in vegetables during storage and processing has drawn much more attention compared to the other products. Spinach, broccoli, peas, non-leafy vegetables, pak-choi, leafy vegetables and beans have been studied for folate retention or losses during various heat treatments (Leichter, et al., 1978; Aramouni and Godber, 1991; 1993b; Hoppner and Lampi, 1993a; Bergstrom, 1994; Lin and Lin, 1999; Dang, et al., 2000; McKillop, et al., 2002). Among these studies, the highest retention was observed in some non-leafy vegetables under stir-frying with soybean oil for 1 min (up to 100%, except for snap bean and sponge gourd) (Lin and Lin, 1999) Folate breaks down under heat and leaches into the cooking water, leading to a large amount of loss. Thus, cooking methods that minimizes direct contact of food with the cooking water would decrease the loss of folate. Pressure-cooking, microwave cooking, and stir-frying have been found to be preferable to boiling for folate retention**.** Addition of supplemental

vitamin C to canned vegetables like okra and tomatoes prior to processing was found to provide additional stability to folate contents during subsequent storage (Sotiriadis and Hoskins, 1982).

Folate retention in Rosehips (Rosa spp.) after drying was studied (Stralsjo, et al., 2003a). It was indicated that degradation of folate after drying depended on the drying time until the water activity was below 0.75, and ascorbic acid could provide a possible protection for folate degradation.

Some researchers tried to increase folate content in vegetables by fermentation. Jagerstad et al. (2004) showed that lactic acid fermentation by one of the 10 selected lactic acid bacteria cultures can increase folate concentration in vegetables by more than 2 times, making the product superior to similar foods produced by the more common preservation techniques. Folate retention varied from 50% to 75% during the overall fermentation. Some commercial canned samples of sauerkraut contained folate (50 ~ 200 μg/kg), mainly as 5-methyltetrahydrofolate.

Folate stability in homogenized fresh fruits and vegetables during storage was evaluated (Phillips, et al., 2005). No change in 5-methltetrahydrofolate content was detected in any of the samples after 12 months of storage at -60 ± 5 °C.

Folates in fruit products were also evaluated. Stralsjo (2003b) studied folate contents in strawberries and folate retention during storage and commercial processing of strawberries. The study showed that effect of cultivar, ripeness, and year of harvest is significant for folate concentration in strawberries. Folate retention was high in intact berries during storage until 3 or 9 days (4 °C). It was indicated that almost no losses of folate occurred when strawberry desserts were made from frozen berries, which is contradictory to the data reported in four European food tables indicating that only 3-30% of folate was retained in various strawberry products like jam and stewed desserts (Holland, et al., 1991; Souci, et al., 1994; Levnedsmiddelstyrelsen, 1996;

Livsmedelsverket, 2002). Stralsjo et al. (2003b) also suggested that fresh strawberries and processed strawberry products were good folate sources. For instance, 250 g (fresh weight) of strawberries (about 125 μg of folate) supplies around 50% of the recommended daily folate intake in various European countries (200-300 μg/day) or 30% of the U.S. recommendation (400 μg/day).

Microbiological Assay

Microbiological assay is a technique to determine the potency or concentration of a chemical substance by its effect on the growth of a microorganism. This effect may be to promote the growth of the microorganism by substances such as certain vitamins and amino acids (growth promoting substances), or to inhibit growth in the case of antibiotics and other substances having similar properties (growth inhibiting substances).

Principle of Microbiological Assay for Vitamins

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

The basis of microbiological assay was outlined as the following (Favell, 1990):

1. A basal medium which provides all growth requirements for the organism except the vitamin to be assayed.
2. Addition of an aliquot of sample.
3. Inoculation of the appropriate organism.
4. Incubation of the mixture, such that the organism reproduces in proportion to the vitamin.
5. Measurement of the growth achieved (turbidity or metabolic products).

History of Microbiological Assay for Vitamins

Early in 1901, Wildiers discovered that certain species of yeasts need substances which he termed “bios” to give full growth. (Wildiers, 1901). But these views did not meet with universal acceptance at the time. Until 1919, R. J. Williams suggested that yeast might be enlisted as an assay organism for the quantitative determination of “bios” based on his discovery that there was great similarity between the properties of “bios” and the “water soluble vitamin” required by the animal organism (Williams, 1919). However, knowledge of the nutritional requirements of microorganisms was not sufficient at that time for Williams to put forward his suggestion to practical use.

Over the next twenty years, there were discoveries in the knowledge of vitamin and amino acid requirements of microorganisms. Vitamin B1(thiamine), vitamin B2 (riboflavin), vitamin B6 (pyridoxine) and choline were found to be essential factors for normal growth of different organisms. Biotin, pyridoxal, pyridoxamine, p-aminobenzoic acid, inositol and pantothenic were discovered to be essential nutritional factors in bacterial and fungal nutrition. There were similarities in nutritional needs exhibited by animals and microorganisms. These discoveries led

to the use of microorganisms in nutritional studies for the quantitative determination of vitamin B-complex and a wide range of amino acids. For example, the discovery that B group vitamins were essential for the growth of *Lactobacillus* and some other organisms led to the development of microbiological assay for B group vitamins during the 1930s.

It was the initiative and pioneering efforts of Esmond E. Snell that contributed greatly to the present-day widespread successful development and popularity of microbiological assay methods. Snell and Strong were the first investigators to make use of a lactic bacterium (*Lactobacillus helveticus or casei*ε) for the quantitative determination of a vitamin. The vitamin in this instance was riboflavin (Snell and Strong, 1939). This assay was served as a prototype assay for each of the B vitamins.

The earliest report of microbiological assay for folate was by Snell's group in 1941(Mitchell, et al., 1941). They used *Streptococcus lactis R* as a test organism to analyze the folate purified from four tons of spinach. The report of this work has been called "A Nutrition Classic**".**

Presently, microbiological assay has been considered to be one of the best and most versatile methods for folate analysis (Tamura, et al., 1997). AOAC Official Method 2004.05 is a microbiological method developed for folate analysis in cereal grain products (AOAC International, 2005).

Traditional microbiological assay is so laborious and time consuming that it is difficult to establish it as a dependable laboratory routine. Many developments have been made to modify the traditional microbiological assay. A 96-well microtiter plate assay with microcomputer analysis was one of the modifications to improve microbiological assay, making it less laborious,

less time consuming, and more reproducible (Newman and Tsai, 1986; Horne and Patterson, 1988; Tamura, 1990; Horne, 1997).

Development of 96-Well Microtiter Plate Assay

Development of microtiter plate technology in 1986 (Newman and Tsai, 1986) facilitated the improvement of microbiological assay for food folates. Subsequently, Horne and Patterson (1988) established a similar plate assay which was simpler, with cryopreservation of the organism *Lactobacillus casei* subsp. *rhamnosus* (ATCC 7469) and a shortened incubation time. A microplate assay for serum and red cell folate using a chloramphenicol resistant strain of *L. casei* was developed by O'Broin and Kelleher (1992). Sarma et al. (1995) reported a microplate assay for folic acid in multivitamin formulations using *S. faecalis* as the test organism. Horne (1997) presented an improved microplate assay using microtiter plates with opaque, black walls to resolve the problem of overestimation of folate concentration of samples in the two perimeter rows of 96-well plates. Techniques for the microplate assay are provided by Tamura (1990).

Assay Organism

The different species of lactic bacteria showed a wide variability in vitamin requirements, which made them efficient assay organisms for microbiological assay of various vitamins. Orla-Jensen et al (1936) discovered that when milk was shaken with an activated charcoal, it was unable to support the growth of many of these lactic bacteria. If riboflavin together with the material eluted from the charcoal adsorbate was returned to the charcoal-treated milk, growth was once more resumed, and was almost as prolific as in the untreated milk. The observation showed that lactic acid bacteria might be useful to assay various growth factors.

Requirements for the growth of lactic bacteria

The following factors affect the growth of lactic acid bacteria:

1) Carbon

Glucose is now universally used as a source of carbon and energy

2) Nitrogen

Peptone or acid hydrolyzed casein plus tryptophan is commonly used as a source of nitrogen.

3) Mineral salts

Different from those used for other bacteria, the mineral salt mixtures used for the lactic organisms have relatively high manganese content. The mineral salt mixture for the cultivation of lactic bacteria was first investigated by Snell et al (1937).

4) Vitamins

Lactic acid bacteria require a wide range of vitamins for growth. It had been demonstrated that riboflavin, pantothenic acid, nicotinic acid, pyridoxine and folic acid are required for the growth of *L. casei*, the important test organism for a majority of the B complex vitamins. Pyridoxal phosphate, nicotinic acid, pantothenate, riboflavin, folic acid are vitamin requirements for *Lactobacillus casei* subsp. *rhamnosus* (ATCC 7469).

5) Buffer

The optimum pH for the growth of a majority of lactic bacteria lies between 6 and 7. In practice, a pH of 6.8 is usually employed, but growth will proceed until a pH of 4 or less is attained.

Lactic acid formed during the growth and fermentation by the lactic bacteria will lead to sudden and rapid changes in pH levels in the medium, which will seriously interfere with or even entirely inhibit the normal growth of the lactic bacteria. Thus, buffer is needed in order to prevent this from happening. In AOAC Official Method 2004.05. Total Folates in Cereals and Cereal Foods, Microbiological Assay - Trienzyme Procedure, phosphate buffer (pH 6.8) is used as an assay buffer for this purpose.

Microbiological Assay for Folate Analysis

Medium

The first microbiological assays for folate were attempted in the 1940s (Snell and Peterson, 1940; Mitchell, et al., 1941; Stokstad, 1941; Sauberlich and Baumann, 1948). The basic composition of the assay medium essentially remained the same since that time, although many improvements have been investigated. Freeze-dried media for folate asays using L. casei are available from Difco Laboratories (Detroit, MI). Difco folic acid casei medium contains: charcoal treated pancreatic digest of casein, dextrose, sodium acetate, dipotassium phosphate, monopotassium phosphate, L-asparagine, L-cysteine HCl, magnesium sulfate (anhydrous), DL-tryptophan, polysorbate 80, xanthine, sodium chloride, ferrous sulfate, manganese sulfate, adenine sulfate, glutathione (reduced), guanine HCl, uracil, pyridoxine HCl, p-aminobenzoic acid, riboflavin, calcium pantothenate, nicotinic acid, thiamine HCL and biotin. Final pH is 6.7 ± 0.1.

Test organism

Lactobacillus casei subsp. *rhamnosus* ATCC 7469, *Enterococcus hirae* ATCC 8043, and *Pediococcus acidilactici* ATCC 8081 have been used for microbiological assay of folate. The responses of these three microorganisms to the folates available in biological matrix are shown in Table 2.4.

Lactobacillus casei subsp. *rhamnosus* responds to various natural folate forms present in biologicals, and does not respond to pteric acid—a common folate degradation product (Eitenmiiler and Landen, 1998).

Enterococcus hirae has the limitation in that it does not respond to 5-methyl-H_4 folate, the most common folate present in milk, other foods, tissue, and serum, and it responds to pteroic acid—a common folate degradation product (Voigt and Eitenmiller, 1978).

Pediococcus acidilactici has the most limited response and can grow on only mono-, di-, and triglutamates of 5- or 10-HCO-H_4 folate. It does not respond to methyl-substituted folates (Ball, 1994a).

None of the organisms efficiently responds to γ-glutamyl folate with greater than 3 glutamic acid residues. Among the three bacteria, *Lactobacillus casei* subsp. *rhamnosus* has greatest capacity for response to theγ-glutamyl folate polymers. However, its response is limited to no greater than 3 glutamates with much lower response to higher polymeric folates (Eitenmiiler and Landen, 1998).

Lactobacillus casei subsp. *rhamnosus* is considered to be the best available bacteria for folate assay, because of its response to the most forms of natural folate, its greatest capacity in response to the γ-glutamyl folate polymers compared to other bacteria, and its lack of response to pteroic acid—a common folate degradation product.

Lactobacillus casei subsp. *rhamnosus* (*ATCC 7469)*

Lactobacillus casei subsp. rhamnosus (ATCC 7469) is the test organism used in AOAC Official Method 2004.05 Total Folate in Cereals and Cereal Food, Microbiological Assay – Trienzyme Procedure (45.2.09) (AOAC International, 2005).

Based on the report by the taxonomic subcommittee on *Lactobacilli* and closely related organisms (Hansen, 1968), *Lactobacillus casei* subsp. *rhamnosus* ATCC 7469 was presented to the subcommittee as a type strain for *Lactobacillus casei* subsp. *rhamnosus* by Rogosa et al (Rogosa, et al., 1953). It was recommended by the subcommittee on July 22, 1966. Some information was listed for ATCC 7469 *Lactobacillus casei* subsp. *rhamnosus*:

- History: From R. Tittsler; originally from F.M. Strong, U. of Wisconsin, assay strain.
- Morphology: Short rods in chains
- Temperature relationships: Develops at 10°C and 48°C.
- Survival at 63 °C: Does not survive for 30 minutes
- Vitamin requirements: Pyridoxal phosphate, nicotinic acid, pantothenate, riboflavin, folic acid are required.

Trienzyme Extraction

The trienzyme extraction is a combined enzymatic digestion by Pronase^{R}, α-amylase and conjugase (γ-glutamyl hydrolase) for extracting folate from foods prior to microbiological assay. By the trienzyme treatment, carbohydrate and protein-bound folates are released from the food matrix. Conjugase cleaves polyglutamate folates to mono- or diglutamates which the test organism *L. casei ssp. rhamnosus* (ATCC 7469) can utilize for growth.

Pronase^{R}

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

α-Amlyase

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

Conjugase

Folate conjugase (γ-glutamyl hydrolase) catalyzes the hydrolysis of pteroylpolyglutamates to oligoglutamates or monoglutamates. There are various natural sources of folate conjugase enzyme (Keagy, 1985; Goli and Vanderslice, 1992; Lim, et al., 1998). Among them, chicken pancreas and hog kidney are the most commonly used (Tamura, 1990), and the enzyme from chicken pancreas was found to be more efficient for folate extraction ((Pedersen, 1988). The optimum pH for the conjugase from chicken pancreas is 7.8-8.5.

Development of the Trienzyme Extraction

The traditional extraction method with conjugase alone very likely underestimates the folate content in certain food items, because folate was bound to the protein and starch in the

food matrix. Over the years, enzyme treatments were investigated in order to liberate the carbohydrate and protein-bound folates from the food matrix in order to estimate the proper folate level in foods. Yamada (1979) reported a significant increase in measurable folate in high protein products (breast milk, hog liver, and cod) treated with Pronase^{R} and conjugase. Khalsa (1982) also found a 40% increase in measurable folate in human milk treated with protease and conjugase compared to conjugase digestion alone. Cerna and Kas (1983) indicated that α-amylase treatment was essential for obtaining proper folate assays of foods rich in starch. Compared to folate conjugase alone, Pedersen(1988) obtained 9.3% higher folate contents in starch containing food items with a simultaneous treatment of α-amylase and chicken pancreas conjugase. In 1990, a trienzyme treatment was first introduced by Eitenmiller and his colleagues (DeSouza and Eitenmiller, 1990; Martin, et al., 1990). The trienzyme extraction, including digestion with protease, α-amylase and chicken pancreas conjugase, increased the measurable folate levels in a wide variety of foods. Ryu (1994) found that the measurable folate levels in feed ingredients predigested with the trienzyme method increased by 22.3% compared to digestion by conjugase alone.

The trienzyme treatment did not draw much attention until 1997 when two groups of investigators reported that the trienzyme treatment was necessary to assay folate in cereal foods (Pfeiffer, et al., 1997; Tamura, et al., 1997). Tamura et al. (1997) found an average increase of 45% in folate contents in the 210 dairy products after trienzyme treatment compared to conjugase alone. After 1997, the trienzyme extraction became widely investigated for use in folate extraction of various food items. Lim et al. (1998) applied the trienzyme treatment to human milk and found an average of 85% increase of measurable folate compared to folate conjugase treatment alone. Rader (1998) reported a 20-30% increase of pre-fortification levels of

folate in enriched cereal-grain products treated with trienzyme digestion compared to conjugase treatment alone. Additional work by Rader et al. (2000) assayed the total folate in cereal grain products using the trienzyme extraction and microbiological assay. Shresta et al. (2000) extracted spinach and fortified bread and ready to eat cereal and found the trienzyme treatment was a significant improvement over the single enzyme treatment. Reports of Konings (Konings, 1999; Konings, et al., 2001) showed a 20% increase in total folate contents of dairy products using the trienzyme extraction compared to conjugase alone. Johnston et al. (2002a; 2002b) measured folate contents in dairy products and fast foods using the trienzyme extraction and obtained markedly higher values than those in the published literature.

Mandatory folic acid fortification of cereal-grain products emphasized the need for validated methods for determining total folate content in foods, particularly enriched cereal products. A microbiological assay protocol utilizing a trienzyme extraction procedure was developed to fulfill this need and a collaborative study was carried out (DeVries, et al., 2001). Thirteen laboratories participated in the collaborative study of 10 required and 10 optional cereal grain products including flour, bread, cookies, baking mixes and ready to eat breakfast cereals. The relative standard deviation between the laboratories ranged from 7.4 -21.6% for eight fortified products compared with values of 11-20%. Based on the results of this collaborative study, the microbiological assay with the trienzyme extraction was recommended for First Approval Status with American Association of Cereal Chemists (AACC Method 86-47) (DeVries, et al., 2001; AACC, 2000) and adopted as First Action Official Method with Association of Official Analytical Chemists (AOAC International, 2005) for folate analysis in cereal grain products. An international inter-laboratory performance study of food folate analysis suggested that it was important to standardize the methods of folate extraction and detection, and

the use of reliable reference materials should be encouraged (Puwastien, et al., 2005). The above research clearly shows the significance of the trienzyme extraction to the accuracy of the various determinative assays for food folate that are now in use.

Optimum Condition for Trienzyme Digestion

Optimum conditions for trienzyme extraction are still under investigation. It was suggested that appropriate methods of trienzyme treatment may be established for each food item to obtain the maximum folate contents (Tamura, et al., 1997). Aiso and Tamura (1998b; 1998a) investigated optimal pH and incubation time for α-amylase and protease treatment for fresh beef, white bread, cow's milk and fresh spinach, and found that the optimum conditions for enzyme treatment varied depending on food items. Further investigation was needed to optimize the trienzyme extraction for folate analysis in different type of food matrices.

Optimum pH and order of enzyme addition for trienzyme extraction have been investigated over these years. It was indicated that pH 7.8 provided much more efficient extraction than that with a lower pH (Konings, 1999; Konings, et al., 2001). The best order of enzyme addition was proven to be Pronase^{R} first, followed by α-amylase, then conjugase (Rader, et al., 1998). Optimum incubation time of the enzyme digestion still remains unclear. In AOAC Method 2004.05, a 3 h digestion with Pronase^{R} is used followed by a 2 h with α-amylase. Then the sample is digested for16 h with chicken pancreas conjugase for cereals and cereal foods. However, Pfeiffer et al.(1997) found that measurable folate decreased after long term incubation compared with short term incubation. Thus, a shorter incubation time has been suggested to prevent the destruction of labile folate in long term incubation. To find out the optimum incubation time of each enzyme that completely releases the bound folate from food matrix while

avoiding the destruction of labile folate meanwhile is significant to the improvement of the trienzyme extraction. The optimum incubation time may be related to the unique characteristics of the food matrix and the extent of the binding of folate to the matrix.

Trienzyme extraction for vegetables

Vegetables are primary source of folate, and most of the optimization studies trienzyme extraction have been completed on leafy vegetables. Working with spinach, Pandrangi and LaBorde (2004) found an optimum incubation time of 8 h for protease digestion, while α-amylase digestion did not appreciably affect measurable folate. The study of Iwatani et al. (2003) indicated that the application of the trienzyme extraction to spinach and Chinese broccoli did not increase measurable folate values of the analyzed vegetables. They determined the folate content in some common Australian vegetables and reported that the total folate content of 22 vegetables ranged from 68~425 μg/100g. Research of Shrestha et al (2000) also indicated that spinach treated with trienzyme digestion didn't give higher folate value than those treated with chicken pancreas conjugase alone. Yon and Hyun (2003) determined the folate content of foods commonly consumed in Korea and found that folate values by trienzyme treatment were higher in all foods analyzed with the exception of a few vegetables such as cucumber, eggplant, and banana compared to samples treated by conjugase alone. However, pH, addition order of enzymes and other procedures of the trienzyme extraction in these studies were different from AOAC Method 2004.05. Some researchers agreed that the trienzyme extraction did not always give higher folate values compared to the single conjugase treatment (Johnston, et al., 2002a; Johnston, et al., 2002b; Yon and Hyun, 2003). It depended on the food item, the difference in food matrics and forms of folate that can be labile when longer incubation periods are used

(Hyun and Tamura, 2005). Hence, lose of labile folate during long term of incubation may cause lower measurable folate by trienzyme extraction. Therefore, Optimization of the incubation time for the trienzyme extraction is necessary. In the original work of Desouza and Eitenmiller (1990), it was clearly stated that Pronase^{R} digestion was not necessary for all types of foods.

Other Assay Methods for Folate Analysis

Liquid chromatography (LC) methods have been used for food folates detection (Vahteristo, et al., 1996; Pfeiffer, et al., 1997; Ndaw, et al., 2001). Advanced techniques such as LC with fluorescence detection (Doherty and Beecher, 2003), and stable isotope liquid chromatography-Mass spectrometry (LC-MS) assay (Pawlosky and Flanagan, 2001; Pawlosky, et al., 2001; Nelson, et al., 2003; Thomas, et al., 2003) have been developed. LC methods are useful to differentiate between the different folates. The primary advantage of the LC analysis is the ability to quantify the specific folate forms such as the γ-glutamyl folate polymers. Such specificity is not obtained by other methods. However, the complexity of the procedures such as lengthy multi-enzyme extraction, extensive sample clean-up and concentration using ion-exchange or affinity columns, and gradient elution chromatographic profiles, makes these methods unsuitable for many quality control laboratories.

Ligand binding assays were also developed for folate analysis, including radio immunoassay, enzyme linked immunosorbent assay (ELISA), radio-labelled protein binding assay (RPBA), and enzyme protein binding assay (EPBA). Radio assays are quicker, less expensive, less subject to variation, simple to perform, and have a high sample throughput compared to LC (Mandella and Depaola, 1984; Finglas, et al., 1988). The major limitation of the ligand binding assays is the improper response of the individual folate forms to the folate binding

protein used. Careful control of the pH of the assay buffer and a good choice of the folate calibrant is necessary for the food folate measurements (Finglas, et al., 1993).

Method Validation

The International Organization for Standardization (ISO) definition of validation is "Confirmation by examination and provision of objective evidence that the particular requirements for a specified intended use are fulfilled." (Fleming, et al., 1996; EURACHEM Guide, 1998). Method validation can be interpreted as "the process of defining an analytical requirement, and confirming that the method under consideration has performance capabilities consistent with what the application requires." (EURACHEM Guide, 1998).As part of method development, method validation is necessary for examination of the fitness of an analytical system for a particular analytical purpose (Thompson, et al., 2002). Method validation is considered to be a tool to demonstrate that a specific analytical method actually measures what it is intended to measure, and is suitable for its intended purpose (ICH-Q2A, 1995; Fleming, et al., 1996; Huber, 1998; Taverniers, et al., 2004).

Accuracy

Accuracy means the closeness of the test result to the "true" or accepted value. The difference of the reported value from the accepted value is the bias under the reported condition (AOAC International, 2002a). Accuracy can be determined by use of certified reference materials (AOAC International, 1998).

Accuracy can be also evaluated by recovery. For quantitative methods, recovery of added analyte over an appropriate range of concentrations may be taken as an indication of trueness

(AOAC International, 1998). Recovery is the fraction or percentage of the analyte that is recovered when the test sample is conducted through the entire method. Reference material with the matrix of interest is the best test sample for determining recovery (AOAC International, 2002a). An acceptable recovery criterion is a function of concentration as presented in the "AOAC Requirements for Single Laboratory Validation of Chemical Methods" (AOAC International, 2002b).

Recovery is calculated by the following equation:

$$R(\%) = [(Cs-Cp)/Ca]\times 100,$$

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

The concentration should cover the range of concern including one concentration at target level, one at low end, and one at the high end. The amount spiked should be a substantial fraction of, or more than, the amount present in the unspiked material (AOAC International, 1998).

Precision

Precision measured within a laboratory is designated as repeatability precision (Horwitz, 2003), which refers to the degree of agreement of results when the conditions are maintained as constant as possible with the same analyst, reagents, equipment, and instruments performed within a short period of time (AOAC International, 2002a). Precision includes simultaneous and consecutive replicates (Horwitz, 2003). Simultaneous replicates (intra-assay) represent the minimum within-laboratory variability(AOAC International, 2002a). Larger within-laboratory

variability can be induced by consecutive replicates (inter-assay) conducted in the same laboratory on identical test samples on different days (AOAC International, 2002a).

The Horwitz Function is a criterion for precision evaluation. Dr. William Horwitz has been well-known for his contribution to the statistics of inter-laboratory method performance studies. By observing the results of over 50 inter-laboratory collaborative studies conducted by the Association of Official Analytical Chemists over 100 years on various commodities for numerous analytes, Horwitz et al (Horwitz, et al., 1980; Horwitz, 1982; Boyer, et al., 1985) found a relationship between the mean coefficient of variation (CV), expressed as powers of 2, with the mean concentration measured, expressed as powers of 10. This relationship is now the famous Horwitz function, formulated as:

$$CV = 2^{(1-0.5\log C)}$$

where C is the mass fraction expressed as a power of 10.

Remarkably, the Horwitz function seemed to be a general law irrespective of the identity of the analyte, or the nature of the matrix or analytical method. Most data from collaborative studies on various analytes fell between 0.5 and 2.0 times that predicted by the Horwitz function. This "Horwitz Band" is now regarded as a criterion to evaluate a precision for a successful collaborative trial (Thompson and Lowthian, 1997).

Horwitz et al (1980) also found that the mean coefficient of variation (CV) of within-laboratory or analyst repeatability is usually between one-half and two thirds of the CV of collaborative study. Thus, a maximum reference CV has been used as a criterion for within-laboratory precision evaluation (Cherlet, et al., 2003), which is two thirds of the CV of collaborative study:

$$CV_{maximum} = 2/3 \times 2^{(1-0.5\log C)}$$

where C is the mass fraction expressed as a power of 10.

Horwitz criterion of acceptance, determined as $HORRAT_r$ value, was used to evaluate the repeatability precision. The acceptable $HORRAT_r$ value is 0.3-1.3 for single laboratory precision (Horwitz, 2003). Calculations were conducted by the following equations:

$\%RSD_{r\ observed} = SD*100/Mean$

$\%\ RSD_{r\ predicted} = 2/3 \times 2^{(1-0.5\log C)}$ (with C the analyte concentration in mass fraction: g/g)

$HORRAT_r = \%RSD_{r\ observed} / \%\ RSD_{r\ predicted}$

In the above equation, SD is standard deviation; RSD_r is relative standard deviation for repeatability precision.

Calibration Curve

Calibration curve is graphic representation of measuring signal as a function of quantity of analyte (AOAC International, 1998). A sufficient number of standard solutions are needed to define the response in relation to concentration. The curve should be statistically tested and expressed.

Limit of Detection and Limit of Quantitation

Limit of detection is the lowest content that can be measured with reasonable statistical certainty (AOAC International, 1998). Limit of quantitation is the content equal to or greater than the lowest concentration point on the calibration curve (AOAC International, 1998).

An alternative definition of limit of detection and limit of quantitation is based upon the variability of the blank. The mean value of the blank reading ($n \geq 20$) plus 3 times the standard deviation of the mean is taken as the limit of detection. The mean value of the blank reading ($n \geq$

20) plus 10 times the standard deviation of the mean is taken as the limit of quantitation. Both are expressed in analyte concentration (AOAC International, 1998; AOAC International, 2002a).

Parallelism Test

The parallelism test is used to determine whether an assay's accuracy depends on the anlyte concentration in the sample used. This characteristic is tested by diluting a standard sample, and determining whether results agree after correction for the dilution. Failure of the parallelism test may indicate extreme sensitivity of the assay to matrix composition (Mikkelsen and Corton, 2004). Thus, parallelism test is to evaluate if the matrix components other than the analyte of interest affect an assay's accuracy. A criterion has been set for the acceptance of the parallelism test: the determination can be regarded as reliable only if the results calculated from at least three dilutions do not differ by more than ± 10% from their common mean (Strohecker and Henning, 1965).

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Table 2.1 Substituent groups and their positions in folate coenzymes

Name	Abbreviation	Position	
		N-5	N-10
Pteroylglutamic Acid	Folic acid	—	-H
7,8-Dihydrofolate	H_2 folate	-H	-H
5-Methyl-5,6-dihydrofolate		$-CH_3$	-H
5,6,7,8-Tetrahydrofolate	H_4 folate	-H	-H
5-Methyltetrahydrofolate	5-CH_3-H_4 folate	$-CH_3$	-H
5-Formyltrtrahydrofolate	5-CHO-H_4 folate	-CHO	-H
10-Formyltetrahydrofolate	10-CHO-H_4 folate	-H	-CHO
5,10-Methenyltetrahydrofolate	5,10-CH^+=H_4 folate	=CH-$^{\pm}$ bridge	—
5,10-Methylenetetrahydrofolate	5,10-CH_2H_4 folate	-CH_2-bridge	—
5-Formiminotetrahydrofolate	5-CHNH-H4 folate	-CHNH	-H

Table 2.2 Recommended dietary allowances for folate in µg DFE / day for children and adults

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

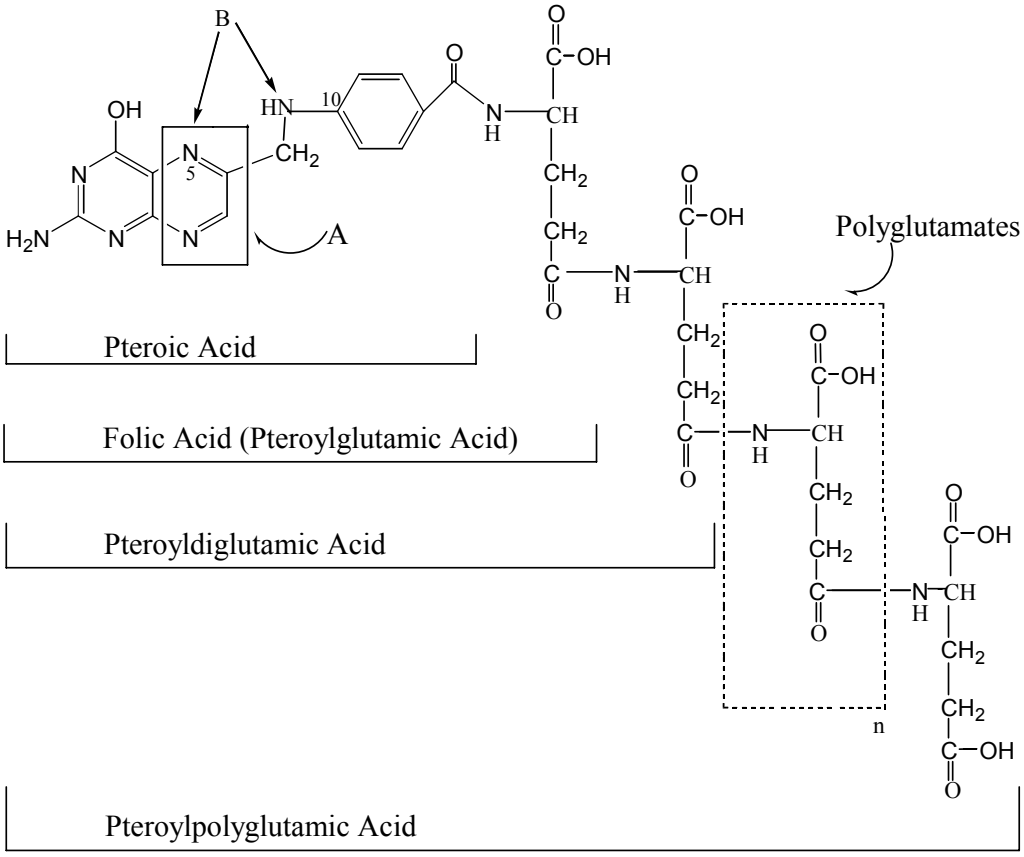
Table 2.3 Tolerable upper intake levels for folate in µg DFE / day for children and adults

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

Table 2.4 Responses induced by folate derivatives relative to folic acid

Folate	*Lactobacillus casei* subsp. *rhamnosus*	*Enterococcus hirae*	*Pediococcus acidilactici*
Folic acid	+	+	-
H_2 folate	+	+	-
H_4 folate	+	+	+
5-HCO-H_4 folate	+	+	+
10-HCO-H_2 folate	+	+	-
10-HCO-H_4 folate	+	+	+
5-CH_3-H_4 folate	+	-	-
5,10-CH^+=H_4 folate	+	+	-
5-CHNH-H_4 foate	+	+	+
$PteGlu_2$	+ (100%)	+	-
$PteGlu_3$	+ (100%)	-	-
$PteGlu_4$	+ (65%)	-	-
$PteGlu_5$	+ (20%)	-	-
$PteGlu_6$	+ (3.6%)	-	-
$PteGlu_7$	+ (2.4%)	-	-
Pteroic acid	-	+	-

Table adapted from Voigt and Eitenmiller (1978).



A – Pterin ring oxidation – reduction

B – One carbon fragment attachment

n – Number of glutamates

Figure 2.1 Structure of folate and folic acid

CHAPTER 3

SINGLE LABORATORY METHOD PERFORMANCE EVALUATION FOR THE ANALYSIS OF TOTAL FOOD FOLATE BY TRIENZYME AND MICROPLATE ASSAY[1]

[1]Chen, L. and R.R. Eitenmiller. To be submitted to *Journal of Food Science.*

ABSTRACT:

Single laboratory method performance parameters including the calibration curve, accuracy, recovery, precision, limit of detection (LOD) and limit of quantification (LOQ) were evaluated for the analysis of total food folate by the trienzyme extraction and microplate assay with *Lactobacillus casei* subsp. *rhamnosus*. Standard Reference Material (SRM) 1546 (meat homogenate), SRM 2383 (baby food composite), SRM 1846 (infant formula), Certified Reference Material (CRM) 121 (wholemeal flour) and CRM 485 (mixed vegetables), representing a broad selection of food matrices, were used to evaluate the performance of the method. A generated four parameter logistic equation of the calibration curve was $y = (0.0705\text{-}1.0396) / (1 + (x / 0.0165)^{1.3072}) + 1.0396$ ($p < .0001$). The test of parallelism demonstrated that matrix components in the food extracts did not affect the accuracy. Measured values of the SRMs and CRMs were within their certified or reference values. Recoveries for all reference materials met 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. LOD and LOQ values were 0.3 and 0.6 μg/100g, respectively. The results showed that trienzyme digestion using α-amylase, Pronase^{R} 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.

Key words: microbiological assay, trienzyme, folate, method validation, microplate

Introduction

Microbiological assay is one of the oldest and most versatile methods for the determination of total food folate. This assay measures the growth of *Lactobacillus casei* subsp. *rhamnosus* (ATCC 7469) turbidimetrically in a folate deficient medium. A 96-well microtiter plate assay with microcomputer analysis improves microbiological assay, making it less laborious, less time consuming, and more reproducible (Horne 1997; Horne and Patterson 1988; Newman and Tsai 1986; Tamura 1990). A recent collaborative study indicated excellent agreement between the results of the method using test tubes versus microtiter plates for the determination of total folate in cereal products by microbiological assay, following trienzyme extraction (DeVries and others 2005). Trienzyme extraction, introduced by Eitenmiller and his colleagues (DeSouza and Eitenmiller 1990; Martin and others 1990), is a combined enzymatic extraction for folate analysis with protease, α-amylase and conjugase (γ-glutamyl hydrolase). Food folate is liberated from non-specific binding by carbohydrates and proteins with α-amylase and protease digestion. Conjugase removes glutamic acid residues from γ-glutamyl folates with three or more glutamic acid residues. Deconjugation of folate is necessary because *Lactobacillus casei* subsp. *rhamnosus* (ATCC 7469) will not respond to poly γ-glutamyl folates with three or more glutamic acid residues (Goli and Vanderslice 1992). The microbiological assay with the trienzyme extraction was recommended for First Approval Status with American Association of Cereal Chemists (AACC Method 86-47) (AACC 2000; DeVries and others 2001) and adopted as First Action Official Method by the Association of Official Analytical Chemists (AOAC International 2005) for folate analysis in cereal grain products. A recent international inter-laboratory performance study of food folate analysis showed the importance of standardization the methods of folate extraction and detection. Further, the use of reliable reference materials was strongly

encouraged (Puwastien and others 2005). Many studies have proved the significance of the trienzyme extraction to the accuracy of microbiological and liquid chromatographic assays for food folate that are now in use. The microbiological assay using trienzyme extraction was evaluated and confirmed to be a reliable method for determining total folate in cereal products (DeVries and others 2001; DeVries and others 2005), however, little documentation of method performance parameters is available using the method for other food matrices. Although AOAC Official Method 2004.05 was collaborated for enriched cereals, the digestion was originally developed for general assay of total food folate and not limited to cereals (DeSouza and Eitenmiller 1990; Martin and others 1990). In order to verify that microbiological assay with the trienzyme extraction is a suitable method for the determination of total folate in a broad selection of food matrices, we evaluated the performance parameters of this method for assay of total folate using available standard reference materials. This study evaluated the calibration curve and matrix effect (parallism), accuracy, recovery, precision, limit of detection and limit of quantitation for determination of total food folate with trienzyme extraction and microplate assay in a broad selection of food matrices. Single laboratory method performance evaluation was conducted according to the AOAC guidelines (AOAC International 1998, 2002a, 2002b).

Materials and Methods

Standard Reference Materials (SRM) and Certified Reference Materials (CRM)

SRM 1546 (meat homogenate), SRM 2383 (baby food composite) and SRM 1846 (infant formula) were purchased from the National Institute of Standards & Technology (NIST), Gaithersburg, MD.

European Commission certified reference materials: CRM 121 (wholemeal flour) and CRM 485 (mixed vegetables) were purchased from Resource Technology Corporation, Laramie, WY.

Folic acid standard was purchased from USP (US Pharmacopeia).

Trienzyme Extraction

The extraction of folate by trienzyme digestion follows AOAC Official Method 2004.05 - Microbiological Assay with Trienzyme Procedure for Total Folates in Cereals and Cereal Foods (AOAC International 2005). Here is the brief description of the procedure: 0.25-1.0 g of sample in pH 7.8 phosphate buffer (0.1M, containing 1% ascorbic acid) is preheated at 100°C for 15 min. The sample is then cooled to room temperature and 1 mL of Pronase^{R} (2 mg/mL, Calbiochem, #53702, San Diego, CA) is added, followed by incubation at 37°C for 3 h. At the end of the Pronase^{R} digestion, the sample is heated for 3 min at 100°C, cooled and digested with 1 mL of α-amylase (20 mg/mL, Fluka, #10065, St Louis, MO) for 2 h at 37°C. Conjugase digestion is followed by adding 4 mL of chicken pancreas conjugase (5 mg/mL, Difco, #245910, Sparks, MD) and incubating at 37°C for 16 h. At the end of the incubation, the digest is heated at 100°C for 3 min, cooled, adjusted to pH 4.5 with HCl, taken to volume of 100 mL with water and filtered through ashless filter paper (Whatman No. 2V, 12.5 cm).

Hexane Extraction

In order to remove fat, which is known to stimulate the growth of *Lactobacillus casei* subsp. *rhamnosus (ATCC 7469),* 20mL hexane was added with vortexing to SRM 1846 (infant formula), SRM 1546 (meat homogenate), and SRM 2383 (baby food composite) prior to trienzyme

extraction. Fat was extracted by the hexane and removed by pipetting out the hexane layer 15 min later. Residual hexane was removed by nitrogen flush.

Microplate Assay

Total folate was assayed microbiologically using *Lactobacillus casei* subsp. *rhamnosus (ATCC 7469),* according to the procedures outlined by Tamura (1990).

Method Performance Parameters

1. Calibration Curve

Working standards are prepared freshly for analysis by diluting the folic acid stock standard solution to the desired range of 1.6×10^{-3} - 0.1 µg/100 mL. Working standards and the sample extracts are microplate assayed in the same manner, as described by Tamura (1990).

Twenty-five standard curves were obtained over a 10-month period. Each standard curve represented assay from a different day. By nonlinear regression in SAS (SAS 2002), calibration curves were fitted into a Four-Parameter Logistic (4PL) equation:

$$y = (A – D) / [1 + (X/C)^B] + D$$

where y is the absorbance reading at 595 nm of microplate assay; X, concentration of folate in µg/100mL; A, response at zero concentration; D, response at infinite concentration; C, concentration resulting in a response halfway between A and D (ED_{50}), µg/100mL; B, slope parameter.

Calibration curves were plotted using Sigma Plot software. The 4PL function represents the sigmoidal relationship between the measured response and the logarithm of concentration (Findlay and others 2000)

2. Parallelism Test

CRM 121 (wholemeal flour), CRM 485 (mixed vegetables), SRM 1846 (infant formula), SRM 2383 (baby food composite) and SRM 1546 (meat homogenate) were diluted by the dilution factors shown in Table 3.1. Test curves were obtained by plotting the absorbance at 595 nm of the samples and the standard solutions on the y-axis versus $\log_{10}$ of the dilution factors on the x-axis. The dilution factors of the standard solutions are 2, 4, 8, 16, 32, 64,128, based on the procedure of microplating.

3. Accuracy

Accuracy was evaluated by analyzing the reference materials, comparing the analytical values to the accepted values (certified / reference / information value). Twenty-five replicates were assayed for each reference material. Bias is the difference of the analytical value from the accepted value (AOAC International 2002a).

4. Recovery

Based on the AOAC guidelines for recovery evaluation (AOAC International 1998), CRM 121 (wholemeal flour), CRM 485 (mixed vegetables), SRM 1846 (infant formula), SRM 2383 (baby food composite) and SRM 1546 (meat homogenate) were spiked with folic acid at low, target and high levels (50%, 100%, 200%). Recovery was calculated by the following equation(AOAC International 2002a):

$R(\%) = [(Cs-Cp)/Ca]\times100,$

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

5. Repeatability Precision

Precision measured within a laboratory is designated as repeatability precision (%RSDr), including simultaneous and consecutive replicates (Horwitz 2003).

5.1 Simultaneous replicates

Five replicates of each reference material were assayed simultaneously. For each reference material, mean and standard deviation (SD) of the five replicate assays were calculated. Repeatability relative standard deviation (% RSD_r) was calculated as:

% RSD_r = (SD × 100) / mean

5.2 Consecutive replicates

Each reference material was analyzed on 5 different days. Mean, standard deviation and repeatability relative standard deviation (% RSD_r) were calculated.

5.3 *$HORRAT_r$ calculation*

The Horwitz criterion of acceptance, determined as the $HORRAT_r$ value, was used to evaluate the repeatability precision. The acceptable $HORRAT_r$ value is 0.3-1.3 for single laboratory precision (Horwitz 2003). Calculations were conducted by the following equations:

$\%RSD_{r\ observed}$ = SD*100/Mean

$\%\ RSD_{r\ predicted} = 2/3 \times 2^{(1-0.5\log C)}$ (with C the analyte concentration in mass fraction: g/g)

$HORRAT_r = \%RSD_{r\ observed} / \%\ RSD_{r\ predicted}$

In the above equation, SD is standard deviation, and $\%RSD_r$ is relative standard deviation for repeatability precision.

6. Limit of Detection and Limit of Quantitation

Limit of detection is estimated by transforming blank absorbance plus 3 times the standard deviation to a concentration on the calibration curve, and the limit of quantitation is estimated in a similar manner with 10 times the standard deviation (AOAC International 1998, 2002a, 2002b).

The blank, consisting of sodium phosphate buffer (0.1N, pH 7.8), ascorbic acid and folic acid Casei medium was assayed microbiologically using *Lactobacillus casei* subsp. *rhamnosus (ATCC 7469)*, according to the procedures outlined by Tamura (1990). Twenty-four replicates were assayed totally.

7 Statistical Analysis

Non-linear regression procedure in statistical analysis system (SAS 2002) was used to generate the Four-Parameter Logistic (4PL) equation of the calibration curve. Linear regression procedure in statistical analysis system (SAS 2002) was used to investigate the linear portion of the calibration curve.

Sigma Plot (Version 9.0) was used to plot the calibration curve. Microsoft Excel was used to plot the parallelism test curves.

Results and Discussion

Calibration Curve

The 4PL equation for the calibration curve was:

$$y = (0.0705-1.0396) / (1 + (x / 0.0165)^{1.3072}) + 1.0396$$

A representative calibration curve is shown in Figure 3.1 from 0.0016 to 0.1 μg/100mL which represents the usual concentration range for the dose-response curve. The non-linear regression analysis of data from the 25 runs showed that the curve fit the 4PL equation ($p<.0001$). The ED_{50} was 0.0165 μg/100mL and represents the concentration resulting in a response half way between zero and infinite concentration. The microplate reader automatically generates the 4PL equation of the calibration curve for each assay. Although the parameters of the 4PL equation calibration curve are close to each other between assays, the calibration curve is set up on each microplate.

Examination of the most linear portion of the calibration curve by linear regression in SAS 9.0 (SAS 2002) in the concentration range of 0.0016~0.025 μg/100mL gave the equation: $y = 24.4 x + 0.1$ ($P < .0001$, $R^2 = 0.9570$). This portion of the calibration curve is usually used for calculation, because the response is proportional to the concentration.

Parallelism Test

The parallelism test is used to determine if an assay's accuracy depends on the concentration of the analyte in the sample extract. This characteristic is tested by assay of dilutions of the sample extract and determining whether results agree after correction for the dilution factor. Failure of the parallelism test may indicate sensitivity of the assay to components in the sample extract (Mikkelsen and Corton 2004). Thus, a test of parallelism evaluates effects of matrix components other than the analyte of interest on accuracy.

Reference materials: CRM 121 (wholemeal flour), CRM 485 (mixed vegetables), SRM 1846 (infant formula), SRM 2383 (baby food composite) and SRM 1546 (meat homogenate) were used to represent common foods. Figure 3.2 shows matrices test curves for the five reference materials to be generally parallel to the calibration curve, indicating the responses of microplate assay were free from potential stimulating or inhibiting factors presented in the matrix.

Folate concentrations obtained at different dilutions were shown in Table 3.1. The determination can be regarded as reliable only if the results calculated from at least three dilutions do not differ by more than ± 10% from their common mean (Strohecker and Henning 1965). Folate levels of 51~55 μg/100g, 299~331 μg/100g, 139 ~ 145 μg/100g, 1.2 ~1.4 and 14.8 ~ 16.9 μg/100g were obtained for CRM 121 (wholemeal flour), CRM 485 (mixed vegetables), SRM 1846 (infant formula), SRM 1546 (meat homogenate) and SRM 2383 (baby food composite), respectively, at 4 different dilutions. All determinations were shown to be within ± 10% of the mean for each reference material. The parallelism test demonstrated that the analytical results were reliable and depended on the folate concentration in the sample.

Accuracy

Accuracy indicates the closeness of the test result to the "true" or accepted value. The difference of the reported value from the accepted value is the bias under the reported condition (AOAC International 2002a). Positive bias shows that the analytical value was less than accepted value; whereas, negative bias indicates that the analytical value was higher than the accepted value. Accuracy can be determined by use of standard reference materials or certified reference materials (AOAC International 1998).

Among the 5 reference materials, analytical values of CRM 485 (mixed vegetables) and SRM 1846 (infant formula) had the largest bias, 4 and -4 μg/100g respectively. Analytical values of SRM 2383 (baby food composite) and SRM 1546 (meat homogenate) had smaller bias with 0.1 and -0.16 μg/100g, respectively (Table 3.2).

The ratio of analytical value to accepted value expressed as a percentage (% of accepted value) is used to evaluate accuracy ((LaCroix and Wolf 2002; Rolim and others 2005). The % of accepted value for all reference materials were within 95 ~ 115%, indicating the closeness of analytical and accepted values for the five matrices.

Recovery is the fraction or percentage of the analyte that is recovered when the test sample is caused through the entire assay. Recovery is also used for evaluating accuracy. A reference material with the matrix of interest is the best test sample for determining recovery (AOAC International 1995, 2002a). For quantitative methods, recovery of added analyte over an appropriate range of concentrations may be taken as an indication of trueness (AOAC International 1998).

At spike levels of 50%, 100%, 200% of the accepted value, recoveries ranged from 80 to 118%. Recoveries for each reference material at each concentration are provided in Table 3.3. Recoveries for the specific concentrations were within the acceptable range provided by AOAC International (AOAC International 2002b), confirming the accuracy of this method.

Repeatability Precision

The Horwitz function (Boyer and others 1985; Horwitz 1982; Horwitz and others 1980), expressed as $\%RSD = 2^{(1-0.5\log C)}$, is a primary criterion for evaluating reproducibility precision. Repeatability precision measured within a single laboratory is approximately one-half to two-

thirds of the among-laboratory precision (Horwitz 2003). The HORRAT value is the definitive parameter of the Horwitz criteria of acceptance for method performance studies. The HORRAT value is calculated by taking the observed %RSD calculated from the data divided by the predicted %RSD calculated from the Horwitz function. The acceptable single laboratory precision range, calculated as the $HORRAT_r$ value, is 0.3-1.3 (Horwitz 2003).

Repeatability precision of simultaneous replicates (intra-assay) and consecutive replicates (inter-assay) are presented in Table 3.4 and 3.5. The $HORRAT_r$ values of intra-assay and inter-assay precisions for all the reference materials were within the acceptable range of 0.3-1.3.

Limit of Detection and Limit of Quantitation

The limit of detection was 0.3μg/100g, and the limit of quantitation was 0.6μg/100g.

Conclusion

Evaluation of single laboratory method performance parameters showed that the trienzyme extraction combined with microplating techniques was suitable for total folate analysis in a broad selection of food matrices. The method is not only suitable for total folate analysis in cereal products, but reliable for analysis of total folate in vegetables, meats, milk-based formula and other foods.

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Table 3.1 Parallelism test for matrix effect evaluation [a]

Sample	Dilution factor	Folate	Mean ± SD (μg/100g)	± 10% of mean
CRM 121 (wholemeal flour)	1: 1600	55		
	1: 3200	51		
	1: 6400	53		
	1: 12800	53	53 ± 1.7	47.7 ~ 58.3
CRM 485 (mixed vegetables)	1: 9600	329		
	1: 19200	299		
	1: 38400	302		
	1: 76800	331	315 ± 17.1	283.5 ~ 346.5
SRM 1846 (infant formula)	1: 4000	142		
	1: 8000	139		
	1: 16000	141		
	1: 32000	145	142 ± 2.6	127.8 ~ 156.2
SRM 1546 (meat homogenate)	1: 100	1.4		
	1: 200	1.3		
	1: 400	1.2		
	1: 800	1.2	1.3 ± 0.11	1.17 ~ 1.41
SRM 2383 (baby food composite)	1: 800	16.6		
	1: 1600	14.8		
	1: 3200	16.9		
	1: 6400	15.5	16 ± 1.0	14.4 ~ 17.6

[a] n = 3

Table 3.2 Comparison of the accepted values of total folate in the standard (certified) reference materials to the analytical values

Sample	Accepted Value	Analytical Value	Bias[d]	RSD %	% of Accepted Value
	µg/100g				
CRM 121 [a] (wholemeal flour)	50 ± 7	48.2 ±2.7	1.8	5.6	96
CRM 485 [a] (mixed vegetables)	315 ± 28	311 ±19.8	4	6.4	99
SRM 1846 [b] (infant formula)	129 ± 28	133 ±10.0	-4	7.5	103
SRM 1546 [c] (meat homogenate)	1.2	1.36 ± 0.2	-0.16	14.7	113
SRM 2383 [c] (baby food composite)	15	14.9 ±1.3	0.1	8.7	99

[a] Certified value - Combination of results provided by European Commission and collaborating laboratories, values meet European Commission criteria for certification.

[b] Reference value - Results of collaborating laboratories, values do not meet NIST criteria for certification

[c] Information value - Results of collaborating laboratories, noncertified values with no uncertainties reported as there is insufficient information to make an assessment of the uncertainties.

[d] Bias = accepted value – analytical value, study based on 25 replicate assays

Table 3.3 Recoveries of folic acid from the standard (certified) reference materials

Sample	Concentration[a] µg/100g	Recoveries at Different Spike Levels[b] %			Recovery Limits %
		50	100	200	
CRM 121 (wholemeal flour)	50 ± 7	101 ± 3.2	102 ± 5.1	110 ± 4.3	70-125
CRM 485 (mixed vegetables)	315 ± 28	85 ± 3.4	101 ± 3.6	112 ± 2.5	75-120
SRM 1846 (infant formula)	129 ± 28	104 ± 5.6	110 ± 2.6	116 ± 1.9	75-120
SRM 1546 (meat homogenate)	1.2	80 ± 4.1	95 ± 4.3	115 ± 3.5	70-125
SRM 2383 (baby food composite)	15	99 ± 3.4	118 ± 1.5	109 ± 3.5	70-125

[a] CRM or SRM accepted value

[b] n = 3

Table 3.4 Intra-assay precision [a]

Sample	Mean µg/100g	Standard deviation	RSD_robserved %	RSD_rpredicted %	$HORRAT_r$
CRM 121 (wholemeal flour)	49.4	2.28	4.62	11.84	0.39
CRM 485 (mixed vegetables)	308.9	11.11	3.60	8.97	0.40
SRM 1846 (infant formula)	131.1	5.87	4.48	10.27	0.44
SRM 1546 (meat homogenate)	1.27	0.13	10.24	20.76	0.49
SRM 2383 (baby food composite)	14.4	0.90	6.25	14.19	0.44

[a] n = 5

Table 3.5 Inter-assay precision [a]

Sample	Mean µg/100g	Standard deviation	RSD_robserved %	RSD_rpredicted %	$HORRAT_r$
CRM 121 (wholemeal flour)	48.5	2.78	5.73	11.84	0.48
CRM 485 (mixed vegetables)	309.3	14.40	4.66	8.97	0.52
SRM 1846 (infant formula)	133.7	7.51	5.62	10.27	0.55
SRM 1546 (meat homogenate)	1.38	0.19	13.77	20.76	0.66
SRM 2383 (baby food composite)	14.8	1.48	10.00	14.19	0.70

[a] n = 5

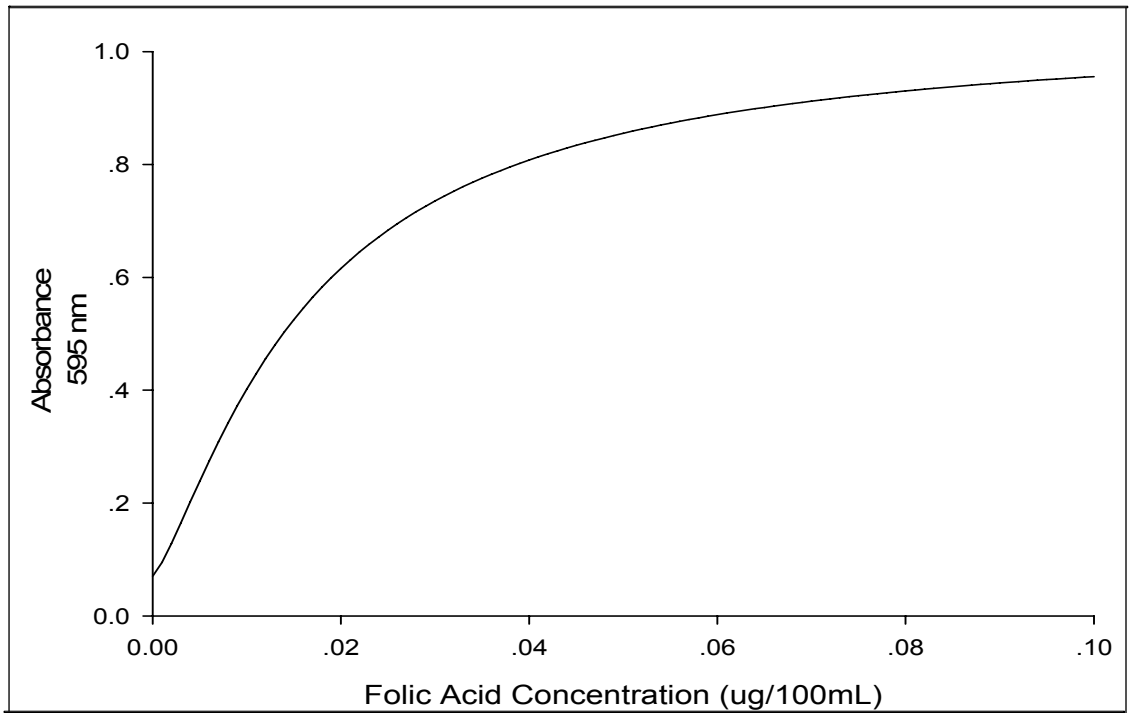

Figure 3.1 Calibration curve of microplate assay for folate determination: four parameter logistic (4PL) equation fit: y = (0.0705-1.0396) / (1 + (x / 0.0165) ^ 1.3072) + 1.0396

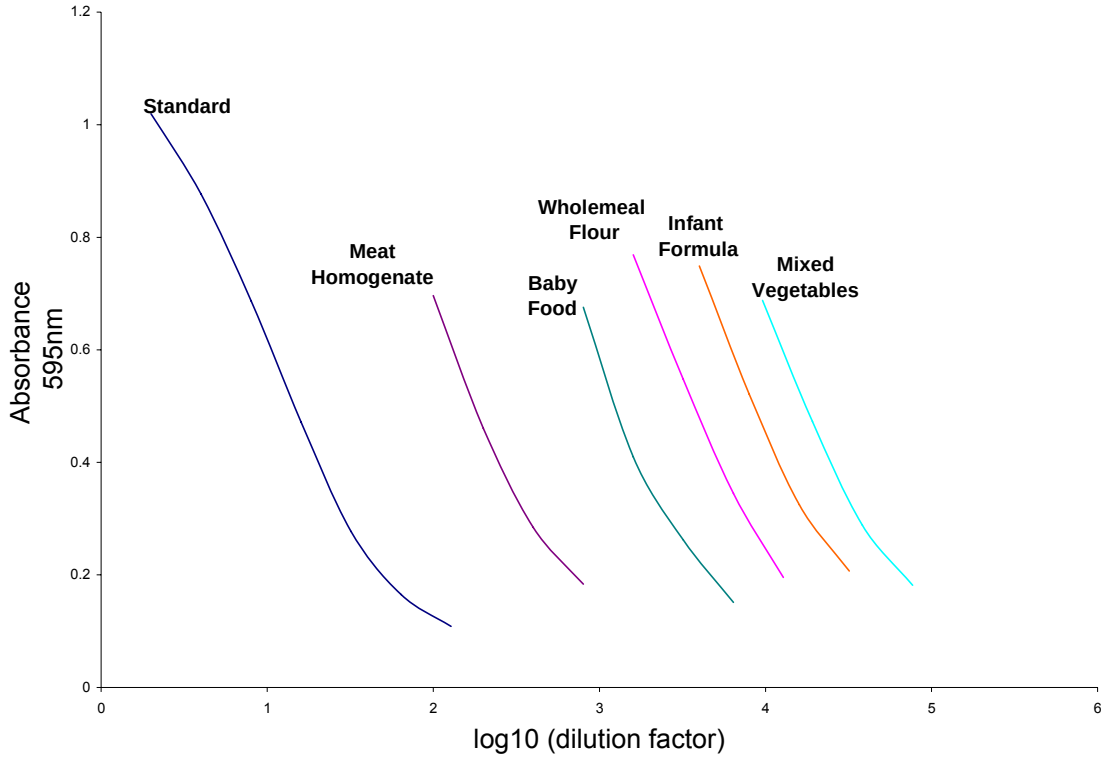

Figure 3.2 Test curves for the five reference materials

CHAPTER 4

OPTIMIZATION OF THE TRIENZYME EXTRACTION FOR THE MICROBIOLOGICAL ASSAY OF FOLATE IN VEGETABLES[1]

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[1]Chen L. and R.R. Eitenmiller. To be submitted to *Journal of Agriculture and Food Chemistry.*

ABSTRACT: Response surface methodology (RSM) was applied to optimize the trienzyme digestion for extraction of folate from vegetables. Trienzyme extraction is a combined enzymatic digestion by protease, α-amylase and conjugase (γ-glutamyl hydrolase) to liberate the carbohydrate and protein-bound folates from food matrices for total folate analysis. It is the extraction method used in AOAC Official Method 2004.05 for assay of total folate in cereal grain products. Certified Reference Material (CRM) 485 Mixed Vegetables was used to represent the matrix of vegetables. Regression and ridge analysis were performed by statistical analysis software . The predicted second-order polynomial model was adequate ($R^2 = 0.947$), without significant lack of fit ($p > 0.1$). Both protease and α-amylase have significant effects on the extraction. Ridge analysis gave an optimum trienzyme digestion time: Pronase^R, 1.5 h; α-amylase, 1.5 h; conjugase, 3 h. The experimental value for CRM 485 under this optimum digestion was close to the predicted value from the model, confirming the validity and adequacy of the model. The optimized trienzyme digestion condition was applied to 5 vegetables and yielded higher folate levels than the trienzyme digestion parameters employed in AOAC Official Method 2004.05.

INTRODUCTION

Trienzyme extraction, introduced by Eitenmiller and his colleagues (*1, 2*), is a combined enzymatic digestion by Pronase^{R}, α-amylase and conjugase (γ-glutamyl hydrolase) to liberate the carbohydrate and protein-bound folates from food matrices prior to total folate analysis by microbiological assay using *L. casei ssp. rhamnosus* (ATCC 7469). It is the extraction method used in AOAC Official Method 2004.05 (*3*) for total folate analysis of cereal foods.

Optimum pH, order of enzyme addition, incubation time and other conditions of trienzyme extraction have been investigated for food folate determination in different matrixes(*4-12*). Vegetables are primary sources of food folate. Working on spinach, Pandrangi et al (*10*) found an optimum incubation time of 8 h for protease digestion, while α-amylase digestion did not appreciably affect measurable folate. Australian researchers reported that single enzyme extraction with conjugase gave higher measurable folate levels than trienzyme extraction for leafy vegetables (*11, 12*). Digestion pH and order of enzyme addition varied from AOAC Official Method 2004.05.

Response surface methodology (RSM) is a collection of statistical and mathematical techniques useful for developing, improving, and optimizing processes (*13*). Originally described by Box and Wilson (*14*), RSM evaluates the effects of several process variables and their interactions on response variables. Fundamental and theoretical aspects of RSM are well understood (*15-17*). RSM is less laborious and time-consuming than other optimization approaches, requiring fewer experimental trials to evaluate multiple parameters and their interactions. RSM has been widely used for optimizing conditions in agricultural and biological research (*18-24*). Applying RSM to vitamin analysis, Lee et al (*18*) optimized the extraction parameters (amount of 60% KOH, saponification time at 70°C and final ethanol concentration)

for the quantitative determination of vitamin E in tomatoes and broccoli. This work showed the potential of RSM techniques to improve vitamin extraction techniques.

Our objective was to optimize the trienzyme digestion for folate extraction from vegetables using RSM. Certified Reference Material (CRM) 485 Mixed Vegetables was used to represent the vegetable matrix.

MATERIALS AND METHODS

Certified Reference Material. European Commission CRM 485 (Mixed Vegetables) was purchased from Resource Technology Corporation, Laramie, WY. Sweet potatoes, White potatoes, yellow sweet corn, carrots and frozen green peas were purchased from retail stores in Athens, GA. The samples were trimmed according to common household practice and only the edible portions were analyzed. The samples were cut into small pieces, homogenized in a blender, and analyzed immediately.

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

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

Purity of the standard solution was determined by diluting the stock standard solution (0.2 mg/mL) with phosphate buffer (0.1 M, pH 7.0) to a final concentration of 0.01 mg/mL (1:20 dilution), measuring the absorbance of diluted standard at 282 nm using phosphate buffer (0.1M, pH 7) as a blank. Calculate the purity using the following equation:

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

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

Where C_1=0.01 mg/mL, C_2=analyzed concentration of diluted stock standard (mg/mL), A=absorbance, $\varepsilon=27.0\times10^3$, b=1cm, M=molar mass of folic acid (441.40). Reference (*25*) gave the physical properties of folates.

Trienzyme Extraction. The extraction of folate by the trienzyme digestion follows AOAC Official Method 2004.05 (*3*). In brief, the procedure involves homogenizing 1 g of sample in 20 mL of 0.1M phosphate buffer, pH 7.8, containing 1% ascorbic acid plus water to give 50 mL. After preheating at 100°C for 15 min, the sample is cooled to ambient temperature and 1 mL of Pronase^R (Calbiochem, #53702, San Diego, CA) solution (2 mg/mL in water) is added, followed by incubation at 37°C for 3 h. At the end of the Pronase^R digestion, the sample is heated for 3 min at 100°C, cooled and digested with α-amylase (Fluka, #10065, St Louis, MO) solution (1 mL of a 20 mg/mL solution in water) for 2 h at 37°C. Conjugase digestion is followed by adding 4 mL of chicken pancreas conjugase (Difco, #245910, Sparks, MD) solution (5 mg/mL in water) and incubating at 37°C for 16 h. At the end of the incubation, the digest is heated at 100°C for 3 min, cooled, adjusted to pH 4.5 with HCl, taken to volume of 100 mL with water and filtered through ashless filter paper (Whatman No. 2V, 12.5 cm).

Microplate Assay. Total folate was assayed microbiologically using the 96-well microplate technique according to the procedures outlined by Tamura (*26*).

Experimental Design. RSM was applied to optimize the trienzyme extraction for folate in vegetables. A three level experimental design (*27*) was used to investigate effects of three independent variables (Pronase^R digestion time, X_1; α-amylase digestion time, X_2; conjugase digestion time, X_3) on the dependent variable (Folate content, Y) for CRM 485 Mixed Vegetables. The independent variables (digestion time of each enzyme) were coded at 3 levels (-1, 0 and 1). Digestion time of each level was selected on the basis of preliminary experiments for proper range with the predicted optimum point in the center (Table 4.1). The complete experimental design consisted of 15 experimental points including three replications of the center points.

Data Analysis. The experimental data was fitted to the following second-order polynomial equation by statistical analysis system (*28*) through the response surface regression (RSREG) procedure:

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

Where Y is response (folate content, μg/100 g sample); β_0, β_i, β_{ii}, β_{ij} are constant coefficients; X_i is the uncoded independent variable. The model was predicted through regression analysis and analysis of variance (ANOVA). RIDGE MAX was one part of the RSREG SAS output to compute the estimated ridge of maximum response for increasing radii from the center of the original design (*18*). Response surface and contour plots were created using Sigma Plot software (Version 9.0) by holding one variable constant in the estimated second-order polynomial equation.

Verification of Model. Assay of CRM 485 by the optimized extraction was compared to the predicted value. Folate contents of other vegetables were assayed with the optimized extraction and compared to data determined by the AOAC Official Method 2004.05 (*3*).

RESULTS AND DISCUSSION

Fitting the Models. Experimental data for each set of variable combinations were obtained (Table 4.1) and fitted to the second-order polynomial equation (eq 1) by RSREG. Using the estimated values of constant coefficients (Table 4.2), the regression model was predicted as:

$$Y = 216.8 + 50.6X_1 + 47.8X_2 + 23.8X_3 - 20.8X_1^2 - 23.0X_2^2 - 3.9X_3^2 + 10.0X_1X_2 - 1.8X_1X_3 + 0.6X_2X_3 \quad \text{(eq 2)}$$

(X_1, Pronase^R digestion time; X_2, α-amylase digestion time; X_3, conjugase digestion time). With a small *p* value (0.01) from ANOVA (Table 4.3) and a suitable coefficient of determination ($R^2 = 0.947$), the predicted regression model (eq 2) was significant and sufficient to represent the actual relationship between the response (folate content) and the significant variables. β_1, β_2, β_3, constant coefficients of incubation time of protease, α-amylase and chicken pancreas, respectively, are positive (Table 4.2), indicating linear effects to increase Y (folate content). β_1, β_2, β_3 indicate linear effect of variables. β_{11}, β_{22}, β_{33} indicate quadratic effect of variables. It was shown from Table 4.2 that both linear and quadratic effects of variables were the primary determining factors of the responses ($p < 0.05$). β_{12}, β_{13}, β_{23} indicate interactions between these independent variables. Results suggest that there is no significant interaction between these independent variables (Table 4.2).

Since conjugase digestion is necessary for folate quantification using microbiological assay, effects of Pronase^R and α-amylase were of the primary concern of this study. The results indicated that both Pronase^R and α-amylase were the important variables, exerting a statistically

significant effect (Pronase^R, $p<0.05$; α-amylase, $p<0.01$) on the measured folate levels (Table 4.4). Conjugase digestion in the experimental scale (1~5 h) of this study was the least important factor ($p = 0.14$), which confirmed the previous findings that deconjugation occurred primarily within the first 1 h of incubation (*7*). However, incubation with conjugase for more than 1 h ensures the deconjugation of the poly-γ-glutamyl folates.

Analysis of Response Surfaces. The relationship between independent and dependent variables is illustrated by the three-dimensional representation of the response surface (Figure 4.1). For Pronase^R and α-amylase, folate levels increased with the incubation time in the first hour, reached a maximum level in 1~1.5 h, followed by a slow decline (Figure 4.1 a). For Pronase^R and conjugase (Figure 4.1 b), measurable folate levels increased with incubation time until a maximum folate content was observed at 1 h for Pronase^R and 3 h for conjugase. After 1.3 h incubation for Pronase^R and 3.3 h for conjugase, a gradual decline was observed. A similar trend was observed for α-amylase (Figure 4.1 c).

The results indicated that liberation of matrix-bound folate by Pronase^R and α-amylase is necessary for folate analysis of vegetables. However, longer incubation can lead to destruction of folate by increasing the exposure of folate to oxidation and other deleterious conditions potentially present in the extraction media.

Optimization and Model Verification. The optimum incubation time for trienzyme digestion was determined by the ridge maximum analysis. Ridge analysis generates the estimated ridge of maximum response for increasing radii from the center of original design (*13*). The ridge maximum analysis predicted that maximum folate contents was 319 μg/100g at digestion of 1.5 h for Pronase^R, 1.5 h for α-amylase, followed by 3 h digestion with conjugase.

Model verification was performed by extracting and determining total folate content in Mixed Vegetables (CRM 485) using the optimized incubation time. The actual experimental value was 317 μg/100g, close to the predicted value of 319 μg/100g, confirming the validity and adequacy of the predicted model.

Moreover, the optimized incubation time of trienzyme extraction was applied to analyze folate contents in sweet potatoes, white potatoes, peas, corn and carrots (Table 4.5) and compared to the folate levels measured by AOAC Official Method 2004.05. The optimized trienzyme digestion gave higher measurable folate in all samples tested (peas, $p<0.0001$; corn, $p<0.01$; carrots, $p<0.01$; sweet potatoes, $p<0.05$) compared to AOAC Official Method 2004.05. For white potatoes, although the optimized trienzyme digestion gave higher measurable folate compared to AOAC Official Method 2004.05, the effect was not significant. By optimized digestion, analytical values for the vegetables tested are somewhat higher than the USDA Nutrient Database (*29*), except for white potato (16 vs 11, sweet potato; 17 vs 18, white potato; 71 vs 53, peas; 51 vs 46, corn; 34 vs 19, carrot).

The study shows that extraction of folate can be maximized using RSM techniques. Time and cost savings can be achieved in folate analysis through use of optimized digestions.

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Table 4.1 Response surface design and experimental data

	Variables (incubation time, h)			Folate (μg/100g)
Treatment no. [a]	Pronase[R]	α-Amylase	Conjugase	CRM 485
1	2(1)[b]	2(1)	3(0)	299
2	0(-1)	2(1)	3(0)	262
3	2(1)	0(-1)	3(0)	259
4	0(-1)	0(-1)	3(0)	262
5	1(0)	2(1)	5(1)	295
6	1(0)	2(1)	1(-1)	291
7	1(0)	0(-1)	5(1)	258
8	1(0)	0(-1)	1(-1)	259
9	2(1)	1(0)	5(1)	290
10	2 (1)	1(0)	1(-1)	304
11	0(-1)	1(0)	5(1)	259
12	0(-1)	1(0)	1(-1)	259
13	1(0)	1(0)	3(0)	309
14	1(0)	1(0)	3(0)	315
15	1(0)	1(0)	3(0)	319

[a] Treatments were run in a random order.

[b] (-1), (0), and (1) are coded levels.

Certified value of CRM 485 is 315 ± 28 μg/100g.

Table 4.2 Regression coefficients of the predicted quadratic polynomial model

Constant coefficient[a]	Estimate value	Standard error	Computed t-value	Pr > | t |
β_0	216.8	15.6	13.87	<.0001
β_1	50.6	12.9	3.92	0.0112
B_2	47.8	12.9	3.70	0.0139
β_3	23.8	7.9	2.99	0.0305
β_{11}	-20.8	4.7	-4.40	0.0070
β_{22}	-23.0	4.7	-4.88	0.0046
β_{33}	-3.9	1.2	-3.29	0.0218
β_{12}	+10.0	4.5	2.20	0.0789
β_{13}	-1.8	2.3	-0.77	0.4757
β_{23}	+ 0.6	2.3	0.28	0.7941

[a] β_0 represents intercept and β_1, β_2, β_3 represent constant coefficients of incubation time of protease, α-amylase and chicken pancreas, respectively.

Table 4.3 Analysis of variance for the 2nd-order response surface model

Source of variation	df	Sum of squares
Model	9	7346.9**
Linear	3	3012.8***
Quadratic	3	3878.9***
Cross product	3	455.3 n.s.
Lack of fit	3	361.8 n.s.
Pure error	2	50.7
Total error	5	412.4
R^2	-	0.947

*** Significant at 1% level; ** significant at 5% level; * significant at 10% level.

n.s. = Not significant.

Table 4.4 Analysis of variance showing significance of the variables on responses.

Independent variables	DF	Sum of squares
Pronase^{R}	4	3557.6**
α-Amylase	4	3851.7***
Chichen pancreas	4	962.2 $^{n.s.}$

*** Significant at 1% level; ** significant at 5% level; * significant at 10% level.

$^{n.s.}$ = Not significant.

Pronase^{R} = digestion time at 2mg/mL, α-amylase = digestion time at 20mg/mL,

Conjugase = digestion time at 5mg/mL.

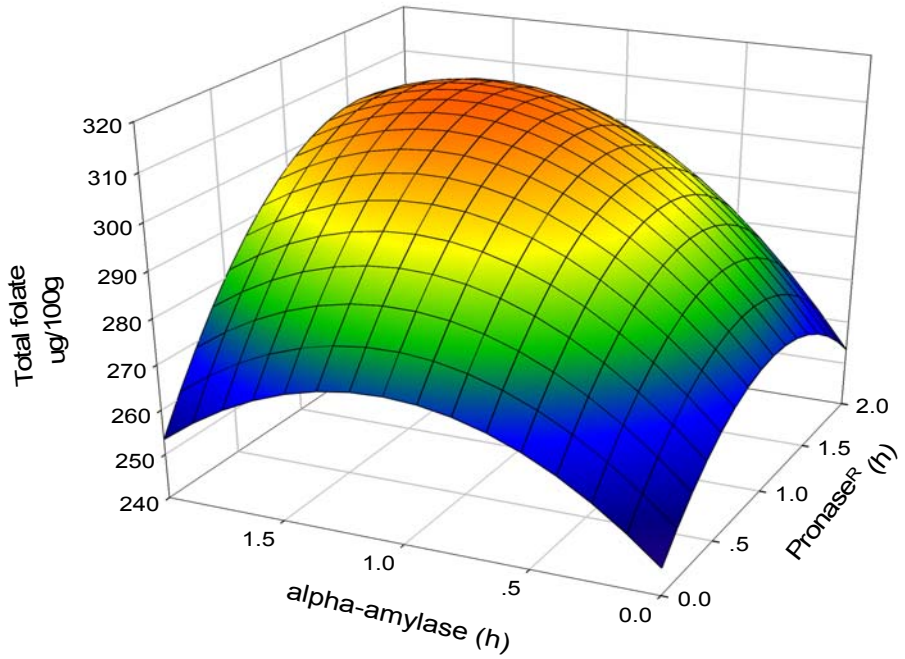
Table 4.5 Comparison of folate contents measured by the optimized extraction and AOAC Method 2004.05.

Vegetables (n=3)	Optimized digestion	AOAC Method 2004.05
	μg/100g	
Sweet potato (flesh and skin, raw)	16 ± 1.4**	13 ± 1.3**
White potato (flesh and skin, raw)	17 ± 0.6 n.s.	16 ±2.1 n.s.
Peas (green, frozen, unprepared)	71 ± 0.5****	59 ± 0.5****
Corn (sweet, yellow, raw)	51 ± 2.0***	38 ± 1.9***
Carrot (raw)	34 ± 2.3***	26 ± 0.7***

**** Significant at 0.01% level; *** Significant at 1% level; ** Significant at 5% level.

* Significant at 10% level; n.s. = Not significant.

Figure 4.1 a Response surface and contour plot for the effects of Pronase^{R} and α-amylase digestion time on total folate assay of CRM 485 (Mixed Vegetables).


Total folate
ug/100g
320
310
300
290
280
270
260
250
240
1.5
1.0
.5
0.0
alpha-amylase (h)
0.0
.5
1.0
1.5
2.0
Pronase^R (h)

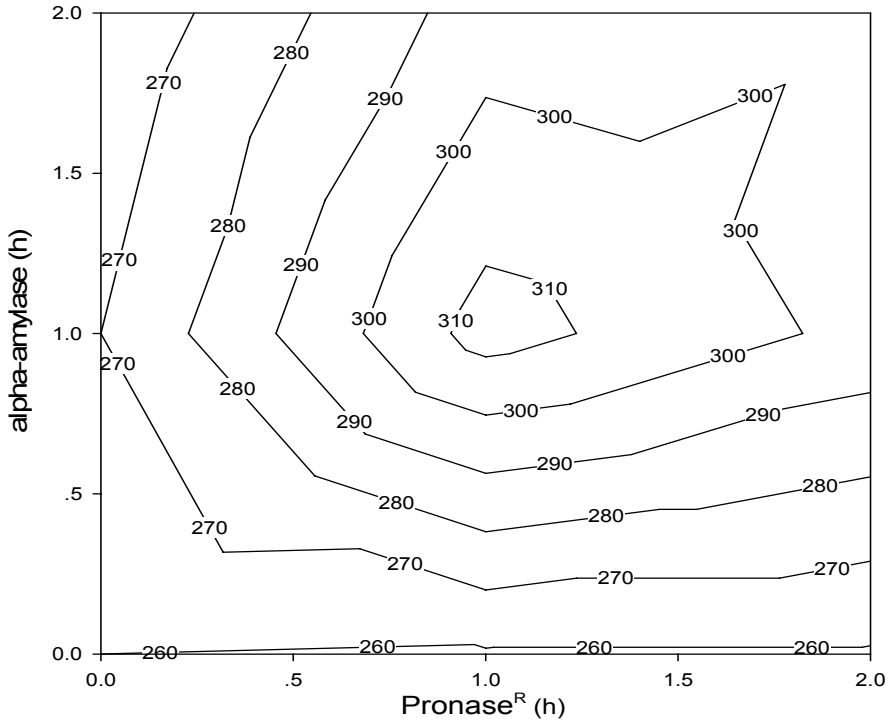
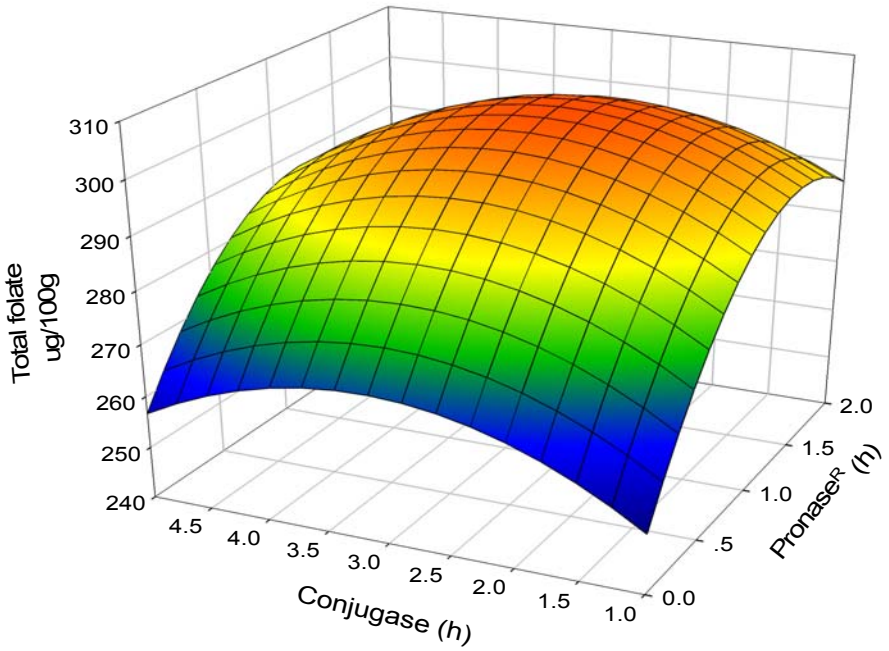

alpha-amylase (h)
2.0
1.5
1.0
.5
0.0
270
280
290
300
310
260
0.0
.5
1.0
1.5
2.0
Pronase^R (h)

Figure 4.1 b Response surface and contour plot for the effects of Pronase^{R} and conjugase digestion time on total folate assay of CRM 485 (Mixed Vegetables).


Total folate
ug/100g
310
300
290
280
270
260
250
240
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
Conjugase (h)
0.0
.5
1.0
1.5
2.0
Pronase^R (h)

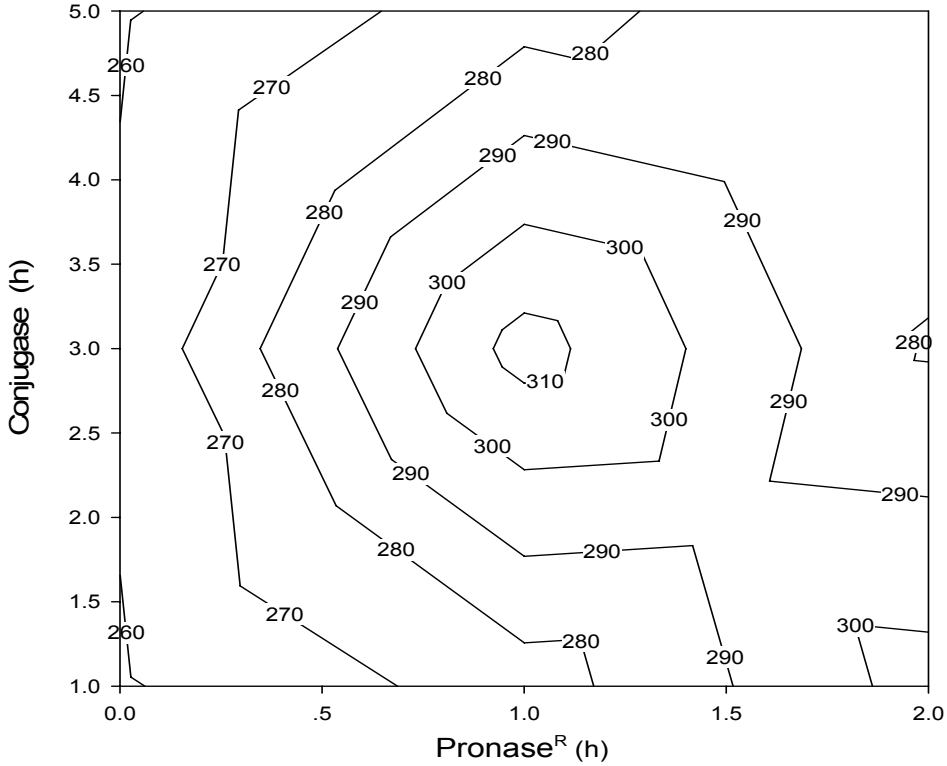
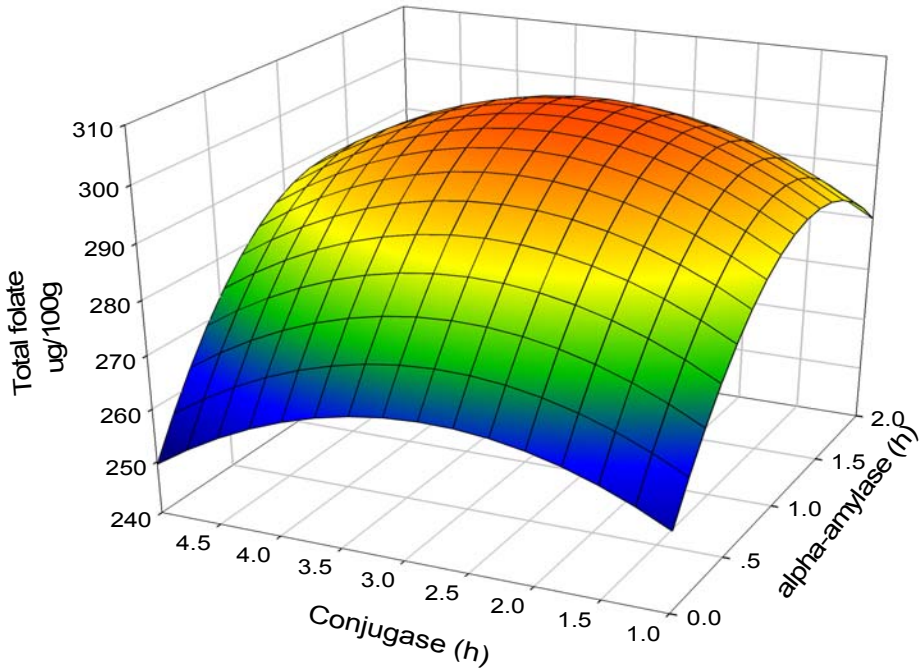
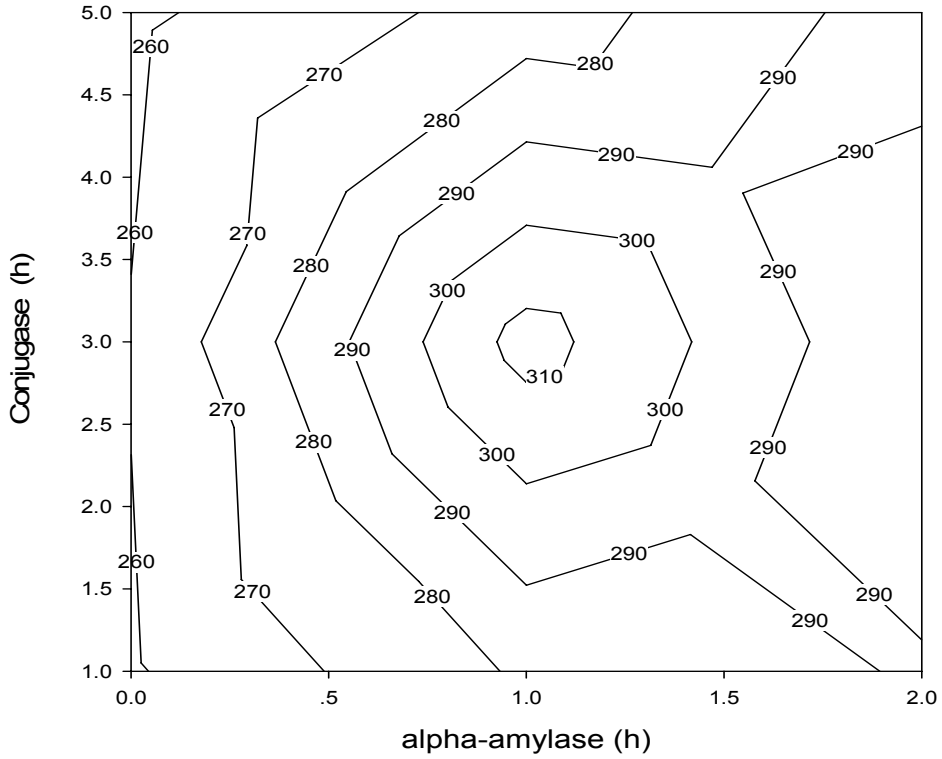

Conjugase (h)
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.0
.5
1.0
1.5
2.0
Pronase^R (h)
260
270
280
290
300
310

Figure 4.1 c Response surface and contour plot for the effects of α-amylase and conjugase digestion time on total folate assay in CRM 485 (Mixed Vegetables).


Total folate
ug/100g
310
300
290
280
270
260
250
240
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
Conjugase (h)
0.0
.5
1.0
1.5
2.0
alpha-amylase (h)


Conjugase (h)
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.0
.5
1.0
1.5
2.0
alpha-amylase (h)
260
270
280
290
300
310

CHAPTER 5

FOOD FOLATE CONTENTS IN FRUITS, VEGETABLES AND THEIR COMMERCIAL PRODUCTS[1]

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[1]Chen, L., L. Kota, J. Chun, J. Lee, J. Exler and R.R. Eitenmiller. To be submitted to *Journal of Food Composition and Analysis.*

Abstract

In order to provide reliable folate data for fruits, vegetables and their commercial products, 250 fruit samples and 171 vegetables samples were collected through the National Food and Nutrient Analysis Program (NFNAP) or other United States Department of Agriculture (USDA) food sampling programs. The extraction procedure followed AOAC Official Method 2004.05 using the trienzyme extraction. Folate contents were determined microbiologically using *L. casei ssp. rhamnosus* (ATCC 7469). Internal quality control, enzyme blank and recoveries were simultaneously assayed along with the samples. All values of internal quality controls had standard deviations less than 10% of the mean. Recoveries for most of the fruits and vegetables were over 90%. Folate in most of the fruits and fruit products ranged from 10~80 μg/100g. Atemoya showed the highest folate content (77 μg/100g), followed by breadfruit (59), guava (49), lychee (48), passion fruit, yellow and purple (48; 46, respectively). Folate contents in vegetables and their products varied from 3 to 307 μg/100g. Edamame showed the highest folate content (307, μg/100g), followed by leafy vegetables (137-249), beans (28), broccoli (67), corn (38), green peas (31), carrots (26), orange juice (25). Most tropical fruits (30-77) are high or concentrated natural sources of folate.

1. Introduction

Folate includes folic acid (pteroylglutamate) and poly-γ-glutamyl conjugates that exhibit the biological activity of folic acid. Naturally existing folates are mostly poly-γ-glutamyl conjugates. Most plant-derived foods, especially fruits and vegetables, contain high to moderate levels of natural folate. Koehler et al (1997) reported that fruits and vegetable provided 20.8 % and 23.2 % of folate intake of older adults, respectively. They also found that fruits and vegetables provided 23 of the top 50 folate sources. Increasing the consumption of fruits and vegetables or enrichment of the diet with folic acid can improve dietary folate status, thereby, decreasing risks of cardiovascular disease and neural tube defects in the general population (Brouwer et al., 1999; Chen et al., 2005; Koebnick et al., 2001). A diet high in fruit and vegetables was also recommended to potentially reduce cancer risk (Bailey, 2003). Most naturally occurring folate derivatives in foods are labile, sensitive to oxidation, light, temperature and extremes of pH. Thus, folate stability is affected by food processing. Factors that influence stability during processing and storage include variation in food matrices, oxygen availability, chemical environment, extent of heating and forms of folate in the food (Eitenmiiler and Landen, 1998). Several studies have reported the influence of processing and storage on folate contents in fruits and vegetables (Desouza and Eitenmiller, 1986; Leichter et al., 1978; Lin and Lin, 1999; Malin, 1977; Melse-Boonstra et al., 2002; Miyamoto et al., 1973; Mullin et al., 1982; Phillips et al., 2005; Stralsjo et al., 2003). Iwatani et al (2003) reported folate contents for Australian vegetables. However, complete information on folate contents of fruits, vegetables and their commercial products is generally lacking for world-wide diets.

The objective of this study was to provide reliable folate data for fruits, vegetables and their commercial products present in the U.S. consumer's diet. It represents analysis of 250 fruit samples and 170 vegetable samples collected through USDA sampling protocols.

2. Materials and methods

2.1. Sampling and pretreatment

250 fruit samples and 170 vegetable samples were collected through the United States Department of Agriculture National Food and Nutrient Analysis Program (USDA/NFNAP). The NFNAP sampling program ensures nationwide, representative food samples for nutrient analysis. USDA samples were composited at Virginia Polytechnic Institute and State University, Food Analysis Laboratory Control Center (FALCC) according to NFNAP protocols. Full details of the NFNAP sampling program that assures a representative sample are available (Pehrsson et al., 2003). The samples were delivered frozen, stored at -50°C and transferred to the refrigerator one day before the assay.

2.2. Analytical quality control

2.2.1. Use of certified reference materials

Accuracy was determined by analysis of European Commission Certified Reference Material CRM 485 (mixed vegetables) purchased from Resource Technology Corporation, Laramie, WY.

2.2.2. Internal quality control sample

Pillsbury all-purpose, bleached, enriched flour purchased at the local grocery was used as the control material for internal quality control. The quality control flour was transferred into 4 oz nalgene bottles and refrigerated at 4 °C. The flour was used through out the study.

2.2.3. Determination of recovery

Samples were spiked with folic acid at 50-150% of the expected folate amount in the original sample matrix. The expected folate levels in the samples were estimated from existing databank or literature values, if available. Otherwise, similar products with available literature values were used to estimated the spiking level. Recovery was calculated by the following equation (AOAC International, 2002a):

$R(\%) = [(Cs-Cp)/Ca]\times100,$

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

2.2.4. Control

A control (enzyme blank) without any food extract was carried through the assay for each sample. The control was used to determine the contribution of the enzymes to the growth response of *L. casei ssp. rhamnosus* (ATCC 7469).

2.3. Standard stock solution

Standard folic acid was purchased from the USP (US Pharmacopoeia). Twenty milligrams of the folic acid was weighed into a 200mL Pyrex conical flask containing 20 mL of ethanol and 50 mL of the distilled water. The pH was adjusted to 10.0 with 0.1N NaOH to help dissolve the folic acid. The final pH was adjusted to 7.0 with 0.05N HCl. The volume was made up to 100mL with water. Aliquot was transferred to 10 mL Pyrex tubes and stored in the refrigerator at 4 °C. A new standard should be prepared after 6 months.

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

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

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

where C_1=0.01 mg/mL, C_2=analyzed concentration of diluted stock standard (mg/mL), A=absorbance, $\varepsilon=27.0\times10^3$, b=1cm, M=molar mass of folic acid (441.40). Reference (Budavari, 1996) gave the physical properties of folates.

2.4. Trienzyme extraction for total folate

The extraction of folate by trienzyme digestion follows AOAC Official Method 2004.05 − Microbiological Assay with Trienzyme Procedure for Total Folates in Cereals and Cereal Foods (AOAC International, 2005). Here is the brief description of the procedure: 0.25-1.0 g of sample in pH 7.8 phosphate buffer (0.1M, containing 1% ascorbic acid) is preheated at 100°C for 15 min. The sample is then cooled to room temperature and 1 mL of Pronase^R (2 mg/mL,

Calbiochem, #53702, San Diego, CA) is added, followed by incubation at 37°C for 3 h. At the end of the Pronase^{R} digestion, the sample is heated for 3 min at 100°C, cooled and digested with 1 mL of α-amylase (20 mg/mL, Fluka, #10065, St Louis, MO) for 2 h at 37°C. Conjugase digestion is followed by adding 4 mL of chicken pancreas conjugase (5 mg/mL, Difco, #245910, Sparks, MD) and incubating at 37°C for 16 h. At the end of the incubation, the digest is heated at 100°C for 3 min, cooled, adjusted to pH 4.5 with HCl, taken to volume of 100 mL with water and filtered through ashless filter paper (Whatman No. 2V, 12.5 cm).

Although AOAC Official Method 2004.05 was collaborated for enriched cereals, the digestion was originally developed for general assay of total food folate and not limited to cereals (DeSouza and Eitenmiller, 1990; Martin et al., 1990).

2.5. Microplate assay

Total folate was assayed microbiologically with *Lactobacillus casei* subsp. *rhamnosus (ATCC 7469),* according to the procedures outlined by Tamura (1990).

2.6. Method performance evaluation

Performance parameters including accuracy, recovery and precision of the microplate assay with trienzyme extraction were evaluated following the AOAC guidelines (AOAC International, 1998; AOAC International, 2002a; AOAC International, 2002b; Horwitz, 2003).

3. Results and discussion

3.1. Method performance evaluation

3.1.1. Accuracy

Accuracy is the closeness of the test result to the "true" or accepted value (AOAC International, 2002a; 2002b). By analyzing 25 replicates of CRM 485, the test result was 311 ± 19.8 μg/100g, within the certified value of 315 ± 28 μg/100g, indicating an acceptable accuracy.

3.1.2. Recovery

Recovery is the fraction or percentage of the analyte that is recovered when the test sample is conducted through the entire method (AOAC International, 2002b). By spiking CRM 485 with folic acid at the level of 160, 320, 640 μg/100g, recoveries were obtained as 85±3.4, 101±3.6, 112±2.5, respectively, with 3 replicate assays at each concentration. An acceptable recovery criterion is a function of concentration as presented in the "AOAC Guidelines for Single Laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals" (AOAC International, 2002a). The AOAC recovery limit at this concentration was 75-120%. Our results were within the acceptable range, confirming the accuracy of the method.

Recovery values for most of the fruits and vegetables were over 90% (Table 5.2). The % mean recoveries ± S.D. in 10 fruits (n=27) were 94.8 ± 4.37; the % mean recoveries ± S.D. in 6 vegetables (n=30) were 93.2 ± 2.14. These recoveries were used to correct the measurable folate contents in the samples assayed in this study.

3.1.3. Precision

Precision measured within a laboratory is designated as repeatability precision ($\%RSD_r$), which refers to the degree of agreement of results when the conditions are maintained as constant as possible with the same analyst, reagents, equipment, and instruments performed within a short period of time (AOAC International, 2002a; Horwitz, 2003). Repeatability precision includes intra-assay precision and inter-assay precision. The $\%RSD_r$ for the microbiological assay with trienzyme extraction for CRM 485 (mixed vegetable) is presented in Table 5.1.

Criterion of acceptance, determined as the $HORRAT_r$ value, was used to evaluate the $\%RSD_r$ values. The acceptable $HORRAT_r$ value is 0.3-1.3 for single laboratory precision (Horwitz, 2003). Calculations were conducted by the following equations:

$\%RSD_{r\ observed} = SD*100/Mean$

$\%\ RSD_{r\ predicted} = 2/3 \times 2^{(1-0.5\log C)}$ (with C the analyte concentration in mass fraction: g/g)

$HORRAT_r = \%RSD_{r\ observed} / \%\ RSD_{r\ predicted}$

The observed $\%RSD_r$ (3.60%, 4.66%) for intra-assay and inter-assay precision were less than the predicted $\%RSD_r$ (8.97%). The $HORRAT_r$ values of both repeatability precisions were within the acceptable range of 0.3-1.3, indicating an acceptable precision of this assay.

3.2 Internal quality control

Internal Quality Control is defined "as a set of procedures undertaken by the laboratory staff for the continuous monitoring of operation and the results of measurements in order to decide whether the results are reliable enough to be released" (Thompson et al., 2002). IQC only monitors intra-laboratory precision.

Enriched flour was used as a control material in this experiment to continuously check the precision of the analytical data obtained day to day in the laboratory. Figure 5.1 is the control charts for folic acid and total folate in enriched flour. Standard deviation at 10% of the mean sets the control limit of upper and lower control lines. All values are within the upper and lower control limit with standard deviations less than 10% of the mean, indicating the analytical process is "in control"

3.4. Folate contents in fruit, vegetables and their commercial products

3.4.1. Folate contents in fruits and fruit products

Table 5.3 presents folate levels in fruits and fruit products. Folate amounts in most of the fruits were less than 50 μg/100g. Atemoya showed the highest folate content (77), followed by breadfruit (59), guava (49), lychee, fresh (48), passion fruit, yellow (48) and passion fruit purple (46), avocado, Florida, variety II (41), feijoa (38), strawberries (30), mango, Kiett (30). Most of the fruits with higher folate levels were tropical or subtropical fruits. Among the commonly consumed fruits, strawberries, fresh (30), mango, Kiett (30) and mango, Tommy Atkin (23), cantaloupes (24), kiwi (22), raspberries, black (22) and red (21), oranges, fresh (21) contained comparably higher folate contents, ranging within 20~30 μg/100g. Blackberries (18), honeydew melon (16) and bananas (12) contained folate ranging within 10~20 μg/100g. Other fruits like grapes, grapefruits, blueberries, peaches, cherries, plums apples contained comparably low levels of folates within 1~10 μg/100g. Among fruit products, orange juice ranked the highest with an average folate content of 25 μg/100g. Orange juice is the most commonly consumed fruit juice in the United States. Subar et al (1989) reported that orange juice was the first ranking food folate source for adults aged 19 to 74 years old. Stralsjo et al (2003) suggested that fresh strawberries

and commercial strawberry products (strawberry jam, stewed strawberry sauce and strawberry syrup) were good folate sources.

3.4.2. Folate contents in vegetables and vegetable products

Folate contents in vegetables and various commercial vegetable products are given in Table 5.4. Most of the leafy greens showed folate levels above 100 μg/100g: turnip greens (249), spinach (170), collards (144), mustard greens (137), except for lettuce, iceberg (28) and butterhead (63). For non-leafy green vegetables, broccoli showed the highest folate content (67), followed by corn, raw (38) and frozen, cooked (35), and carrots, raw (26). Most of the other non-leafy vegetables contained folate contents within the range of 10-20 μg/100g: cucumber, raw (14); green pepper, raw, sweet (15); white potatoes, raw (16), boiled with skin (13), boiled without skin (10); sweet potatoes, raw (16), cooked (13); tomatoes, raw and unpeeled (10), red ripe and canned whole (13). For legumes, edamame, a green soybean, contained the highest mean folate content (307) among all the samples tested. Green peas (frozen, unprepared) contained 59 μg/100g. Green bean products contained folate between 11-31 μg/100g, depending on the processing method: refried green beans (11), frozen green beans (28), baked green beans (18), canned green beans (31). Various tomato products including ketchup, sauce, paste and puree contained folate amount within 10 ~ 16 μg/100g. Vegetable juice cocktail showed a mean folate content of 31 μg/100g.

Table 5.5 ranks the fruits and vegetables by folate content. Edamame ranked the first place, followed by various leafy vegetables, broccoli and green peas. Folate amount in freezed-dried vegetables (137) and dehydrated white potatoes (62) ranked within the top-ten.

Leskova et al (2006) reviewed effects of various heat treatments on folate retention in vegetables, fruit products, legumes, meat and fish products. Spinach, broccoli, pak-choi, peas, beans, and other leafy, non-leafy vegetables have been studied for folate retention or losses during various heat treatments (Aramouni and Godber, 1991; Bergstrom, 1994; Dang et al., 2000; Hoppner and Lampi, 1993a; Hoppner and Lampi, 1993b; Leichter et al., 1978; Lin and Lin, 1999; McKillop et al., 2002). Folate broke down under heat and leached into the cooking water, leading to a large amount of loss. Prolonged exposure of vegetables to heat or water will lower the folate retention (Melse-Boonstra et al., 2002). Our data supported the previous findings. It was indicated in Table 5.4 that cooked corn, boiled white potatoes and cooked sweet potatoes showed lower folate contents than uncooked products. Frozen, cooked and canned green beans contained similar levels of folate. Also, canned whole tomatoes had similar folate levels compared to unpealed raw tomatoes. Ascorbic acid was found to provide additional protection to folate stability in canned vegetables (Sotiriadis and Hoskins, 1982). Measurable folates in dehydrated or dried vegetables were shown higher than the raw ones.

4. Conclusions

This study provides folate contents for fruits, vegetables and their commercial products. Fruit and vegetables provide good sources of folate. Especially, leafy vegetables, broccoli, corns, beans, green peas, carrots, orange juice and strawberry are significant sources of folate.

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Table 5.1 Repeatability precision (n=5)

Repeatability precision	Mean (µg/100g)	Standard deviation	RSD_robserved (%)	RSD_rpredicted (%)	$HORRAT_r$
Intra-assay precision	308.9	11.11	3.60	8.97	0.40
Inter-assay precision	309.3	14.40	4.66	8.97	0.52

Table 5.2 Analytical recovery values for fruit, vegetables, and various products

Sample	Recovery (%)
Fruit	
Apples (n=4)*	85 ± 5
Blackberries (n=2)	100
Blueberries (n=2)	100
Cantaloupes (n=4)	95 ± 5
Fruit juice (n=2)	96
Oranges (n=4)	92 ± 2
Orange juice (n=3)	96 ± 5
Peaches (n=2)	96
Pear juice (n=2)	96
Strawberries (n=2)	92
Mean ± S.D.	95 ± 4.4
Vegetable	
Beans, green (n=4)	96 ± 5
Mixed vegetable (n=10)	93 ± 7
Potatoes, white (n=4)	95
Potatoes, sweet (n=8)	93 ± 2.9
Spinach (n=2)	92
Vegetable, freeze dried (n=2)	90
Mean ± S.D.	93 ± 2

* n = number of trials

Table 5.3 Folate contents of fruit and fruit products

Samples	Food folate µg/100g	Range
Apples		
Golden Delicious (2)*	3	2 - 4
Red Delicious (2)	3	2 - 4
Unspecified (18)	3	2 - 4
Asian pears (2)	8	7 - 9
Atemoya (2)	77	69 - 85
Avocadoes		
Florida, variety I (2)	29	9 - 49
Florida, variety II (2)	41	31 - 51
Bananas (18)	12	9 - 14
Berries		
Blackberries (1)	18	-
Blueberries		
Fresh (3)	8	5 - 12
Drained solids (2)	6	3 - 9
Drained liquids (1)	1	-
Raspberries		
Red (2)	21	20 - 22
Black (2)	22	18 - 26
Salmonberries (1)	17	-
Strawberries (4)	30	27 - 33
Blood Orange (2)	17	14 - 20
Breadfruit (2)	59	39 - 78
Cantaloupes (10)	24	18 - 30
Carambola		
Sweet (2)	7	6 - 8
Tart (2)	16	14 -18
Cherimoya (2)	18	17 - 19
Cherries, raw (4)	5	4 - 6
Coconut, dried, sweetened (3)	3	2 - 6
Dates (4)	14	11 - 18
Feijoa (2)	38	34 - 43
Figs (4)	11	10 - 12
Grapefruit, raw (6)	8	6 - 10
Grapes		
Red flame seedless (2)	9	9 - 10
Thompson seedless (2)	7	7 - 8
Guava, common (2)	49	47 -51
Honeydew melon (6)	16	12 - 21
Japanese Persimmon		
Fuyu (2)	20	19 - 21
Hachiya (2)	13	12 - 14

Table 5.3 (continued)

Samples	Food folate µg/100g	Range
Kiwi (2)	22	21 - 23
Kumquat (2)	17	15 - 18
Lychee		
Fresh (2)	48	46 - 50
Dried (2)	14	13 - 16
Mamey Sapota (2)	8	6 - 10
Mango		
Tommy Atkin (2)	23	20 - 25
Kiett (2)	30	29 - 31
Melon		
Canary (2)	18	13 - 22
Crenshaw (2)	5	4 - 6
Casaba (2)	8	7 - 9
Nectarines (4)	4	3 - 5
Oranges, fresh (18)	21	19 - 23
Passion fruit		
Purple (2)	46	43 - 48
Yellow (2)	48	32 - 65
Peaches		
Fresh (6)	4	3 - 5
Canned, lite syrup (2)	5	4 - 6
Dried (8)	8	5 - 11
Pepino Melon (2)	7	5 - 9
Perimmon, dried (2)	6	5 - 7
Pineapple, dried (2)	1	0.8 - 1.2
Plums (4)	4	3 - 5
Prickly Pear (2)	17	15 - 19
Pummelo, white, fresh (2)	11	8 - 14
Sapote		
Black (2)	7	6 - 8
White (2)	21	17 - 24
Tamarillo (2)	22	21 - 24
Other fruit products		
Fruit juice (7)	24	22 - 26
Fruit juice beverage (8)	4	1 - 7
Fruit punch, canned (4)	1	1 - 2
Lemonade, frozen concentrate, sugar sweetened (4)	9	6 - 12
Orange juice (4)	25	23 - 27
Orange juice, grape juice, and non-carbonated cola mixture (2)	8	7 - 9
Pear juice/syrups (9)	3	1 - 5
Applesauce (5)	2	1 - 3

* ( ) = number of samples

Table 5.4 Folate contents of vegetables and vegetable products

Samples	Food folate µg/100g	Range
Broccoli, raw (6)	67	64 - 71
Carrots, raw (3)	26	25 - 27
Collards, raw (3)	144	141 - 147
Corn		
Raw (3)	38	36 - 40
Frozen, cooked (4)	35	28 - 42
Canned solids (2)	43	37 - 48
Cucumber, raw (6)	14	12 - 16
Edamame (4)	307	201 - 404
Green Beans		
Cooked (6)	28	22 - 34
Canned, solids (2)	31	24 - 38
Baby food (2)	24	21 - 26
Frozen, cooked (4)	28	23 - 30
Hearts of palm, canned (2)	39	34 - 44
Legume products		
Refried beans (4)	11	6 - 14
Baked beans (2)	18	17 - 19
Lettuce		
Iceberg, raw (6)	28	26 - 30
Butterhead, raw (3)	63	61 - 65
Mushroom soup, cream, condensed (2)	4	2 - 6
Mustard greens, raw (2)	137	134 - 140
Nopales, raw (4)	3	1 - 5
Peas, green		
Frozen, unprepared (3)	59	58 - 60
Canned, baby food (1)	31	-
Peppers,		
Chili, sun dried (2)	51	39 - 62
green, sweet, raw (6)	15	14 - 16
Pepper sauce, chilli (2)	6	5 - 7
Potatoes, white		
Raw (25)	16	12 - 20
Boiled, cooked with skin (2)	13	12 - 14
Boiled, cooked without skin (2)	10	9 - 11
Dehydrated (2)	62	61 - 63
French fried, frozen (4)	23	14 - 32
Potatoes, sweet		
Raw (2)	16	13 - 18
Cooked (4)	13	11 - 15
Spinach, raw (6)	170	144 - 196
Squash, baby food (2)	8	8 - 9

Table 5.4 (continued)

Samples	Food folate μg/100g	Range
Thyme, fresh (2)	45	43 - 46
Tomatoes		
Raw, unpeeled (6)	10	8 ~ 12
red ripe, canned whole (2)	13	11 ~ 15
Tomato products		
Ketchup, bottled (4)	10	8 ~ 11
Sauce, canned (4)	11	8 ~ 14
Paste, canned (4)	15	12 ~ 18
Puree, canned (4)	13	11 ~ 15
Chili sauce, canned (4)	16	12 ~ 20
Turnip greens, raw (3)	249	240 ~ 258
Vegetables		
Juice, cocktail (4)	31	25 ~ 37
Mixed (4)	21	19 ~ 23
Freeze-dried (3)	137	104 ~ 170

* ( ) = number of samples

Table 5.5 Ranking of fruit and vegetables by folate content*

Fruit or vegetable	Food folate (μg/100g)	Fruit or vegetable	Food folate (μg/100g)
Edamame	307	Carambola, tart	16
Turnip greens, raw	249	Potatoes, white, raw	16
Spinach, raw	170	Potatoes, sweet, raw	16
Collards, raw	144	Tomato chili sauce, canned	16
Mustard greens, raw	137	Green pepper, sweet, raw	15
Freeze-dried vegetables	137	Tomato paste, canned	15
Atemoya	77	Cucumber, raw	14
Broccoli, raw	67	Dates	14
lettuce, Butterhead, raw	63	Lychee, dried	14
Potatoes, white, dehydrated	62	Japanese Persimmon, Hachiya	13
Green peas, frozen	59	Potatoes, white, boiled, cooked with skin	13
Breadfruit	59	Potatoes, sweet, cooked	13
Pepper, chili, sun dried	51	Tomatoes, red ripe, canned whole	13
Guava, common	49	Tomato puree, canned	13
Passion fruit, yellow	48	Banana	12
Lychee, fresh	48	Figs	11
Passion fruit, purple	46	Pummelo, white, fresh	11
Thyme, raw	45	Beans, refried	11
Corn, canned solids	43	Tomato sauce, canned	11
Avocadoes, Florida, variety II	41	Tomato Ketchup	10
Hearts of palm, canned	39	Tomatoes, raw, unpeeled	10
Feijoa	38	Potatoes, white, boiled, cooked without skin	10
Corn, raw	38	Grapes, red flame seedless	9
Corn, frozen, cooked	35	Lemonade, frozen concentrate, sugar sweetened	9
Green peas, canned, baby food	31	Asian pears	8
Green beans, canned solid	31	Blueberries, fresh	8
vegetable juice, cocktail	31	Grapefruit, raw	8
strawberries	30	Mamey Sapota	8
Mango, Kiett	30	Melon, Casaba	8
Avocadoes, Florida, variety I	29	Orange juice, grape juice, and non-carbonated cola mixture	8
Beans, cooked	28	Peaches, dried	8

Green beans, frozen, cooked	28	Squash, baby food	8
Lettuce, iceberg, raw	28	Carambola, sweet	7
Carrots, raw	26	Grapes, Thompson seedless	7
Orange juice	25	Pepino Melon	7
Cantaloupes	24	Sapote, black	7
Fruit juice	24	Blueberries, drained solids	6
Green beans, baby food	24	Perimmon, dried	6
Mango, Tommy Atkin	23	Pepper sauce, chilli	6
Potatoes, French fried, frozen	23	Cherries, raw	5
Kiwi	22	Melon, Crenshaw	5
Tamarillo	22	Peaches, canned, lite syrup	5
Apple banana	22	Fruit juice beverage	4
Raspberries, black	22	Nectarines	4
Oranges, fresh	21	Peaches, fresh	4
Raspberries, red	21	Plums	4
Sapote, white	21	Mushroom soup, cream, condensed	4
Mixed vegetables	21	Apples, golden delicious	3
Japanese Persimmon, Fuyu	20	Apples, red delicious	3
Cherimoya	18	Apples, unspecified	3
Blackberries	18	Nopales, raw	3
Bean, baked	18	Coconut, dried, sweetened	3
Melon, Canary	18	Pear juice	3
Prickly pear	17	Applesauce	2
Salmonberries	17	Pineapple, dried	1
Honeydew melon	16	Blueberries, drained liquids	1
Blood orange	17	Fruit punch, canned	1
Kumquat	17		

* Mean values from Table 3 and Table 4

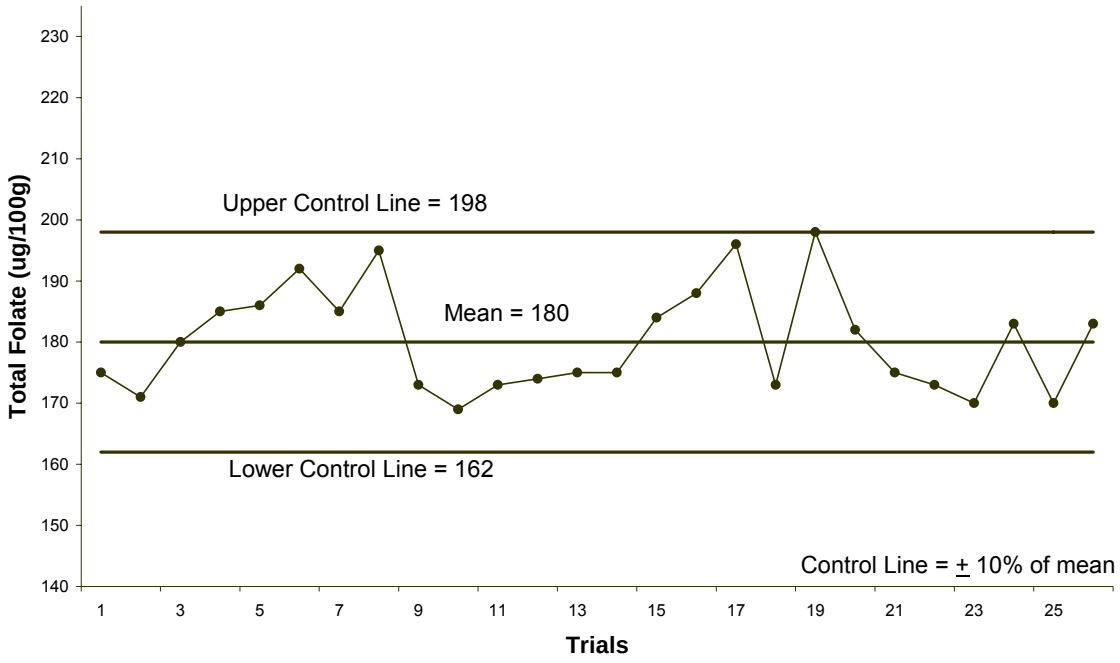

Figure 5.1 Control chart for folate analysis in enriched flour.

CHAPTER 6

CONCLUSIONS

1. Evaluation of single laboratory method performance parameters showed that the trienzyme extraction combined with a 96-well microplate assay was suitable for total folate analysis in a broad selection of food matrices. Accuracy, precision and recovery met acceptance criteria as presented in AOAC guidelines. LOD and LOQ values were 0.3 and 0.6 μg/100g, respectively. The method is not only suitable for total folate analysis in cereal products, but reliable for analysis of total folate in vegetables, meats, milk-based formula and other foods.
2. The optimum incubation time of trienzyme extraction for folate from vegetables was estimated as: 1.5 h for Pronase^{R}, 1.5 h for α-amylase, followed by 3 h digestion with conjugase. Both Pronase^{R} and α-amylase are necessary for folate extraction from vegetables. By analyzing folate contents in sweet potatoes, white potatoes, peas, corn and carrots, the optimized trienzyme digestion gave higher measurable folate compared to AOAC Method 2004.05.
3. From an overview of folate contents for fruits, vegetables and their commercial products, fruits and vegetables are good sources of folate. Most tropical fruits are high or concentrated natural sources of folate. Edamame, leafy vegetables, green beans, broccoli, corn, green peas, carrots, and orange juice are good sources of dietary folate.