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ARTICLE

Determination of the Folate Content in Cladodes of Nopal (*Opuntia ficus indica*) by Microbiological Assay Utilizing *Lactobacillus casei* (ATCC 7469) and Enzyme-Linked Immunosorbent Assay

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Prickly pear cactus has been an important food source in Mexico since ancient times due to its economical and ecological benefits and potential nutraceutical value. Nevertheless, studies on the nutritional aspects and health benefits have been scarce. The purpose of this study was to assess, apparently for the first time, the folate contents of cladodes of nopal by a microbiological assay, using *Lactobacillus casei* (ATCC 7469) in extracts that were enzymatically treated to release the bound vitamin, employing single, dual, and trienzymatic procedures, and using the enzyme-linked immunosorbent assay (ELISA). We used *Opuntia* cladodes of different length sizes. The microbiological assay showed some differences among enzyme treatments and sizes of nopal; the trienzyme treatment (α-amylase-protease-conjugase) was more efficient in determining the folate content in nopal, giving 5.0 ng/g in the small size cladodes at 54 h of testing time, while ELISA showed no significant differences in the folate content among sizes of cladodes (5.5−5.62 ng/g at 0 min testing time). Both techniques may be used for the assessment of folate content in cladodes, but ELISA is more rapid and reliable.

KEYWORDS: Folate; nopal; *Lactobacillus casei*; trienzymatic; ELISA

INTRODUCTION

Prickly pear cactus is endemic to Mexico and has become an alternative crop in semiarid areas (*1*) because of its peculiar adaptation to water scarcity and sun irradiation due to crassulacean acid metabolism (CAM) (*2*). CAM is characterized by high water use efficiency and the ability to store considerable quantities of water, which help the plant to survive against severe environmental conditions (*3*). Traditionally, cactus contributes considerably to the human diet in Mexico and still serves as a therapeutic agent (*4*); the genus *Opuntia* is considered a nutritional source in several countries of Latin America (*5*). Cactus plants are used as fruits and vegetables, for medicinal and cosmetic purposes, as forage and building materials, and also as a source for natural pigments (*6*). Nopal has recently received considerable attention in the scientific community for its bioactive components, which might provide health benefits beyond basic nutrition (*7*). The cladode composition varies depending on the edaphic factors at the cultivation site and the age of the plant (*8*); its nutritional value is considered to be intermediate between lettuce and spinach (*9*). According to several studies on cactus fruit and cladodes, they are a good source of key nutrients such as betalains, amino compounds (taurine), vitamins (ascorbic acid), and antioxidants (*2*, *10*). Also, they are rich in β-carotene and chlorophyll and contain sugars, protein, lipids, and minerals (calcium, potassium, and magnesium) (*11*). However, no information is yet available on the folate content of cactus, although this vitamin is usually found in green vegetable tissues rich in chlorophyll such as spinach and lettuce (*6*). Folates are substrates and coenzymes involved in the transport and enzymatic processing of one-carbon units in a set of reactions such as amino acid and nucleic acid metabolism (*12*); they are tripartite molecules comprised of a pterin moiety, a ρ-aminobenzoate unit, and a γ-linked glutamate chain with 1−8 residues (*13*). Low folate intake in humans leads to impaired cell division exhibited as megaloblastic anemia and the presence of neural tube defects (*14*). There is strong evidence of a relationship between an inadequate folate status, elevated homocysteine concentration, and a risk of coronary heart disease and certain forms of colorectal, breast, pancreatic, bronchial, and cervical cancer as well as leukemia (*15*). Plants, fungi, and many microorganisms synthesize folates de novo, but humans and other higher animals do not and require a dietary supply (*16*). Plants are the primary source of folate in human nutrition, namely, green leafy vegetables, lettuce, spinach, certain fruits such as oranges and grapefruit juices, beans and legumes, wheat germ including yeast, egg yolk, liver, and folate-fortified breakfast cereal products (*17*). Given the significance of this vitamin in human nutrition and that the folate deficiency is a

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worldwide health problem and that foods and plants are major sources of folates for humans, it is very important to identify its presence in accessible and economic food sources. Thus, the aim of this study was to analyze the folate content in nopal by microbiological assay through *Lactobacillus casei* (ATCC 7469) and by enzyme-linked immunosorbent assay (ELISA).

MATERIALS AND METHODS

Plant Material. Cladodes of nopal Verde Valtierrilla variety were obtained from a commercial orchard in Salamanca, Gto., México, and selected according to size for commercialization: 17, 20, and 30 cm in length for small, medium, and large samples, respectively. Fresh spinach was purchased at a local supermarket and utilized as a control because of its wide use and its high folate content as reported in the literature (*14*). Both cladodes and spinach were tested by the microbiological assay and ELISA.

Preparation of the Cryoprotected Microorganisms. Cryoprotected cultures were prepared from lyophilized *L. casei* (ATCC 7469) obtained from the American Type Culture Collection (Manassas, VA) and used as an assay organism following the procedure described by Grossowicz et al. (*18*). The culture was reconstituted according to the manufacturer's directions and inoculated in test tubes (5 mL) of the broth and incubated overnight at 37 °C; 0.1−0.3 mL of the culture was transferred to a second tube and reincubated for 5−7 h. *L. casei* assay medium was distributed in a flask (150 mL) with 30 ng/L folic acid, and 0.1−0.3 mL of cryoprotected microorganisms was incubated overnight at 37 °C. The absorbance was measured at 650 nm through 60 min intervals, and when the growth rate decreased, the culture was poured into a flask with 300 mL of medium without folic acid, and the absorbance was recorded during 60 min intervals. Then, it was centrifugated at 6500 rpm for 10 min at 4 °C and washed to remove the remaining folate. The pellets were reconstituted with 10 mL of medium assay and diluted 100-fold, and the absorbance was measured until 0.2 nm, was diluted with a volume of glycerol/water (80/20), and was stored at −70 °C until analysis.

Microbiological Assay: Extraction and Enzyme Treatments. For the determination of the folate content by microbiological assay, samples were prepared using extraction and enzyme treatments as reported previously by Pandrangi and LaBorde (*14*) with modifications. The difference was the use of carboxipeptidase G of *Pseudomonas* sp. as the conjugase source instead of rat serum. The extraction of folate in cladodes and spinach was performed in triplicate; the samples were weighed, cut into pieces, and homogenized in a blender with 50 mL of 0.1 M phosphate buffer containing 114 mM ascorbic acid (final pH 4.1). The homogenate was heated in a water bath at 100 °C for 10 min, cooled, and stored at −70 °C for about 2 h until analysis. The extracts were treated by single (conjugase), dual (protease conjugase), and trienzyme (α-amylase-protease-conjugase) procedures. We used conjugase to hydrolyze polyglutamyl folate to monoglutamyl forms, both α-amylase (*Aspergillus niger* E.C. 3.2.1.184) and protease (*Streptomyces griseus* E.C. 3.4.2.4.312), to allow digestion of carbohydrate and protein matrices of foods where folates were possibly trapped. For single treatment, 335 μL of nopal homogenate was mixed with 1580 μL of phosphate buffer (pH 7.0) and incubated with 85 μL of conjugase at 37 °C for 3 h. A dual enzyme treatment was performed by mixing 250 μL of homogenate with 250 μL of citrate buffer (pH 4.0) and 500 μL of protease and incubated at 37 °C for 8 h. The extract was heated to stop the reaction, and 335 μL was mixed with 1580 μL of phosphate buffer (pH 7.0) and 85 μL of conjugase, and then, the mixture was incubated at 37 °C for 3 h. The trienzyme was carried out by mixing 250 μL of homogenate with an equal amount of citrate buffer (pH 3.0) and 500 μL of α-amylase, followed by incubation at 37 °C for 6 h. The extract was heated, and 250 μL of cooled homogenate was mixed with 750 μL of citrate buffer (pH 4.0) and 500 μL of protease and then incubated at 37 °C for 8 h. The homogenate was heated again, 335 μL of cooled extract was mixed with 1580 μL of phosphate buffer (pH 7.0), 85 μL of conjugase was added, and the mixture was incubated at 37 °C for 3 h. For all treatments, the extracts were heated at 100 °C for 5 min in a boiling water bath to stop the reaction, cooled, and centrifuged at 10000 rpm at 4 °C for 15 min. The residues were suspended in 2 mL of extraction buffer and recentrifuged for 15 min. The supernatants were mixed with 2 mL of extraction buffer and filtered through a microfilter (0.22 μm). The folate concentration in each homogenate after enzyme digestion was determined by using a 96-well microplate with *L. casei*. Folate contents are given on a wet basis.

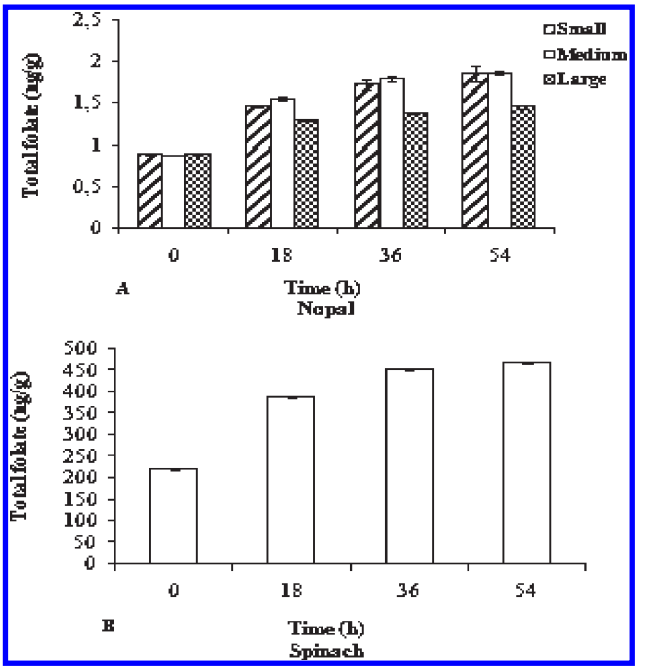

Figure 1. Total folate content (ng/g) in nopal cladodes (small, medium, and large sizes) (**A**) and in spinach (**B**) after a single enzyme treatment and during different times (h) of incubation with *L. casei*. Results are given on a wet basis. Each value represents the mean of three determinations.

Quantification of Folates by ELISA. This procedure was done according to the ELISA test for quantitative determination of folic acid in food (Diagnostic Automation, Inc., United States). Briefly, the extracts of nopal and spinach were prepared using double-distilled water; the pH was adjusted to 6−7, and then, 0.5 mL of Carrez-I and Carrez-II solutions was added and centrifuged at 7500 rpm at 4 °C for 10 min, and the supernatant was stored at −20 °C.

One hundred microliters of samples and 50 μL of mouse antifolic acid antibody were added into the microplate, mixed, and incubated at room temperature for 60 min in a shaker. After the liquid was discarded and the microplate was washed three times and dried, 100 μL of antimouse IgG HRP was added, mixed shortly, and incubated at room temperature for 60 min. The supernatant was eliminated, washed, and dried as above-mentioned. One hundred microliters of tetramethylbenzidin was added, mixed, and incubated at room temperature for 20 min in dark conditions. When a blue pigmentation appeared, the reaction was stopped, and then, the color changed to yellow, and the microplate was mixed before the absorbance was measured at 450 nm. The color intensity was inversely proportional to the concentration of folates present in the samples; this parameter was stable for only 30 min; therefore, the absorbance was measured in 5 min intervals up to 30 min. The folate contents are given on a wet basis.

Statistical Analysis. Each assay was determined by triplicate. Multifactor analysis of variance was performed, and means were compared using Tukey's multiple range test ($p = 0.05$) Statgraphics Centurion XV 2007 (Stat Point Inc.).

RESULTS AND DISCUSSION

Microbiological Assay. Folate concentrations in nopal samples determined by a single treatment are given in **Figure 1**. According to size, in the conjugase alone treatment, the highest values were found at medium size (1.87 ng/g), whereas 2.2 ng/g to small sizes were shown in the dual enzyme treatment, both at 54 h (**Figure 2**). Our results showed that there was no significant difference in the measured folate between single and dual treatments, while the total folate content found in the trienzyme treatment (**Figure 3A**) showed the highest values (5.0 ng/g) in the small cladodes.

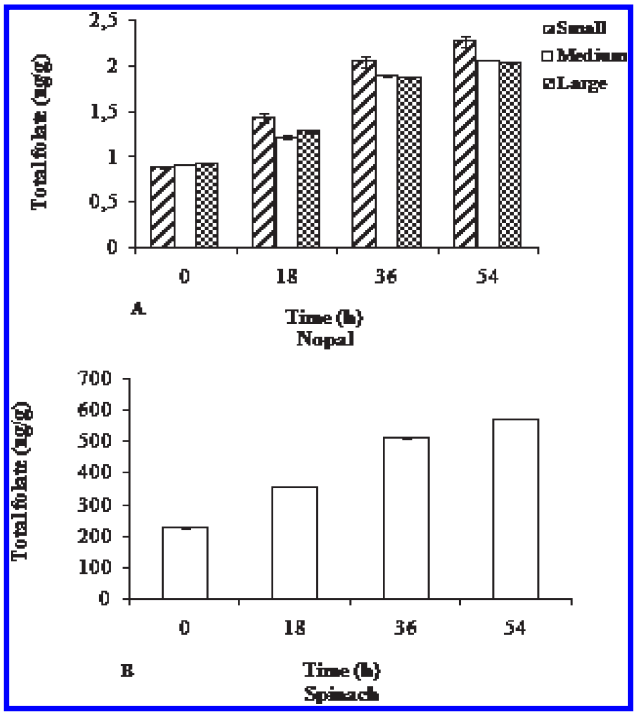

Figure 2. Total folate content (ng/g) in nopal cladodes (small, medium, and large sizes) (**A**) and in spinach (**B**) after a dual enzyme treatment and during different times (h) of incubation with *L. casei*. Results are given on a wet basis. Each value represents the mean of three determinations.

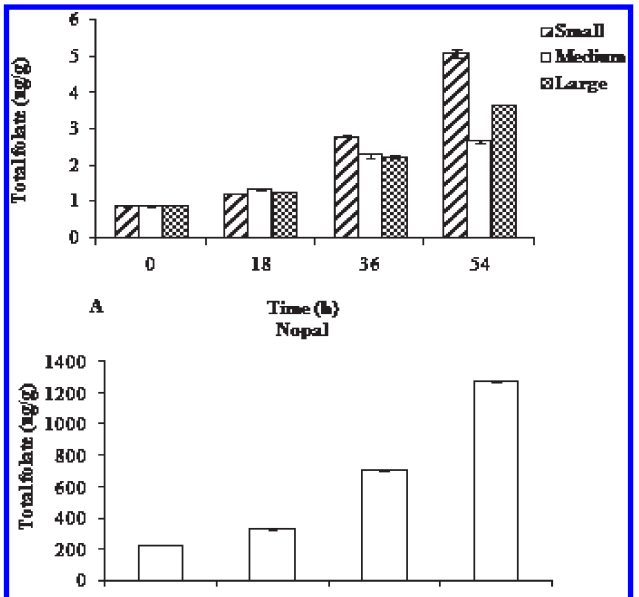

Figure 3. Total folate content (ng/g) in nopal cladodes (small, medium, and large sizes) (**A**) and in spinach (**B**) after a trienzyme treatment and during different times (h) of incubation with *L. casei*. Results are given on a wet basis. Each value represents the mean of three determinations.

These values indicate that the enzymatic treatment is an important step in the microbiological assay before folate determination in order to obtain the proper folate content in foods. The trienzyme treatment (**Figure 3B**) using spinach for comparison purposes showed remarkably higher values than the single and dual enzyme procedures (**Figures 1B** and **2B**, respectively).

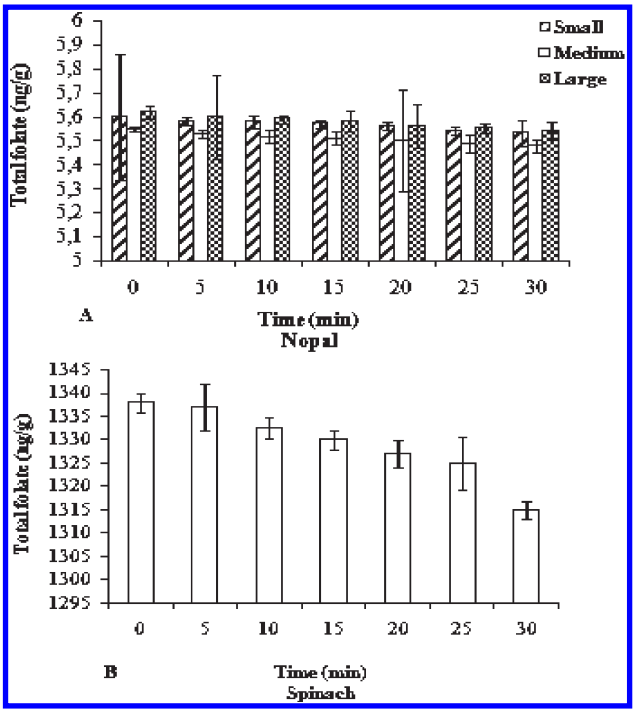

Figure 4. Total folate content (ng/g) in nopal cladodes (small, medium, and large sizes) (**A**) and in spinach (**B**) evaluated by ELISA. Results are given on a wet basis. Each value represents the mean of three determinations.

Our results confirm that treatment with folate conjugase alone is usually not effective to liberate food-bound folate, in agreement with Arcot et al. (*19*). The use of additional enzymes, proteolytic or amylolytic, has been shown to liberate folate from the rest of the components, maximizing the folate activity in certain foods. Johnston et al. (*20*) reported folate contents of 56 fast foods, such as hamburgers, sandwiches, pizzas, Mexican foods, and breakfast items, and the highest values were obtained by a trienzyme treatment. Aiso and Tamura (*21*) reported that a trienzyme procedure consisting of α-amylase and protease before conjugase resulted in higher folate values than a dual enzyme treatment; however, as compared with folate alone and dual treatments, some authors (*20*, *22*) did not find increases with a trienzyme assay. The type of conjugase used, enzyme treatment, incubation time, and extraction pH also play important roles in the assessment, as reported by Devi et al. (*23*).

We utilized times up to 54 h of incubation because folate contents in nopal and spinach samples did not change after this time. In contrast to our assessments, in other studies, the folate content was higher using shorther incubation periods. The differences may be attributes to the influence of growth conditions of crop, type of cultivar, and degree of folate bound to matrix constituents (*14*). The form of the folates is another factor, since some of them may be labile at long incubation periods (*24*).

ELISA. The folate content obtained by the ELISA with all cladode sizes showed no significant differences at each tested time (**Figure 4A**). However, large cladodes tended to have higher values in relation to small and medium samples. Folate contents decreased with longer tested times; however, these changes were not significant. Spinach gave decreasing values of folates at longer times as well (**Figure 4B**); 1338 and 1315 ng/g at 0 and 30 min, respectively, values that were statistically different at these two extremes.

The comparison of folate values between the two methods (trienzymatic at 54 h for small sizes cladodes and ELISA) showed some slight and nonsignificant differences. The microbiological

assay has been described as the best method for folate assessment because it responds to the widest variety of folate derivatives and has a low cost for equipment set up. On the other hand, ELISA appears to be a rapid and reliable technique for the measuremet of food folates. Immunoassays are highly sensitive and specific as a result of the interaction of an antibody molecule with its target; a high affinity interaction occurs even in complex matrices; however, some of its limitations are related to its cost and short shelf life, and in some specific cases, variations in results may be obtained (*17*). In brief, on the basis of our results provided by the two techniques, we believe that any of them (trienzymatic and ELISA procedures) may be used to determine folates in cladodes.

According to our knowledge, this is the first assessment of folates in nopal cladodes. It may also be interesting to point out that under all conditions and sizes, cladodes showed very low folate content in relation to spinach. Thus, nopal cladodes are a poor source of folates; their recommended dietary allowance (RDA), in dietary folate equivalents, is 400 μg/day for adults and 600 μg/day for pregnant women (*25*).

LITERATURE CITED

(1) Mondragón, J. C.; Bordelon, B. B. Apomixis in crosses of Mexican pear cactus preliminary molecular identification. *Rev. Fitotec. Mex.* **2002**, *3*, 247–252.

(2) Feugang, J. M.; Konarski, P.; Zou, D.; Stintzing, F. C.; Zou, C. Nutritional and medicinal use of cactus pear (*Opuntia* spp.) cladodes and fruits. *Front. Biosci.* **2006**, *1*, 2574–2589.

(3) Anaya-Pérez, M. A. History of the use of *Opuntia* as forage in México. In *Cactus* (*Opuntia spp.*) *as Storage*; Mondragón-Jacobo, C., Pérez González, S., Eds.; FAO: Rome, Italy, 2001; pp 5−12.

(4) Lüttge, U. Ecophysiology of crassulacean acid metabolism (CAM). *Ann. Bot.* **2004**, *93*, 629–652.

(5) Shapiro, K.; Gong, W. C. Use of herbal products for diabetes by Latinos. *J. Am. Pharm. Assoc.* **2002**, *42*, 278–279.

(6) Lee, Y.-C.; Pyo, Y.-H.; Ahn, C.-K.; Kim, S.-H. Food functionality of *Opuntia ficus-indica* var. cultivated in Jeju Island. *J. Food Sci. Nutr.* **2005**, *10*, 103–110.

(7) Stintzing, F. C.; Carle, R. Cactus stems (*Opuntia* spp.): A review on their chemistry, technology, and uses. *Mol. Nutr. Food Res.* **2005**, *49*, 175–194.

(8) Tesoriere, L.; Fazzari, M.; Allegra, M.; Livrea, M. A. Biothiols, taurine and lipid-soluble antioxidants in the edible pulp of Sicilian cactus pear (*Opuntia ficus-indica*) fruits and changes of bioactive juice components upon industrial. *J. Agric. Food Chem.* **2005**, *53*, 7851–7855.

(9) Batista, A. M.; Mustafa, A. F.; McAllister, T.; Wang, Y.; Soita, H.; Mckinnon, J. J. Effects of variety on chemical composition, *in situ* nutrient disappearance and *in vitro* gas production of spineless cacti. *J. Sci. Nutr. Agric.* **2003**, *83*, 440–445.

(10) Delgado-Vargas, F.; Paredes-López, O. Anthocyanins and Betalains. In *Natural Colorants for Food and Nutraceutical Uses*; CRC Press: Boca Raton, FL, 2003; pp 167-21.

(11) Betancourt-Domínguez, M. A.; Hernández-Pérez, T.; García-Saucedo, P.; Cruz-Hernández, A.; Paredes-López, O. Physico-chemical changes in cladodes (Nopalitos) from cultivated and wild cacti (*Opuntia* spp.). *Plant Foods Hum. Nutr.* **2006**, *61*, 115–119.

(12) Lermo, A.; Fabiano, S.; Hernández, S.; Galve, R.; Marco, M. P.; Alegret, S.; Pividori, M. I. Immunoassay for folic acid detection in vitamin- fortified milk based on electrochemical magneto sensors. *Biosens. Bioelectron.* **2009**, *24*, 2057–2063.

(13) Iniesta, M. D.; Pérez-Conesa, D.; García-Alonso, J.; Ros, G.; Periago, M. J. Folate content in tomato (*Lycopersicon esculentum*). Influence of cultivar, ripeness, year of harvest, and pasteurization and storage temperatures. *J. Agric. Food Chem.* **2009**, *57*, 4739–4745.

(14) Pandrangi, S.; LaBorde, L. F. Optimization of microbiological assay of folic acid and determination of folate content in spinach. *Int. J. Food Sci. Technol.* **2004**, *39*, 525–532.

(15) Quinlivan, E. P.; Hanson, A. D.; Gregory, J. F. The analysis of folate and its metabolic precursors in biological samples. *Anal. Biochem.* **2006**, *348*, 163–184.

(16) Díaz de la Garza, R.; Gregory, J. F., III; Hanson, A. D. Folate biofortification of tomato fruit. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 4218–4222.

(17) Arcot, J.; Shrestha, A. Folate: Methods of analysis. *Trends Food Sci. Technol.* **2005**, *16*, 253–266.

(18) Grossowicz, N.; Waxman, S.; Schreiber, C. Cryoprotected *Lactobacillus casei*: An approach to standarization of microbiological assay of folic acid in serum. *Clin. Chem.* **1981**, *27*, 745–747.

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

(20) Johnston, K. E.; Lofgren, P. A.; Tamura, T. Folate concentrations of fast foods measured by trienzyme extraction method. *Food Res. Int.* **2002**, *35*, 565–569.

(21) Aiso, K.; Tamura, T. Trienzyme treatment for food folate analysis: optima pH and incubation time for α-amylase and protease treatments. *J. Nutr. Sci. Vitam.* **1998**, *44*, 361–370.

(22) Yon, M.; Hyun, T. H. Folate content of foods commonly consumed in Korea measured after trienzyme extraction. *Nutr. Res. (N.Y.)* **2003**, *23*, 735–746.

(23) Devi, R.; Arcot, J.; Sotheeswaran, S.; Ali, S. Folate contents of some selected fijian foods using trienzyme extraction method. *Food Chem.* **2008**, *106*, 1100–1104.

(24) Hyun, T. H.; Tamura, T. Trienzyme extraction in combination with microbiologic assay in food folate analysis: an update review. *Exp. Biol. Med.* **2005**, *230*, 444–454.

(25) Food and Nutrition Information Center. Dietary reference intakes (DRI) and recommended dietary allowances (RDA); **2004**; http://www.nal.usda.gov/fnic/etext/000105html.

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