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Optimization of Tri-enzyme Extraction Procedures for the Microbiological Assay of Folate in Red Kidney Bean and Roasted Peanut Using Response Surface Methodology

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Abstract Total folate content was determined by microbiological assay using *Lactobacillus casei* spp. *rhamnosis* (ATCC 7469) with a 96-well microplate technique. Using roasted peanut and red kidney beans as representative legume samples, response surface methodology (RSM) was supplied to optimize the trienzyme procedures for the determination of folate in legumes. After response surface regression (RSREG), the second-order polynomial equation was fitted to the experimental data. Ridge analysis showed that the optimal digestion times were <2 hr for Pronase® and α-amylase, and <5 hr for conjugase to obtain maximal folate values for legume samples. This study confirms that established digestion times for cereal products (AOAC Method 2004.05) of 3 for protease and 2 hr for α-amylase are applicable to legumes. Conjugase treatment can be reduced to 5 from 16 hr and the conjugase level to 5 from 20 mg per sample, providing significant cost saving.

Keywords: trienzyme extraction, folate, optimization, response surface response, analysis, legume

Introduction

Many analytical methods including bioassay, microbiological, high performance liquid chromatography (HPLC), ligand-binding, and radioimmuno assay have been used for the analysis of food folates (1). Despite progress in the development of HPLC methods, currently, the Association of Official Analytical Chemists (AOAC) Official Methods for the analysis of folates are microbiological assay by *Lactobacillus casei* (spp. *rhamnosis*) ATCC 7469 (2). Before microbiological assay, food samples must be hydrolyzed with protease and α-amylase to liberate folates from food matrices and treated with γ-glutamyl hydrolase (conjugase) to cleave poly γ-glutamyl folates to mono- or di-glutamate forms that can be utilized by *L. casei*.

Optimum of pH, order of enzyme addition, incubation time, and other condition of trienzyme extraction have been investigated for folate analysis in different food matrixes. Asio and Tamura (3) evaluated the conditions of pH and incubation time for the treatment with protease and α-amylase in 4 food items including fresh beef, white bread, milk, and spinach. They found that optimal pHs for α-amylase treatment of beef and milk were 7.0 and 5.0, respectively, whereas those for bread and spinach were not distinctive at pHs from 2.0 to 7.0. Recently, working on spinach, Pandrangi and LaBorde (4) reported that a dual-enzyme treatment (protease and conjugase) is sufficient to determine folate. Australian researchers (5) showed that single enzyme extraction with conjugase gave higher measurable folate contents than trienzyme digestion for leafy vegetables. DeSouza and Eitenmiller (6) observed a remarkable increase in folate content after trienzyme treatments. It appears that conditions of the enzyme treatment might be different for each type of food matrices and therefore, to extract folate effectively, incubation conditions for all 3 enzymes must be optimized according to the type of food being analyzed.

Response surface methodology (RSM), originally described by Box and Wilson (7), enables evaluation of the effects of many factors and their interactions on the response variables. The main advantages of RSM are the reduced number of experimental trials needed to evaluate multiple parameters and their interactions: therefore, it is less laborious and time-consuming than other approaches required to optimize a process. RSM has been widely applied for the optimizing conditions in agricultural and biological researches. Applying RSM to vitamin analysis, Lee *et al.* (8) optimized the extraction parameters for the quantitative determination of vitamin E in tomato and broccoli. Recently, Chen and Eitenmiller (9) adopted the RSM techniques to the optimization of tri-enzyme extraction procedure for the analysis of folate in vegetables.

The purpose of this study was to understand the relationships between trienzyme extraction and its parameters including incubation time of each Pronase®, α-amylase, and conjugase and to optimize the trienzyme extraction procedures for the quantitative determination of folate in legumes.

Materials and Methods

Standard preparation Accurately, 20 mg of the folic acid (US Pharmacopoeia, Rockville, MA, USA) was transferred into the 100-mL conical flask containing 20 mL of ethanol and 50 mL of distilled water. The pH was adjusted to 10.0 with 0.1 N NaOH to dissolve the folic acid

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thoroughly and the final pH of this solution adjusted to 7.0 with 0.05 N HCl. The volume of standard solution was made up to 100 mL with water and aliquots of the standard solution were flushed with nitrogen gas and stored at 4°C. The purity of standard solution was calculated immediately using a molar extinction coefficient of 27,000 at 282 nm. This stock solution was stable at least 6 months.

Trienzyme extraction and total folate analysis Red kidney bean and roasted peanut as representative samples were brought from local supermarkets in Athens, GA, USA. Peanut and peanut butter were defatted using hexane to prevent fatty acid stimulation of the growth response of *L. casei*. (10) The concentration of each enzyme and pH of extraction buffer in folate analysis were based on AOAC Method 2004.05 (11). Each of servings was ground in a small coffee grinder, and the accurately 1 g samples were homogenized with 0.1 M potassium phosphate extraction buffer (pH 7.8) containing 1% ascorbic acid. These homogenates were heated at 100°C for 15 min, immediately cooled to ambient temperature. The trienzyme extraction was completed by adding 1.0 mL of Pronase® solution (2 mg/mL, Calbiochem, San Diego, CA, USA) and digesting at 37°C. At the end of Pronase® digestion, the samples were heated for 3 min at 100°C, cooled, and digested with 1 mL of α-amylase solution (20 mg/mL, Fluka, St. Louis, MO, USA) at 37°C. Conjugase digestion is followed by adding 1 mL of chicken pancrease solution (5 mg/mL) at 37°C. The various digestion times of each enzyme for the folate extraction were chosen according to Table 1. At the end of trienzyme digestion, the digest is heated at 100°C for 3 min, cooled, adjusted to pH 4.5, taken to volume of 100 mL with water and filtered through filter paper. Total folate was determined microbiologically using 96-well microplate technique according to the methods of Tamura (12).

Experimental design RSM was employed for optimizing the trienzyme digestion time in folate determination from red kidney bean and roasted peanut as representative samples. The folate values were assumed to be affected by 3 independent variables: the digestion time of Pronase®, α-amylase, and conjugase. A fractional 3-level-3-factor experimental design with 3 replicates at the centerpoint was adopted (13). The 3 factors (digestion time variables), level, and experimental design in terms of coded and uncoded were given in Table 1.

Data analysis The response surface regression (RSREG) procedure of statistical analysis system (SAS Institute, Cary, NC, USA) was used to fit the experimental data to the quadratic polynomial equation to obtain coefficients of the equations

$$Y=\beta_0+\Sigma_{i=1}^{3}\beta_i X_i+\Sigma_{i=1}^{3}\beta_{ii}X_i^2+\Sigma_{i=1}^{2}\ \Sigma_{j=i+1}^{3}\beta_{ij}X_iX_j \quad (1)$$

where Y is response (total folate content, μg/100 g sample); β_0, β_i, β_{ii}, and β_{ij} are constant coefficients and X_i is the uncoded independent variables. RIDGE Max in the RSREG SAS output was used to compute the estimated ridge of optimum response by increasing radii from the center of the origin. Response plots were created using Sigma Plot (version 9.0) by holding constant one variable of the second-order polynomial equation.

Verification of model Optimization of trienzyme extraction times of Pronase®, α-amylase, and conjugase was calculated using the predictive equation from RSM. Folate contents of representative samples (red kidney bean and roasted peanut) were analyzed with the optimized conditions and compared to data assayed by the AOAC Official Method 2004.05 (11).

Results and Discussion

Fitting the models An optimization of trienzyme extraction

Table 1. Extraction variables, levels, and experimental data

Trial[1)]	Variables (incubation time, hr)			Total folate (μg/100 g)	
	Pronase® X1	α-Amylase X2	Conjugase X3	Red kidney bean	Roasted peanut
1	3(1)[2)]	3(1)	3(0)	145.1	80.6
2	1(-1)	3(1)	3(0)	150.9	78.4
3	3(1)	1(-1)	3(0)	156.7	94.5
4	1(-1)	1(-1)	3(0)	176.3	103.3
5	2(0)	3(1)	5(1)	149.3	84.6
6	2(0)	3(1)	1(-1)	116.3	79.6
7	2(0)	1(-1)	5(1)	187.3	106.3
8	2(0)	1(-1)	1(-1)	158.4	89.6
9	3(1)	2(0)	5(1)	159.1	91.6
10	1(-1)	2(0)	1(-1)	119.3	77.7
11	3(1)	2(0)	5(1)	168.9	107.3
12	1(-1)	2(0)	1(-1)	124.7	101.8
13	2(0)	2(0)	3(0)	182.0	105.9
14	2(0)	2(0)	3(0)	183.2	110.8
15	2(0)	2(0)	3(0)	175.7	110.0

[1)]Treatments were run in a random order.
[2)](-1), (0), and (1) are coded levels.

Table 2. Regression coefficients of the second-order polynomial equations

Coeffcient[1)]	Estimated value	
	Red kidney bean	Roasted peanut
β_0	62.97	45.59
β_1	57.75	16.13
β_2	0.25	38.38
β_3	39.36	12.94
β_{11}	-17.63	-7.63
β_{22}	3.50	2.75
β_{33}	-5.86	-12.16
β_{12}	0.75	1.13
β_{13}	0.63	-1.38
β_{23}	-5.59	-1.67

[1)]β_0 represents intercept and β_1, β_2, and β_3 represent constant coefficients of digestion time of Pronase®, α-amylase, and conjugase, respectively.

times for the determination of folate in red kidney bean and roasted peanut samples was tried using RSM. After the RSREG procedure, the second-order polynomial equation 1 (Eq. 1) was fitted to the experimental data (Table1). The regression coefficients of Eq. 1 are given Table 2.

The effects of variables as linear, quadratic, or interaction coefficients on responses (folate contents) were tested for adequacy and fitness by analysis of variance. In general, the analysis of variance showed that the model appeared to be adequate with no significant lack of fit and with a satisfactory coefficient of determination (R^2=0.9543 for red kidney bean, R^2=0.9210 for roasted peanut) (Table 3).

From statistical analysis, all 3 variables had a significant effect on folate contents in red kidney bean sample (Pronase®, $p<0.1$; α-amylase, $p<0.05$; conjugase, $p<0.01$). The digestion time of Pronase® and conjugase were the important variables for the roasted peanut samples, exerting a statistically significant effects (Pronase®, $p<0.1$; conjugase, $p<0.05$) on the measured folate levels (Table 4).

Analysis of response surfaces The relationship between variables and responses was also investigated by the 3-dimensional representation of the response surface by holding constant one variable of the second-order polynomial equation. In red kidney bean, folate levels increased with digestion time of tri-enzyme until maximum folate content was observed at 1.8 for Pronase®, 1.1 for α-amylase, and 3.6 hr for conjugase, respectively, and after maximum digestion time, a gradual decline was observed (Fig. 1). A similar trend was showed for roasted peanut samples. Folate levels increased with digestion time of tri-enzyme until maximum folate content was observed at 1.5 for Pronase®, 1.4 for α-amylase, and 4.2 hr for conjugase, respectively (Fig. 2). The results indicated that release of matrix-bound folate by tri-enzymes is necessary for folate analysis legumes. However, prolonged digestion can lead to destruction of folate by increasing the exposure of folate to oxidation and other deleterious conditions potentially present in the extraction buffers.

Optimum condition and model verification The method of ridge analysis computes the estimated ridge of optimum response by increasing radii from the center of the original design (14). The ridge analysis showed that the optimal digestion times were <2 hr for Pronase® and α-amylase, and <5 hr for conjugase to obtain maximal folate values for legume samples.

Model verification was performed by determining total folate content in red kidney bean and roasted peanut samples using the following digestion conditions: 2 for Pronase®, 2 for α-amylase, and 5 hr for conjugase. Slightly different digestion conditions were used for the model verification. The predicted values from the results of RSM and ridge analysis (185 for red kidney bean and 112 μg/100 g for roasted peanut) were closed to actual experimental values of 178 for red kidney bean and 119 μg/100 g for roasted peanut, respectively, confirming the validity and adequacy of the predicted models. Moreover, comparison of the optimized digestion to AOAC Method 2004.05 to

Table 3. Analysis of variance for the second-order response surface model

Source of variation	DF	Red kidney bean		Roasted peanut	
		Sum of squares	*p*-value	Sum of squares	*p*-value
Model	9	6,983.5	0.0074	1,945.9	0.0267
Linear	3	4,097.3	0.0031	1,054.5	0.0134
Quadratic	3	2,822.0	0.0071	810.8	0.0230
Cross product	3	64.3	0.8111	80.8	0.5422
Lack of fit	3	296.3	0.1656	153.0	0.1231
Pure error	2	38.0	-	14.0	-
Total error	5	334.3	-	167.0	-

Table 4. Analysis of variance showing significance of the variables on responses

Independent variables[1)]	DF	Red kidney bean		Roasted peanut	
		Sum of squares	*p*-value	Sum of squares	*p*-value
Pronase®	4	1,302.9	0.0563	507.2	0.0880
α-Amylase	4	1,835.8	0.0290	1,215.8	0.0162
Conjugase	4	4,209.9	0.0049	412.6	0.1239

[1)]Digestion time at 2 for protease, 20 for α-amylase, and 5 mg/mL for conjugase.

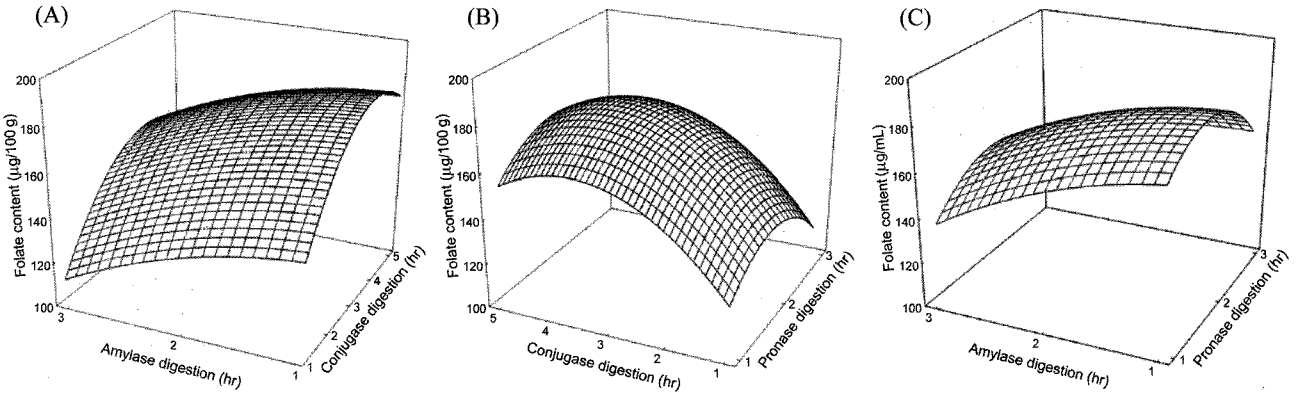

Fig. 1. Response surface plots of folate contents in red kidney bean. (A) Effects of α-amylase and conjugase treatment time under constant treatment time of protease (2 hr). (B) Effects of protease and conjugase treatment time under constant treatment time of α-amylase (2 hr). (C) Effects of α-amylase and protease treatment time under constant treatment time of conjugase (3 hr).

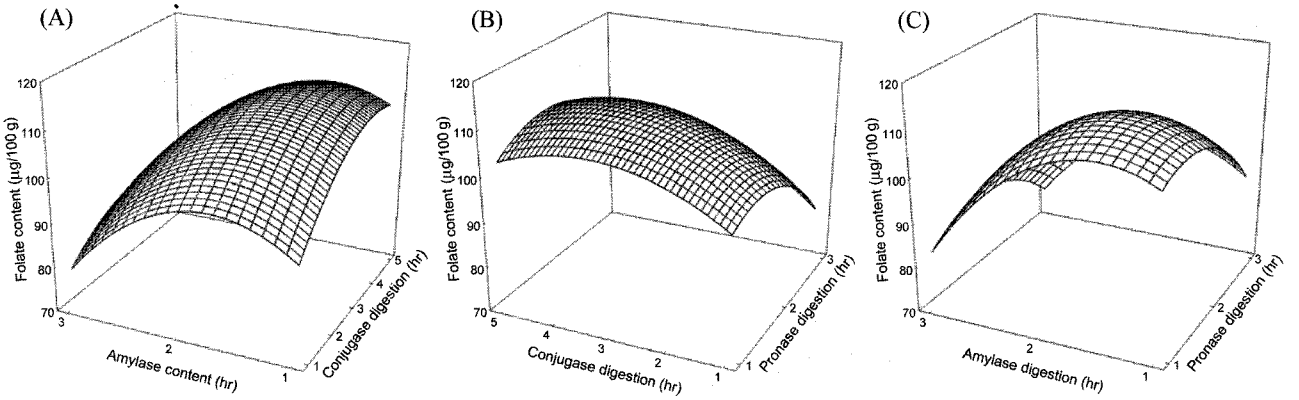

Fig. 2. Response surface plots of folate contents in roasted peanut. (A) Effects of α-amylase and conjugase treatment time under constant treatment time of protease (2 hr). (B) Effects of protease and conjugase treatment time under constant treatment time of α-amylase (2 hr). (C) Effects of α-amylase and protease treatment time under constant treatment time of conjugase (3 hr).

total folate analysis of red kidney beans and roasted peanuts showed that the procedures gave statistically similar values ($p>0.1$) (Table 5). The optimized digestion times of trienzyme extraction was applied to analyze total folate contents in red kidney bean, great northern bean, lima bean, chickpea, roasted peanut, and peanut butter (Table 6). Chickpea contained higher total folate levels (brand c: 301 ±7.7 mg/100 g) compared to other legumes assayed by the optimized condition. The analytical method validation

Table 5. Comparison of AOAC Method 2004.05 digestion to the optimized trienzyme digestion

Independent variables	Total folate[1] (μg/100 g)	
	Red kidney bean	Roasted peanut
AOAC Method 2004.05[2]	179.4±2.6	81.0±8.9
Optimized condition[3]	176.9±11.2	88.9±12.3

[1] n=14 (red kidney bean), n=8 (roasted peanut).
[2] Trienzyme digestion condition: 3 for protease, 18 for α-amylase, and 16 hr for conjugase.
[3] Trienzyme digestion condition: 2 for protease, 2 for α-amylase, and 5 hr for conjugase.

Table 6. Total folate contents of dry bean, pea, and peanut products

Sample	Folate content (μg/100 g)	Sample	Folate content (μg/100 g)
Red kidney bean (dried)		Chick pea (dried)	
Brand a	155.9±1.1	Brand a	273.3±1.1
Brand b	145.4±9.5	Brand b	296.4±27.5
Brand c	145.4±4.6	Brand c	301.0±7.7
Brand d	144.4±0.8		
Brand e	178.2±4.0		
Great northern bean (dried)		Peanut (roasted, salted)	
Brand a	154.7±11.6	Brand a	59.9±6.4
Brand b	107.4±5.2	Brand b	119.9±9.2
Brand c	125.3±8.1	Brand c	61.0±0.1
Brand d	113.9±6.6	Brand d	80.5±2.3
Lima bean (dried)		Peanut butter (smooth style, salted)	
Brand a	79.9±7.1	Brand a	67.6±8.6
Brand b	111.4±4.2	Brand b	61.6±6.3
Brand c	111.7±20.3	Brand c	69.9±5.1
Brand d	79.1±16.8	Brand d	77.2±7.1

Table 7. Precision and recovery for red kidney beans

Parameter[1)]	Precision		Accuracy
	Repeatability	Reproducibility	Recovery (%)
Mean	177.2	177.5	99.3
SD	14.9	15.6	6.2
CV (%)	8.4	8.6	6.3

[1)]Mean, n=5 (μg/100 g); SD, standard deviation. CV, coefficient of variation.

parameters including accuracy and precision were calculated to further ensure the method's validity (Table 7). In order to obtain above parameters, the optimized digestion conditions from the results of RSM and ridge analysis were used. Precision is made up of 2 components: repeatability (intra-day assay) and reproducibility (inter-day assay). The accuracy was evaluated by analyzing a spiked sample (200 μg/100 g). The mean recovery was 99.3±6.2% with a range of 90 to 107%. In general, the results of validation parameters were reliable and satisfactory.

This study confirms that established digestion times for cereal products (AOAC Method 2004.05) of 3 for protease and 2 hr for α-amylase are applicable to legumes. Conjugase treatment can be reduced to 5 from 16 hr and the conjugase level to 5 from 20 mg per sample, providing significant cost saving.

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