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Optimization of the Trienzyme Extraction for the Microbiological Assay of Folate in Vegetables

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Response surface methodology was applied to optimize the trienzyme digestion for the 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, 1.5 h; α-amylase, 1.5 h; and 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 five vegetables and yielded higher folate levels than the trienzyme digestion parameters employed in AOAC Official Method 2004.05.

KEYWORDS: Trienzyme; folate; analysis; vegetable

INTRODUCTION

Trienzyme extraction, introduced by Eitenmiller and his colleagues (*1*, *2*), is a combined enzymatic digestion by Pronase, 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 *Lactobacillus casei* ssp. *rhamnosus* (ATCC 7469). It is the extraction method used in AOAC Official Method 2004.05 (*3*) for the total folate analysis of cereal foods.

The 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*). The 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

CRM. European Commission CRM 485 (mixed vegetables) was purchased from Resource Technology Corp. (Laramie, WY). Sweet potatoes, white potatoes, yellow sweet corn, carrots, and frozen green peas were purchased from retail stores in Athens, Georgia. 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 throughout 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). Enzyme blank

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values are almost always quite low and produce nondetectable growth at dilution levels used for most food products. It is, however, necessary to include an enzyme blank with each batch of assays to ensure the absence of folate addition from the enzyme preparations. Also, new commercial enzyme preparations need to be assayed before use to ensure the absence of folate at levels that would hinder their use.

Standard Stock Solution. Standard folic acid was purchased from the USP (US Pharmacopoeia). Twenty milligrams of the folic acid was weighed into a 200 mL Pyrex conical flask containing 20 mL of ethanol and 50 mL of distilled water. The pH was adjusted to 10.00 with 0.1 N NaOH to help dissolve the folic acid. The final pH was adjusted to 7.00 with 0.05 N HCl. The volume was made up to 100 mL with water. An 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.

The 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 (0.1 M, pH 7) as a blank. The purity was calculated using the following equations:

$$\text{purity of standard (\%)} = 100 \times (\text{analyzed concentration } C_2/\text{known concentration } C_1)$$

$$\text{calculated concentration } C_2 = (A_{\text{std}} - A_{\text{blank}}) \cdot M/(\epsilon \cdot b)$$

where $C_1 = 0.01$ mg/mL, C_2 = analyzed concentration of diluted stock standard (mg/mL), A = absorbance, $\epsilon = 27.0 \times 10^3$, $b = 1$ cm, and M = molar mass of folic acid (441.40) (*25*).

Trienzyme Extraction. The extraction of folate by the trienzyme digestion followed AOAC Official Method 2004.05 (*3*). In brief, the procedure involved homogenizing 1 g of sample in 20 mL of 0.1 M phosphate buffer, pH 7.8, containing 1% ascorbic acid plus water to give 50 mL. After it was preheated at 100 °C for 15 min, the sample was cooled to ambient temperature and 1 mL of Pronase (Calbiochem, #53702, San Diego, CA) solution (2 mg/mL in water) was added, followed by incubation at 37 °C for 3 h. At the end of the Pronase digestion, the sample was 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 was followed by the addition of 4 mL of chicken pancreas conjugase (Difco, #245910, Sparks, MD) solution (5 mg/mL in water) and incubation at 37 °C for 16 h. At the end of the incubation, the digest was heated at 100 °C for 3 min, cooled, adjusted to pH 4.5 with HCl, taken to a volume of 100 mL with water, and filtered through ashless filter paper (Whatman #2V, 12.5 cm).

Microplate Assay. The 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 digestion time, X_1; α-amylase digestion time, X_2; and 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 three levels (−1, 0, and 1). The 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 1**). The complete experimental design consisted of 15 experimental points including three replications of the center points.

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

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

Table 1. Response Surface Design and Experimental Data

treatment no.[a]	variables (incubation time, h)			folate (μg/100 g) CRM 485	
	Pronase	α-amylase	conjugase	mean	range
1	2 (1)[b]	2 (1)	3 (0)	299 ± 12.6	286–312
2	0 (–1)	2 (1)	3 (0)	262 ± 16.8	245–279
3	2 (1)	0 (–1)	3 (0)	259 ± 15.4	243–275
4	0 (–1)	0 (–1)	3 (0)	262 ± 13.2	248–275
5	1 (0)	2 (1)	5 (1)	295 ± 11.9	283–307
6	1 (0)	2 (1)	1 (–1)	291 ± 14.5	276–306
7	1 (0)	0 (–1)	5 (1)	258 ± 16.3	241–275
8	1 (0)	0 (–1)	1 (–1)	259 ± 12.5	246–272
9	2 (1)	1 (0)	5 (1)	290 ± 14.2	275–304
10	2 (1)	1 (0)	1 (–1)	304 ± 16.9	287–321
11	0 (–1)	1 (0)	5 (1)	259 ± 13.8	245–272
12	0 (–1)	1 (0)	1 (–1)	259 ± 11.9	247–271
13	1 (0)	1 (0)	3 (0)	309 ± 15.7	293–325
14	1 (0)	1 (0)	3 (0)	315 ± 15.8	299–331
15	1 (0)	1 (0)	3 (0)	319 ± 14.8	304–334

[a] Treatments were run in a random order; means represent three independent trials. [b] Incubation time, h (code). Certified value of CRM 485 is 315 ± 28 μg/100 g.

Table 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	<0.0001
β_1	50.6	12.9	3.92	0.0112
β_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 the intercept, and β_1, β_2, and β_3 represent constant coefficients of the incubation times of protease, α-amylase, and chicken pancreas, respectively.

where Y is the response (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 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. The assay of CRM 485 by the optimized extraction was compared to the predicted value. The 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 1**) and fitted to the second-order polynomial equation (eq 1) by RSREG. Using the estimated values of constant coefficients (**Table 2**), the regression model was predicted as:

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

Table 3. Analysis of Variance for the Second-Order Response Surface Model

source of variation	df	sum of squares
model	9	7346.9[a]
linear	3	3012.8[b]
quadratic	3	3878.9[b]
cross-product	3	455.3[c]
lack of fit	3	361.8[c]
pure error	2	50.7
total error	5	412.4
R^2		0.947

[a] Significant at the 5% level. [b] Significant at the 1% level. [c] Not significant.

Table 4. Analysis of Variance Showing Significance of the Variables on Responses[a]

independent variables	df	sum of squares
Pronase	4	3557.6[b]
α-amylase	4	3851.7[c]
chicken pancreas	4	962.2[d]

[a] Pronase = digestion time at 2 mg/mL, α-amylase = digestion time at 20 mg/mL, and conjugase = digestion time at 5 mg/mL. [b] Significant at the 5% level. [c] Significant at the 1% level. [d] Not significant.

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

Because enzyme digestion is necessary for quantification using microbiological or LC assays, effects of Pronase, α-amylase, and chicken pancreas were studied at digestion times of 1−2 h for Pronase and α-amylase and 1−5 h for conjugase. The results indicated that both Pronase and α-amylase were the important variables, exerting a statistically significant effect (Pronase, $p < 0.05$; α-amylase, $p < 0.01$) on the measured folate levels (**Table 4**). Conjugase digestion in the experimental scale (1−5 h) of this study was the least important variable ($p = 0.14$), 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. However, overnight or extended digestion with conjugase is not necessary and, with some matrices, possibly detrimental.

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

The results indicated that liberation of matrix-bound folate by Pronase 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.

Table 5. Comparison of Folate Contents Measured by the Optimized Extraction and AOAC Method 2004.05

	μg/100 g ± SD	
vegetables ($n = 3$)	optimized digestion	AOAC method 2004.05
sweet potato (flesh and skin, raw)	16 ± 1.4[a]	13 ± 1.3[a]
white potato (flesh and skin, raw)	17 ± 0.6[b]	16 ± 2.1[b]
peas (green, frozen, unprepared)	71 ± 0.5[c]	59 ± 0.5[c]
corn (sweet, yellow, raw)	51 ± 2.0[d]	38 ± 1.9[d]
carrot (raw)	34 ± 2.3[d]	26 ± 0.7[d]

[a] Significant at the 5% level. [b] Not significant. [c] Significant at the 0.01% level. [d] Significant at the 1% level.

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 were 319 μg/100 g at digestion of 1.5 h for Pronase, 1.5 h for α-amylase, and 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/100 g, close to the predicted value of 319 μg/100 g, 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 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, $p < 0.01$; and sweet potatoes, $p < 0.05$) as compared to AOAC Official Method 2004.05. For white potatoes, although the optimized trienzyme digestion gave a higher measurable folate as 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 U.S. Department of Agriculture 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; and 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 the use of optimized digestions.

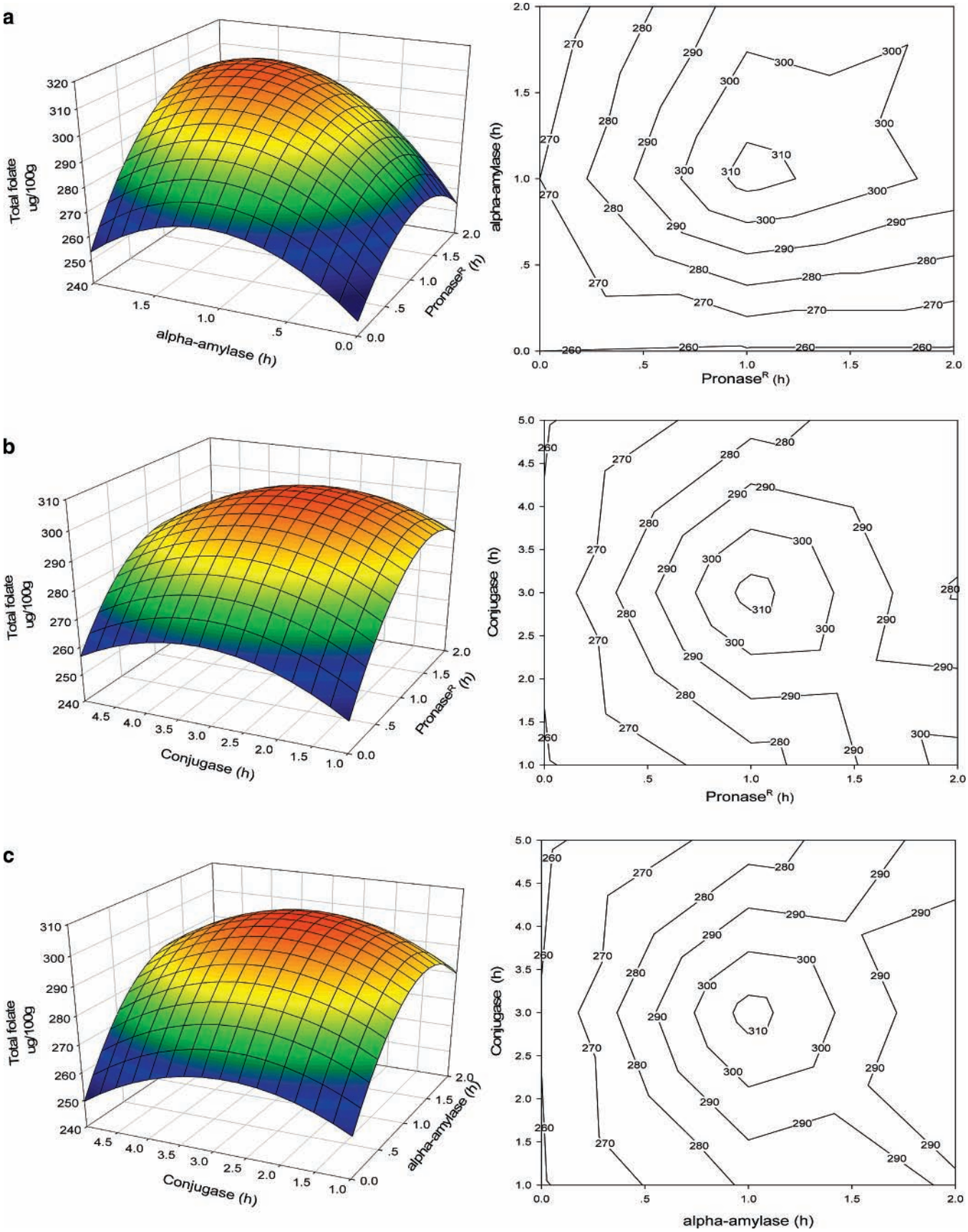

Figure 1. (**a**) Response surface and contour plot for the effects of Pronase and α-amylase digestion time on total folate assay of CRM 485 (mixed vegetables). (**b**) Response surface and contour plot for the effects of Pronase and conjugase digestion time on total folate assay in CRM 485 (mixed vegetables). (**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).

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