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Optimization of Enzyme Extractions for Total Folate in Cereals Using Response Surface Methodology

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The trienzyme digestion including protease, α-amylase, and conjugase (γ-glutamyl hydrolase) for the extraction of total folate from cereal grain products used in AOAC Official Method 2004.05 was optimized using response surface methodology. Digestion times of α-amylase ($p < 0.05$) and conjugase ($p < 0.01$) showed significant effects on the response (total folate). Digestion time for Pronase digestion was not significant ($p > 0.1$). Ridge analysis showed that total times required for the trienzyme digestions ranged from 6.1 h for buckwheat to 8.7 h for CRM 121 (wholemeal flour). The predicted maximal value for CRM 121 (51 μg/100 g) was close to the certified value (50 ± 7 μg/100 g). The optimized extractions for the four cereals suggest that a generalized trienzyme extraction for cereals of 1, 2.5, and 6 h for Pronase, α-amylase, and conjugase, respectively, could be used to approximate total folate in cereals and cereal products. These conditions provide data comparable to AOAC Official Method 2004.05 for the cereals included in this study and would be suitable to approximate total folate in most cereals and cereal products. Compared to the official method, the shortened digestion provides cost savings and minimizes the folate loss possible with prolonged digestions.

KEYWORDS: Trienzyme digestion; total folate; cereals; response surface methodology

INTRODUCTION

Extraction of folates from food and other biologicals relies on enzymatic steps to liberate the folates from the cellular matrix and deconjugate polyglutamate forms to mono- or diglutamates (*1*, *2*). Until the more recent development of chromatographic methods using liquid chromatography (LC), microbiological assay with *Lactobacillus casei* ssp. *rhamnosus* (ATCC 7469) was the most common method to assay food folate (*3*, *4*). AOAC Official Method 2004.05, Total Folates in Cereals and Cereal Foods, Microbiological Assay (*5*), uses *L. casei* ssp. *rhamnosus* and a trienzyme extraction with α-amylase, proteases (Pronase), and conjugase (γ-glutamyl hydrolase). It is generally accepted that the trienzyme extraction frees bound forms of folate and provides an accurate means of total folates when applied to most foods (*6*). This procedure has been successfully applied to the extraction of various foods and biologicals for analysis by LC with traditional detectors or LC coupled with mass spectrometry (*7*−*16*).

Efforts to improve the trienzyme extraction have effectively utilized response surface methodology (RSM) to optimize the digestion protocol. Chen and Eitenmiller (*17*) used RSM to optimize the trienzyme method for the extraction of folate from vegetables. Digestion times were found to be optimal at 1.5, 1.5, and 3 h for Pronase, α-amylase, and conjugase, respectively. The authors reported that the much shorter, optimized digestion times yielded higher folate values for most samples ($p < 0.05$) than the longer times (3, 2, and 16 h for Pronase, α-amylase, and conjugase, respectively) employed in AOAC Official Method 2004.05 (*5*). Recently, Choi et al. (*18*) used RSM to optimize the extraction for legumes using red kidney beans (*Phaseolus vulgaris*) and roasted peanuts (*Arachis hypogaea*) as models. Optimized digestion times were 2, 2, and 5 h for these products. Compared to AOAC Official Method 2004.05 (*5*), analytical folate values were greater with the optimized procedure for roasted peanuts, but not statistically different ($p > 0.1$). Unfortunately, the optimized digestion times acquired from the previous studies may not be applicable to other types of food, because folate extraction is largely dependent on food matrices.

Thus, the objective of this study was to determine optimal digestion times for the trienzyme extraction of folate from various cereals for time savings and reduced cost by applying RSM.

MATERIALS AND METHODS

CRM121 and Nonenriched Flours. CRM121 (wholemeal flour), a European Commision Certified Reference Material, was purchased from Resource Technology Corp. (Laramie, WY). Four representative nonenriched cereals were purchased online at Purcell Mountain Farms: oat flour, triticale flour, buckwheat flour, and whole wheat flour. Collected samples were stored at −50 °C, and the samples were adjusted to ambient temperature before the assay. Also, the samples were mixed to ensure homogeneity with a spatula right before weighing.

Control. An enzyme control without any cereal sample was carried throughout the total folate extraction procedure to determine the contribution of the enzymes to the growth response of the *L. casei* ssp. *rhamnosus* (ATCC 7469).

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Table 1. Digestion Times for Each Coded Level Used in the Response Surface Methodology Study

	variables (incubation time, h)								
cereal	Pronase			α-amylase			conjugase		
CRM121	0.25 (−1)[a]	0.5 (0)	1 (1)	1 (−1)	2 (0)	3 (1)	2 (−1)	4 (0)	6 (1)
oat flour	0.25 (−1)	0.5 (0)	1 (1)	0 (−1)	1 (0)	2 (1)	2 (−1)	4 (0)	6 (1)
triticale flour	0.25 (−1)	0.5 (0)	1 (1)	0.25 (−1)	0.5 (0)	1 (1)	2 (−1)	4 (0)	6 (1)
buckwheat flour	0.2 (−1)	0.5 (0)	1 (1)	0.25 (−1)	0.5 (0)	1 (1)	2 (−1)	4 (0)	6 (1)

[a] (−1), (0), and (1) are coded levels.

Table 2. Response Surface Design and Experimental Data

	coded level			total folate (μg/100 g)			
treatment no. (in random order)	Pronase	α-amylase	conjugase	CRM121	oat flour	triticale flour	buckwheat flour
1	1	1	0	48.3	46.5	66.7	49.1
2	−1	1	0	48.3	46.4	65.1	48.6
3	1	−1	0	45.6	41.6	61.6	41.3
4	−1	−1	0	46.0	40.1	61.3	40.5
5	0	1	1	51.8	48.1	70.4	51.9
6	0	1	−1	46.4	41.9	59.8	42.8
7	0	−1	1	46.4	44.7	64.8	44.8
8	0	−1	−1	42.8	38.2	55.8	39.6
9	1	0	1	49.1	48.5	68.3	48.5
10	1	0	−1	46.0	41.2	63.7	42.3
11	−1	0	1	51.4	48.4	69.1	47.7
12	−1	0	−1	44.6	40.0	60.4	40.1
13	0	0	0	48.9	44.3	64.8	46.8
14	0	0	0	48.8	45.7	66.4	45.1
15	0	0	0	47.8	46.1	67.5	45.2

Standard Stock Solution. Twenty milligrams of USP (U.S. Pharmacopoeia) folic acid was accurately weighed, and the folic acid was transferred into a 200 mL Pyrex conical flask containing 20 mL of 95% (v/v) ethanol and 50 mL of deionized water. To thoroughly dissolve the folic acid, the pH of the solution was adjusted to 10.0 with 0.1 N NaOH, and then the final pH was adjusted to 7.0 with 0.05 N HCl. After the solution was made up to 100 mL, aliquots were transferred to 10 mL Pyrex tubes with screw caps to prevent evaporation of ethanol. The tubes were stored in a refrigerator at 4 °C. The standard is very stable but should be replaced after 6 months.

The purity of the standard solution was determined immediately by measuring the absorbance of diluted standard at 282 nm using phosphate buffer (0.1 M, pH 7.0) as a blank. Phosphate buffer (0.1 M, pH 7.0) was used to dilute the stock standard solution (0.2 mg/mL) in a 1:20 ratio (0.01 mg/mL). With the measured values of the standard stock solution and the blank, the purity was calculated by using the equations

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

where C = concentration of diluted stock standard (mg/mL) and

$$\text{purity of standard} = 100(\text{calcd } E^{1\%}_{1\mathrm{cm}}/\text{ref } E^{1\%}_{1\mathrm{cm}})$$

where ref $E^{1\%}_{1\ \mathrm{cm}} = 611.7$ (*19*).

Trienzyme Extraction and Total Folate Analysis. The trienzyme extraction used in this study followed AOAC Official Method 2004.05 (*5*). To accurately weighed 1 g samples were added 20 mL of 0.1 M, pH 7.8, phosphate buffer and enough deionized water to give a final volume of 50 mL. After preheating at 100 °C for 15 min, the diluted samples were cooled to ambient temperature. One milliliter of Pronase (2 mg/mL in water, Calibiochem 53702, San Diego, CA) was added, followed by incubation at 37 °C for 3 h. The samples were heated at 100 °C for 3 min and then cooled at the end of Pronase digestion to inactivate the enzyme. The samples were digested with 1 mL of α-amylase (20 mg/mL in water, Fluka 10065, St. Louis, MO) at 37 °C for 2 h. Four milliliters of chicken pancreas conjugase (Difco 245910, Sparks, MD) solution (5 mg/mL in water) was added for conjugase digestion. After 16 h of incubation at 37 °C, the digests were heated at 100 °C for 3 min, cooled, and adjusted to pH 4.5 with HCl. The sample digests were taken to a volume of 100 mL with deionized water and filtered through ashless filter paper (Whatman no. 1, 18.5 cm). Total folate was determined microbiologically using a 96-well microplate technique according to the procedures of Tamura (*20*).

Experimental Design. Response surface methodology (RSM) was employed to optimize the trienzyme extraction for folate in cereals. A fractional three-level−three-factor experimental design with three replicates at the centerpoint (*21*) 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 cereal samples including CRM121 (wholemeal flour), oat flour, buckwheat flour, and triticale flour. The digestion times of each enzyme (independent variables) were coded at three levels (−1, 0, and 1), and the digestion time of each level was selected on the basis of preliminary experiments (**Table 1**). Response surface experimental design is provided in **Table 2**. The complete experimental design consisted of 15 experimental points.

Data Analysis. Data analysis was conducted to predict the following second-order polynomial model through the response surface regression (RSREG) procedure of statistical analysis system, SAS (version 9.00, SAS Institute Inc., Cary, NC):

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

In eq 1, Y is response (total folate content, μg/100 g of sample), β_0, β_i, β_{ii}, and β_{ij} are constant coefficients, and X_i are the uncoded independent variables. Regression analysis and analysis of variance (ANOVA) were used to predict the model. RIDGE MAX in the RSREG SAS output was used to compute the estimated ridge of maximum response for increasing radii from the center of the original design (*22*). Ridge analysis computes the estimated ridge of maximum response by increasing radii from the center of the original design (*23*). Sigma Plot (version 9.0, Systat Software, Inc., San Jose, CA) was used to create response plots by holding constant one variable of the second-order polynomial equation. The three-dimensional representation of the response surface is the graphical representation of the regression equation, showing the optimum values of the variables where the response is maximized (*24*). Minitab Statistical Software (version 15, Minitab Inc., State College, PA) was used for Tukey's pairwise comparison at the 5% level.

Table 3. Regression Coefficients of the Predicted Second-Order Polynomial Model

	CRM121			oat flour			triticale flour			buckwheat flour		
constant coeff[a]	est[b]	SE[c]	p[d]	est	SE	p	est	SE	p	est	SE	p
β_0	32.4	3.7	0.0003	29.7	3.4	0.0003	38.7	4.4	0.0003	29.6	3.6	0.0004
β_1	6.3	5.7	0.3211	5.2	6.6	0.4638	7.9	7.3	0.3262	8.4	7.1	0.2884
β_2	5.7	2.1	0.0401	5.8	1.7	0.0201	36.0	7.3	0.0043	18.8	7.1	0.0451
β_3	2.3	1.0	0.0810	3.2	1.2	0.0462	4.4	1.3	0.0220	2.1	1.1	0.1067
β_{11}	−1.6	3.6	0.6834	−2.4	4.5	0.6156	−1.0	4.8	0.8512	−5.0	4.8	0.3531
β_{22}	−1.2	0.4	0.0419	−1.5	0.5	0.0374	−27.2	4.8	0.6545	−11.3	4.8	0.0667
β_{33}	−0.1	0.1	0.3482	−0.2	0.1	0.3125	−0.2	0.1	0.0024	−0.1	0.1	0.3774
β_{12}	−0.1	1.1	0.9091	−0.5	1.4	0.7145	1.8	3.8	0.0827	0.6	3.8	0.8747
β_{13}	−1.1	0.6	0.0970	−0.2	0.7	0.7872	−1.6	0.8	0.3288	−0.4	0.7	0.6292
β_{23}	0.2	0.2	0.3482	−0.05	0.3	0.8700	0.8	0.8	0.1741	1.2	0.7	0.1572

[a] β_0 intercept and β_1, β_2, and β_3 constant coefficients of incubation time of Pronase, α-amylase, and conjugase, respectively. [b] Estimated value. [c] Standard error. [d] Pr > |t|.

Model Verification. The certified value of CRM121 was compared to the analytical values with the optimized extraction. The literature values of oat flour, buckwheat flour, triticale flour, and whole wheat flour were compared to the analyzed values with the optimized extraction. Breakfast cereal samples were assayed, and the values with the optimized extraction were compared to the data obtained with AOAC Official Method 2004.05 (*5*) to confirm that the optimized extraction would work for highly fortified cereal products.

RESULTS AND DISCUSSION

Predicted Second-Order Polynomial Models. Total folate levels measured in CRM121, oat flour, triticale flour, and buckwheat flour from the 15 sets of variable combinations (**Table 2**) were fit into the second-order polynomial equation (eq 1) by the RSREG procedure. The estimated values of constant coefficients are given in **Table 3**, and the regression models for CRM121 (wholemeal flour), oat flour, triticale flour, and buckwheat flour are predicted as follows:

CRM121 (wholemeal flour)

$$Y = 32.4 + 6.3X_1 + 5.7X_2 + 2.3X_3 - 1.6{X_1}^2 - 1.2{X_2}^2 - 0.1{X_3}^2 - 0.1X_1X_2 - 1.1X_1X_3 + 0.2X_2X_3 \quad (2)$$

oat flour

$$Y = 29.7 + 5.2X_1 + 5.8X_2 + 3.2X_3 - 2.4{X_1}^2 - 1.5{X_2}^2 - 0.2{X_3}^2 - 0.5X_1X_2 - 0.2X_1X_3 + 0.05X_2X_3 \quad (3)$$

buckwheat flour

$$Y = 29.6 + 8.4X_1 + 18.8X_2 + 2.1X_3 - 5.0{X_1}^2 - 11.3{X_2}^2 - 0.1{X_3}^2 + 0.6X_1X_2 - 0.4X_1X_3 + 1.2X_2X_3 \quad (4)$$

triticale flour

$$Y = 38.7 + 7.9X_1 + 36.0X_2 + 4.4X_3 - 1.0{X_1}^2 - 27.2{X_2}^2 - 0.2{X_3}^2 + 1.8X_1X_2 - 1.6X_1X_3 + 0.8X_2X_3 \quad (5)$$

In the preceding equations, X_1 is the Pronase digestion time, X_2 is the α-amylase digestion time, and X_3 is the conjugase digestion time.

The data in **Table 3** show that β_1, β_2, and β_3, constant coefficients of incubation time of Pronase, α-amylase, and conjugase, respectively, indicate linear effect of variables. They are all positive, indicating linear effects to increase folate content (Y). From all of the predicted models, β_2 and β_3 had small p values (**Table 3**) and β_1 had a large p value ($p > 0.2$). p values determine the significance of each coefficient, and the higher significance of the corresponding coefficient is indicated by decreasing p values (*25*). Therefore, it can be said that the linear effects of incubation time of α-amylase and conjugase are significant, whereas the linear effect of incubation time of Pronase is not. For quadratic effects, each β_{22} of the predicted models for all cereals except triticale flour is significant ($p < 0.1$) (**Table 3**). For other quadratic coefficients (β_{11} and β_{33}), only triticale flour shows significance ($p < 0.05$).

Table 4. Analysis of Variance for the Second-Order Response Surface Model

		sum of squares			
source of variation	df	CRM 121	oat flour	triticale flour	buckwheat flour
model	9	78.5[a]	153.7[a]	216.6[a]	192.8[a]
linear	3	68.8[a]	143.8[a]	166.2[a]	180.0[a]
quadratic	3	5.9[b]	9.6[b]	42.4[c]	8.7[b]
cross-product	3	3.8[b]	0.3[c]	8.0[b]	4.1[b]
lack of fit	3	2.8[b]	3.9[b]	2.5[b]	4.6[b]
pure error	2	0.8	1.6	3.8	1.8
total error	5	3.6	5.5	6.3	6.4
R^2		0.956	0.966	0.972	0.968

[a] Significant at the 1% level ($p < 0.01$). [b] Not significant ($p > 0.1$). [c] Significant at the 5% level ($p < 0.05$).

Table 5. Analysis of Variance Showing Significance of the Variables on Responses

		sum of squares			
independent variable[a]	df	CRM 121	oat flour	triticale flour	buckwheat flour
Pronase	4	3.5[b]	1.4[b]	8.5[b]	3.4[b]
α-amylase	4	30.6[c]	50.4[c]	70.3[c]	89.3[c]
conjugase	4	48.7[d]	102.8[c]	146.3[c]	104.2[c]

[a] Pronase,digestion time at 2 mg/mL; α-amylase, digestion time at 20 mg/mL; conjugase, digestion time at 20 mg/4 mL. . [b] Not significant ($p > 0.1$). [c] Significant at the 5% level ($p < 0.05$). [d] Significant at the 1% level ($p < 0.01$).

The analysis of variance (**Table 4**) indicates that the predicted models are all significant at the 1% level. Each R^2, the coefficient of determination, is >0.95, indicating that the model was adequate with no significant lack of fit.

From analysis of variance, incubation times of α-amylase ($p < 0.05$) and conjugase ($p < 0.01$) show significant effects on the response (total folate), whereas Pronase digestion was not significant ($p > 0.1$) for the optimization of enzyme extractions for total folate in cereals (**Table 5**).

Analysis of Response Surface. Figure 1 shows the relationship between the incubation time of α-amylase and conjugase (significant, independent variables) and total folate (dependent variable), holding the insignificant, independent variable constant (Pronase = 1 h). Over 80% of maximum folate can be

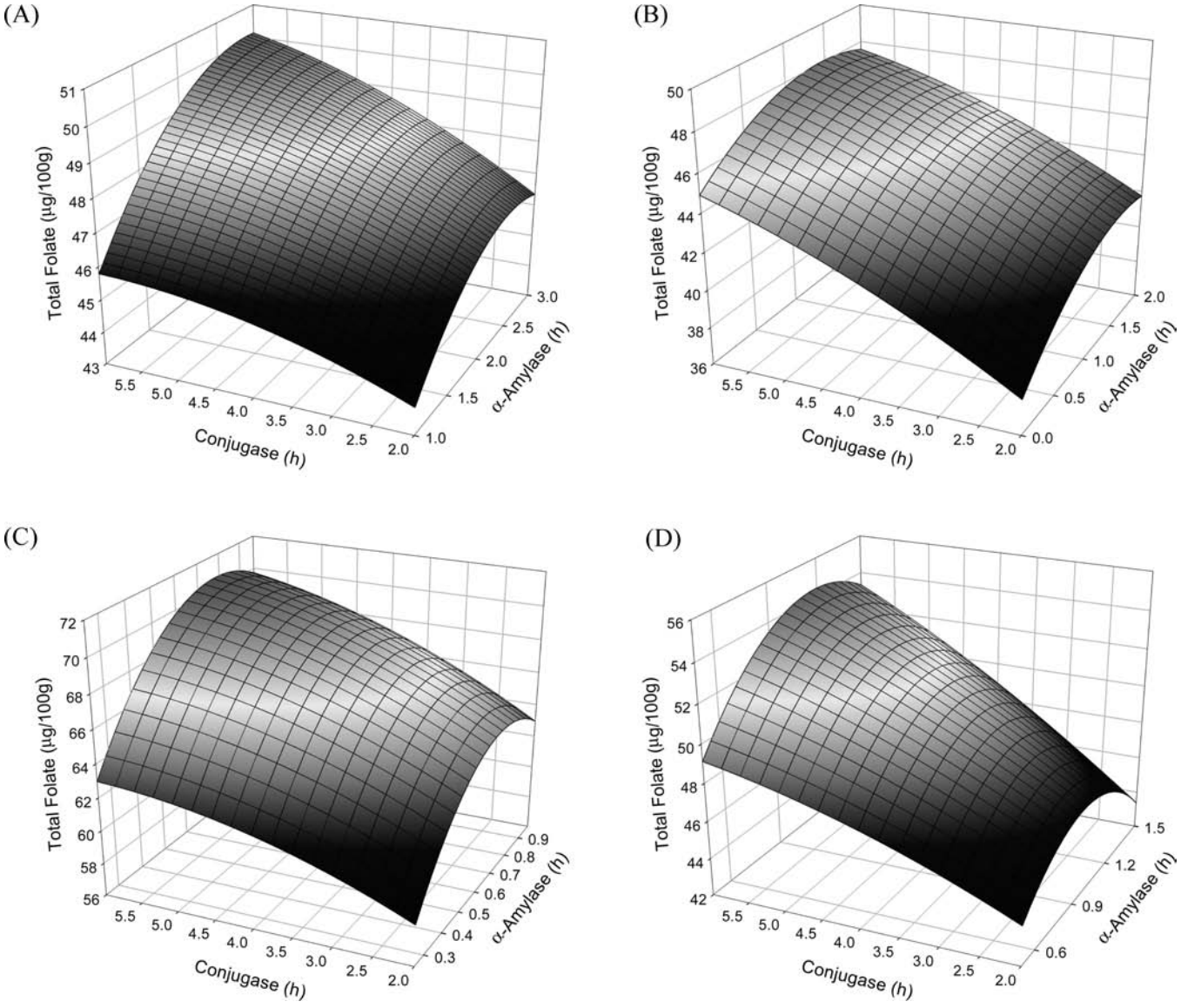

Figure 1. Response surface graphs for the effects of α-amylase and conjugase digestion time on total folate assay of (**A**) CRM 121 (wholemeal flour), (**B**) oat flour, (**C**) triticale flour, and (**D**) buckwheat flour. Pronase digestion was held constant at 1 h.

analyzed within the shortest incubation times for α-amylase and conjugase (coded at −1 level). Deconjugation is primarily known to occur within the first 1 h of incubation (*26*). However, because poly-γ-glutamyl folates commonly exist in foods (*6*), incubation with conjugase for >1 h is still very important to ensure complete deconjugation (*17*, *18*). From previous studies, the optimized conjugase digestion times for vegetables and legumes were 3 and 5 h, respectively (*17*, *18*). With increased digestion times longer than the optimal times, α-amylase and conjugase digestion led to lower measurable total folate in CRM121 (wholemeal flour) and triticale flour. α-Amylase digestion longer than the optimal time led to decreased total folate in buckwheat flour; however, the effect was not noted for conjugase digestion. Similarly, previous studies (*17*, *18*) showed that longer incubation times of Pronase, α-amylase, and conjugase resulted in decreased folate values, most likely from destruction of folate by increasing the exposure of folate to oxidation and other deleterious conditions potentially present during extended extraction.

Optimal Conditions and Model Verification. The optimal incubation times for the determination of total folate for the cereals assayed in this study using Pronase, α-amylase, and conjugase were determined by ridge analysis (**Table 6**). Total digestion times indicated by the optimal times for the three enzyme digestions ranged from 6.1 h for buckwheat flour to 8.7 h for CRM 121 (wholemeal flour), representing times much shorter than the 3, 2, and 16 h digestions (21 h) required by AOAC Official Method 2004.05 for Pronase, α-amylase, and conjugase, respectively. The predicted maximal values were close to published values (*27*, *28*). The predicted maximal value for CRM 121 (51 μg/100 g) was within the acceptable range (50 ± 7 μg/100 g) of the certified value.

Although optimal extractions must be determined for each food matrix, a generalized extraction procedure suitable for approximation of folate in most cereal matrices can be determined from the optimal conditions presented here for the four flours. For example, by choosing the longest optimal incubation time for each enzyme given in **Table 6** that ensures maximal release of folate, a generalized extraction procedure of 0.7 h for Pronase, 2.5 h for α-amylase, and 5.9 h for conjugase digestion should be suitable for most cereals or cereal products. These conditions would ensure maximal release of folate and guard against folate loss that might occur with longer digestions. In practical terms, digestion times of 1, 2.5, and 6 h for Pronase, α-amylase, and conjugase, respectively, represent a laboratory-friendly digestion sequence adaptable to a single work day.

Table 6. Ridge Maximum Analysis with Predicted Maximum Folate

	h, df = 3			total folate (μg/100 g ± SD)	
	Pronase	α-amylase	conjugase	maximum folate[d]	certified or literature value
CRM121	0.5[a]	**2.5**[b]	5.7[c]	51	50 ± 7[e]
oat flour	0.6[a]	1.4[c]	5.8[c]	49	46[f]
buckwheat flour	**0.7**[a]	0.8[c]	4.6[c]	52	54 ± 7[g]
triticale flour	0.6[a]	0.7[c]	**5.9**[c]	71	74 ± 6[g]

[a] Not significant ($p > 0.1$). [b] Significant at the 5% level ($p < 0.05$). [c] Significant at the 1% level ($p < 0.01$). [d] Determined from the response surface regression (RSREG) procedure. [e] Certified reference value. [f] Value from Fineli (Finnish Food Composition database) maintained by National Public Health Institute of Finland analyzed by HPLC (*27*). [g] Values from USDA National Nutrient Database for Standard Reference, release 22 (*28*).

Table 7. Comparison of Folate Contents from Cereals and Breakfast Cereals Measured by a Generalized Optimal Extraction and AOAC Official Method 2004.05

	total folate (μg/100 g ± SD)	
cereals[a]	generalized optimal digestion[b]	AOAC Official Method 2004.05
CRM121	50 ± 3.4	48 ± 2.5
oat flour	48 ± 2.0	47 ± 2.7
buckwheat flour	53 ± 4.0	57 ± 1.0
triticale flour	72 ± 4.1	71 ± 5.4
whole wheat flour	46 ± 3.9	43 ± 2.4
oat breakfast cereal	1224 ± 41.9	1206 ± 23.8
wheat breakfast cereal	625 ± 18.2	612 ± 29.5
wheat, corn, oat breakfast cereal	552 ± 42.0	547 ± 17.2

[a] Triplicate runs. [b] Not significantly different ($p > 0.1$) from AOAC Official Method 2004.05.

The utility of this generalized extraction for cereals was verified by assaying the four flours used in this study by the generalized digestion procedure and by AOAC Official Method 2004.05 (*5*). Additionally, to ensure that the procedure is applicable to highly fortified cereal products, three commercial breakfast cereals with different fortification levels were assayed by the two methods. As shown in **Table 7**, except for buckwheat flour, the generalized procedure gave slightly higher, although not significantly different, values ($p > 0.1$) compared to AOAC Official Method 2004.05.

In summary, application of response surface methodology together with ridge analysis provided optimal digestions for the trienzyme extraction of total folate for CRM121 (wholemeal flour), oat, triticale, and buckwheat flours that provide data comparable to AOAC Official Method 2004.05. The optimized extractions provide considerable time savings compared to the official method and ensure against folate loss that can occur with prolonged digestions. A generalized extraction determined from the optimal digestion procedures for the specific cereals of 1, 2.5, and 6 h for Pronase, α-amylase, and conjugase digestions, respectively, would be suitable to approximate total folate in most cereal matrices.

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