Protein and Apparent Amylose Contents of Milled Rice by NIR-FT/Raman Spectroscopy

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ABSTRACT

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The chemometric calibration of near-infrared Fourier-transform Raman (NIR-FT/Raman) spectroscopy was investigated for the purpose of providing a rigorous spectroscopic technique to analyze rice flour for protein and apparent amylose content. Ninety rice samples from a 1996 collection of short, medium, and long grain rice grown in four states of the United States, as well as Taiwan, Korea, and Australia were investigated. Milled rice flour samples were scanned in rotating cups with a 1,064 nm (NIR) excitation laser using 500 mW of power. Raman scatter was collected using a liquid N_2 cooled Ge detector over the Raman shift range of 175–3,600 cm^{-1}. The spectral data was preprocessed using baseline correction with and without derivatives or with derivatives alone and normalization. Nearly equivalent results were obtained using all of the preprocessing methods with partial least squares (PLS) models. However, models using baseline correction and normalization of the entire spectrum, without derivatives, showed slightly better performance based on the criteria of highest r^2 and the lowest SEP with low bias. Calibration samples (n = 57) and validation samples (n = 33) were chosen to have similar respective distributions for protein and apparent amylose. The best model for protein was obtained using six factors giving r^2 = 0.992, SEP = 0.138%, and bias = –0.009%. The best model for apparent amylose was obtained using eight factors giving r^2 = 0.985, SEP = 1.05%, and bias = –0.006%.

The protein and amylose contents of rice are important factors in the estimation of the quality of rice for various markets. However, the chemical analyses that have been traditionally used for the determination of these factors are environmentally unfriendly, time-consuming, and fraught with errors. The substitution of combustion analysis (Approved Method 46-30, AACC 2000) for the Kjeldahl method (AACC Method 46-11A) in the determination of nitrogen content has reduced some of the environmentally unfavorable aspects of protein determination. However, combustion analysis is still time-consuming and suffers from a small bias relative to the Kjeldahl method. The determination of amylose content by the iodine complexing method requires defatting with methanol, gelatinization, and subsequent colorimetric (Williams et al 1958), amperometric (Larson et al 1953), or potentiometric (Bates et al 1943) titration measurement against a standard curve. Automation of the colorimetric determination of amylose content has greatly improved precision (Webb 1972). However, the accuracy of these methods is so much in doubt that the term "apparent amylose content" is used for the amylose content determined in this manner. This is because long linear chains of α–(1$\rightarrow$4) glucans that occur in the more α–(1$\rightarrow$6) branched amylopectin cannot be distinguished from those that make up the generally linear amylose. Linear glucan chains from both sources form an iodine complex, thus introducing error in the method.

The use of spectral analyses that are more rapid, are not subject to many of these errors, and do not produce chemical waste has been sought. Diffuse reflectance near-infrared spectroscopy (NIRS) has been used as the spectral method to overcome these problems (Delwiche et al 1996). Raman spectroscopy also has the potential to serve this purpose and provide some other advantages. In the past, dispersive Raman spectroscopy has shown sufficient resolution to assess the subtleties of various amylose polymorphic structures (Cael et al 1972). Raman spectroscopy provides structural details on the level of the mid-infrared region but with even fewer overlapping bands. Due to the fact the Raman effect is based on the polarizability of bonds and not their dipoles, it is relatively insensitive to water. Near-infrared Fourier-transform Raman (NIR-FT/Raman) spectroscopy provides some additional advantages over visible wavelength excited dispersive Raman (Chase 1986; Weesner and Longmire 2001). As a Fourier-transform method, it has the advantage of excellent frequency precision. In addition, the use of NIR excitation reduces the interference from fluorescence, which is often experienced when analyzing pigmented materials with Raman spectroscopy. All of these factors suggest that it should be well suited for the analysis of a complex but almost colorless natural material such as rice flour. The use of NIR-FT/Raman spectroscopy for the determination of the amylose content in isolated starch has had some limited success (Phillips et al 1999). Preliminary work with milled rice has demonstrated the potential of the method (Barton et al 1997, 2000; Himmelsbach et al 2000). The purpose of this study was to optimize an NIR-FT/Raman spectroscopic method for the measurement of milled rice flour quality based on determination of protein and apparent amylose contents.

MATERIALS AND METHODS

Samples

Ninety rice samples of individual cultivars of long, medium, and short grains were used. Seventy-eight were obtained from breeders in four states in the United States (Arkansas, California, Louisiana, and Texas) and three were obtained from Taiwan, three from Korea, and six from Australia. All U.S. samples were shelled using a Satake rice machine, model SB, and then immediately milled. Milling was accomplished using a laboratory Satake one pass mill (pearler, model SKD). The first pass was with a 50-g weight in the 5th position; the second pass was with a 50-g weight in the 3rd position. Finally, the samples were ground using a Satake rice grinder. All overseas samples were milled under comparable conditions using commercial procedures.

Samples of isolated amylose and amylopectin from potato (Sigma, St. Louis, MO) were used as reference materials. The amylopectin was used without further purification. The amylose was subjected to continuous Soxhlet extraction with refluxing methanol for 3 hr to remove the residual butanol and then dried overnight in vacuum at 60°C to remove the methanol and then allowed to equilibrate to ambient conditions.

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Reference Analyses

Protein (N × 5.95) was determined by combustion analysis using a Leco model FP-2000 nitrogen analyzer on duplicate 0.5-g assays of ground rice (Approved Method 46-30, AACC 2000). Analysis for protein gave a range of 4.89 to 11.35 ± 0.06% for the samples. Apparent amylose was determined by duplicate colorimetric assays using an autoanalyzer (Webb 1972). Analysis for apparent amylose content gave a range of 0.41 to 24.90 ± 0.25%. All replicates were averaged for use in the data analysis.

Raman Spectroscopy

Raman spectroscopy was conducted on a Nicolet 950 Raman bench using a 1,064-nm NIR laser source, a CaF2 beamsplitter, and a liquid N_2 cooled Ge detector. Samples were placed in "spinning" cups and slowly rotated during data acquisition using a locally manufactured sample-handling device. Raman scatter was collected using the 180º reflective mode with 500 mW of laser power and 128 scans at 16 cm^{-1} resolution. All data was collected using Nicolet Omnic software (v. 3.1). Duplicate spectra were collected on each, then averaged to produce a single spectrum for each sample.

Data Processing

Averaged spectral data files were truncated to 175–3,600 cm^{-1} Raman shift (Stokes region) and preprocessed for chemometic analysis both within and outside of the Unscrambler chemometrics software package. One set of the spectral files was subjected to multipoint baseline removal and normalization using GRAMS/32 (v. 4.10, Galactic Industries Corp., Salem, NH). Baseline removal was accomplished using a locally developed Array Basic macro that automatically found the lowest points within six specified spectral ranges and leveled them to zero intensity. Spectral normalization on this data set was accomplished with another locally developed Array Basic macro that automatically measured the total integrated area of the spectrum from 235 to 3,600 cm^{-1} and normalized it. The integration and normalization eliminated the effects of residual fluorescence and instrumental fluctuations while retaining the signal/noise. Both this set of files and an identical copy of the original files were converted to JCAMP-DX format and imported into the Unscrambler chemometrics software package.

Chemometric Analysis

Chemometric analysis was performed using Unscrambler (v. 7.51, CAMO ASA, Oslo, Norway). Derivatization and normalization were employed to accomplish the same essential tasks as baseline correction and normalization. Copies of the original spectral files were converted to the 1st and 2nd derivatives with both Savitsky-Golay (Savitsky and Golay 1964; Steinier et al 1972; Madden 1978) and Norris (Norris and Williams 1984) methods. The Savitsky-Golay derivatives were fit with a 2nd-order polynomial with the loss of a data point from each end of the spectra. The Norris derivative used the second difference and a segment size of 3, which retains all of the data points. The resulting data was smoothed by a 3-point moving average as required to reduce noise. This preprocessed data plus that preprocessed in GRAMS/32, along with the apparent amylose and protein data, were subjected to a partial least squares type-1 (PLS1) analysis using separate calibration and validation sets. In all cases, the Raman spectra were mean centered. Samples of comparable distributions of values for protein and apparent amylose were used for calibration (n = 57) and validation (n = 33). The validation sets were selected by sorting the samples in increasing order of reference values, selecting every third sample plus an additional sample from each of the low, medium, and high value groups as validation samples. This ensured, in the case of amylose, that at least two samples were selected from the low-amylose group. All of the spectral preprocessing procedures described above were investigated for potential for generating suitable chemometric models for predicting protein and amylose contents of rice flour.

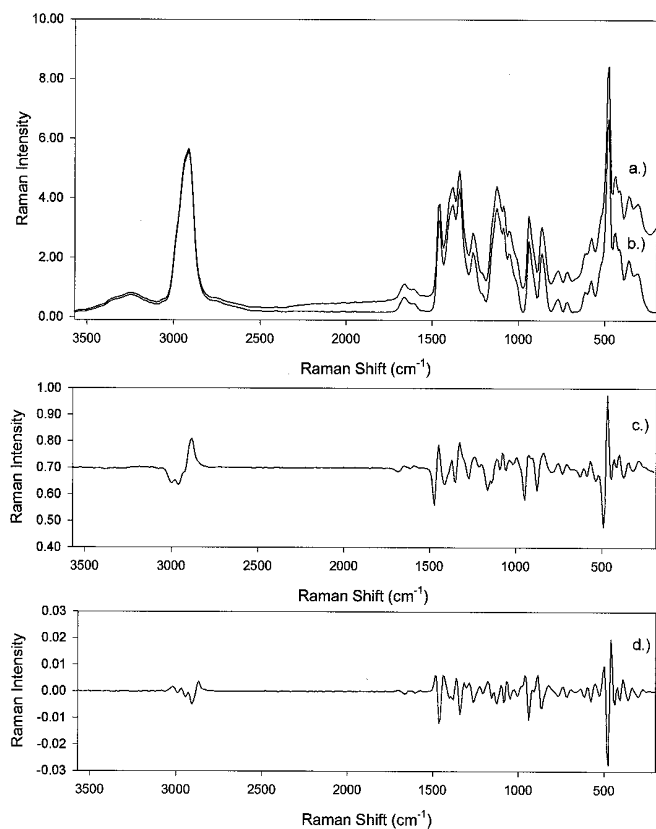

Fig. 1. NIR-FT/Raman spectrum of a rice flour collected at 16 cm^{-1} resolution, averaged (two repetitions) and truncated to the Raman shift range 175–3,570 cm^{-1} (Stokes region) a) without further processing, b) processed with baseline correction and normalization, and c) processed with 1st derivative and d) processed with 2nd derivative.

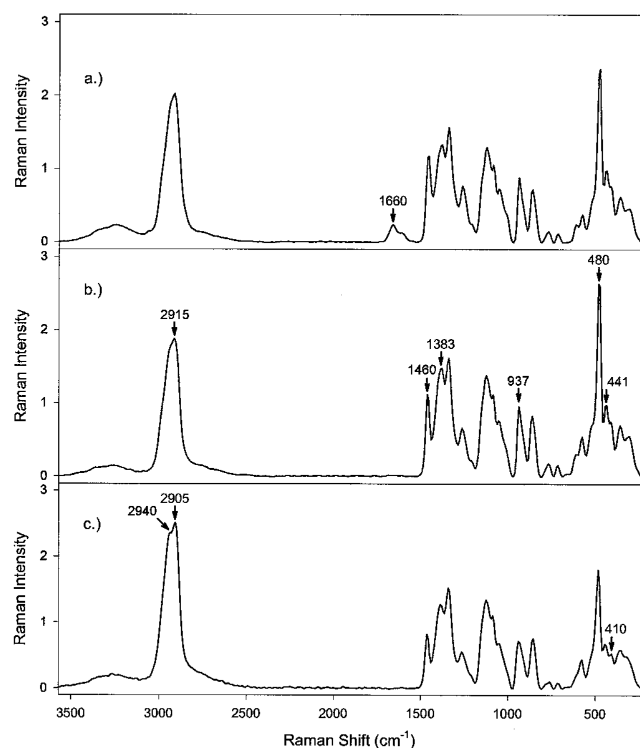

Fig. 2. Stacked plots of NIR-FT/Raman spectra of a) high-protein (11.35%), low-amylose (0.41%) rice flour; b) amylopectin; and c) amylose. Spectral bands that visibly show intensity differences related to levels of protein and amylose are labeled in cm^{-1}.

RESULTS AND DISCUSSION

Preliminary reports on the use of NIR-FT/Raman spectroscopy to measure constituent parameters of rice (Barton et al 1997, 2000; Phillips 1999; Himmelsbach et al 2000) indicated that the method had some advantages over other methods of analysis. However, some disadvantages of the method (poor sampling and residual fluorescence) had to be overcome. In addition, optimization of data treatments had to be conducted to ensure an entirely viable method.

Because Raman spectroscopy employs a laser, only a very small area of the material under investigation is normally sampled. This leads to relatively poor sampling and potential sample heating to the point of charring. Thus, to provide better sampling and to reduce sample heating, a sampling device was built to accommodate and rotate samples contained in cylindrical sample cups (38 mm i.d., 9 mm thick) with optical quartz windows. These are the same cups that are used for the spinning cup sampling device used in Foss-NIRSystems 6500 spectrometer. The use of this device, plus a defocusing lens provided with the Nicolet 950 Raman spectrometer, permitted effective sampling at 500 mW of laser power. In addition, a 16 cm^{-1} resolution was optimal for retaining the essential spectral features and providing sufficient signal/noise with only 128 scans, which took 72 sec for data acquisition.

Figure 1a shows a typical Raman spectrum obtained after averaging two sample repetitions and truncating the spectral range to 175–3,570 cm^{-1} with the instrumental setup described. Relatively sharp bands in the 175–1,800 cm^{-1} region and two broader bands in the 2,500–3,500 cm^{-1} region, plus a sloping baseline, probably due to residual fluorescence, generally characterized this spectrum. Figure 1b shows the same spectrum with multipoint baseline correction applied to remove the effects of fluorescence. Figures 1c and d show the use of the application of the 1st and 2nd derivatives to the spectrum in Fig. 1a. The application of derivatives removed the sloping baseline but at the expense of signal/noise (note the difference in ordinate scales) and the loss of broad components of the spectra. The loss of signal by the application of the 2nd derivative was so severe that the intensity of the strong band at 480 cm^{-1} was reduced to only 0.007 of its original intensity. This greatly increased the detrimental effect of noise on calibrations based on the use of the 2nd derivative as a preprocessing treatment.

Figure 2 shows the comparison of the Raman spectrum of a high-protein low-amylose rice with that of purified samples of amylose and amylopectin. The presence of protein in rice is easily detected (Fig. 2a) by the amide I band at ≈1,660 cm^{-1} with a shoulder at 1,613 cm^{-1}, indicative of aromatic amino acids (Parker 1983). On the other hand, amylopectin (Fig. 2b) and amylose (Fig. 2c) cannot be distinguished by well-separated vibrational bands but primarily by differences in the relative intensity or shape of common bands. Comparison of spectra reveals a CH_2 stretching band at 2,905 cm^{-1} with a shoulder at 2,940 cm^{-1} in amylose compared with a lower intensity band at 2,915 cm^{-1} with a less pronounced shoulder in amylopectin for the same type of vibration. Amylopectin shows greater intensity for bands at 441, 480, 937, 1,383, and 1,460 cm^{-1} that are mainly related to C-C-O, CH, and CH_2 bending vibrations (Parker 1983). Amylose shows a more pronounced band at 410 cm^{-1} due to an unassigned vibration. Because these variations are very subtle in the rice samples and the detection of amylose versus amylopectin is facilitated by the analysis of multiple bands, multivariate techniques are necessary to highlight these differences and make determination of amylose contents by Raman spectroscopy possible. As long as multivariate techniques were being applied to one component, it was convenient to apply to the other. Thus, they were employed for the prediction of both protein and amylose contents of rice.

Table I shows the results obtained by applying the multivariate method of partial least squares (PLS) modeling to various calibration setups for protein content and the associated validation results. These calibrations include those with no correction, baseline correction, 1st derivative, and 2nd derivative. Results were generally im-

TABLE I
Summary of PLS Modeling Results for Protein

Calibration Parameters[a]	No. of Factors	Validation Results		
		r^2	SEP (%)	Bias (%)
No correction, MN, SM, MC	11	0.988	0.171	0.029
Basl. corr., EN, R, SM, MC	6	0.992	0.138	–0.009
No basl. corr. 1st SG Deriv.,-MN, SM,MC	6	0.988	0.164	–0.000
No basl. corr. 2nd SG Deriv., R, MN, 2SM, MC	5	0.985	0.179	–0.039
No basl. corr. 1st N Deriv.,-R, MN, 2SM, MC	7	0.991	0.157	–0.033
No basl. corr. 2nd N Deriv.,-2SM, MN, MC	5	0.989	0.154	–0.011
Basl. corr., EN w/ 1st SG Deriv.-R, SM, MC	4	0.992	0.140	–0.057
Basl. corr., EN w/ 2nd SG Deriv.-R, 2SM, MC	5	0.989	0.163	–0.045
Basl. corr., EN w/ 1st N Deriv.-R, SM, MC	4	0.992	0.144	–0.063
Basl. corr., EN w/ 2nd N Deriv.-R, 2SM, MC	4	0.991	0.148	–0.065

[a] EN = External normalization (GRAMS/32), MN = mean normalization (Unscrambler), MC = mean centering, R = reduced data region (200–1795 and 2505–3570), SM = 3 pt Moving Average Smooth, Basl. Corr. = baseline correction, Deriv. = derivative, SG = Savitsky-Golay, N = Norris.

TABLE II
Summary of PLS Modeling Results for Amylose

Calibration Parameters[a]	No. of Factors	Validation Results		
		r^2	SEP (%)	Bias (%)
No correction, MN, SM, MC	12	0.984	1.07	0.064
Basl. corr., EN, R, MC	8	0.985	1.05	–0.006
No basl. corr. 1st SG Deriv.-R, SM, MN, MC	9	0.983	1.12	0.119
No basl. corr. 2nd SG Deriv.-R, 2SM, MN, MC	6	0.979	1.23	0.268
No basl. corr. 1st N Deriv.-R, 2SM, MN, MC	8	0.981	1.17	–0.039
No basl. corr. 2nd N Deriv.-R, 2SM, MN, MC	8	0.985	1.09	0.150
Basl. corr., EN w/ 1st SG Deriv.-R, 2SM, MC	8	0.983	1.10	0.068
Basl. corr., EN w/ 2nd SG Deriv.-R, 3SM, MC	6	0.974	1.42	–0.056
Basl. corr., EN w/ 1st N Deriv.-R, 2SM, MC	7	0.980	1.16	–0.002
Basl. corr., EN w/ 2nd N Deriv.-R, 2SM, MC	8	0.984	1.06	–0.006

[a] EN = External normalization (GRAMS/32), MN = mean normalization (Unscrambler), MC = mean centering. R = reduced data region (200–1795 and 2505–3570), SM = 3 pt. Moving Average Smooth, Basl. Corr. = baseline correction, Deriv. = derivative, SG = Savitsky-Golay, N = Norris.

proved by eliminating spectral regions where no bands were present. Thus, the regions of the Raman spectra from 1,800 to 2,500 cm^{-1} and from 175 to 200 cm^{-1} were typically excluded from the spectral data set. Also, results were improved by applying three-point moving average smoothing to the data. The best calibration was that with the highest r^2 and the lowest standard error of prediction (SEP) with low bias, based on the test validation set. Thus, the tabulated data in Table I reveals that the best calibration obtained for protein was produced using the baseline correction and normalization routine developed in GRAMS/32. This resulted in using six principal components (PC) or factors giving $r^2 = 0.992$, SEP = 0.138%, and bias = –0.009%. In all respects, this was better than the model produced with no baseline correction but with mean normalization (from Unscrambler), one smoothing treatment, and mean centering of the data. The use of derivatives (either Savistky-Golay or Norris type) without baseline correction of the data generally reduced the number of factors but did not increase the r^2 nor appreciably reduce the SEP compared with that of the original, nonbaseline corrected, data. The only exception to this was the use of the 2nd derivative of the Norris type, which was accomplished by performing the 1st derivative twice. This reduced the number of factors to five, slightly increased the r^2 value, and reduced the SEP with nearly the same bias. Further treatment of the baseline corrected data with derivatives typically reduced the number of factors to four and generally gave comparable SEP values but with consistently increased bias. The lack of improvement of the results with the use of derivatives is probably related to the loss of signal as noted from Fig. 1, thus causing a greater interference from noise. The result obtained with baseline correction is on a par with results that have been reported for NIRS, using 16 factors with 2nd derivative mathematics (r^2 = 0.989, SEP = 0.107%, and bias = 0.004%) (Delwiche et al 1996). The fact that fewer than half the number of factors are required for the Raman method should lead to more robust calibrations. Also, because fewer factors were necessary in the Raman calibration, fewer calibration samples are required. Thus, using 57 samples for calibration permitted using 33 samples out of a total of 90 for validation.

The result of comparing the laboratory measured values for protein content versus that predicted from the Raman spectra using the selected model is shown in Fig. 3. All predicted values were within the 95% confidence lines (dashed lines).

Table II shows the results of using essentially analogous models to predict apparent amylose content that were used for prediction of protein content. Again, the use of baseline correction and normalization produced the best results. Two more factors, eight total, were required, yielding a model with validation statistics of r^2 = 0.985, SEP = 1.05%, and bias = –0.006%. In this case, the best result was obtained without smoothing of the data. This would appear to indicate that spectral resolution is important in the determination of amylose. Again, the use of derivatives did not improve the SEP, even though the number of factors required was reduced. However, the use of the 2nd derivative of the Norris type with nonbaseline corrected data may be an acceptable alternative employing baseline correction. This may be attractive due to its easier implementation. There appears to be no advantage in using the Norris type derivative on the baseline corrected data because almost identical results were obtained without its use. The result obtained with baseline correction and normalization almost mimics the results that have been reported for NIRS using 18 factors with 2nd deriva-

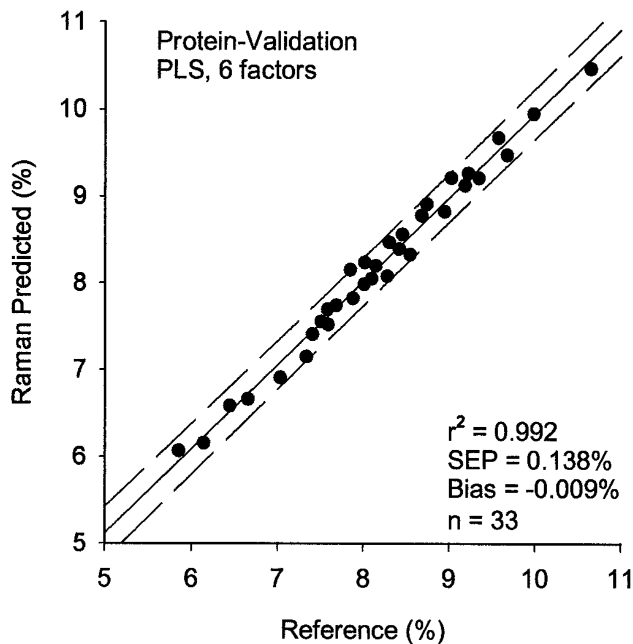

Fig. 3. Plot of % protein predicted by Raman spectroscopy (200–1,795 cm^{-1} and 2,050–3,570 cm^{-1}) vs. that measured by combustion analysis using six factors for calibration model. Dashed lines indicate 95% confidence limits.

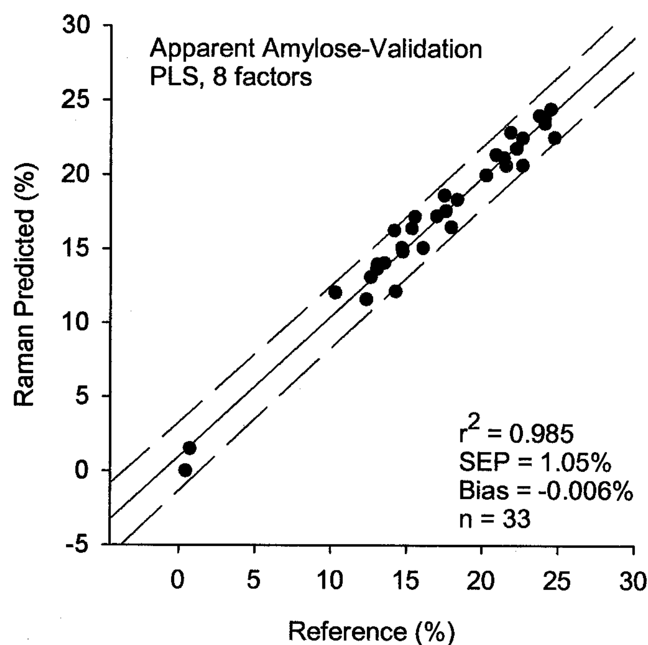

Fig. 4. Plot of % amylose predicted by Raman spectroscopy (200–1,795 cm^{-1} and 2,050–3,570 cm^{-1}) vs. that measured by colorimetric assay using eight factors for calibration model. Dashed lines indicate 95% confidence limits.

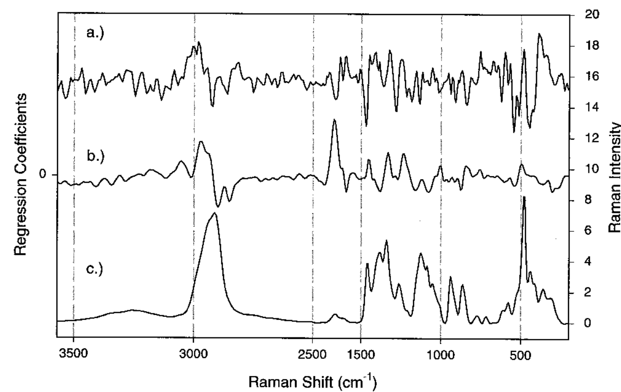

Fig. 5. Plot of regression coefficients (scale on left) vs. Raman Shift for baseline corrected and normalized model for a) apparent amylose (with positive offset) and b) protein compared to c) Raman spectrum of a high-amylose (24.9%) rice sample (with negative offset).

tive mathematics (r^2 = 0.956, SEP = 1.04%, and bias = −0.077%) (Delwiche et al 1996). Again, the Raman approach requires fewer factors for the calibration. The general requirement for using more factors in the amylose model versus the protein model is not surprising because amylose and amylopectin spectra are so similar (Fig. 2).

Figure 4 shows the comparison of laboratory-measured values for apparent amylose content versus that predicted from the Raman spectra using the selected model. Only four samples of waxy (low amylose) rice were available for this study, thus, they were underrepresented in the data set. These were split evenly between the calibration and validation sets. Regardless, the error in prediction of the apparent amylose content of these samples is on a par with the remainder of the sample set.

Figure 5 shows the resulting regression coefficients for the selected amylose model (Fig. 5a) and those for the selected protein model (Fig. 5b) versus the Raman spectrum, eliminating the region between 1,800–2,500 cm^{-1} and (Fig. 5c) of a high-amylose sample (24.9% amylose and 7.59% protein). This generally indicated that the Raman Shift variables identified in Fig. 1 were those with the largest regression coefficients. The exception to this is the low correlation of amylose content to the CH_2 stretching band maximum 2,905 cm^{-1} but positive correlation to the higher frequency side of this band. Thus, the subtle differences band shape appeared to be more important than just band intensity. This type of vibration also seems to be important for protein in addition to the amide I band at 1,660 cm^{-1}.

CONCLUSIONS

Based on these results, a NIR-FT/Raman spectroscopic method optimized by removal of fluorescence effects has the potential to be a viable method for the prediction of the protein and apparent amylose contents of rice flour from diverse sources. If the Raman data is preprocessed with multipoint baseline correction and normalization, fewer factors than for NIRS are required to provide comparable results. The Raman spectra are relatively immune to moisture effects, are easy to interpret, and possess sufficient resolution by which to distinguish the subtle differences between amylose and amylopectin in native starch. In these respects, it has an advantage over NIRS in providing a rigorous, robust, yet still rapid, noninvasive, and environmentally friendly method of analysis for rice flour. This method should be easily extendable to the determination of protein and apparent amylose in flours of other grain types.

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