Apparent Amylose Content of Milled Rice by Near-Infrared Reflectance Spectrophotometry

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ABSTRACT

Amylose content is considered to be the most important factor influencing cooking and processing characteristics of rice. Traditional methods for measurement entail titrimetric, amperometric, or colorimetric measurement of an iodine-binding complex of solubilized starch, but these methods are time-consuming and prone to random error. A method for determining apparent amylose content (AAC), based on the near-infrared (NIR) reflectance spectrum (1,100–2,498 nm) of ground milled rice, was developed on a diverse population of 50 commercial rice varieties or experimental lines grown in the five major rice-producing states of the United States. Three laboratories possessing NIR spectrophotometers participated in a cooperative study. Each laboratory ground, scanned, and developed AAC models by partial least squares analysis, using common sets of samples for calibration (n = 150) and validation (n = 97). Validation set statistics typically indicated r^2 = 0.95 and standard error = 1.0% AAC for models consisting of 16–18 factors. Within-laboratory repeatability error of the NIR method was comparable to the reference colorimetric method. Between-laboratory reproducibility error (0.5% AAC) indicated that the NIR models were consistent across laboratories. Such accuracies are considered sufficient for selection in rice breeding programs.

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Amylose content of rice is considered to be one of the most important factors influencing the cooking and processing characteristics of rice. It is commonly used as an objective index for cooked rice texture (Webb 1991). Low amylose levels are associated with cohesive, tender, and glossy cooked rice. Conversely, high levels of amylose cause rice to absorb more water and consequently expand more during cooking, and the grains tend to cook dry, fluffy, and separate (Juliano 1971). Rice breeders invariably are concerned with, and routinely select for, amylose content in new lines.

The traditional method for quantitation of amylose in rice is based on the starch-iodine-blue value protocol of Williams et al (1958). Colorimetric measurements were performed on the amylose-iodine blue color complex, having first defatted the milled rice flour with methanol followed by gelatinization in alkali solution at 4°C for two days. Later modifications, such as gelatinization at 100°C in alkali solution and neutralization with acetic acid to pH 4.5–4.7, shortened the preparation time to 10 min (Juliano 1971). Adjusting the solution pH by using phosphate buffers to stabilize the starch-iodine-blue color complex was examined in a cooperative study (Juliano et al 1981) in which amperometric (Larson et al 1953) and potentiometric (Bates et al 1943) titration methods were also investigated. Historically, iodine-complexing measurements of amylose in rice have not made a definitive distinction between the linear chained amylose molecule and the longer branches of the branched chained amylopectin molecule. More recently, it has been observed that a portion of the reported amylose level in rice actually arose from the longer branches of amylopectin; hence the term "apparent amylose content" (AAC) is now used to clarify the distinction between what exists and what is actually measured (Takeda et al 1987).

In general, chemical methods for AAC determination in rice have the drawbacks of being time-consuming, slow, and somewhat imprecise due to various factors, including difficulty in developing suitable reference curves from starch mixtures of known amylose-to-amylopectin ratios and problems encountered in solubilization of the milled rice flours. Determination of rice constituent concentrations (inclusive of amylose content) by near-infrared (NIR) spectrophotometry was first described by Satake (1988). Preliminary studies at the USDA Western Regional Research Center also demonstrated that NIR technology showed considerable potential for estimating amylose levels in ground milled rice (Bean et al 1990). Various forms of chemometric modeling were used to quantitatively relate the NIR reflectance spectra to AAC determined by amylose-iodine blue value colorimetry. Three crop years of uniform yield nursery samples from one location were tested along with one year of commercial milled rices. The present study expanded the geographical range to include rices grown in five states of the United States. Further, sufficient material was provided to each of three laboratories for sample grinding and NIR analysis, allowing for interlaboratory comparisons of AAC readings.

MATERIALS AND METHODS

Milled rice samples were obtained from rice breeders' nurseries in five states (Arkansas, California, Louisiana, Mississippi, and Texas) in the rice-growing regions of the United States. All samples were grown during 1992 and were part of a cooperative performance testing program conducted annually at each of the five locations. A common set of 49–50 entries, consisting of commercial varieties and experimental lines, was submitted from each state. This set was representative of the U.S. crop and of the potential new varieties being developed for the rice-growing regions of the United States. The samples were milled on-site, subdivided into three portions of 50 g each, and then sent to three laboratories for NIR analysis: USDA-ARS, ISL (Beltsville, MD); USDA-ARS, WRRC (Albany, CA); and Canadian Grain Commission, GRL (Winnipeg, Canada). Distributions of AAC are shown in Figure 1, where samples used in calibration (upper bars) are distinct from those used in model validation (lower bars). Most samples were of either low AAC (in a range of 14–20%) or intermediate AAC (in a range of 22–27%). These ranges are typical of short- and medium-, and long-grain rice, respectively, that are bred in the United States. Also included in each breeder's set of samples was a waxy rice whose AAC was near zero percent. Two states (California and Texas) included a high-amylose extender line developed at the International Rice Research Institute (IRRI). Samples of this line were not available from the other states.

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Each laboratory followed the same procedure for sample grinding and data collection. For every sample, two portions (15 g each) were ground separately in a laboratory cyclone grinder (model 3010-018, Udy, Fort Collins, CO) equipped with a 0.5-mm screen. Feed-rate was 1.0 g/sec. Approximately 5 g of rice flour from each grind was packed into a cell (38 mm diameter × 8 mm deep) capped on one end with an infrared transmitting quartz window. A log(1/reflectance) spectrum for each grind was collected on a spectrophotometer (model 6500, NIRSystems, Silver Spring, MD) equipped with a rotating drawer. Reflected energy readings were referenced to corresponding readings from a ceramic disk. Grinds were scanned over a wavelength range of 1,100 to 2,498 nm in 2-nm increments, yielding 700 values per spectrum. Each spectrum stored to disk was the log(1/reflectance) transformation of the average of 50 repetitive scans. The spectra from grinds 1 and 2 of each sample were averaged to produce one spectrum per sample for model development.

Upon scanning, the flour of each grind from the ISL laboratory was combined with the unused portion of the grind, sealed in a plastic ziplock bag, and stored at room temperature for later chemical analyses. Reference analyses of AAC were performed on the ISL grinds submitted as blind samples to the USDA-ARS Rice Quality Laboratory in Beaumont, TX. Analyses were conducted between two and six months after spectral collection. A modified version of the colorimetric assay for milled rice amylose (Williams et al 1958, as modified by Juliano 1971) was performed on an autoanalyzer, such that each grind was run in successive triplicate analyses. The average of the triplicate readings was recorded as the AAC for each grind. Sample AAC was then calculated as the average of the values of the respective grinds.

Protein content (N × 5.95) of the ground rice samples was performed by the combustion method on a nitrogen analyzer (Leco FP-428, St. Joseph, MI). Two 150-mg samples were analyzed per grind and then averaged. Sample protein content was the average of the two grinds.

For AAC and protein content reference analyses, correction to a constant moisture basis was not performed. Instead, the AAC and protein contents are reported on an "as is" moisture basis (~9–10%) for samples stored in ordinary laboratory conditions: temperature 20–25°C, rh ~40%.

Modeling Procedure

The method of partial least squares (PLS) analysis was used in model development. Mathematical development of PLS is well documented elsewhere (Lindberg et al 1983, Geladi and Kowalski 1986). The basic purpose for PLS analysis and the related analysis of principal components regression is to reduce the dimension of the wavelength space from the number of values in a spectrum (700 in the present study) to a much smaller number, often 5–20, while retaining the pertinent spectral information. Two major benefits arise from the data-reduction step: 1) array sizes are vastly reduced, improving speed of computations; 2) colinearity between neighboring wavelengths is eliminated, thereby reducing the possibility of computational difficulties during matrix inversion operations. The smaller dimension represents the number of factors, or pseudo-spectra, of the PLS model. The set of factors becomes common to all spectra in the set being modeled. The factors can be either weighted or nonweighted to the analyte (AAC in the present case), depending on the procedure PLS or principal components analysis, respectively. Upon factoring, each sample's spectrum is represented by a unique set of coefficients, called "scores". The number of scores per spectrum equals the number of factors, hence the data reduction. When each score is multiplied by its corresponding factor and then linearly combined with the other score × factor terms, the spectrum is reconstituted with only small discrepancy to the original. As the last step in the PLS analysis, multiple-term least squares regression is performed on the scores.

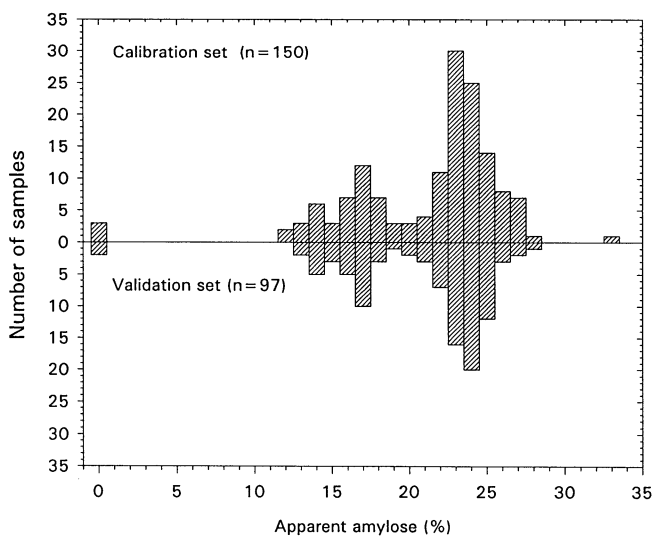

Fig. 1. Distributions of apparent amylose contents of rice samples used in calibration (upper bars) and validation (lower bars) of the near-infrared models. Contents determined by reference colorimetric method.

PLS modeling was performed with a commercial software package (GRAMS/386, Galactic Industries, Salem, NH) run in the Microsoft Windows, v3.1 operating system. Before spectral decomposition, a multiplicative scatter correction (Martens and Naes 1987) was applied to each spectrum for the purpose of minimizing the effect of sample-to-sample particle size variation. The full wavelength region (1,100–2,498 nm) was used during modeling of all three NIR laboratory's spectra. However, in some of the ISL models, subsets of the full region were examined. In some trials, PLS modeling was performed on the three-point central second difference of the spectra. Second differences are often used to reduce the effect of particle size variation among samples and to sharpen the appearance of the NIR absorbers. Together, both effects can substantially improve performance of multiple-term linear regression models. However, the potential for improvement of PLS models is less understood.

Samples were divided into calibration and validation sets. Initially, samples within the parent set were sorted by chemically determined AAC. Starting with the five lowest AAC samples, the first, third, and fifth samples were assigned to the calibration set, while the second and fourth samples were assigned to the validation set. The next group of five samples was similarly assigned, and so on, until the highest whole group of five was assigned, with two samples (having the highest AAC) remaining. The highest and second highest samples were placed in the calibration and validation sets, respectively. As a last step, an arbitrarily selected sample from the validation set was moved to the calibration set, thus bringing the total number of samples in calibration to 150 and in validation to 97. The samples comprising the calibration and validation sets were the same among the three NIR laboratories. Likewise, the laboratories used the same modeling procedure; however, model performance statistics were determined individually.

Two additional forms of the calibration and validation sets were studied. In the first form, calibration and validation were performed on the ISL sets that excluded the five very low amylose samples and the one highest amylose sample. The purpose of using a more limited amylose content range was to demonstrate typical model performance when waxy and amylose extender samples are omitted, anticipating that such samples may be excluded in breeding programs. In the second form, equal numbers of spectra from the three laboratories formed the calibration set. This was accomplished by selecting every third sample from each laboratory's calibration set, starting with the first, second, or third sample (dependent on laboratory). Validation was performed on each laboratory's original validation set. Lastly, the ISL calibration and validation sets were used to develop and validate a PLS model for NIR protein content. The NIR protein model satistics were used to characterize the quality of the NIR spectra, employing a constituent whose ability to be determined by NIR is well accepted.

Model performance was expressed in terms of the standard error of calibration (SEC), standard error of prediction (SEP), the coefficient of determination (r^2), and the bias, all defined in Hruschka (1987). SEC and SEP were calculated on the calibration and validation sets, respectively; r^2 was calculated on either set and specification was given.

Repeatability (within-laboratory) statistics were calculated for both reference and NIR modeling techniques. For the primary reference method, the variances of AAC for the two grinds of each sample were summed over all samples, from which, the average variance was determined. Consequently, the reported error (the square root of the average variance) represented the level of uncertainty in repeatability for the case when a single (successive-triplicate) chemical analysis was performed on a sample, which is assumed to be the normally encountered situation. For the NIR method, a typical PLS model was applied to each individual grind's spectrum of the ISL validation set. Variances of the predicted AAC values were summed and then average was determined. Therefore, the error represented the level of uncertainty associated with an NIR model applied to one spectrum, which assumes that, in practice, only one grind and associated spectrum is used when determining AAC by NIR.

Reproducibility analysis for a between-laboratory measure of consistency of the NIR method was determined on the validation set spectra of the three laboratories. Reproducibility error was the square root of the average variance between laboratories.

RESULTS

PLS modeling performances on the validation sets are summarized in Table I. For each calibration condition, optimal model performance, defined as the best combination of high r^2 (validation) and low SEP, generally occurred at the same number (±1) of factors for each laboratory. As such, the statistics in Table I for each condition are reported at the number of factors collectively deemed as optimal among the three laboratories. These full wavelength-region PLS models, regardless of laboratory or of whether a second-difference pretreatment was performed on the spectra, resulted in r^2 (validation) values of 0.93 or higher. The accompanying SEP values were ~1% AAC, with negligible bias, which are comparable with recently reported results on AAC determination by NIR transmittance (800–1,050 nm wavelength range) through bulk samples of milled rice (Villareal et al 1994).

As many as 16–18 factors were needed for optimal model performance. When the three waxy samples of the calibration set and the two of the validation set were omitted along with the Texas-grown amylose extender sample of the calibration set, the r^2 value of the validation set declined to 0.94, although the SEP remained virtually unchanged. The effect of the second-difference pretreatment was to reduce the number of factors required for optimal performance by two. The standard errors at optimal conditions were equivalent between the standard and second-difference pretreatments.

The validation statistics for the three-laboratory calibration model were slightly poorer than those for the single-laboratory models. Though the NIR instruments were identical, they were not photometrically standardized to a common cell. Therefore, spectral differences on any common rice sample could have been caused by the instruments as well as by the sample storage, grinding, and packing procedures of each laboratory.

For reference purposes, the modeling results for protein content are also listed in Table I. Very good correlations can be developed for protein content with small error (standard error <0.2%), which is typical of cereals, notably wheat (Williams and Norris 1983) and barley (Williams et al 1985).

NIR-predicted versus reference values of AAC are shown in Figure 2 for the 18-factor ISL model applied to the validation set. Because of the negligible bias, model predictions appear to be evenly grouped around the 45° line, the line on which all predictions would lie if the model were perfect. Models that utilized second-difference pretreatments demonstrated predicted versus reference plots that were very similar to Figure 2. Likewise, similar plots were obtained for the WRRC spectra and are therefore omitted. Protein content NIR predictions are plotted against reference values in Figure 3.

When performance of the standard ISL model was examined as a function of the number of PLS factors utilized in the validation set (Fig. 4), performance, as gauged by an increase in r^2 and a decrease in SEP, increased readily up to 10 factors. Beyond this point, a gradual improvement in performance continued, although at a much shallower rate. Even at 20 factors, the PLS model demonstrated improvement. However, there was some concern that the model was overfitting the closed population of samples. Therefore, 18 factors were chosen as optimal. Similar model performances (i.e., r^2, SEP vs. number of factors) were

TABLE I
Summary of Partial Least Squares (PLS) Modeling Results[a]

Calibration Conditions	Laboratory[b]	No. of Factors	Validation Set Statistics			
					(% amylose)	
			n	r^2	SEP	Bias
Standard on $n = 150$	ISL	18	97	0.956	1.04	−0.077
	WRRC	18	97	0.962	0.963	0.038
	GRL	18	97	0.950	1.10	−0.038
2nd difference on	ISL	16	97	0.953	1.06	0.002
$n = 150$	WRRC	16	97	0.967	0.890	−0.018
(gap = 20 nm)[c]	GRL	16	97	0.929	1.32	0.008
2nd difference on 50	ISL	16	97	0.916	1.43	0.109
samples from each	WRRC	16	97	0.943	1.19	−0.029
laboratory ($n = 150$)	GRL	16	97	0.920	1.39	−0.189
(gap = 20 nm)[c]						
Without lows or highs on $n = 146$	ISL	18	95	0.936	0.987	−0.146
Protein (N × 5.95) on $n = 150$	ISL	16	97	0.989	0.107[d]	−0.004[d]

[a] Wavelength region for second difference models is 1,120–2,478 nm; for other models it is 1,100–2,498 nm.
[b] ISL = Instrumentation and Sensing Laboratory, USDA, ARS; WRRC = Western Regional Research Center, USDA, ARS; GRL = Grain Research Laboratory, Canadian Grain Commission.
[c] Gap is defined as the half-width of the three-point central second difference window.
[d] Units of % protein.

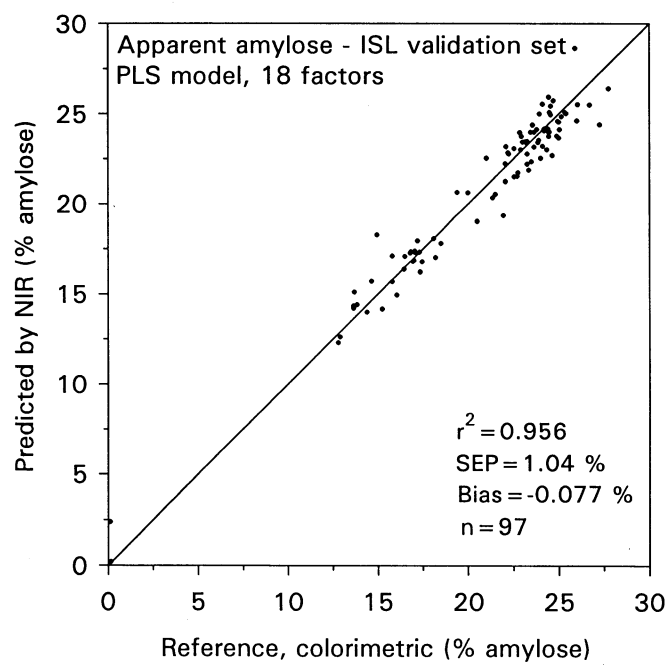

Fig. 2. Comparison of modeled and reference values for apparent amylose of a typical partial least squares model.

noted for the models that utilized a second-difference pretreatment. However, two fewer factors were generally needed to achieve comparable results.

Results for models that utilized portions of the 1,100–2,498 nm region are summarized in Table II. In all cases, the best model was that which utilized spectral information across the full region. The poorest models were associated with the low wavelength (1,120–1,398 or 1,120–1,498 nm) region, in which the SEP exceeded 2% AAC. Utilization of the central (1,500–1,998 nm) region yielded a model that had slightly poorer performance than the full region model. When the central region was further reduced by excluding the 1,900 nm water absorption band, the resulting model (1,500–1,798 nm) demonstrated a decline in r^2 to 0.89 and a rise in SEP by 0.46% AAC units. Models based upon the high wavelength (2,100–2,478 nm) region yielded performance comparable to those of the central region. When the two predominant water absorption band regions (1,400–1,498 nm and 1,800–1,998 nm) were excluded, the resulting model performance was nearly the same as that of the full region, suggesting that moisture variability in samples did not enhance or detract from the model. Generally, the longer and central wavelength regions demonstrated better model performance than did the shorter wavelength regions, although these were not as good as when the full wavelength region was used.

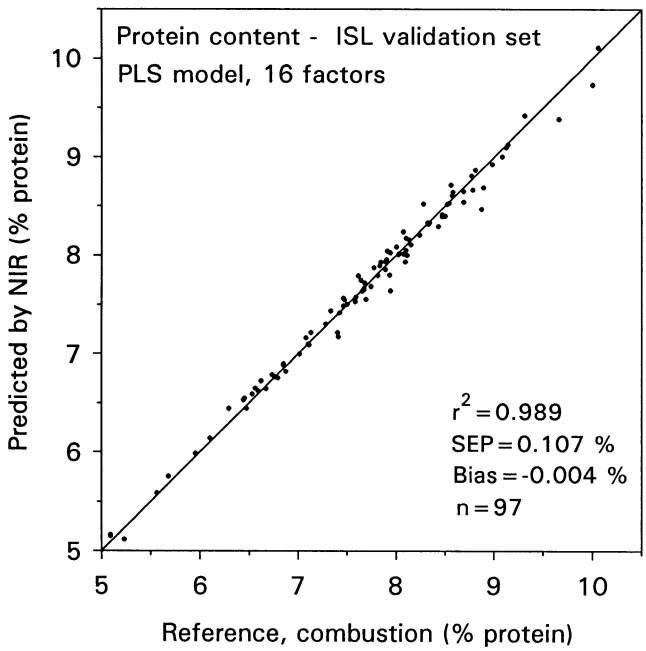

Fig. 3. Comparison of modeled and reference values for protein content of a typical partial least squares model.

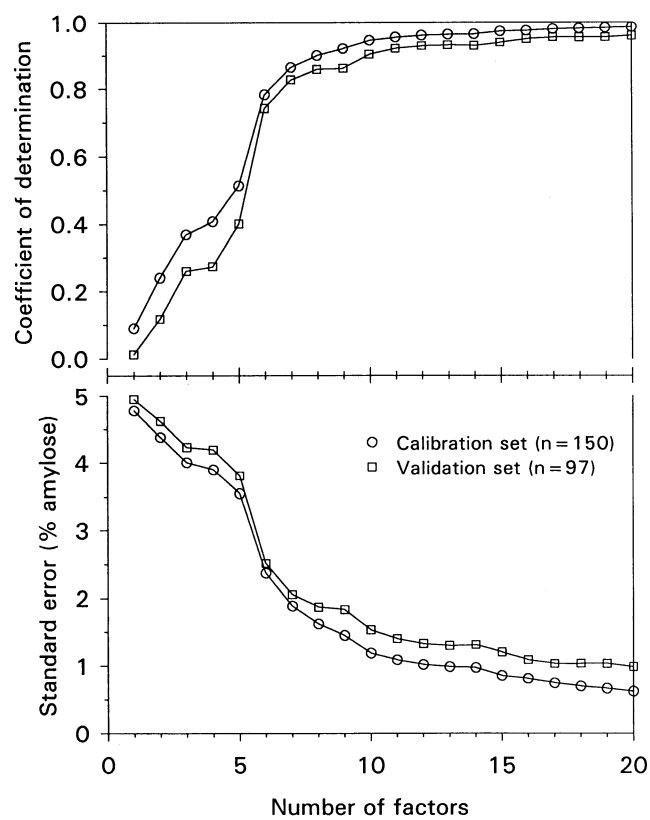

Fig. 4. Model performance as a function of the number of factors. Conditions: partial least squares on log(1/R) 1,100–2,498 nm in 2-nm increments, with multiplicative scatter correction.

The repeatability error of the AAC reference method was 0.527% AAC and that for the 18-factor ISL PLS model was 0.451% AAC. Though the reference method error is slightly larger than the NIR method, a two-tailed *f*-test failed to confirm this difference as significant (at $P = 0.05$).

A plot of the AAC residuals for each laboratory's validation set predictions from the 18-factor standard models is shown in Figure 5. Sixty-four of the 97 sample triplicates consisted of residuals whose signs (positive or negative) were the same within a triplicate, indicative that the NIR method was consistent among laboratories. Removal of each laboratory's bias (Table I) in the residuals did not alter the number of same-sign triplicates. A χ-square value of 87, determined from the hypothesis of a two-chances-in-eight probability of each sample's residual having the same sign for all three laboratories, verified that the consistency between laboratories was significant ($P < 0.001$). The reproducibility error of this NIR model was 0.55% AAC.

DISCUSSION

Interpretation of the PLS factors is extremely difficult, and it was not attempted. However, it is interesting to note that the best models were those that utilized the full wavelength region. Deliberate removal of the water absorption band regions resulted in a very slight decline in performance, suggesting that the NIR method is sensitive to AAC directly rather than indirectly sensed as a complementary component to moisture. The NIR region is largely characterized by overtone and combination frequencies of CH, OH, and NH bonds. Such frequencies are affected by intra- and inter-molecular hydrogen bonding (Luck 1973), which may accentuate the spectroscopic differences between amylose and amylopectin. Second-difference spectra of the starch fractions (isolation, purification, and gelatinization of starch in dimethylsulfoxide, performed according to the method outlined by Briones et al [1968]; fractionation of the gelatinized starch into amylose and amylopectin by the method of Lugay and Juliano [1965])

TABLE II
Modeling Results Summary for Wavelength Region Selection[a]

Region (nm)	No. of Factors	*n*	r^2	(% amylose) SEP	(% amylose) Bias
1,120–2,478	16	97	0.953	1.06	0.002
1,120–1,398	14	97	0.813	2.16	0.244
1,120–1,498	16	97	0.832	2.04	0.069
1,500–1,798	12	97	0.886	1.66	0.088
1,500–1,998	12	97	0.942	1.20	−0.076
2,100–2,478	16	97	0.926	1.35	−0.019
1,120–1,398, 1,500–1,798, 2,000–2,478	16	97	0.945	1.16	0.016

[a] Conditions are ISL model on ISL validation set, second difference spectra (gap = 20 nm). ISL = Instrumentation and Sensing Laboratory, USDA, ARS. Gap is defined as the half-width of the three-point central second difference window.

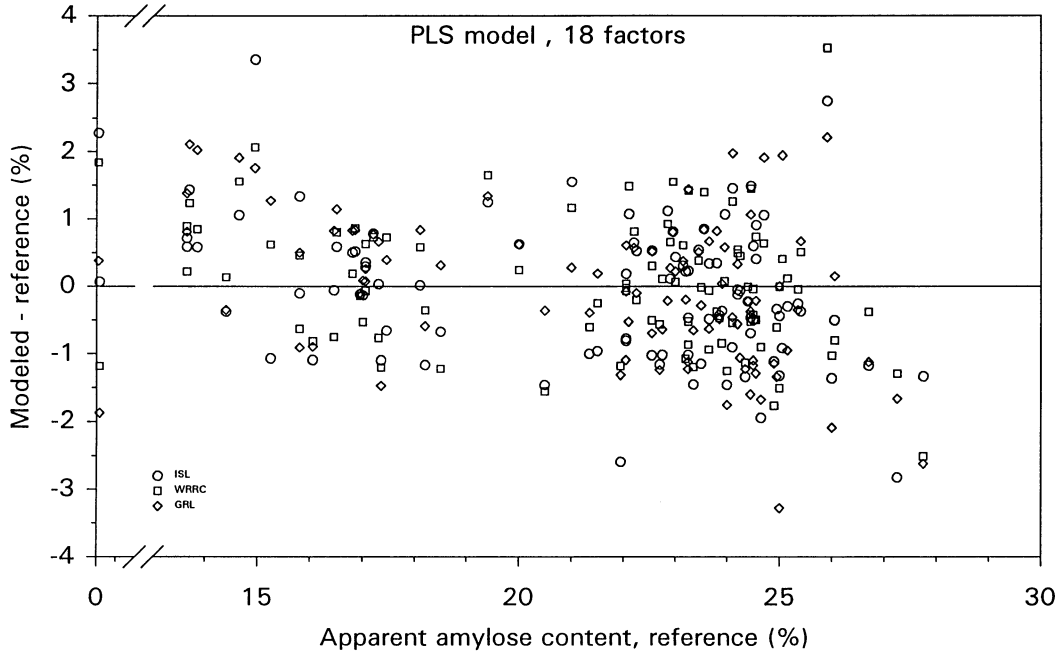

Fig. 5. Residuals of apparent amylose contents of the validation samples (n = 97) for three laboratories. Model conditions: partial least squares on log(1/R) 1,100–2,498 nm in 2-nm increments, with multiplicative scatter correction.

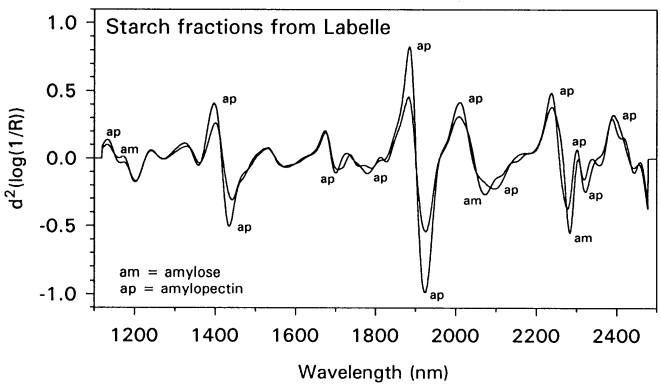

Fig. 6. Second difference spectra (gap = 20 nm) of amylose and amylopectin fractionated from a sample of the Labelle variety.

of the rice variety Labelle are plotted in Figure 6. The greatest non-water-related differences in spectral shape of the two fractions exist in the regions of 1,690–1,840 nm, 2,040–2,190 nm, and 2,250–2,375 nm. Such differences are thought to arise because of spectral sensitivity to differences in crystallinity between the two fractions, especially prominent in the longest wavelength region. Additionally, the spectra may be sensitive to differences in the OH bonds of branched versus linear chains and to other differences in conformation. However, these are believed to be less apparent than the sensitivity to differences in physical structure.

CONCLUSIONS

Determination of AAC in milled rice by NIR can be accomplished with accuracies equivalent (if not better) than a conventional colorimetric method. Robust PLS models have routinely demonstrated standard errors of 1% AAC. Repeatability (within-laboratory) error of the NIR method is ~0.5% AAC, which is also equivalent to the colorimetry method. NIR reproducibility (between-laboratory) error is 0.5% AAC. Such accuracies should be more than sufficient in rice breeding programs, resulting in a selection technique that is less expensive and faster than conventional methods.

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