

# Comparison of Methods for Amylose Screening Among Amylose-Extender (*ae*) Maize Starches from Exotic Backgrounds

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## ABSTRACT

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Breeding for high-amylose corn requires a rapid analytical method for determining starch amylose so that generating wet chemistry values does not pose a major limitation in the volume of materials that can be screened. Two methods for determining apparent amylose content (AAC) were examined and compared with an iodine-binding method involving the solubilization of isolated starch in a sodium hydroxide solution (method 1). These methods included one based on near-infrared transmittance spectroscopy (NIRT) (method 2) and another iodine-binding method involving the solubilizing of starch from ground whole corn with a DMSO-iodine solution (method 3). These methods were chosen because, aside from initial set up costs, they are relatively rapid and inexpensive to perform. The materials evaluated consisted of various exotic corn populations including plant introductions and experimental materials generated from the Germplasm Enhancement of Maize (GEM) project. Crosses were made between these materials and a Corn Belt dent hybrid (OH43 × H99) converted with the amylose-extender (*ae*) allele. Grain from F2 ears, presumed to be homozygous for the *ae* allele based on visual selection of mutant kernels on F1 ears from which they were planted, were then eval-

uated to identify possible modifiers of *ae* conditioning high starch AAC. From a total of 1,006 F2 ears, a core set consisting of 155 samples was established and only these were subjected to starch AAC analysis, using all three methods to compare the methods. Method 2 showed poor correlation to method 1 ( $r = 0.88$ ), however, NIRT did appear to discriminate between samples converted to *ae* vs. those with a normal or possibly segregating endosperm type. Method 3 showed a slightly better correlation with method 1 ( $r = 0.92$ ) and appeared to more fully discriminate among samples with AAC values >65% from those at ≈55%. Results from this study suggest that NIRT may be useful when a quick screening method is needed to discriminate mutant from nonmutant genotypes within grain samples of exotic germplasm especially when visual identification is difficult. In addition, method 3 could be used to replace the more time-consuming method 1 when trying to identify high AAC levels among *ae* genotypes, even though some inconsistency was observed between the two methods. Finally, this study revealed that exotic germplasm may be an important source of new modifiers to the *ae* allele because values as high as 70% AAC were identified.

In recent years, researchers have identified several novel uses for starches isolated from high-amylose corn (*Zea mays* L.) genotypes possessing the recessive *ae* (amylose-extender) allele for both food and nonfood applications (Ferguson 1994). One of the difficulties encountered in the breeding of high-amylose corn, as with any grain chemical constituent, is the identification of a rapid method for determining starch apparent amylose content (AAC) that would be usable in a breeding program where large numbers of grain samples need to be screened. Many early breeding studies reported AAC values determined from isolated starch by chemical gelatinization and formation of the blue iodine amylose complex (Williams et al 1958). More recently, Knutson (1989) suggested a more rapid method based on solubilization of amylose in DMSO, followed by formation of the blue iodine amylose complex. In addition, a nondestructive approach based on near-infrared transmittance spectroscopy (NIRT) demonstrated that there was some predictive capability in using this method even though it was associated with a large error (Campbell et al 1999).

A number of studies have demonstrated the benefits of screening exotic germplasm to identify novel sources of genetic variation for starch properties and other grain carbohydrates. Exotic collections of crops may also continue to be an important breeding resource because they will not likely be associated with consumer acceptance problems as seen with other contemporary breeding techniques. Tracy (1990) emphasized the importance of exploring exotic germplasm for improved agronomic performance as well as improving quality traits since "much of the maize grown outside the U.S. is consumed directly by humans and has undergone centuries of selection for flavors, aromas, and textures." Some examples include the single gene mutations waxy (*wx*) and sugary-1 (*su1*)

that were identified from exotic germplasm. Whistler and Weatherwax (1948) examined 39 exotic populations from North, Central, and South America and found that AAC values were 22.2–28.3%. In another example, 75 foreign plant introductions were examined by Deatherage et al (1955) where AAC contents were 0–36%. Similarly, Morrison et al (1984) determined the AAC content of 20 corn populations of temperate and tropical origins. Although classified as having normal no-mutant starch types, they contained AAC at 24–32%. More recently, exotic germplasm has been an important source of variation for other starch characteristics such as thermal properties and starch granule size (Li et al 1994; Campbell et al 1996). In other studies, the interaction of exotic germplasm with specific mutations affecting starch structure was observed. For example, the sugary enhancer gene (*se*) was identified from crosses between a U.S. sweet corn inbred with a Bolivian corioco interlocking flour corn that interacted with the sugary-1 (*su1*) mutation. This resulted in twice the sugar content compared with genotypes possessing *su1* alone (Gonzales et al 1974). In addition, Campbell et al (1995) investigated a system for observing the influence of exotic germplasm on thermal properties of starch from genotypes possessing the sugary-2 (*su2*) mutation.

The primary objective of this study was to evaluate two rapid starch amylose procedures that could be applicable in a plant breeding situation relative to an earlier method described by Williams et al (1958). The second objective was to observe the effect of the exotic germplasm backgrounds with the recessive amylose extender (*ae*) allele on levels of AAC, which may lead to the identification of new sources of modifying genes for development of high-amylose starch.

## MATERIALS AND METHODS

### Development of Genetic Material

In 1997, at the Truman State University Research Farm near Kirksville, MO, crosses were made using the single-cross hybrid OH43*ae* × H99*ae* as a male parent onto 155 exotic corn entries. Of these exotic materials, 57 were populations obtained directly from the North Central Regional Plant Introduction Station in Ames, IA. The remaining 97 were experimental breeding crosses obtained from the Germplasm Enhancement of Maize (GEM) program as described

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by Pollak and Salhuana (1999), which represented many tropical corn populations crossed with proprietary inbred lines to improve adaptation to the Midwest. The F1 seed obtained from these crosses was advanced in a winter breeding nursery near Ponce, Puerto Rico, with plants producing F1 ears with F2 kernels segregating normal and *ae* in the expected 3:1 (normal to *ae*) ratio. Mutant F2 kernels were visually selected and planted in a 1998 breeding nursery at the Truman State University Research Farm, where all plants were self-pollinated. From this nursery, a total of 1,006 F2 ears, presumed to be homozygous for the *ae* allele, were harvested and air-dried to a moisture content of  $\approx 13\%$  before analysis. Entry identification for the germplasm utilized in developing crosses and the numbers of F2 ears included in this study are shown in Tables I and II.

### Amylose Determination

**Method 1.** For the AAC method described by Williams et al (1958), duplicate samples of 27.1 mg of starch were chemically gelatinized by dissolving in 5 mL of 2N NaOH and 5 mL of H<sub>2</sub>O. The method for starch extraction is described by Li et al (1994). Samples were then diluted to 100 mL using distilled H<sub>2</sub>O. Aliquots of 10 mL were added to a volumetric flask followed by addition of 50 mL of H<sub>2</sub>O, two drops of a 0.1% phenolphthalein ethanol solution, followed by pH neutralization using 0.5N HCL, addition of 2 mL of a 0.2% I<sub>2</sub> + 2.0% KI solution and dilution to 100 mL. After 30 min of color development, samples were measured in duplicate at 600 nm using a spectrophotometer (model DU-520, Beckman, Fullerton, CA) and absorbance values converted to percent amylose using a standard curve. Following the collection of data, the coefficient of variation (CV) for method 1 was 2.2%.

**Method 2.** For the NIRT method, predicted AAC values were determined using either an unmodified calibration described by Campbell et al (1999) or a modified version of this calibration. Both methods were developed using partial least squares (PLS) analysis. The unmodified calibration was constructed from >400 yellow-dent reference

grain samples that were divided into separate calibration and validation sets. Grain samples were scanned using a NIRT grain analyzer (Infratec 1255, Foss North America, Eden Prairie, MN) at wavelengths of 850–1,048 nm and consisted of 100 data points. Scans were collected using a path length of 30 mm through sample cups, each representing one subsample. A log (1/transmittance) spectrum from each grain sample was used in the development of the prediction model (average of at least 10 subsamples). All calibration modeling was performed using the software program Unscrambler (Camo A/S V6.11, Trondheim, Norway). Modification of the calibration involved incorporating the scans and AAC values (method 1) from 155 exotic grain samples from the present study into the original calibration. This was done so that the calibration might better recognize spectral variation within the exotic material such as unique kernel colors, size, and textures not typically observed in the yellow-dent germplasm that was originally used. The modified calibration was evaluated using a cross-validation approach where the analysis was run numerous times; each time a sample was left out and treated as a validation sample to eventually generate an entire validation set. The predictive capability of the models was evaluated using the correlation coefficient (*r*) between predicted and actual values. In addition, the AAC prediction of exotic samples by the unmodified calibration was evaluated using the standard error of performance (SEP), while the modified calibration was evaluated using the standard error of cross-validation (SECV) (Martens and Naes 1989). The CV% associated with method 2 was 3.8%.

**Method 3.** For the method described by Knutson (1989),  $\approx 15$  grams of grain were ground to pass a 0.5-mm screen using a sample mill (Tecator Cyclotec, Foss North America). In duplicate,  $\approx 50$  mg of corn flour was weighed and solubilized overnight in 5.5 mL of 90% DMSO containing  $6 \times 10^{-3}$  M iodine while in a 70°C water bath. Following solubilization, samples were vortexed, centrifuged, and 100- $\mu$ L aliquots were placed into separate tubes. For AAC analysis, 1 mL of 90% DMSO containing  $6 \times 10^{-3}$  M iodine solution and 8 mL

**TABLE I**  
Pedigree, PI Number<sup>a</sup> and Number of Ears Harvested from 57 Exotic Maize Samples Obtained from the North Central Regional Plant Introduction Station<sup>b</sup>

Accession or Breeding Cross	PI Number	No. of Ears	Accession or Breeding Cross	PI Number	No. of Ears
Yankee Cheat	PI 213771	9	San Lorenzo Pueblo	PI 218142	5
Speckled Mandan	PI 213798	4	Taos Pueblo	PI 218152	1
Sac Blue	PI 213768	3	Mandan Black	PI 213806	5
Albuquerque Pink	PI 213767	7	Mandan Red Flour	PI 213808	1
Potawatomi Mixed	PI 213765	5	Tama Flint	PI 217411	11
Rhee Flint	PI 213764	3	Zuni Blue	PI 213799	10
White Thunder	PI 213763	2	Mandan Yellow Flint	PI 213800	7
Gehu Yellow	PI 213762	3	Bear Island Chippewa	PI 213801	7
Separation-Onehorn	PI 213761	1	Cargill N. Temp. Zn. Coroico	PI 451692	2
Onehorn	PI 213760	6	Tesuque Pueblo	PI 218137	2
Many Horses	PI 213759	2	Acoma Pueblo	PI 218140	7
Arapaho White	PI 213753	2	Bronze Beauty	Ames1836	1
Binger Blue	PI 213754	6	Wilbur's Rhode Island Flint	Ames2748	2
Bighorse Spotted	PI 213755	3	Blue Corn	PI 420246	4
Quapaw Red	PI 213757	6	Koko'ma	PI 420249	10
Shawnee	PI 213758	8	Turtle Mountain White	PI 472021	4
De-aur-le	PI 213743	5	Onaveno/cristalino flint	PI 474209	5
Yellow Eagle	PI 213745	7	Dulcillo de Noroeste	PI 474213	8
Walter Fire	PI 213747	4	Mohawk Round Nose	PI 483087	1
Concho Brown	PI 213748	11	Wampum Flint	PI 483088	8
Stilwell	PI 213750	9	Loreto 1	PI 488381	1
Globe Variegated	PI 213736	9	Maiz Blanco	PI 512024	3
Red Navajo	PI 213737	2	Havasupai, Grand Canyon	PI 317674	5
Blue Navajo	PI 213738	2	Yankton Sioux	PI 317681	3
Yellow Navajo	PI 213739	8	Jemez Pueblo	PI 218171	7
Oklahoma Bronze	PI 213742	5	San Xavier del Bac	PI 218179	7
Sehsapsing	Ames3507	1	Zia Pueblo	PI 218188	2
Bighead	PI 213732	2	Cochiti Pueblo	PI 218131	2
Kokoma	PI 213733	1			

<sup>a</sup> USDA Plant Introduction accession number

<sup>b</sup> Total number of F2 ears used in study: 1006; Core set (one F2 ear per exotic cross) analyzed by methods 1,2, and 3: 155; Remaining samples analyzed only by method 2: 851

of distilled H<sub>2</sub>O were added. The samples were vortexed and the absorbance was read at 600 nm following 30 min of color development. The blue complex solution (1 mL) was then used to determine carbohydrate by the method of DuBois et al (1956) to estimate starch content (CV = 6.3% for method 3). Apparent amylose values were compared between methods 1 and 3 using standard error of deviation (SED) calculated similarly to SEP (Martens and Naes 1989).

### Grain Density

Grain density values were obtained from material grown in 1999. A total of 100 kernels were weighed and the displacement of these kernels was measured in a graduated cylinder containing 90% ethanol.

## RESULTS AND DISCUSSION

### Evaluation of Exotic Core Set Using Method 1

To establish a common set of material for comparing the three analytical methods and to observe the breadth of variability within the germplasm, a core set was established by visually selecting one sample with good ear and grain quality (filled ears, full kernels, absence of mold, etc.) from each of the 155 exotic crosses. It should be noted that although method 1 was used as a reference to compare with other analytical methods, using it does not imply that it is the best means of quantifying starch amylose. Other methods such as gel permeation chromatography may more accurately quantify amylose, amylopectin, and intermediate fractions (Wang et al 1993). For this study, however, we were interested in colorimetric AAC analysis based on pure starch as described in method 1. This is because method 1 and variations of it are currently used among several producers and end-users of specialty starch grains for establishing grain types such as amylomaize V, VI, and VII.

The frequency distribution of AAC values using method 1 for this core set of 155 samples is shown in Fig. 1. Previous studies (Bear et al 1958; Loesch and Zuber 1964) have shown that genotypes possessing the *ae* allele typically have AAC values of 45-70% as the result of modifying genes from different genetic backgrounds. For this study, AAC values were 25-70%; however, samples with values of 25-45% were not likely homozygous for the recessive *ae* allele due to misclassification of segregating F<sub>2</sub> kernels. The presence of nonmutant kernels occurring within materials reported in this study confirms the observations made by Haunold and Lindsey (1964) that show the difficulty in visually selecting *ae* kernels when segregation occurs within exotic backgrounds.

### Comparison of Methods 1 and 2

Predicted AAC values using method 2 are compared with AAC values determined using method 1 in the scatter plot shown in Fig. 2. For a breeding program, the advantage of method 2 over method 1 is that values can be obtained from unground samples in minutes, allowing for the screening of large volumes of samples. In addition, samples remain intact and can be used to advance the genetic materials. Performance of the unmodified model using 14 factors on predicting AAC for the 155-sample core set was associated with a correlation coefficient ( $r = 0.88$  and  $SEP = 5.26$ ) that were slightly worse than those reported in a previous study ( $r = 0.94$ ,  $SEP = 4.19$ ) which included only yellow-dent samples (Campbell et al 1999). This may be explained by the fact that exotic materials vary more widely in other grain characteristics such as seed size, color, and texture, which may have confounded the prediction model.

From the scatter plot it is clear that the NIRT method does not resolve AAC values of 50-70%, which would preclude its use in discriminating an Amylomaize V genotype (>50% AAC) from an

TABLE II  
Pedigree and Number of Ears Harvested from 97 Samples Obtained from the GEM Project<sup>a,b</sup>

Accession or Breeding Cross	No. of Ears	Accession or Breeding Cross	No. of Ears	Accession or Breeding Cross	No. of Ears
Cuba117:S1516	8	UR13085:N0215	5	SCROGP3:N20	6
CH04030:S0917	6	AR13035:S11b	9	AR13026:N08b15	6
AR01150:N04	7	UR13085:S1912	4	CUBA110:N1711c	9
SCROGP1:N1310	8	AR17026:N10	2	AR13026:N08d06	12
DK212T:S0620	9	AR13026:S15	8	BARBGP2:N08a18	14
DREP269:S0622	7	DKB844:S16	9	BR51403	15
DREP150:N2011d	11	AR13035:S11b01	5	ANTIG03:N1218	10
PASCO14:N04	9	AR16035:S0219	8	DREP150:N2012	9
DKB844:N11b18	10	ANTIG01:S02	6	AR16021:S0915	6
CASH:N1410	9	UR05017:S0409	8	AR17056:S1219	10
FS8B(S):S0301	9	GOQUEEN:N16	8	UR10001:N17	6
CUBA164::S1517	8	CH04030:S09	3	CUBA164:S1511b	7
UR10001:S1813	8	GUAD05:N0613	9	UR13085:0204	10
AR03056:N0902	11	GUAD05:N06	6	AR17026:N1019	6
AR13026:N08a02	10	CHIS775:N19	2	DKB830:N11b20	10
CHIS775:N1912	6	DK212T:N11a	5	CH05015:N1507	10
FS8A(T):N1801	7	BVIR155:S2011a	4	DKXL212:N11a01	10
UR11002:S1409	6	UR11002:N0302	3	SCROGP3:N2017	5
GOQUEEN:N1603	6	SCROGP3:N14	8	FS8B(T):N1813	9
AR13026:N08c06	3	GOQUEEN:N1612	6	DKXL380:S08a15	12
DREP269:S06	8	DKXL212:S09	5	CHIS775:N1920	13
PASCO14:N04	5	ANTIG03:N1216	2	FS8B(T):N1802p	13
AR03056:N0906	8	BVIR103:S04	9	PASCO14:S01	10
DKXL370:S08c	8	AR16035:S0209	10	DKXL370:N11a20	7
DKXL380:N11a	7	CH04030:N0301	9	AR16021:S08a01	4
CUBA173:S04	4	DKB844:S1612	12	CASH:N14	4
UR13088:S0619	5	AR01150:S01	8	CUBA173:S0422	4
UR13061:S22	8	DKXL380:S08B	7	UR13010:N0613	9
DKXL370:S08d	4	GUAD05:N0620	13	FS8B(T):N11a08a	5
CH04030:N0306	3	SCROGP3:N2001	14	UR13010:S1309	4
AR16035:S19	6	GUAT209:S13	10		
CH05015:N12	9	DK212T:N11a10	9		
UR13088:S0604	6	PRICGP3:N1218	12		

<sup>a</sup> Germplasm Enhancement of Maize project.

<sup>b</sup> Total number of F<sub>2</sub> ears used in study: 1006; Core set (one F<sub>2</sub> ear per exotic cross) analyzed by methods 1,2, and 3: 155; Remaining samples analyzed only by method 2: 851

Amylomaize VII (>70%) in a breeding program, for example. The scatter plot did reveal, however, that samples composed of kernels apparently nonmutant or segregating for the *ae* allele could be differentiated from those that were apparently fully homozygous for *ae*. The use of NIRT as a diagnostic tool in identifying homozygous *ae* samples from those that are normal or segregating for the *ae* allele, therefore, was further investigated. Predicted values were generated for the remaining 851 samples (not including the 155-sample core set) by the use of the modified NIRT amylose calibration. Again, this modified calibration consisted of reference samples used in the unmodified calibration in addition to spectral and AAC (method 1) data from the 155-sample core set examined in this study. It was believed that this modified calibration would be better suited for predicting AAC in grain samples from exotic germplasm because exotic germplasm was included in the calibration model. Performance statistic for the modified calibration with 15 factors were  $r = 0.89$ ,  $SECV = 4.1$ . The frequency distribution of NIRT-predicted AAC values of the remaining 851 samples using

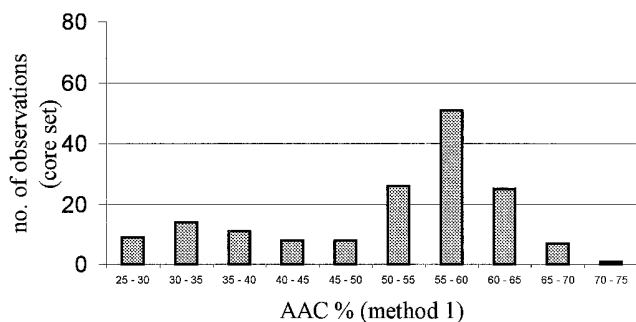


Fig. 1. Frequency distribution of apparent amylose content (AAC) values determined using method 1 for the 155-sample core set.

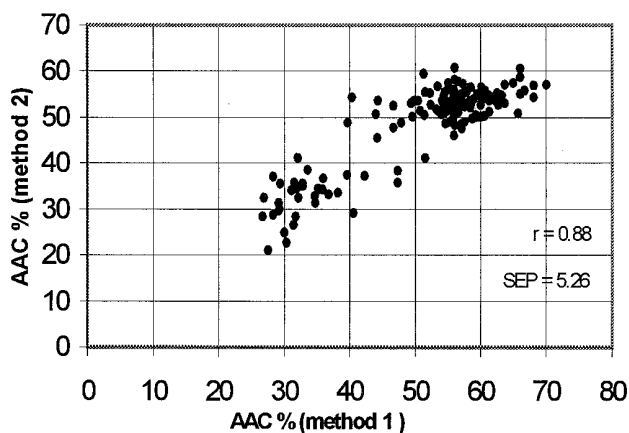


Fig. 2. Scatter plot of apparent amylose content (AAC) values determined using method 2 vs. method 1 of the 155-sample core set.

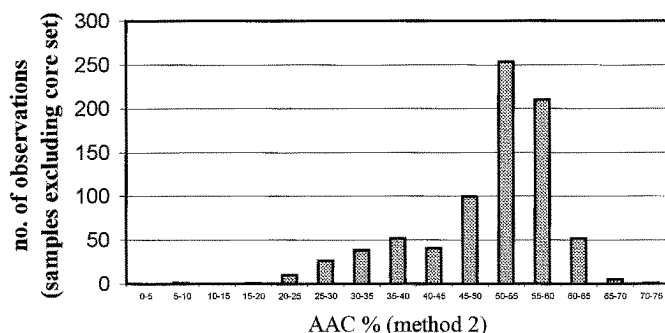


Fig. 3. Frequency distribution of apparent amylose content (AAC) values determined by method 2 of the remaining set of 851 exotic samples that excluded the core set, using a modified amylose calibration.

the modified calibration are shown in Fig. 3. As seen previously, the bimodal distribution suggests a group of mostly homozygous *ae* samples and another group composed of samples that are apparently normal or segregating for the *ae* allele. Using NIRT-predicted values, 160 samples with the highest AAC values were selected from the 851-sample set and reanalyzed using method 1. The frequency distribution of the AAC values is shown in Fig. 4. The results in this case no longer revealed a bimodal distribution, suggesting that samples not fully homozygous for *ae* were eliminated. Therefore, results from this comparison indicate that NIRT may be useful in eliminating samples not converted to the recessive *ae* allele or perhaps heterozygous for the *ae* allele.

### Comparison of Methods 1 and 3

A comparison of AAC values using method 1 versus method 3 for the 155-sample core set is shown in a scatter plot in Fig. 5. A comparison of the two methods revealed values of  $r = 0.92$  and  $SED = 4.92$ . In contrast to NIRT, the scatter plot suggests that method 3 can better resolve differences within 50-70%.

The advantage of method 3 over method 1 is that it is more rapid because the starch isolation step has been eliminated. It should be noted that although both methods are based on the affinity of iodine to amylose, there are several fundamental differences that could lead to discrepancies. First, because method 3 is based on the use of milled flour, AAC values would represent the total starch as opposed to extractable starch as with method 1. Second, starch of the milled flour samples is estimated using a total carbo-

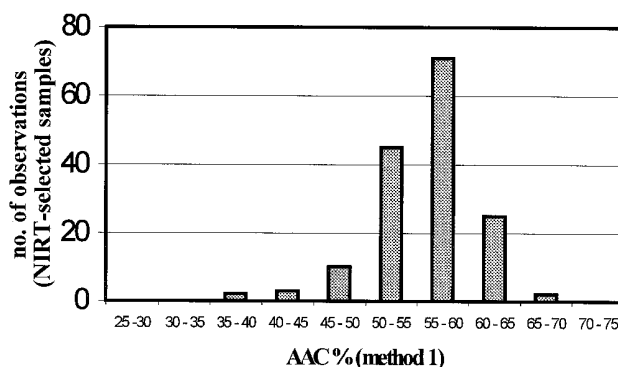


Fig. 4. Histogram showing distribution of apparent amylose content (AAC) values determined by method 1 for 160 samples previously selected as having the highest AAC values by method 2 from the 851 exotic samples that excluded the core set.

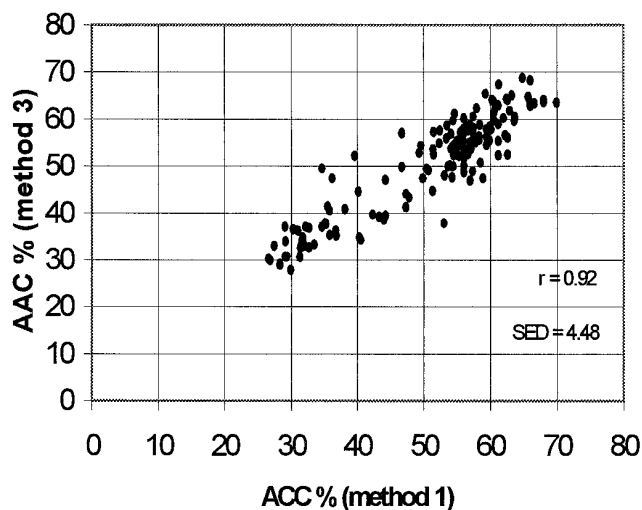


Fig. 5. Scatter plot comparing apparent amylose content (AAC) values from the 155-sample core set determined using method 1 vs. AAC values using method 3.

hydrate test. Since relative amounts of nonstarch carbohydrates may vary, especially among mutant genotypes, this may account for differences. And finally, the coefficients of variation indicated that the reproducibility of data for method 3 (CV = 6.3%) was not as good as method 1 (CV = 2.2%), which could result in a lower than expected correlation between the two methods.

### Advanced Progeny of Selected Germplasm

To further study the germplasm, a select group of F2 grain samples with the highest AAC values was chosen from the 1998 growing season. In a 1999 breeding nursery, these samples were advanced by self pollination to the F3 generation, where grain samples from 10 F3 ears per sample were examined using method 1 to determine whether the elevated amylose phenotype would also be observed among the progeny. Table III shows AAC values determined from individual F2 ears from the initial screening using method 1 in addition to means and ranges of the 10 F3 progeny ears relative to a OH43ae × H99ae check. In both the 1998 and 1999 growing seasons, the average AAC values of the selected materials averaged 8.1 and 7.9%, respectively, greater than the OH43ae × H99ae check. Several 1999 individual ear samples within the GUAT209:S13 × (OH43ae × H99ae) and Concho Brown × (OH43ae × H99ae) genotypes also exceeded 70%. In addition, a positive correlation ( $r = 0.86$ ,  $P < 0.01$ ,  $df = 9$ ) was observed between the 1998 samples and their corresponding progeny ears, indicating that the variations in observed AAC values are likely due, in part, to actual genetic differences.

In addition, kernel density values were determined for 1999 F3 ears. With only one exception, kernel density measures were equal to or greater than the OH43ae × H99ae check. Also, in all cases, 100 kernel weight values for the exotic materials were less than that observed for the OH43ae × H99ae check. These observations suggest that selection for increased AAC or introgression of exotic germplasm may be responsible for altering other general grain quality characteristics.

### CONCLUSIONS

The data presented here show that even though methods 2 and 3 are correlated to method 1, the extent of this correlation would limit how each is used. Method 2, based on near-infrared analysis, clearly does not resolve AAC differences within the ae genotypic class. However, it could be useful as a rapid means of eliminating samples that are normal or segregating normal kernels for further analysis. This would be especially useful when visual identification of genotypic classes is difficult. Although the correlation between methods 1 and 3 was greater, the CV% was larger for method 3. Method 3, therefore, may be useful as a rapid screen of materials to identify the upper half of AAC samples from a larger set. Method 1, the most lengthy procedure, would then be necessary to confirm high AAC values from the previous group.

In addition, the data clearly demonstrates that exotic germplasm can be an important source of modifying genes conditioning high

starch amylose content. Future studies should be conducted to determine whether the modifying genes in these backgrounds are independent of one another and, if so, whether they could function synergistically to elevate AAC to even greater levels.

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