Results of Collaborative Testing Using a Simplified, Rapid Colorimetric Alpha-Amylase Assay for Evaluation of Sprouted Wheat[1]

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ABSTRACT

The procedure and equipment required for a simplified colorimetric α-amylase assay are described, as are the results of two collaborative studies utilizing this method for the evaluation of sprout damage in wheat. Collaborative testing, using wheat having from 0 to 8% sprouted kernels, demonstrated that the different levels of sprout damage were distinguishable, despite statistically significant sampling error for whole wheat samples. In addition, collaborators within a laboratory were able to achieve reproducible results on their own equipment. The use of a standardized, commercially available instrument designed to perform this test should provide a reliable, rapid procedure for the evaluation of sprout damage.

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Recognition of the importance of α-amylase in germination, malting, and sprouting has resulted in the development of many techniques to measure activity of this enzyme. Its ability to hydrolyze starch has been utilized in tests that measure 1) decrease in viscosity of a starch suspension, 2) increase in reducing power of a starch solution, and 3) decrease in the color of iodinated β-limit dextrin (AACC 1975). Although these tests are useful for many purposes, they are generally complicated and time-consuming and lack the precision and sensitivity required for many analytical situations. A new generation of techniques has been reported that attempt to mitigate the limitations of the previous tests. These new methods include measurement of change in nephelos of a starch suspension (Campbell 1980), use of coupled enzyme reactions (Guilbault and Rietz 1976), and automated fluorometric determination of α-amylase using a special starch substrate (Marchylo and Kruger 1978).

A significant development in the search for a convenient assay for α-amylase was reported by Rinderknecht et al in 1967. This technique utilized a dyed starch as substrate for the enzyme. The result of the reaction is a colored solution, the absorbance of which can be conveniently measured using a colorimeter and is linearly related to the α-amylase concentration.

Many modifications to this technique have resulted in a test used extensively in clinical assays of serum and urine as a diagnostic indicator of pancreatic and associated diseases. The method is fast, sensitive, and reproducible. In addition, the finding that the Cibacron-blue amylose (CBA) substrate was resistant to attack by β-amylase (Barnes and Blakeney 1974) made the technique a viable alternative for specific determination of α-amylase in cereals.

Barnes and Blakeney (1974) reported the successful use of a commercial preparation of CBA for α-amylase determination in cereals. Following up on this work, Mathewson and Pomeranz (1977, 1979) reported a colorimetric method for evaluating sprouting in wheat based on α-amylase content. Although we felt that this method would be of considerable value for many applications, consultation with action and regulatory agencies in the U.S. Department of Agriculture and with representatives of grain trade and grain producers indicated that in marketing channels in particular, a different procedure was desirable. The method should be rapid (~5 min), very simple, and sequential so that samples could be run on a single-sample basis to keep elevator traffic flowing efficiently. In addition, a desirable procedure would be adaptable to testing large numbers of samples so as to be useful both in marketing channels and in plant breeding programs. This report describes the results of two collaborative studies designed to evaluate a procedure developed to meet all of these requirements.

MATERIALS AND METHODS

Wheat Samples

Four samples of Western White wheat were from the Western Wheat Quality Laboratory, U.S. Department of Agriculture, Pullman, WA 99163. These wheats were graded by federal inspectors as having 0, 3.4, 5.5, and 8.0% sprouted kernels. A fifth sample was prepared to contain 1.7% sprouted kernels by mixing equal amounts of the 0- and 3.4%-sprouted samples. These samples contained 0.019, 0.106, 0.200, 0.441, and 0.705 dextrinizing units per gram, determined as reported by Mathewson and Pomeranz (1979).

Reagents

The substrate is a potato amylose dyed with Cibacron-blue F3GA (Hoffmann-LaRoche, Nutley, NJ 07110). This powdered dyed starch was formed into 60-mg tablets for ease in handling. One tablet is required for each test.

Phosphate buffer was prepared by mixing 810 ml of 0.05*M* monobasic sodium phosphate (6.00 g/L) and 190 ml of dibasic sodium phosphate (7.10 g/L). To this liter of buffer, 0.20 g of $CaCl_2 \cdot 2H_2O$ was added. The pH of this solution was 6.20.

Although not available for this study, the substrate is now available (D&S Instrument Ltd., Pullman, WA 99163) in the form of tablets containing the mono- and dibasic sodium phosphate and $CaCl_2 \cdot 2H_2O$ so that buffer preparation is eliminated and the only liquid required is distilled water.

Equipment

The equipment for this study (Fig. 1) included a mill, balance, dry-block heater with 20 × 125-mm screw-cap culture tubes, stirring motor, laboratory timer, filtering unit, and colorimeter. All of the individual items are available commercially. For routine testing, the temperature of the heat block was set to 60°C. The balance should be accurate to 0.01 g. The stirring motor was activated only while the timer was on. The filtering unit, described by Mathewson et al (1978), contained two filter papers. The first was a Teflon filter (TE-38, Schleicher & Schuell Inc., Keene, NH 03431) that retains particles larger than 5 μm in diameter and ensures an optically clear solution. A glass fiber filter (25-glass, Schleicher & Schuell Inc., Keene, NH) placed on top of the Teflon

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filter traps the larger particles of ground grain and insoluble substrate. The glass fiber filter was replaced for each test, but the Teflon filter can be used for 75–100 tests. The colorimeter was set to read absorbance at 620 nm. An adaptor for ½-in. tubes was used in the sample compartment.

An Alpha-Amylase Analyser is manufactured by D&S Instrument Ltd., Pullman, WA 99163. Although not utilized in the collaborative portion of this work, the instrument is designed to carry out the procedure described below.

Procedure

Five basic steps are required: 1) grinding the whole grain, 2) weighing a sample of the ground material, 3) simultaneous extraction and reaction, 4) filtering the reaction mixture, and 5) determining the absorbance at 620 nm of the reaction solution.

Grinding the whole grain can be accomplished with any suitable laboratory mill. We used a Udy-Cyclone mill with a 1-mm screen. The ground grain was blended and 200 mg was transferred to a culture tube containing 10.0 ml of phosphate buffer maintained at 60° C. The water-grain mixture was shaken horizontally 20 times to wet and disperse the sample, and the tube was then replaced in the heat block in the right front position. The stirring motor was lowered into position so that the stirring rod was inside the culture tube. The reaction sequence was initiated by setting a specified amount of time on the timer. For routine analysis of wheat, the reaction time is 3.0 min. The timer was set at 3 min and 10 sec. The extra 10 sec allowed time for picking up a substrate tablet and positioning it for dropping into the culture tube containing the sample to be analyzed. When the timer reached exactly 3 min, the substrate tablet was dropped into the culture tube. The stirring action disperses the tablet while mixing the whole mixture so that extraction of α-amylase and reaction with CBA occur simultaneously. At the end of the 3-min period, the stirring motor stopped and was raised out of the tube and swung aside. The contents of the reaction tube were then poured into the filter unit. A Vacutainer (Becton-Dickinson, Rutherford, NJ 07070) was pushed onto the needle on the under side of the filter unit. The vacuum in the Vacutainer immediately pulls all the liquid through the filter, separating the substrate from the enzyme and stopping the reaction. The colored reaction fluid was then contained in a clear sealed tube that could be placed directly in the colorimeter for determination of absorbance at 620 nm.

RESULTS AND DISCUSSION

We have previously shown that results from the colorimetric test are linearly related to α-amylase and that the technique can accurately measure α-amylase activity in wheat samples (Mathewson and Pomeranz 1979). Furthermore, colorimetric test results have been shown to be highly correlated with both falling number and amylograph results (Mathewson et al 1981). We wanted to evaluate the simplified procedure for its reliability in differentiating among wheats having different levels of sprouting. To do this, two collaborative studies were organized using several samples of Western White wheat. These wheats were selected on the basis of their α-amylase activity, which ranged from about 0.10 to 0.70 dextrinizing units per gram. That range of α-amylase was selected on the basis of extensive work by Finney et al (1981) indicating that this range of α-amylase was important for the evaluation of Western White wheats for use in Japanese sponge cakes.

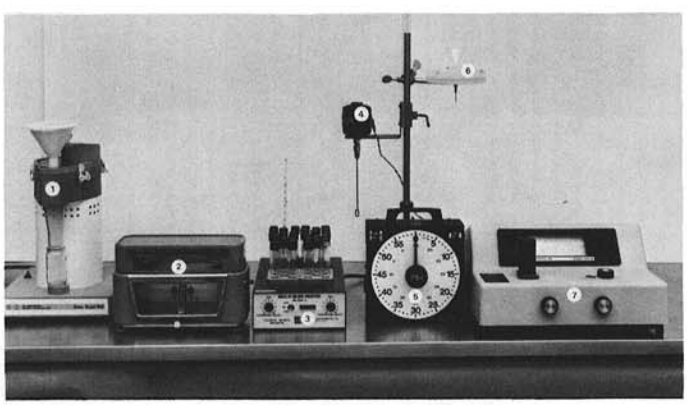

Fig. 1. Equipment necessary to perform the simplified colorimetric procedure. **1,** mill; **2,** balance; **3,** heat block; **4,** stirrer motor; **5,** timer; **6,** filter unit; **7,** colorimeter.

Intralaboratory Study

In the first study, half of each of five wheat samples was ground, blended, and resampled (hereafter referred to as "ground"). The remaining half was sampled whole, then ground immediately before testing (hereafter referred to as "whole"). Each of 10 laboratory technicians from our laboratory, who had no prior experience with this procedure, received 10 portions of ground samples and 10 portions of whole samples (~100 g each). Each group of 10 samples contained duplicates of the original five samples. The participants selected the samples at random, ground the whole samples, and measured the α-amylase content of all samples in random order in duplicate. Although these technicians were instructed to mix the samples they ground, no effort was made to ensure that they blended each sample to homogeneity; they simply mixed the ground wheat to their satisfaction. The purpose of this design was to estimate sources of error, to determine whether this method could reliably distinguish between samples, and to estimate the extent of sampling error associated with sampling from whole wheat compared to sampling from a homogeneous blend of ground wheat. The participants in this first study all used the same equipment (Fig. 1).

Table I shows the results of the analyses of variance for the whole and ground samples. The F-value for level of sprout damage for both whole and ground wheats is highly significant ($P < 0.0001$), indicating that the absorbance of the final reaction fluid is different for the different levels of sprout damage. To further study these differences, an analysis (Gabriel 1978) was done to determine whether the different levels of sprout damage could be distinguished from one another using the CBA method. The results of this analysis (Fig. 2) show that each level could be distinguished in both whole and ground wheat. Thus, the method was able to distinguish among the levels of α-amylase in each sample for both whole and ground wheats.

Presumably, grinding all of the sample, blending it, and then resampling should result in a smaller sampling error than would sampling whole grain, then grinding and testing it. This is, in fact,

TABLE I
Analysis of Variance: Data from Intralaboratory Study

Source	Degrees of Freedom	Sum of Squares	Mean Square	F Value	P>F
Whole Wheat					
Level	4	12.605	3.151	633.80	0.0001
Collaborator	9	0.057	6.39×10^{-3}	1.63	0.25
Level × collaborator	36	0.179	4.97×10^{-3}	1.27	0.25
Sample (level × collaborator)	50	0.196	3.92×10^{-3}	3.18	0.0001
Determination	100	0.123	1.23×10^{-3}		
Total	199				
Ground Wheat					
Level	4	11.755	2.938	2,989.20	0.0001
Collaborator	9	0.031	3.44×10^{-3}	3.51	0.003
Level × collaborator	36	0.035	9.72×10^{-4}	2.08	0.01
Sample (level × collaborator)	50	0.024	4.80×10^{-4}	0.58	0.975
Determination	100	0.081	8.10×10^{-4}		
Total	199				

what was observed (Table I). The sample (level × collaborator) variance is significant for whole wheat (F = 3.18) but not for the ground wheat (F = 0.58). Nevertheless, the sampling error for both ground and whole samples was small compared to differences among sprout levels. Even though the interaction between level and collaborator is significant in ground wheat (Table I), each level of sprout damage is still distinguishable (Fig. 2B).

Estimates of the variance components are presented separately for each level of sprout damage (Table II). The total variance was partitioned into three components; that due to differences among collaborators, that due to heterogeneity of the sample, and that due to failure of the test itself to give the same value for repeat determinations on the same sample. The total variance is generally larger for the whole wheat than for ground wheat. Examining the individual components reveals that the difference is due primarily to sampling error in the whole wheat samples. The variance values for ground and whole wheat are similar for collaborator and determination errors but quite different for the sample error. In terms of average contribution to total error, collaborator variance contributed about 20% in both ground and whole wheat. Heterogeneity of the sample contributed 33% of the total for whole wheat but only 8% for ground wheat. Although the percent contribution of determination variance appears to be much larger for ground samples (71.5%), the actual variance components are similar for both ground and whole samples; the high percentage value is due primarily to the decrease in sample variance.

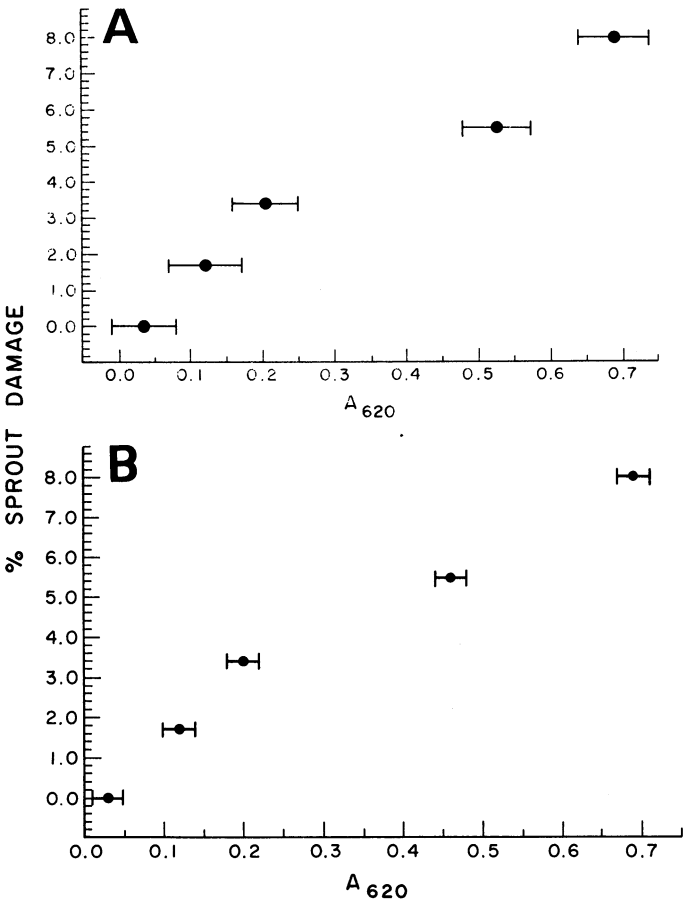

Fig. 2. Intralaboratory study: 95% comparison intervals for "whole" (**A**) and "ground" (**B**) wheat samples.

Although the percentages by themselves are of limited value in interpreting these data, they may be useful in determining trends. In most cases, the determination variance was the largest contributor to total variance. To determine the significance of the various sources of error, a standard deviation (SD) and coefficient of variation (CV) were calculated for each source (Table III). The total SD (SD_t) and CV (CV_t) which were calculated from the total variance, measure the reproducibility of the test, under the conditions described, and account for collaborator differences, sampling error, and determination error. If the collaborator variance is removed, so that only the contributions of sampling and determination errors are included, neither the SD (SD_{s+d}) nor the CV (CV_{s+d}) is greatly affected. In other words, using the same equipment, data produced by different collaborators for all samples was consistent. This was true for both whole and ground samples. If the contribution of sampling error is then removed so that only determination error is included (SD_d and CV_d), one can see that the sample error contributed significantly to the SD and CV in whole samples but not in ground samples. Although the determination variance is large compared to the other variance components, it still represents a relatively small percentage of the mean. The CV for level 1 is consistently higher than for levels 2–5 (Table III). This is probably due to the fact that the colorimeter (Spectronic 20, Bausch and Lomb, Rochester, NY) has limited accuracy and precision at very low absorbance levels. The mean absorbance at 620 nm for level 1 was ~0.033, and the SD associated with this absorbance range is likely to be high, thus inflating the CV.

This study demonstrated the feasibility of this procedure for evaluating wheat using α-amylase as the index of sprouting. The different levels of sprouting were all distinguishable from one another. The determination variance component was the largest

TABLE II
Distribution of Variance Components: Data from Intralaboratory Study

Sprouting Level	Variance Component,[a] ×10⁻⁴			
	Total	Collaborator	Sample	Determination
		Whole Wheat		
1	2.12	0.23 (10.8)	0.41 (19.4)	1.48 (69.8)
2	5.00	0.76 (15.4)	1.39 (28.1)	2.79 (56.4)
3	19.00	8.70 (45.7)	4.20 (22.0)	6.10 (32.3)
4	52.90	10.20 (19.2)	17.20 (32.6)	25.50 (48.2)
5	69.70	0.00 (0.0)	44.00 (63.1)	25.70 (36.9)
(Mean)		(18.2)	(33.0)	(48.7)
		Ground Wheat		
1	1.88	0.46 (24.5)	0.00 (0.0)	1.42 (75.5)
2	1.27	0.25 (19.4)	0.34 (26.5)	0.69 (54.1)
3	5.20	0.33 (6.2)	0.53 (10.1)	4.39 (83.6)
4	11.50	3.30 (28.4)	0.37 (3.2)	7.80 (68.4)
5	34.40	8.30 (24.0)	0.00 (0.0)	26.10 (75.9)
(Mean)		(20.5)	(8.0)	(71.5)

[a]Values in parentheses are percent of total.

TABLE III
Standard Deviation (SD) and Coefficient of Variation (CV): Data from Intralaboratory Study

Sprouting Level	SD_t[a]	CV_t[a]	SD_{s+d}[b]	CV_{s+d}[b]	SD_d[c]	CV_d[c]
			Whole Wheat			
1	0.015	42.8	0.014	40.4	0.012	35.2
2	0.022	18.0	0.020	16.5	0.017	13.4
3	0.044	20.9	0.032	15.4	0.025	11.9
4	0.073	13.8	0.065	12.4	0.050	9.5
5	0.084	12.0	0.084	12.0	0.051	7.3
			Ground Wheat			
1	0.014	42.8	0.012	36.7	0.012	36.7
2	0.012	9.4	0.010	8.4	0.008	6.9
3	0.023	11.2	0.022	10.8	0.021	10.2
4	0.034	7.4	0.028	6.2	0.028	6.1
5	0.059	8.4	0.051	7.3	0.051	7.3

[a]Calculated from total variance.
[b]Calculated from sample and determination variation.
[c]Calculated from determination variation.

contributor to total variance; however, this contribution was still a relatively small percentage of the final test result. The fact that collaborator and sample variance components were smaller indicates that both these sources of error were well controlled. In all cases, the differences attributable to the error components were considerably smaller than the differences between levels of sprouting. When the same equipment is used, the variance among collaborators is small, indicating that standardization of this procedure is possible. Sampling of whole grain is a source of error and sampling should be done by taking the 200-mg subsample of ground wheat from a larger portion (~300 g) sampled from the whole grain. The method is rapid and simple enough that untrained technicians produced results that reliably determined the relative amounts of sprouting.

Interlaboratory Study

A second collaborative study was designed utilizing the same samples but involving participants from nine laboratories—seven in the United States and two in Canada. The necessary equipment was obtained by each collaborator. We emphasized the need for standardized equipment and, to the extent possible, each collaborator complied by obtaining those pieces of equipment not on hand. Even so, some differences in equipment remained, such as the use of several types of colorimeters. The format of the test was the same as for the intralaboratory study except that each collaborator received 10 portions of ground wheat only.

The analysis of variance for data obtained in this interlaboratory study is shown in Table IV. As in the intralaboratory study, the different levels of sprouted wheat produced distinguishably different absorbance values (see also Fig. 3). Collaborator differences were also significant. This was certainly not unexpected because each collaborator used his own equipment setup, and these were not identical. But, even though the interaction of level and collaborator was significant, the levels of sprout damage were distinguishable from one another. Table V shows the breakdown of the variance components. An obvious difference in these interlaboratory data, compared to the intralaboratory data, is that collaborator error is by far the largest contributor to total variance (~68%), followed by determination and sample errors. If the SD and CV are calculated as before (Table VI), removal of the collaborator error makes a large difference in both SD_{s+d} and CV_{s+d}. Much less of a difference is then seen in omitting the sample variance. Thus, collaborator error is a major contributor to total error in the interlaboratory study, whereas in the intralaboratory study, which used only one set of equipment, it was of least significance. The sample variance represents approximately the same percentage of the total variance as it did for ground samples in the intralaboratory study. We were interested to note that the determination variance component was quite similar in the intra- and interlaboratory studies. Thus, if the basic equipment is set up properly, it can provide consistent results within a laboratory.

These data indicate once again that within a laboratory, different operators can achieve reproducible results that reliably distinguish among the different levels of sprouting. The major source of error in this study was attributable to differences among collaborators. Standardization of the equipment, such as providing a common built-in spectrophotometer to give equivalent response characteristics and automating control of the timed sequences, would probably decrease error among different operators. An instrument of this type (Fig. 4) has been developed and is commercially available (D&S Instrument, Ltd., Pullman, WA). Table VII shows data generated in our laboratory with this

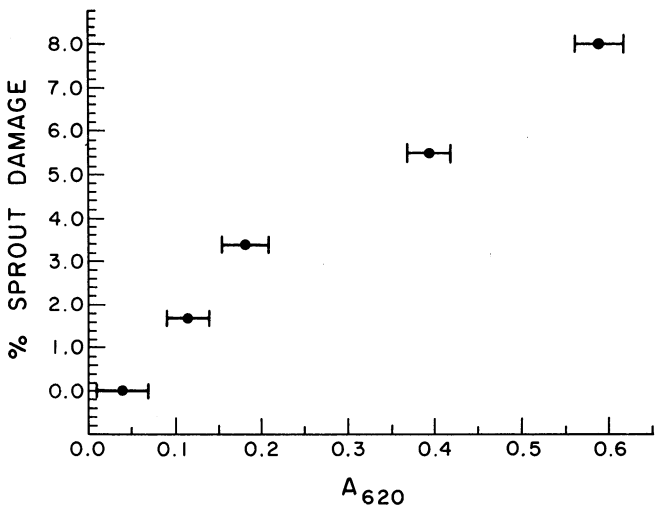

Fig. 3. Interlaboratory study: 95% comparison intervals for "ground" wheat samples.

TABLE IV
Analysis of Variance: Data from Interlaboratory Study

Source	Degrees of Freedom	Sum of Squares	Mean Square	F Value	P>F
Level	4	7.246	1.811	282.08	0.0001
Collaborator	8	0.324	4.05×10^{-2}	21.87	0.0001
Level × collaborator	32	0.205	6.41×10^{-3}	3.47	0.0001
Sample (level × collaborator)	45	0.083	1.84×10^{-3}	3.66	0.0001
Determination	90	0.045	5.00×10^{-4}		
Total	179				

TABLE V
Distribution of Variance Components: Data from Interlaboratory Study

Sprouting Level	Variance Component,[a] × 10^{-4}			
	Total	Collaborator	Sample	Determination
1	5.70	3.70 (66.3)	0.00 (0.0)	1.90 (33.7)
2	8.60	5.70 (66.1)	1.03 (12.1)	1.88 (21.9)
3	30.10	21.10 (70.0)	0.00 (0.0)	9.00 (30.0)
4	59.30	38.90 (65.6)	14.30 (24.2)	6.10 (10.2)
5	97.60	72.90 (74.6)	18.40 (18.8)	6.40 (6.5)
(Mean)		(68.5)	(11.0)	(20.5)

[a] Values in parentheses are percent of total.

TABLE VI
Standard Deviation (SD) and Coefficient of Variation (CV): Data from Interlaboratory Study

Sprouting Level	SD_t[a]	CV_t[a]	SD_{s+d}[b]	CV_{s+d}[b]	SD_d[c]	CV_d[c]
1	0.024	59.7	0.014	34.8	0.014	34.3
2	0.029	25.3	0.017	14.7	0.014	11.8
3	0.055	30.1	0.030	16.5	0.030	16.5
4	0.077	19.4	0.045	11.4	0.025	6.2
5	0.098	16.8	0.050	8.4	0.025	4.2

[a] Calculated from total variance.
[b] Calculated from sample and determination variation.
[c] Calculated from determination variation.

TABLE VII
Data on Interlaboratory Study Samples Using Commercial Alpha-Amylase Analyzer

Level	SD[a]	CV[a]	Mean Absorbance at 620 nm
1	0.004	4.7	0.073
2	0.012	6.9	0.169
3	0.017	7.2	0.239
4	0.032	7.6	0.428
5	0.039	6.1	0.629
Mean		6.5	

[a] Standard deviation and coefficient of variation calculated from sample and determination variation.

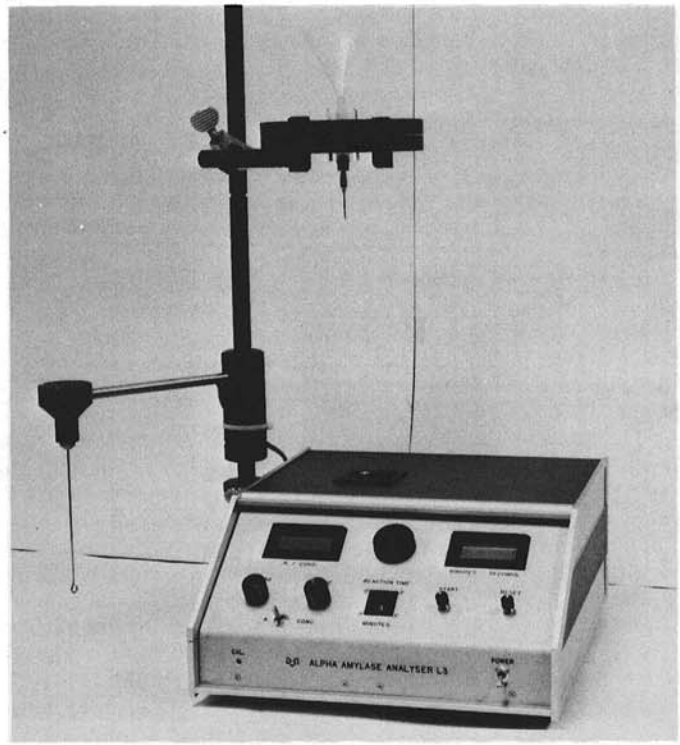

Fig. 4. A commercially available α-amylase analyzer.

instrument on the same ground samples sent to the participants in the interlaboratory study. The SD and CV in this table were calculated from data resulting from running each of the 10 samples six times. The SD and CV were calculated from sample and determination variance components. The average CV for all levels was 6.5%. Even with an absorbance of only 0.073, the SD for level 1 was only 0.004 and the CV was 4.7. The colorimeter in this instrument is accurate to 0.001 absorbance units and gives much more precise, stable readings than those obtained with the Spectronic 20, resulting in a CV for blend 1 comparable to those of blends 2–5.

The previously reported laboratory procedure (Mathewson and Pomeranz 1979), which is more complex but more precise, was used to compare results for α-amylase activity on these samples with the results from the intralaboratory study. This comparison is presented in Table VIII. Excellent agreement between the methods was obtained.

Our results demonstrate that the simplified method provides a promising alternative to present subjective evaluation of wheat for sprout damage in marketing channels. In both the inter- and intralaboratory studies, different levels of sprouting were consistently distinguishable, and although errors in sampling were significant when whole wheat was used, the sampling error did not prevent differentiation among the levels of sprouting. Error among collaborators was significant when different equipment setups were used, but the determination error for the test itself was consistent throughout both studies. These results show that this simplified

TABLE VIII
Comparison of Results on Ground Samples from Intralaboratory Study, Using Laboratory CBA[a] Method and Simplified CBA Method

Sprouting Level	α-Amylase Activity[b]	
	Laboratory Procedure[c]	Simplified Procedure
1	0.019	0.024
2	0.106	0.113
3	0.200	0.198
4	0.441	0.457
5	0.705	0.698

[a] Cibacron-blue amylose.
[b] In dextrinizing units per gram.
[c] Method of Mathewson and Pomeranz (1979).

method can provide a reliable, sensitive, and meaningful assay of α-amylase. The availability of standardized instrumentation should increase the precision of the test by decreasing both collaborator and determination error. The modified method meets the requirements of speed, simplicity, and ability to test on a single sample basis as stipulated by action and regulatory agencies for testing in marketing channels. It is also well suited for testing large numbers of samples in screening and plant breeding programs.

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