

A Rapid Microassay for Determination of Peroxidase in Wheat and Flour¹

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ABSTRACT

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A rapid sensitive microplate assay for the determination of peroxidase activity in wheat, flour, or individual wheat kernels has been developed using a commercially prepared 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) solution. The assay derives a six-point calibration curve based on commercially available horseradish peroxidase ($r^2 > 0.990$), while simultaneously analyzing eight extracts in triplicate. The coefficients of variation (CV) of triplicate assays of a single extract from any of the three sources investigated were generally

<2.0%. Multiple extracts ($n = 5$) of either whole meal or flour yielded assays with an average CV < 5.0%. Preparation of the calibration standards with commercially available peroxidase stabilizing buffer allowed the standards to be used for five days without any deterioration in the assay's reproducibility. This assay is ideally suited for high-throughput operations such as millstream analysis or plant breeder screening evaluations.

Peroxidase enzymes (EC 1.11.1.7) catalyze the oxidation of a number of substrates in the presence of hydrogen peroxide and are of particular interest to cereal scientists. During wheat kernel development, peroxidase plays a role in restricting seed elongation (Chanda and Singh 1997). In breadmaking, arabinoxylans in wheat and rye undergo oxidative coupling of ferulic acid residues, causing an increase in dough viscosity on mixing in the presence of peroxidase and hydrogen peroxide (Schooneveld-Bergmans et al 1999). Peroxidase also plays a significant role in carotenoid bleaching during dough mixing (Hawthorn and Todd 1955; Hsieh and McDonald 1984) and may be responsible for undesirable browning in noodle products (Baik et al 1995; Kruger et al 1992). Wheat contains up to 12 peroxidase isozymes that vary in activity depending on tissue, developmental stage, and physiological state (Kruger and LaBerge 1974a,b; Iori et al 1995).

Researchers (Honold and Stahmann 1968; Kruger and LaBerge 1974a; Zmrhal and Machackova 1978; Paterson et al 1990; Converso and Fernandez 1995) have used a variety of spectrophotometric techniques to assay peroxidase activity in wheat throughout the years but there is no universally accepted system. Currently, only Approved Method 22-80 (AACC 2000) is a subjective evaluation of dye color in a solution of ascorbic acid, hydrogen peroxide, and 2,6-dichloro-indophenol. The AACC method uses only a three-color scale to determine whether peroxidase is present or absent and it is not a quantifiable assay. The most recent method (Delcros et al 1998) employed guaiacol as a substrate for kinetic measurement (465 nm) of wheat flour or dough extracts. A more recent assay (Morgan 1999) was a variation of the Honold and Stahmann (1968) method.

This article describes a rapid, sensitive assay ideal for wheat, flour, or single-kernel analysis. It employs a 96-well microplate system and is ideal for assisting plant breeders in screening for this key enzyme.

MATERIALS AND METHODS

Wheat, Flour, and Millstreams

Wheat samples representing Canada Western Red Spring (CWRS), Canada Prairie Spring White (CPSW), and Canada Prairie Spring Red (CPSR) classes were collected from across western Canada

during the 1999–2003 harvests and stored at 4°C. Flour samples were prepared on an Allis-Chalmers laboratory mill according to Black et al (1980). Whole meal samples were produced by grinding wheat samples (20 g) in a cyclone mill (Udy Corp., Fort Collins, CO) equipped with an 0.80-mm sieve and stored at 4°C until assayed. Individual CWRS millstreams were prepared using a composite sample of CWRS, collected during the 2002 western Canada harvest survey, on the GRL tandem Buhler mill according to Martin and Dexter (1991).

Ground wheat or flour (0.50 g) was placed in a polypropylene 16- × 104-mm centrifuge tube (Nalgene, Rochester, NY) with 5.0 mL of cold (4°C) sodium acetate buffer (0.1M, pH 4.2), capped, and immediately vortexed (10 sec) to ensure suspension of all material. The centrifuge tubes were placed on a rotating mixer in a cold room maintained at 4°C and continuously tumbled for 30 min. The tubes were centrifuged (10,000 × *g*, 10 min) at 4°C and the supernatant was decanted and retained for analysis. Supernatants were filtered through a 25-mm, 1.0-μm glass fiber membrane syringe filter (Acrodisc, Pall Gelman, East Hills, NY) into a borosilicate glass tube and kept on ice in a closed container to minimize exposure to light. Immediately before analysis, assay aliquots were prepared by diluting 100 μL of the extract with 900 μL of cold 0.1M sodium acetate buffer, pH 4.2.

Individual kernels were analyzed by breaking the kernel into small pieces using a custom-built steel mortar and pestle unit. The crushed material was placed into a pretared 1.5-mL microcentrifuge tube and the weight was recorded. An aliquot (1.0 mL) of cold, 0.1M acetate buffer, pH 4.2, was added, and the tube was sealed and immediately vortexed (5 sec). The tube subsequently was placed on a continuously agitating mixer for 30 min in a cold room at 4°C. The centrifuge tube was centrifuged for 10 min at 10,000 × *g* and the supernatant was withdrawn, filtered (as described above), and stored on ice until assayed. The dilution factor for the single-kernel extract was adjusted to reflect the individual kernel's weight.

Initially, a stock solution of horseradish peroxidase (Sigma P-8375) was prepared by dissolving an amount equivalent to 400 purpurogallin units in 100 mL of 0.1M acetate buffer, pH 5.0. The stock solution was protected from light and kept at 4°C except when preparing the calibration standards. Calibration standards (6) ranging from 2.0 to 7.0 × 10⁻³ purpurogallin units were prepared fresh daily and kept on ice until used. All assays were performed using a 96-well microplate reader (ThermoMax model; Molecular Devices, CA) and their SoftMax Pro for Windows software (v. 3.0). Standards (25 μL), extraction buffer (25 μL) serving as the blank, and a daily CWRS control extract (25 μL) each were pipetted into their designated wells of the first, third, and fifth columns. Due to the rapid conversion of the substrate by the enzyme, the three extract aliquots from the eight samples being

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analyzed were placed in their designated wells of the second, fourth, and sixth columns (Fig. 1). This allowed eight separate samples to be analyzed simultaneously. Preliminary data (not shown) demonstrated that this layout maximized reproducibility of the assay and allowed the second half of the plate to be used for additional analyses, thereby reducing assay costs.

The assay was initiated by the rapid, simultaneous delivery of 150 μL of 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) substrate (Pierce 1-Step ABTS, product no. 37615), equilibrated at room temperature, to each well within a column using an eight-channel electronic pipette. The reaction was stopped after 1 min by dispensing 100 μL of 2M sulfuric acid to each well in a column using the eight-channel pipette. Addition of the stopping reagent followed the exact same sequence as the addition of substrate to ensure maximum reproducibility. The plate was placed immediately in the microplate reader, which initiated a 5-sec mix before analyzing absorbance at 405 nm. The software automatically subtracted the blank values, performed linear regression analysis on the calibration standards, and provided the individual activities for each of the three wells plus a full statistical analysis on the mean for each sample. The microplate tray then was reversed and the same assay procedure repeated. This allowed 16 samples to be analyzed per microplate within 10 min, facilitating large numbers of samples to be batch-extracted.

The use of four different peroxidase stabilizing buffers also was evaluated to improve the robustness of the test. These included

	1	2	3	4	5	6
A	Std. 1	Sample 1	Std. 1	Sample 1	Std. 1	Sample 1
B	Std. 2	Sample 2	Std. 2	Sample 2	Std. 2	Sample 2
C	Std. 3	Sample 3	Std. 3	Sample 3	Std. 3	Sample 3
D	Std. 4	Sample 4	Std. 4	Sample 4	Std. 4	Sample 4
E	Std. 5	Sample 5	Std. 5	Sample 5	Std. 5	Sample 5
F	Std. 6	Sample 6	Std. 6	Sample 6	Std. 6	Sample 6
G	CWRS Control	Sample 7	CWRS Control	Sample 7	CWRS Control	Sample 7
H	Blank	Sample 8	Blank	Sample 8	Blank	Sample 8

Fig. 1. Optimal sample layout for a 96-well microplate to maximum reproducibility and sample throughput.

peroxidase, horseradish, conjugate-stabilizing solution (#516534; Calbiochem, La Jolla, CA); Guardian peroxidase stabilizer/diluent (#37548; Pierce, Rockford, IL); peroxidase stabilizing buffer (#P-9209; Sigma-Aldrich, Oakville, ON), and a buffer prepared in the laboratory according to SOP-105 from Virusys Corporation (Sykesville, MD). All stock solutions were protected from the light and kept at 4°C.

Statistical Analysis

Data were analyzed using the Statistical Analysis System (v. 8.2; SAS Institute Inc., Cary, NC). Analysis of variance (ANOVA) was determined using Proc GLM and Proc MIXED. To calculate the respective percentage contributions of various parameters to the variance of the assay, the nested and random options of Proc MIXED were used according to Littell et al (2002).

RESULTS AND DISCUSSION

Method Optimization

In their investigation of dough-mixing effects on peroxidase activity in wheat flour, Delcros et al (1998) extracted flour samples (1.0 g) with 20 mL (1:20 ratio) of 0.1M acetate buffer, pH 4.2. Evaluation of the new assay using CWRS and CPSW wheat and

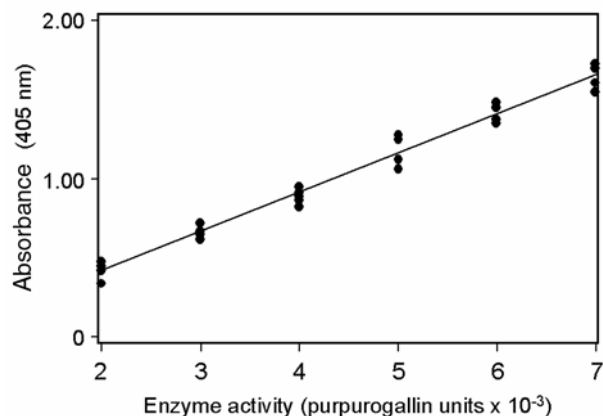


Fig. 2. Relationship between absorbance and the concentration of horseradish peroxidase standards over a five-day work week.

TABLE I
Influence of Extract Dilution on Peroxidase Activity (corrected for dilution) on Four Separate Extractions of Each Sample

Wheat Class Samples ^a	Type	Dilution	Mean Activity (purpurogallin units/g)	Coefficient of Variation (%)
CPSW	Flour	40	74.5	4.9
CPSW	Flour	100	237.6	9.6
CPSW	Wheat	40	119.0	4.3
CPSW	Wheat	100	337.7	8.3
CWRS	Flour	40	90.8	5.1
CWRS	Flour	100	298.1	7.7
CWRS	Wheat	40	132.8	16.0
CWRS	Wheat	100	490.2	10.1

^a CPSW, Canada Prairie Spring White; CWRS, Canada Western Red Spring.

TABLE II
Influence of Different Peroxidase Stabilizing Buffers on an Enzyme Calibration Curve Over a Five-Day Period^a

Calibration Curve	Laboratory Buffer	Calbiochem	Pierce	Sigma-Aldrich
Average slope (absorbance/units $\times 10^{-3}$)	0.279a	0.291a	0.342b	0.243c
CV of average slope (%) ^b	6.67	6.31	10.65	5.66
Average r^2 of slope	0.984a	0.992b	0.990b	0.991b
Mean peroxidase activity of control sample	384.4a	326.4b	295.3c	445.9d
Standard deviation of control sample	23.5	22.1	19.1	16.0
CV of control sample (%)	6.13	6.77	6.46	3.58

^a Values with the same letter are not significantly different ($P < 0.05$).

^b CV, coefficient of variation; peroxidase activity expressed in purpurogallin units/g.

flours, employing the Delcros et al (1998) ratio, yielded extracts that reacted too quickly with the ABTS substrate to give meaningful results (data not shown). Subsequent dilution of the initial (1:20) extracts on a 1:1 basis with the acetate buffer resulted in a 40-fold dilution with measurable activity (Table I).

Subsequent investigation of samples (0.5 g) extracted with the acetate buffer (5 mL) in which an aliquot of the supernatant was further diluted 1:9 with acetate buffer ($\times 100$ dilution) yielded a significantly greater activity for all extracts (Table I). In all cases, the amount of activity increased approximately threefold, although no clear pattern was noted in the coefficient of variation for the assay. Investigation of higher dilution ratios (data not shown) yielded no significant change in activity. This implied that the reaction was substrate-limited at the lower dilution levels; therefore, the 100-fold dilution was used in all subsequent analyses.

TABLE III
Influence of Stabilizing Buffer Incorporated with Normal 0.1M Acetate Buffer on Peroxidase Activity in Four Canada Western Red Spring (CWRS) Wheat Samples^a

Sample	Stabilizing Buffer	Mean Activity (purpurogallin units/g)	SD	CV (%)
CWRS 1	No	380.7	16.7	4.4
	Yes	443.8	9.4	2.1
CWRS 2	No	280.5	14.9	5.3
	Yes	293.3	12.1	4.1
CWRS 3	No	212.3	13.9	6.6
	Yes	198.2	15.0	7.6
CWRS 4	No	291.0	13.1	4.5
	Yes	329.9	18.4	5.6

^a Separate extracts ($n = 4$) for each sample analyzed in three wells each.

Initial investigations using the peroxidase stock solution (0.1M acetate buffer, pH 4.2) to prepare calibration standards for use over a three-day period exhibited a significant decline in the stock and standards' enzyme activity (data not shown). To address the decline in enzyme activity, the peroxidase calibration stock solution was prepared in each of the three commercial buffers and one laboratory-equivalent peroxidase stabilizing buffer rather than in the acetate buffer. Individual calibration standards were prepared by diluting the appropriate volume of stock-stabilized solution with cold 0.1M acetate buffer, pH 4.2, 1 hr before use. Using the Sigma-Aldrich stabilizing buffer yielded a significantly greater absorbance response than using the laboratory and other commercial buffers and remained stable over a five-day period (Fig. 2). No significant day effect was detected by ANOVA. The linear regression calibration curves prepared with the different stabilizing buffers over the five-day period indicated that the standard deviation of the calibration slope and its corresponding coefficient of variation were smallest using the Sigma-Aldrich stabilizing buffer and largest using the Pierce buffer (Table II). All three commercial stabilizing buffers displayed excellent coefficients of determination ($r^2 = 0.99$) with no discernible differences. Analysis of the CWRS control sample over this period indicated that the Sigma-Aldrich stabilizing buffer yielded the most consistent results with the lowest coefficients of variation (CV), approximately half that of the other buffers, for the replicated analyses. Therefore, the use of Sigma-Aldrich stabilizing buffer in the preparation of the peroxidase calibration stock solution was incorporated into the assay protocol.

The positive effect demonstrated by the stabilizing buffer on the horseradish peroxidase standards suggested that it would be beneficial in preserving the enzyme extracted from the wheat and flour. The stabilizing buffer, however, is extremely expensive;

TABLE IV
Reproducibility and Repeatability of Peroxidase Assay on Whole Meal Samples of Canada Western Red Spring (CWRS) Wheat

Day	Extract	CWRS Sample 1			CWRS Sample 2		
		Mean ^a	SD	CV (%)	Mean ^a	SD	CV (%)
1	1	421.3	9.9	2.3	166.0	6.7	4.0
1	2	431.0	5.7	1.3	173.8	1.2	0.7
1	3	414.9	3.9	0.9	189.6	1.9	1.0
1	4	409.2	9.2	2.2	178.1	3.1	1.7
1	5	463.1	3.1	0.7	194.5	2.2	1.1
Average ^b		427.9	21.3	5.0	180.4	11.6	6.4
2	1	369.1	8.0	2.2	171.9	2.6	1.5
2	2	408.7	4.7	1.2	174.8	3.2	1.8
2	3	428.0	19.5	4.6	183.1	3.7	2.0
2	4	434.5	9.2	2.1	172.7	5.7	3.3
2	5	447.7	13.0	2.9	184.0	3.3	1.7
Average ^b		417.6	30.5	7.3	177.3	5.8	3.3
3	1	426.9	1.8	0.4	187.1	2.6	1.4
3	2	475.8	4.4	0.9	181.0	3.1	1.7
3	3	438.4	21.3	4.9	198.4	0.5	0.3
3	4	429.3	3.1	0.7	189.3	1.8	1.0
3	5	417.3	6.0	1.4	189.1	1.5	0.8
Average ^b		437.6	22.7	5.2	189.0	6.3	3.3

^a Mean value of the same extract in three wells (purpurogallin units/g).

^b Values for five extracts performed in a day.

TABLE V
Analysis of Variance Results of Proc GLM and MIXED on Canada Western Red Spring Reproducibility of Whole Meal Samples

Parameter	DF	Type III SS	Mean Square	F Value	Pr > F	Parameter Estimates ^a	
						Estimate	% of Variance
Sample	1	1,354,902	1,354,902	6,461	<0.0001	30,104	98.73
Day	2	3,838	1,919	9.15	0.0003	23.39	0.08
Extract (day)	8	8,024	1,003	4.78	0.0001	170.8	0.56
Replicate (extract)	10	823	82	0.39	0.9454	4.7×10^{-16}	0.00
Residual	192.5	0.63

^a Convergence criteria met.

therefore, only limited amounts would be practical when numerous samples were being analyzed. Four CWRS samples with varying peroxidase activity each were extracted four times using the normal 0.1M acetate buffer and compared with extracts prepared using the buffer augmented with Sigma-Aldrich stabilizing buffer at a 5:1 ratio. The results of the assay are shown in Table III. The use of ANOVA indicated that the presence of the stabilizing buffer had a significant positive effect ($P < 0.05$) on the enzyme activity and, therefore, it was incorporated into the extracting buffer for all subsequent tests. It is interesting that the sample with the lowest activity did not demonstrate the same positive influence on the enzyme activity as those samples with higher activity levels, although the reason for this difference is not known.

The reproducibility and repeatability of the optimized assay was subsequently assessed using two different CWRS whole meal samples, representing high and low peroxidase levels (Table IV). The assay's repeatability for a single extract was excellent for both samples, with the largest CV for the three wells analyzed over the three days being 4.9% and most (60%) being <2.0%. Examination of the extract's daily mean activity ($n = 5$) for both samples also highlighted excellent reproducibility because the largest daily CV for any sample was only 7.3%. Further statistical investigation of the multiday analysis of the two CWRS samples data using a GLM nested model indicated that sample, day, and

TABLE VI
Reproducibility of Peroxidase Assay Performed on Canada Western Red Spring Wheat Flour Samples

Extract	Mean ^a	SD	CV (%)
Cultivar 1			
1	307.6	3.9	1.3
2	320.5	3.5	1.1
3	285.2	16.6	5.8
4	327.4	1.5	0.5
5	324.8	4.4	1.3
Average ^b	313.1	17.4	5.5
Cultivar 2			
1	216.9	3.3	1.5
2	239.3	2.4	1.0
3	232.1	3.1	1.3
4	226.6	1.6	0.7
5	239.7	2.9	1.2
Average ^b	230.9	9.5	4.1
Cultivar 3			
1	410.0	3.2	0.8
2	408.3	6.0	1.5
3	445.1	7.1	1.6
4	398.1	5.3	1.3
5	409.8	5.3	1.3
Average ^b	414.3	17.9	4.3

^a Mean value of the same extract in three wells (purpurogallin units/g).

^b Values for five extracts performed in a day.

TABLE VII
Evaluation of Single-Kernel Peroxidase Activity for Two Canada Western Red Spring Wheat Cultivars

Activity ^a	Cultivar	
	AC Superb	Laura
Number	40	40
Minimum activity	126.8	92.4
Maximum activity	628.4	303.6
Range in activity	501.6	211.2
Mean activity	270.4	158.3
Average SD (three wells)	4.4	2.4
Average CV (%) (three wells)	1.6	1.5

^a Minimum and maximum activity represent the mean value of the same extract in three wells (purpurogallin units/g); mean activity is mean value for all kernels (purpurogallin units/g); SD is standard deviation; CV is coefficient of variation.

extract displayed significant effects (Table V). Only the replicate analysis of individual extracts was not significant. This finding was not unexpected due to the high degree of repeatability of the assay itself. Analyzing the data using a Proc MIXED model, employing the random option (Littell et al 2002), provided better insight into the source of variance within the data (Table V, right side). The samples were responsible for 98.7% of the variance in the assay results, which is highly desirable. The residual error was the second-largest source of variance, representing only 0.63%, whereas the extract itself contributed only 0.56%. The influence of day was even smaller (<0.1%). The suitability of this assay is clearly demonstrated because the samples are the key source of variance, with <1.3% being added by all other factors.

The reproducibility of the assay for flour samples was evaluated using three different CWRS cultivars (Table VI). The repeatability of single flour extracts displayed the same excellent results as those previously observed for whole meal. All but one sample extract displayed CV < 2.0%. The mean activity values ($n = 5$) for the three cultivars had very acceptable and consistent CV values (4.1–5.5%). All three cultivars tested displayed significantly ($P < 0.05$) different peroxidase levels.

Plant breeders have extremely limited amounts of seed material at the earliest generations and this has perennially plagued their screening evaluations. Individual kernels from two CWRS cultivars, AC Superb and Laura, were evaluated using the modified extraction procedure described previously and the results, adjusted for individual kernel weights, are shown in Table VII. The average CV of each single kernel's triplicate analysis displayed the same high degree of repeatability as observed for both the whole meal and flour samples. The standard deviations for AC Superb and Laura were 4.4 and 2.4, representing CV = 1.6 and 1.5%, respectively, consistent with those using whole meal or flour. Examination of the data showed a much larger range in individual kernel

TABLE VIII
Peroxidase Activity by Cultivar and Western Canada Crop Regions from the 2003 Harvest Survey

Cultivar	Number of Samples	Mean Activity (purpurogallin units/g)	Crop Region
AC Barrie	19	249.5 ± 28.2	1,2,3,4,5,6,7,8
AC Cadillac	6	198.8 ± 29.2	2,5,6,7,9
AC Domain	7	208.5 ± 38.5	2,3
AC Elsa	15	148.7 ± 19.4	4,5,6,7,8
AC Intrepid	5	190.2 ± 21.1	2,4,7
AC Superb	7	209.8 ± 40.3	1,3,6,8
AC McKenzie	5	210.3 ± 23.1	3,4,6,8

TABLE IX
Mean Peroxidase Levels in Individual Canada Western Red Spring Wheat Mill Streams

Mill Stream	Peroxidase Activity (purpurogallin units/g)
Break 1	442.2fg
Break 2	543.3d
Break 3	663.2c
Break 4	705.6bc
Quality 1	512.3de
Sizing 1	473.0ef
Middling 1	460.2e–g
Middling 2	478.6d–f
Middling 3	415.6fg
Middling 4	396.5g
Middling 5	458.3e–g
Middling 6	521.4de
Bran flour	669.3c
Fine bran	979.8a
Bran	744.4b
Shorts	919.4a

^a Means ($n = 3$) with the same letter are not significantly different, $P < 0.05$, (least significant difference = 69.0).

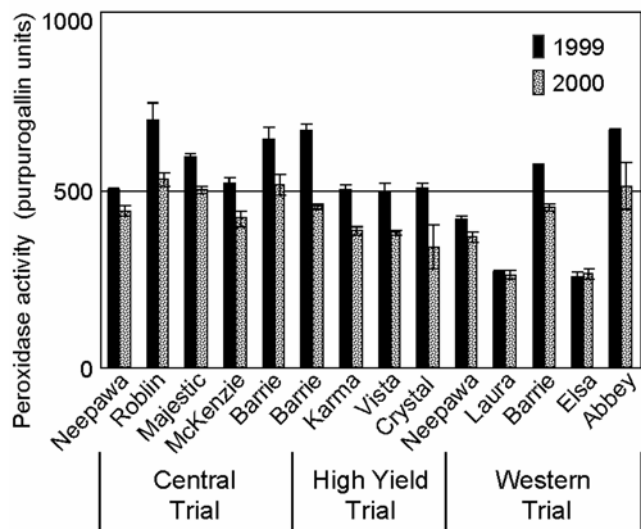


Fig. 3. Variations in whole meal samples of specific cultivars used in three different registration trials during the 1999 and 2000 crop years.

activity for AC Superb (126.8–628.4 activity units) than for Laura (92.4–303.6 units) and significantly different mean activities ($P < 0.05$).

Method Application

The ease of use of this assay combined with its rapidity make it an ideal tool for quality studies. Peroxidase produces undesirable discoloration in oriental noodles and this method was used to evaluate different CWRS cultivars retained from plant breeding trials. Analysis of whole meal from individual cultivars from the 1999 and 2000 crop years can be seen in Fig. 3. The data demonstrated the usefulness of this assay in discerning differences among different cultivars. AC Barrie, which is currently the dominant cultivar grown in western Canada, along with Roblin and AC Abbey, displayed significantly higher peroxidase levels than did cultivars such as AC Laura and AC Elsa.

The assay's ability to analyze multiple samples quickly was of benefit in confirming the varietal differences in peroxidase activity when examining producer-submitted samples from the 2003 crop year from various crop regions across western Canada (Table VIII). AC Elsa again displayed significantly lower peroxidase activity than the majority of other CWRS cultivars.

In preparing specialty flours for commercial noodle markets, the ability to blend individual millstreams to meet the specifications of the manufacturer is critical. The analysis of peroxidase activity of individual millstreams (Table IX) highlights the assay's ability to quickly discern significant differences and offers the miller the opportunity to blend accordingly. There are discernible breaks in peroxidase activity within the streams such that elimination of the shorts, fine bran, bran, and bran flour would significantly reduce the activity levels within the resulting flour. The flour's enzyme activity also could be further reduced by the removal of third- and fourth-break flours.

CONCLUSIONS

Currently, the AACC has no quantitative assay for peroxidase activity in wheat. A rapid assay for peroxidase activity in wheat, flour, or individual wheat kernels has been developed using a com-

mercially prepared ABTS solution. The assay displays excellent repeatability and good reproducibility and is ideally suited for handling a high throughput of samples. Calibration standards were valid for five days, highlighting the ease of the assay over a regular work week. Bulk sample extractions are easily accommodated because eight samples can be analyzed within 1 min using a standard microplate reader.

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