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ALPHA-AMYLASE

ASSAY PROCEDURE

(AMYLASE SD METHOD)

K-AMYLSD 04/19

HIGH SENSITIVITY METHOD FOR THE MEASUREMENT OF α-AMYLASE IN CEREAL GRAINS AND FOOD PRODUCTS

(160/320 Manual Assays per Kit) or
(640 Auto-Analyser Assays per Kit)

Method based on:
AOAC Method 2002.01
AACC Method 22-02.01
ICC Standard No. 303

INTRODUCTION:

The quality of wheat for baking is critically dependent on the level of α-amylase (1,4-α-D-glucan glucanohydrolase, EC 3.2.1.1), which can be present as "late maturity α-amylase", or due to pre-harvest sprouting (PHS) caused by high rainfall and humidity at the time of harvesting. In bread-making, the level of α-amylase must be sufficient to produce saccharides which can be absorbed and utilised by yeast, but not so high as to cause excessive starch dextrinisation, which can lead to sticky crumb and problems in processing.

The most commonly used method to measure α-amylase in wheat grain is the Hagburg Falling Number method but the values are also influenced by rheological properties of starch in the grain.[1,2] The most specific method for the measurement of α-amylase is the Ceralpha method which employs a defined substrate, 4,6-*O*-benzylidene-α-4-nitrophenyl-maltoheptaoside, in the presence of thermostable α-glucosidase (**K-CERA**).[3] This substrate is soluble and the assay can be automated for analysis of α-amylase in wheat grain samples, however, the major limitation of the method is sensitivity. Due to the low levels of α-amylase present in the grain, measurement requires extended incubation periods.

The Amylase SD method (**K-AMYLSD**) provides a highly specific and sensitive assay for the measurement of α-amylase, especially in "pre-harvest sprouted" (sprout damaged) or "late maturity α-amylase" wheat grains. The method can also be used to measure α-amylase in food products such as confectionery, soft drinks, brewing and fermentation, jams, sauces, conserves, ice creams and baby foods. The substrate (4,6-*O*-ethylidene-α-4-nitrophenyl-maltoheptaoside) used in the Amylase SD assay is specific for α-amylase and is absolutely resistant to hydrolysis by *exo*-enzymes such as β-amylase, amyloglucosidase and α-glucosidase.[4,5]

PRINCIPLE:

The Amylase SD procedure (employing Amylase SD reagent) for the assay of α-amylase employs the defined oligosaccharide substrate 4,6-*O*-ethylidene-α-4-nitrophenyl-maltoheptaoside (EtPNPG7) in the presence of excess levels of a thermostable α-glucosidase (which has no action on the native substrate due to the presence of the "blocking group"). On hydrolysis of the oligosaccharide by *endo*-acting α-amylase, the excess quantities of α-glucosidase present in the mixture give instantaneous and quantitative hydrolysis of the *p*-nitrophenyl maltosaccharide fragment to glucose and free *p*-nitrophenol. The assay format is shown in Scheme 1 (page 10) and the linearity of the assay is shown in Figure 1 (page 11).

Essentially, an aliquot of a cereal flour extract of the "pre-harvest sprouted" grain is incubated with Amylase SD reagent mixture under defined conditions, and the reaction is terminated (and

colour developed) by the addition of a weak alkaline solution. The absorbance at 400 nm or 405 nm is measured (Figure 4, page 12) and this relates directly to the level of α-amylase in the sample analysed.

Amylase SD Reagent mixture can be used to quantitatively assay α-amylases in wheat grain. The assay can be used over a broad pH range (5.2 to 7.0) and at temperatures of up to 60°C. The optimal pH for activity of wheat α-amylases is 5.2-5.4 (Figure 6, Page 13).

ACCURACY:

Standard errors of less than 5% are achieved routinely.

KITS:

Kits suitable for performing 160/320 assays in manual format (or 640 assays in auto-analyser format) are available from Megazyme. The kits contain the full assay method plus:

Bottle 1: (x2) Amylase SD Reagent. Lyophilised powder.
Stable for > 5 years below -10°C.

Bottle 2: Concentrated Extraction Buffer (50 mL).
Stable for > 4 years at room temperature.

Bottle 3: Concentrated Stopping Reagent (25 mL).
(Manual procedure only).
Stable for > 4 years at room temperature.

Bottle 4: Milled Wheat Control.
Amylase SD activity as shown on the vial label.
Stable for > 4 years at room temperature.

SPECIFICITY:

The assay is absolutely specific for α-amylase.

A. MANUAL ASSAY PROCEDURE:

PREPARATION OF REAGENTS:

BOTTLE 1 (Amylase SD Reagent):

Ethylidene-end blocked *p*-nitrophenyl maltoheptaoside (EtPNPG7, 156 mg).
Thermostable α-glucosidase (125 U at pH 6.0), per vial.

Dissolve the entire contents of one of Bottle 1 in 8.0 mL of freshly boiled and cooled distilled water. Divide into 2-3 mL aliquots and store frozen between use. At 0-5°C the dissolved substrate is stable for 7 days; in the frozen state it is stable for at least 12 months.

BOTTLE 2 (Concentrated Extraction Buffer):

1 M sodium malate
1 M sodium chloride
40 mM calcium chloride
0.09% sodium azide

Dilute the entire contents of Bottle 2 (50 mL) (plus a crystalline precipitate which may be present) to 1 L with distilled water before use. The pH should be 5.4; adjust if necessary. Stable at 0-5°C for 12 months.

BOTTLE 3 (Concentrated Stopping Reagent):

(20% [w/v] tri-sodium phosphate solution, pH 11.0).
Dilute the entire contents (25 mL) to 500 mL with distilled water. Stable at room temperature for 3 months.

BOTTLE 4 (Milled Wheat Control):

Milled wheat of standardised α-amylase activity (as specified on the vial label). Use as supplied in the extraction and assay procedure. It is recommended that the user standardises at least one batch of their own wheat flour to be employed as a secondary reference flour.

PREPARATION OF ADDITIONAL EXTRACTION BUFFER:

Malic acid (Sigma M0875; 1 M)	134.1 grams/litre
Sodium hydroxide	70.0 grams/litre
Sodium chloride (1 M)	58.5 grams/litre
Calcium chloride dihydrate (40 mM)	5.8 grams/litre
Sodium azide (Sigma S2002; 0.09%)	0.9 grams/litre

Add malic acid, sodium chloride and sodium hydroxide to 800 mL of distilled water, allow to cool to room temperature and add the calcium chloride. Adjust the pH to 5.4 by dropwise addition of sodium hydroxide (4 M) or HCl (4 M). **Then** add the sodium azide. Adjust the volume to 1 L. Store at room temperature. **For use, dilute 50 mL of this concentrated buffer solution to 1 L with distilled water.**

CAUTION

Dissolve the reagents and adjust the pH to 5.4 before adding the sodium azide. Adding sodium azide to an acidic solution results in the release of a poisonous gas. Sodium azide can be deleted from the buffer but, in this case, the buffer should be stored at 4°C and preferably heated to ~ 90°C and cooled before use. This will inactivate any α-amylase that could be produced by any micro-organisms that may grow in the buffer in the absence of the sodium azide preservative.

Powdered malic acid is an irritant and thus should be handled with due care.

PREPARATION OF ADDITIONAL STOPPING REAGENT:

1% [w/v] Tri-sodium Phosphate Solution, pH 11.0:

Dissolve 10 g of tri-sodium phosphate in 1 L of distilled water and adjust the pH to approx. 11.0.
Stable at room temperature for at least 3 months.

EQUIPMENT (RECOMMENDED):

1. Glass test tubes (12 mL and 20 mL capacity).
2. Polypropylene tubes (13 mL capacity).
3. Pipettors (e.g. Gilson Pipetman®) 0.1 mL to dispense substrate and 0.2 mL adjustable to dispense grain or flour extract.
4. Adjustable-volume dispenser:
   - 0-10 mL (for Extraction Buffer).
   - 0-5 mL (for Stopping Reagent).
5. Top-pan balance.
6. Spectrophotometer set at 400 nm.
7. Vortex mixer (optional).
8. Thermostated water bath set at 40°C.
9. Stop clock.
10. Microfuge or Whatman GF/A glass fibre filter paper circles (9 cm diameter).
11. Activated charcoal (Merck Millipore cat. no. 1021861000).

CONTROLS AND PRECAUTIONS:

1. α-Amylase is an enzyme present at high levels in all body fluids. It is thus recommended that disposable gloves are used when handling and dispensing the substrate mixture.
2. It is essential that the water used to dissolve the Amylase SD reagent is of high purity. If freshly distilled water is not available, heat the water to boiling and cool it to less than 30°C before using. Algal growth in water in wash bottles can produce sufficient α-amylase to significantly reduce the long-term stability of the reagent dissolved in such water.
3. The freeze-dried substrate is extremely stable at room temperature. However, when dissolved it should be stored at 0-5°C during use and below -10°C between use. If the number of assays performed at any one time is limited, it is recommended that the substrate be divided into 2-3 mL aliquots and stored in the frozen state.
4. On storage at 0-5°C, the blank absorbance values will increase from 0.03 to about 0.05 in 5 days, this does not affect the performance of the substrate, but obviously these values must be determined at the same time as the assay is performed. Blank absorbance values as high as 0.50 do not affect the reliability or accuracy of the assay.
5. The spectrophotometer employed should be standardised with a *p*-nitrophenol standard in 1% (w/v) tri-sodium phosphate (ε_{mM} = 18.1). *p*-Nitrophenol solution (10 mM) can be obtained from Sigma Chemical Company (cat no. N7660). An aliquot of this solution when diluted 200-fold in 1% (w/v) tri-sodium phospahte gives an absorbance of 0.905 at 400 nm.

6. The assay format should be standardised with the enclosed milled wheat. The activity of this flour is shown on the enclosed vial.
7. The time of extraction of **milled wheat** should be carefully controlled (10 min).

NOTE:
For each sample extract, it is recommended that a specific sample blank assay is performed. The sample blank consists of **0.1 mL of Amylase SD Reagent solution** + **1.0 mL of Stopping Reagent** mixed thoroughly followed by the addition of **0.4 mL of milled grain extract**.

USEFUL HINTS:

1. If the absorbance values for a particular assay are greater than 1.60, the enzyme extract should be diluted with the appropriate buffer and re-assayed.
2. The number of assays which can be performed per kit can be doubled by employing semi-micro spectrophotometer cuvettes and using **0.05 mL** of Amylase SD Reagent solution, **0.2 mL** of milled grain extract and **1.0 mL** of Stopping Reagent.

ENZYME EXTRACTION:

Milled Grain Samples:

1. Mill wheat, barley or other grain (approx. 10-50 g sample) to pass a 0.5 mm screen (e.g. with a Fritsch centrifugal mill).
2. Accurately weigh **0.5 g of milled grain** into a polypropylene tube of 13 mL capacity.
3. To each tube add **8.0 mL of Extraction Buffer solution** (pH 5.4) and stir the flask contents vigorously.
4. Allow the enzyme to extract over **10 min at 40°C** with occasional mixing.
5. Filter an aliquot of the solution through a Whatman GF/A glass fibre filter paper, or centrifuge an aliquot of the extract (e.g. 2 x 1 mL) at 11,000 *g* for 3 min in a microfuge. Use the clear filtrate/ supernatant in the assay. **Assay the enzyme activity within 2 h.**

ASSAY PROCEDURE:

1. Dispense **0.1 mL aliquots of Amylase SD Reagent solution** (unbuffered) into test tubes and pre-incubate the tubes and contents at 40°C for 5 min.
2. Pre-incubate the extract at 40°C for 5 min.

3. To each tube containing **Amylase SD Reagent solution** (0.1 mL), add **0.4 mL of pre-equilibrated milled grain extract** directly to the bottom of the tube. Incubate at 40°C for exactly **10 min** (from time of addition).
4. At the end of the **10 min** incubation period, add exactly **1.0 mL of Stopping Reagent** and stir the tube contents vigorously.
5. Read the absorbance of the solutions and the reaction blank at **400 nm** against distilled water.

CALCULATION OF ACTIVITY (Manual Assay Procedure):

One Unit of activity is defined as the amount of enzyme, in the presence of excess thermostable α-glucosidase, required to release one micromole of *p*-nitrophenol from EtPNPG7 in one minute under the defined assay conditions, and is termed an **Amylase SD Unit.**

Amylase SD Units/g milled grain:

$$= \frac{\Delta E_{400}}{\text{Incubation Time}} \times \frac{\text{Total Volume in Cell}}{\text{Aliquot Assayed}} \times \frac{1}{\varepsilon_{mM}} \times \frac{\text{Extraction Vol.}}{\text{Sample Weight}} \times \text{Dilution}$$

where:

ΔE_{400} = Absorbance (reaction) - Absorbance (blank)

Incubation Time = 10 min

Total Volume in Cell = 1.5 mL

Aliquot Assayed = 0.4 mL

ε_{mM} of *p*-nitrophenol (at 400 nm) in tri-sodium phosphate (1% w/v) = 18.1

Extraction Volume = 8 mL per 0.5 g (milled grain sample)

Dilution = Dilution of the original extract (if required)

Thus:

Amylase SD Units/g milled grain:

$$= \frac{\Delta E_{400}}{10} \times \frac{1.5}{0.4} \times \frac{1}{18.1} \times \frac{8}{0.5} \times \text{Dilution}$$

$$= \Delta E_{400} \times 0.331 \times \text{Dilution}$$

B. AUTOMATED ASSAY PROCEDURE:

NOTE:
The automated Amylase SD procedure has been optmised using a ChemWell®-T auto-analyser. The assay files containing the programmed settings for the ChemWell®-T auto-analyser and used to perform the Amylase SD assay are available to download from the Megazyme website.

EQUIPMENT (RECOMMENDED):

1. ChemWell®-T auto-analyser fitted with a 405 nm filter.
2. Polypropylene tubes (13 mL capacity).
3. Pipettors, 1 mL (e.g. Gilson Pipetman®) to dispense enzyme extract.
4. Adjustable-volume dispenser:
   - 0-10 mL (for Extraction Buffer).
5. Top-pan balance.
6 Vortex mixer (optional).
7. Stop clock.
8. Microfuge or Whatman GF/A glass fibre filter paper circles (9 cm diameter).

PREPARATION OF REAGENTS:

BOTTLE 1 (Amylase SD Reagent):

Ethylidene-end blocked *p*-nitrophenyl maltoheptaoside (EtPNPG7, 156 mg).
Thermostable α-glucosidase (125 U at pH 6.0), per vial.

Dissolve the entire contents of one of Bottle 1 in 8.0 mL of freshly boiled and cooled distilled water. Divide into 2-3 mL aliquots and store frozen between use. At 0-5°C the dissolved substrate is stable for 7 days; in the frozen state it is stable for at least 12 months.

BOTTLE 2 (Concentrated Extraction Buffer):

1 M sodium malate
1 M sodium chloride
40 mM calcium chloride
0.09% sodium azide

Dilute the entire contents of one of Bottle 2 (50 mL) (plus a crystalline precipitate which may be present) to 1 L with distilled water before use. The pH should be 5.4; adjust if necessary. Stable at 0-5°C for 12 months.

BOTTLE 4 (Milled Wheat Control):

Milled wheat of standardised α-amylase activity (as specified on the vial label). Use as supplied in the extraction and assay procedure. It is recommended that the user standardises at least one batch of their own wheat flour to be employed as a secondary reference flour.

PREPARATION OF STOPPING REAGENT:

500 mM Sodium Carbonate, pH 11.0.

Dissolve 53 g of sodium carbonate (anhydrous) in 1 L of distilled water and adjust the pH to approx. 11.0. Store in a sealed bottle to prevent the formation of carbonate. Stable at room temperature for at least 3 months.

ENZYME EXTRACTION AND ASSAY:

Milled Grain Samples:

1. Mill wheat, barley or other grain (approx. 10-50 g sample) to pass a 0.5 mm screen (e.g. with a Fritsch centrifugal mill).
2. Accurately weigh **0.5 g of milled grain** into a polypropylene tube of 13 mL capacity.
3. To each tube add **8.0 mL of Extraction Buffer solution** (pH 5.4) and allow the enzyme to extract over **10 min at 40°C** with occasional mixing.
4. Immediately centrifuge an aliquot of the extract (e.g. 2 x 1 mL) at 11,000 *g* for 3 min in a microfuge.
5. Use the clear supernatant in the automated Amylase SD assay procedure using a ChemWell®-T auto-analyser. **Assay the enzyme activity within 2 h.**
6. Perform the assay using the **K-AMYLSD (SAMPLE), K-AMYLSD (BLANK)** ChemWell®-T assay files and the **K-AMYLSD (CALC)** ChemWell®-T indices file.

Automated Assay Parameters:

Assay volumes:	Amylase SD reagent:	0.025 mL
	Sample (extract):	0.075 mL
	Stopping reagent:	0.100 mL
Reaction time:	10 min at 37°C	
Wavelength:	405 nm	
Assay type:	stopped reaction	
Reaction direction:	increase	

CALCULATION OF ACTIVITY (Automated Assay Procedure):

One Unit of activity is defined as the amount of enzyme, in the presence of excess thermostable α-glucosidase, required to release one micromole of *p*-nitrophenol from EtPNPG7 in one minute under the defined assay conditions, and is termed an **Amylase SD Unit.**

Amylase SD Units/g milled grain:

$$= \frac{\Delta E_{405}}{\text{Incubation Time}} \times \frac{\text{Total Volume in Cell}}{\text{Aliquot Assayed}} \times \frac{1}{\varepsilon_{mM}} \times \frac{\text{Extraction Vol.}}{\text{Sample Weight}} \times \text{Dilution}$$

where:

ΔE_{405} = Absorbance (reaction) - Absorbance (blank)

Incubation Time = 10 min

Total Volume in Cell = 0.2 mL

Aliquot Assayed = 0.075 mL

ε_{mM} of *p*-nitrophenol (at 405 nm) in 500 mM sodium carbonate, pH 11 = 12.345

Extraction Volume = 8 mL per 0.5 g (milled grain sample)

Dilution = Dilution of the original extract (if required)

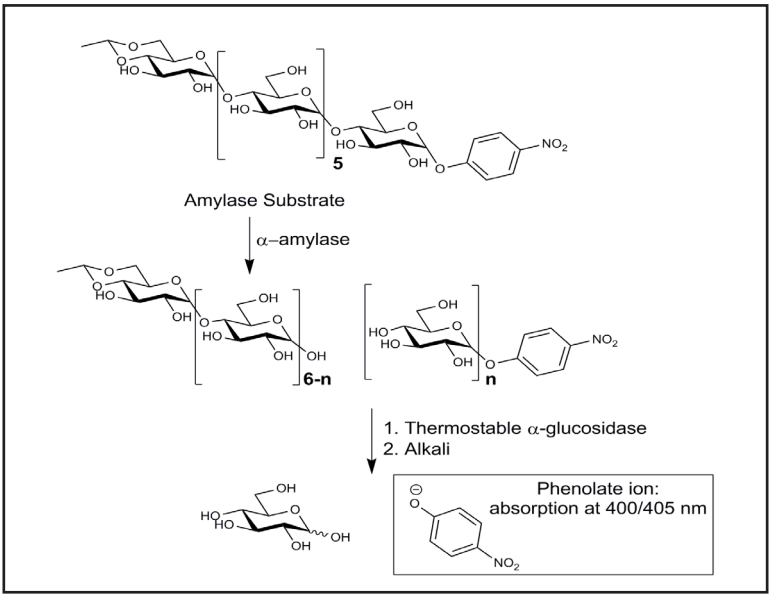
Thus:

Amylase SD Units/g milled grain:

$$= \frac{\Delta E_{405}}{10} \times \frac{0.2}{0.075} \times \frac{1}{12.345} \times \frac{8}{0.5} \times \text{Dilution}$$

$$= \Delta E_{405} \times 0.346 \times \text{Dilution}$$

NOTE:
The absorption coefficient (ε_{mM}) of 12.345 was experimentally determined under the conditions of the automated Amylase SD assay using a ChemWell®-T auto-analyser.



Scheme 1. **Theoretical basis of the Amylase SD assay procedure.** Immediately α-amylase cleaves a bond within the blocked *p*-nitrophenyl maltosaccharide substrate, the non-blocked reaction product containing the *p*-nitrophenyl substituent is instantly cleaved to glucose and free *p*-nitrophenol by the excess quantities of thermostable α-glucosidase which is an integral part of the substrate mixture, and free *p*-nitrophenol is released. The reaction is terminated and the phenolate colour is developed on addition of Stopping Reagent (pH ~ 11.0).

	Absorbance (400 nm)										
Sample	DAY 1		DAY 2		DAY 3		DAY 4		MEAN	STDEV	%CV
A	0.133	0.136	0.127	0.128	0.122	0.125	0.121	0.120	0.126	0.006	**4.52**
B	0.160	0.155	0.156	0.158	0.145	0.140	0.149	0.159	0.152	0.007	**4.79**
C	0.254	0.239	0.259	0.249	0.258	0.239	0.254	0.249	0.250	0.008	**3.18**
D	0.407	0.395	0.409	0.413	0.393	0.390	0.410	0.403	0.402	0.009	**2.19**
E	1.391	1.403	1.409	1.405	1.397	1.385	1.398	1.406	1.399	0.008	**0.59**

Table 1. **Reproducibility of the Amylase SD assay (Manual Assay Procedure) for the measurement of wheat α-amylase.** Duplicate analyses of single extracts made on four separate days.

APPENDIX:

A. Linearity of Amylase SD assay with substrate concentration and incubation time.

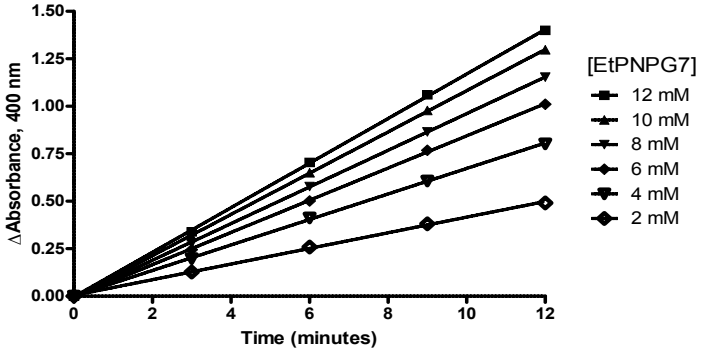


Figure 1. **Linearity of the Amylase SD assay with wheat α-amylase in sodium malate buffer (pH 5.4).** Assays were performed with substrate concentrations of 2, 4, 6, 8, 10 and 12 mM in the final reaction mixture. Reactions were terminated at various times by adding tri-sodium phosphate solution (1.0 mL, 1% w/v, pH 11.0).

B. Effect of the concentration of thermostable α-glucosidase in the reagent solution on determined α-amylase values.

From the results shown in Figure 2, it is evident that the concentration of α-glucosidase required to saturate the reaction is 12 Units/mL in the substrate solution.

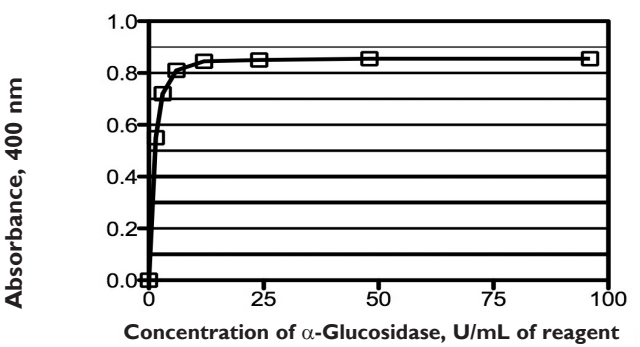


Figure 2. **The effect of the concentration of α-glucosidase in the substrate reagent solution on the determined absorbance values.**

C. Stability of reagent mixture at 60°C.

The stability of the reagent solution was determined by incubating aliquots of this solution at 60°C for 0-20 min. These solutions were then used to assay the activity of fungal α-amylase (at 40°C). From the data shown in Figure 3, it is evident that the reagent is very stable at 60°C. Over a 20 min incubation period, blank absorbance values increased by less than 0.01 absorbance units, and the determined activity decreased by less than 3% (of the non pre-incubated reagent).

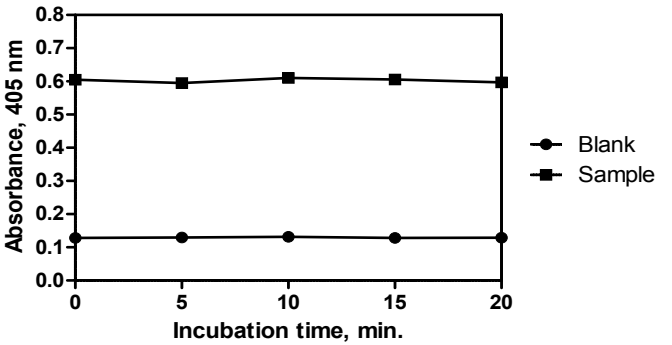


Figure 3. **Temperature stability of Amylase SD assay reagent.** Aliquots of the reagent were stored at 60°C for 0-20 min, cooled to room temperature, and used to assay wheat α-amylase at 40°C.

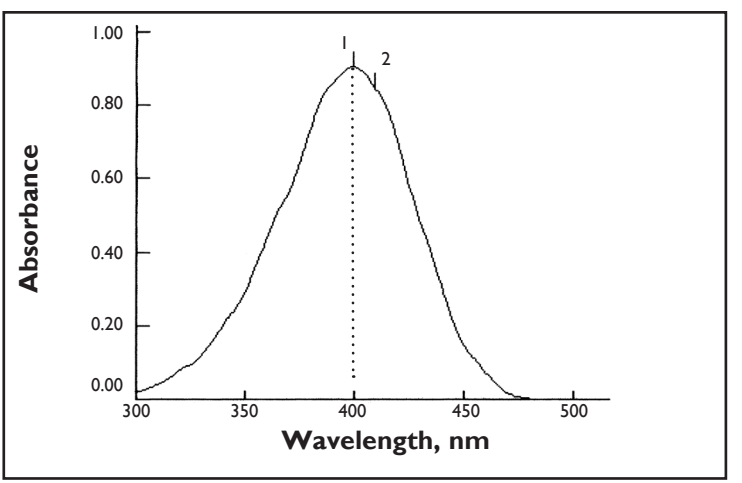


Figure 4. **Absorbance curve for *p*-nitrophenol in tri-sodium phosphate solution (1% w/v) at pH 11.0.**

D. Correlation between Amylase SD and Falling Number methods.

Wheat grain samples with a range of predetermined falling numbers were tested using the Amylase SD assay (manual procedure) to assess the correlation between Amylase SD units and falling numbers.

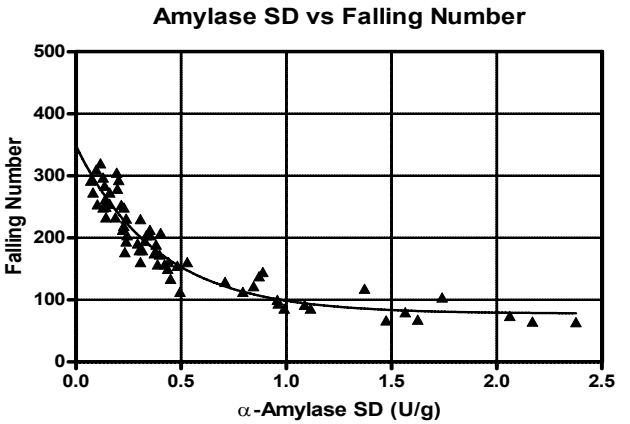


Figure 5. **Correlation between Amylase SD and Falling Number methods.**

E. pH Activity Curve for Wheat α-Amylase.

pH Activity curve for milled wheat α-amylases were determined using the Amylase SD Reagent. The pH curve was prepared using malate and maleate buffers (100 mM, pH 4.0-7.5) containing 5 mM calcium chloride.

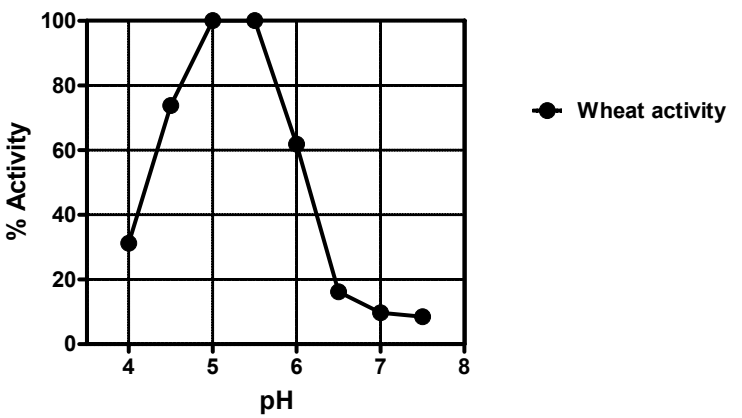


Figure 6. **pH activity curve for wheat α-amylases.** α-Amylase was assayed with the Amylase SD Reagent using the standard manual assay procedure.

NOTES:

REFERENCES:

1. Hagberg, S. (1960). A rapid method for determining α-amylase activity. *Cereal Chemistry,* 37, 218-222.
2. Perten, H. (1964). Application of the Falling Number method for evaluating α-amylase activity. *Cereal Chemistry,* 41, 127-139.
3. McCleary, B. V. & Sheehan, H. (1987). Measurement of cereal α-amylase: a new assay procedure. *Journal of Cereal Science,* 6, 237-251.
4. McKie, V. A. & McCleary, B. V. (2015). A rapid, automated method for measuring α-amylase in pre-harvest sprouted (sprout damaged) wheat. *Journal of Cereal Science,* 64, 70-75.
5. Cornaggia, C., Ivory, R., Mangan, D. & McCleary, B. V. (2015). Novel assay procedures for the measurement of α-amylase in weather damaged wheat. *Journal of the Science of Food and Agriculture*, 96(2), 404-412.

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