Investigating wheat starch functionality associated with weather triggered low falling number and improvement in the quantification of reducing sugars

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AUTHORIZATION TO SUBMIT THESIS

This thesis of Yijing Shao, submitted for the degree of Master of Science with a Major in Food Sciences and titled "Investigating wheat starch functionality associated with weather triggered low falling number and improvement in the quantification of reducing sugars," has been reviewed in final form. Permission, as indicated by the signatures and dates below, is now granted to submit final copies to the College of Graduate Studies for approval.

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ABSTRACT

In this program, we studied a critical issue in wheat from starch aspects and improved a reducing sugar assay that could be potentially utilized in the potato industry for quality control. Both wheat and potato are important to the US agricultural economy, especially in the Pacific Northwest Region. In recent years, wheat growers have faced huge economic loss due to low falling number (FN) wheat. Wheat with low FN is considered having a high α-amylase activity, which is associated with poor end-use quality. The research of low FN wheat has mainly focused on α-amylase; however, its substrate, starch, did not receive much attention. We hypothesized that starch in the low FN wheat might have structural changes that could decrease flour paste property and influence FN measurement. Starch from three soft white wheat lines with normal and low FNs were examined. Starch in the low FN wheat was found having altered granule size distribution and structure. The changes led to a reduction in paste viscosity and influence the interaction between α-amylase and starch that could further decrease FN.

Quantifying reducing sugar is a common practice in carbohydrate research as well as in the food industry. Multiple colorimetric methods are available to measure the reducing sugar content. One of the assays developed by Somogyi and Nelson can accurately measure reducing sugars in a board range of concentrations. The drawback of this method is that it is time-consuming when analyzing a large number of samples. Therefore, we aimed to improve this method so it can be used to quantify reducing sugars with ease and high efficiency. The traditional Somogyi-Nelson assay, which is conducted in test tubes, was adopted into a 96-well microplate. This modification greatly improved the analytic capacity and efficacy and maintained the advantages of the traditional assay at the same time.

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1.1. ABSTRACT

The falling number (FN) test is a widely accepted wheat quality measurement. It quickly determines the liquefaction of the flour due to the hydrolysis of gelatinized starch by α-amylase. The wheat with low FN is considered having a high α-amylase activity, which is associated with poor end-use quality; therefore, the price is discounted greatly. The low FN issue has caused severe economic loss, especially in the Pacific Northwest region of the United States in recent years. The effect of α-amylase activity on FN has been well studied; however, it is unclear whether starch has any change that could reduce flour paste viscosity and alter the interaction between starch and α-amylase to further reduce FN. Therefore, we hypothesized that starch structure change has an impact on flour pasting property (i.e., FN) and the change might be induced by a weather stress (e.g., rain or cold temperature). This study examined three soft white spring wheat cultivars grown in southern Idaho in 2013 (normal FN year) and 2014 (low FN year with pre-harvest rains). Starch in the low FN wheat (2014) was not damaged, but had developmental changes, including increased population of B-type granules and altered structure, which led to a reduction in paste viscosity and higher susceptibility to α-amylase in the FN measurement. Results of this study suggest that the developmental change in starch structure is associated with the change of flour pasting property (i.e., FN) and highlights the impact of substrate (starch) on FN measurement.

1.2. INTRODUCTION

The "falling number" (FN) test was developed by Hagberg (1960) to determine the speed of liquefaction of flour caused by α-amylolysis of gelatinized starch (Hagberg, 1960; Perten, 1964). The measurement yields the time in seconds required for the stirrer-viscometer to drop 70 mm in a tube of hot flour paste (Hagberg, 1960, 1961). The heating condition of FN measurement was developed to mimic dough baking in which the temperature rises quickly from 55 - 65 °C (starch gelatinization temperature) to 70 - 85 °C (enzyme inactivation

temperature) in 30 s (Perten, 1964). As temperature increases, α-amylase activity increases and it quickly hydrolyzes the starch, which leads to the decrease of paste viscosity. When the FN falls below 150 s, the bread crumb may be too sticky, which is undesired. If the FN is greater than 350 s, it is also likely that the bread volume is diminished and malts will be needed to balance the enzyme activity (Perten, 1964).

The FN test has been approved as a standard method of measuring grain α-amylase activity by the International Association for Cereal Chemistry (ICC) and American Association of Cereal Chemists (AACC International, 2010b), as well as adopted by the International Organization for Standardization (ISO) for evaluating cereal quality. The test is also an internationally accepted measurement for grain receipt and trade in Canada, Australia, U.S. and other countries. In the U.S., exporters in the Pacific Northwest region – the major supplier of soft white wheat to the Asian market – started to include FN results in the export specifications in 1994, due to complaints by Asian customers after receiving inferior quality of wheat (Idaho Wheat Commission, 2016). Thus, the FN test has become a risk management tool for exporters. It did not have a strong influence in the domestic market until 2014 when PHS occurred in Idaho (Idaho Wheat Commission, 2016).

In the Pacific Northwest of the U.S., an FN of 300 s is a typical cutoff value. Grains with a low FN are discounted progressively (Delwiche, Vinyard, & Bettge, 2015) and sold for animal feeds because food processors decline to use them for making human foods. A low FN indicates high α-amylase activity in grains and consequently more starch damage, resulting in poor end use quality (Batey, Curtin, & Moore, 1997; Edwards, Ross, Mares, Ellison, & Tomlinson, 1989; Farrand, 1964). Such a discount has caused a serious economic crisis. Wheat growers in Idaho lost millions of dollars due to low FN wheat in 2014 (Idaho Wheat Commission, 2017). It is worth noting that the FN test was developed in the 1960s when the most important quality concern was bread (e.g., bread crumb) and dough properties. At present, the test is used as a risk management tool for a broad range of products, such as sponge cake and Asian noodles, which have different demands for flour characteristics from bread.

The low FN issue is suggested to be caused by two phenomena: pre-harvest sprouting (PHS) or late-maturity α-amylase (LMA; also called pre-maturity α-amylase, PMA). PHS refers to the germination of grains due to pre-harvest rain that causes an elevation in α-

amylase activity and alteration in grain quality (Hagemann & Ciha, 1984). LMA refers to a genetic defect resulting in high levels of α-amylase in the absence of pre-harvest sprouting. LMA-affected grains synthesize high isoelectric point (pI) α-amylase, and such abnormal synthesis is triggered by temperature shocks at the late grain filling stage, especially during 25 - 30 days after anthesis (Farrell & Kettlewell, 2008; Mares & Mrva, 2008). LMA production is often induced by cool and wet conditions (Gale, Salter, & Lenton, 1987; Mrva & Mares, 1994); heat shocks have also been observed to cause LMA synthesis (Major, 1999).

The research of low FN wheat has mainly focused on α-amylase and genetic markers. However, FN and the associated end-use product quality are also affected by the constituents (i.e., starch, protein, non-starch polysaccharides, and lipids) in the flour (Abdel-Aal, Hucl, Chibbar, Han, & Demeke, 2002; Courtin & Delcour, 2002; Dexter, Matsuo, & Kruger, 1990; Every, Simmons, Al-Hakkak, Hawkins, & Ross, 2002; Goesaert et al., 2005; Graybosch, Guo, & Shelton, 2000; Johansson, 2002; Ross, Flowers, Zemetra, & Kongraksawech, 2012). Starch, as the substrate of α-amylase and the major component of wheat flour, has received relatively little attention in FN. Perten (1964) suggested that the effect of α-amylase activity depends on the properties of both enzyme and its substrate – starch. Moreover, starch is the major contributor to the flour paste viscosity (Morris, King, & Rubenthaler, 1997). Thus, the change in starch properties could have a direct impact on viscosity of wheat flour as well as the interaction between the enzyme and substrate, resulting in different rates of liquefaction of flour in the FN measurement.

In 2014, Idaho experienced a huge economic loss due to low FN wheat; this incidence was ascribed to PHS because of rainfalls during the harvest season. It is clear that α-amylase activity increases in the sprouted wheat and the effect of α-amylase activity on FN has already been well studied. However, it is unclear if a weather stress (e.g., rain or cold temperature) could also change the wheat starch structure and alter its interaction with α-amylase, which lead to a further reduction in flour paste viscosity. Therefore, we hypothesized that starch structure change may have an impact on flour pasting property (i.e., FN) and the changes of starch could be induced by a weather stress (e.g., rain or cold temperature). We examined two soft white spring wheat cultivars and one experimental line grown at the same location in southern Idaho in 2013 and 2014. The year 2013 did not have any significant weather stress, and in general, wheat had expected (normal) FNs. The year 2014 had rainfalls during the

harvest season, and a large proportion of the harvested wheat had low FNs. We isolated starch and examined their structure characteristics and functionalities to test our hypothesis that starch properties are influenced by a weather stress and impact on FNs.

1.3. MATERIALS AND METHODS

1.3.1. Materials

Two soft white spring wheat cultivars, UI Stone (Chen et al., 2013) and Alturas (Souza, Guttieri, & O'Brien, 2004), and one soft white spring wheat elite line, SA043, were selected for this study. UI Stone and Alturas were the parents of SA043. All wheat cultivars used in this study were grown at the Aberdeen Research and Extension Center, University of Idaho (Aberdeen, ID) in 2013 and 2014. Wheat kernels were milled into wholemeal flour using a lab grinder with a 0.8 mm screen (Perten Lab Mill 3100, Perten Instruments, Inc., Springfield, IL). The wholemeal flour was stored in double zip lock bags at room temperature.

1.3.2. Starch isolation

Starch was isolated from the wholemeal flour following the method of Shinde, Nelson, and Huber (2003) with modifications. A flour sample (35 g, dry weight) was suspended in 0.02 M hydrochloric acid (350 mL) for 10 min to quickly inactive enzymes in the sample. Sodium bisulfite (0.175 g) and thiomersal (0.0035 g) were then added to the slurry, followed by the addition of the tris-(hydroxymethyl) aminomethane buffer (0.05 M) to adjust the pH to 7.6. The slurry was then combined with the protease solution, which was prepared by dissolving 0.175 g protease (XIV type, Sigma-Aldrich, St. Louis, MO) in 0.02 M hydrochloric acid (10 mL) with 5 min stirring. The slurry was kept at 4 °C for 24 h with continuous gentle shaking and then filtered through a 150-μm sieve to remove large particles such as bran fragments. The filtered solution was centrifuged at 14,000 × *g* for 20 min, and the resulting supernatant was discarded. A light microscope was used to examine the supernatant to assure that starch granules were recovered by centrifugation. The pellet was suspended in 80% (w/v) cesium chloride (50 mL), and the suspension was centrifuged at 14,000 × *g* for 20 min; this step was repeated once more. The starch pellet was washed three

times with deionized water (100 mL) and passed through a 75-μm sieve to remove cell walls and insoluble fiber. The starch was collected by centrifugation at 14,000 × *g* for 20 min, resuspended in absolute ethanol (50 mL), recovered using glass microfiber filter paper (retention size 1.2 μm, Whatman, Maidstone, UK) on a Buchner funnel, and air-dried in a fume hood for 48 h.

1.3.3. Measurement of falling number of wheat flours

A flour sample (7.0 g, 14% moisture) was mixed with 25 mL of distilled water and analyzed by a Falling number machine (Perten Falling number 1700, Perten Instruments, Inc., Springfield, IL) following the AACC Approved Method 56-81.03 (AACC International, 2010b). Each sample was analyzed in duplicate.

1.3.4. Quantification of starch in flours

The total starch content of a flour sample was quantified using the Total Starch Assay Kit (Megazyme Inc., Wicklow, Ireland). In brief, the starch was gelatinized in 2 M potassium hydroxide for 20 min in an ice bath and hydrolyzed by thermostable α-amylase and amyloglucosidase at 50 °C for 30 min. The generated glucose was quantified by using the glucose oxidase and peroxidase based reaction (part of the total starch kit). In total six samples were analyzed and each sample was analyzed in triplicate.

1.3.5. Quantification of crude protein in flours

The crude protein content of a flour sample was analyzed with a combustion analyzer (LECO Combustion Analyzer CN 628, Leco Corporation, St. Joseph, MI) following AACC Approved Method 46-30.01 (AACC International, 2010a). Each sample was analyzed in duplicate.

1.3.6. Starch granule morphology

The morphology of starch granules was examined with a scanning electron microscope (SEM, Model Quanta 200 F, Field Emission Instruments Company, Hillsboro, OR). Dried

starch was sprayed on a piece of carbon tape mounted on a SEM stub and gold coated using a Sputter coater (Technics Hummer V, Anatech, San Jose, CA). Coated starch samples were examined at 10 kV accelerating voltage under high vacuum ($1.4 – 5.9 \times 10^{-4}$ Pa). Each starch samples were prepared in duplicate for examination. Images were taken using the xT Microscope Control software (Quanta Version 4.1.7, Field Emission Instruments Company, Hillsboro, OR).

1.3.7. Starch granule size

A starch sample (0.030 g, dry weight) was mixed with 40 µL of 10% (v/v) Tween 80 solution and then diluted into a 2% (w/v) starch suspension with deionized water in a 2-mL microtube. The suspension was mixed on a vortex for 5 s, and 10 µL of the suspension was diluted with 400 mL water in a beaker and examined with a particle size analyzer (AccuSizer 780, Particle Sizing Systems, Santa Barbara, CA) with continuous stirring. Each starch sample was measured in six replicates.

1.3.8. Amylose content and molecular weight distribution

The apparent amylose content was measured by iodine binding following the method of Morrison and Laignelet (1983) with modifications. In brief, starch (100 mg, dry weight) was heated in urea-dimethylsuplphoxide solution (UDMSO) (10 mL, 1:9 v/v). The solution was then diluted with deionized water (95 mL) and mixed with iodine solution (2 mL, 0.2% iodine in 2% potassium-iodine). The reaction mixture was transferred to a 96-well microplate (Tissue culture plate, Falcon, Corning Incorporated, Corning, NY), and the absorbance was measured at 635 nm using a microplate reader (Spectra Max 190 Microplate Reader, Molecular Devices, Sunnyvale, CA). The result was calculated based on the calibration curve (% apparent amylose = $0.0228 \times \text{Absorbance}_{635nm} + 0.1063$, $R^2 = 0.996$), which was prepared over the range 0 – 35% amylose using mixtures of amylose (from potatoes, Sigma-Aldrich, St. Louis, MO) and waxy maize starch (Tate & Lyle, London, UK). Both amylose and waxy maize were dehydrated before use. In total six starch samples were analyzed and each sample was analyzed in triplicate.

Wheat starch was debranched following the method of Lin et al. (2014; 2006) with modifications and analyzed by the HPSEC system. Starch was dispersed in water (10% of the final volume of starch-DMSO suspension) and then mixed with pure dimethyl sulfoxide (DMSO) to make a 1% (w/v) starch suspension in 90% (v/v) DMSO. The suspension was heated in a boiling water bath for 1 h followed by continuous stirring at room temperature (23 °C) for 12 h. The starch was precipitated by adding ethanol to the starch-DMSO suspension and pelleted by centrifugation at 5,000 × *g* for 20 min. The starch pellet was re-dissolved in water, mixed with DMSO (final concentration 90% [v/v]), and heated in a boiling water bath for 10 min. The gelatinized starch was precipitated with ethanol and centrifuged as described above. The pellet was air-dried at room temperature for 15 h. The dried, gelatinized starch (10 mg) was dissolved in 0.1 M sodium acetate buffer (pH 5.0) and heated in a boiling water bath for 30 min with stirring before debranching. An aliquot (900 µL) of starch solution was mixed with 95 µL enzyme solution I consisting of 1 µL of pullulanase (from *Bacillus licheniformis,* 40 U/mg on pullulan at pH 5 and 40 °C, Megazyme Inc., Wicklow, Ireland) and 1 µL of isoamylase (from *Pseudomonas sp.*, 280 U/mg protein on oyster glycogen at pH 4.0 and 40 °C, Megazyme Inc., Wicklow, Ireland) in 93 µL of 0.1 M sodium acetate buffer (pH 5.0), and then incubated in a 40 °C water bath for 1 h. Enzyme solution II (5 µL), made of 1 µL of pullulanase and 1 µL of isoamylase in 6 µL of 0.1 M sodium acetate buffer, was later added, and the sample was incubated in a 40 °C water bath for another 6 h. The solution was heated in a boiling water bath for 10 min to inactivate the enzyme, and an aliquot (200 µL) was diluted with deionized water (800 µL). Thirty microliters of 0.1M barium acetate was then added to remove the sulfate in the debranched sample. The final solution was centrifuged at 3,000 *g* for 3 min. Each starch sample was debranched in duplicate and the supernatant of the final solution was injected into the HPSEC system. The separation was accomplished by a set of Shodex OHpack columns (Showa Denko K. K., Tokyo, Japan) with guard columns, SB-804 and SB-802, which were maintained at 50 °C. The sample was eluted with 0.1M sodium nitrate containing 0.02% (w/v) sodium azide at a flow rate of 0.4 mL/min. A calibration curve of retention volume and log MW was made with a series of pullulan standards with MW of 342 (Pullulan Standard 320, Fluka-Sigma-Aldrich, Buchs, Switzerland), 5,900, 22,800, 112,000, and 212,000 Da (Shodex Standard P-82, JM Science Inc., Grand Island, NY).

1.3.9. Amylopectin chain-length distribution

Amylopectin was isolated from starch following the method of Bertoft, Källman, Koch, Andersson, and Åman (2011). The freeze-dried amylopectin (10 mg) was debranched in duplicate as described above to analyze chain-length distribution. The debranched amylopectin was filtered through a syringe filter (0.45 μm pore size) and injected into a set of gel permeation columns packed with Superdex 75 resin (30 cm length and 2 cm in diameter) and Superdex 30 resin (30 cm length and 2.2 cm in diameter, GE Healthcare Life Science, Little Chalfont, UK). The gel permeation system was eluted with water containing 0.02% (w/v) sodium azide at a flow rate of 0.4 mL/min. Eluate was collected every minute and analyzed by total carbohydrate analysis (Fox & Robyt, 1991) in triplicate. A calibration curve of retention volume and log MW was made with a series of pullulan standards as described above.

1.3.10. Starch pasting properties

A starch sample (3.0 g, dry weight) was mixed with deionized water (25.00 g ± 0.05 g) and measured with a Rapid Visco Analyzer (RVA; Newport Scientific Inc., Warriewood, Australia) following the standard 2 profile of the AACC Approved Method 76-21.01 (AACC International, 2010c) with a minor modification. The cooling time was extended to reach the plateau while measuring the final viscosity. Briefly, starch suspension was held at 50 °C for 1 min, heated to 95 °C at 6 °C/min, held at 95 °C for 5 min, cooled down to 50 °C at 6 °C /min, and held at 50 °C for 9 min. Each starch sample was analyzed in duplicate.

1.3.11. Starch thermal properties

Starch thermal properties were determined by a DSC (Discovery DSC, TA Instrument, New Castle, DE). Starch (10 mg, dry weight) and deionized water (50 μL) were added into a stainless steel pan and equilibrated for three hours prior to the measurement. The reference pan contained 50 μL of deionized water. Both sample and reference pans were cooled to 20 °C and then heated to 120 °C at a rate of 8 °C/min. Each starch sample was analyzed in triplicate.

1.3.12. Statistical Analysis

Two-sample *t*-tests were performed to compare the characteristics of starches isolated from the same cultivar between years 2013 and 2014 (Minitab 17, Minitab Inc., State College, PA). One-way ANOVA followed by Tukey's pairwise comparison was performed to compare each pasting property of starches isolated from the three cultivars of two years. The significant level was $\alpha = 0.05$.

1.4. RESULTS

1.4.1. Quantification of starch and protein in flours

The starch content of the flour samples ranged from 63 to 66%. UI Stone had a significant increase in starch content (from 63.1% to 66.0%, $p < 0.05$) in 2014 compared to 2013; Alturas (64.0% and 64.7%) and SA043 (63.5% and 63.9%) did not have a significant difference between 2013 and 2014. All three cultivars had the same protein content of 11.8% in 2013. In 2014, UI Stone had a protein content of 10.9%, while the other two cultivars had the same protein content, 11.8%, as in 2013.

1.4.2. Flour falling numbers

The FN of UI Stone, Alturas, and SA043 grown in 2013 were 326 s, 306 s, and 337 s, respectively; however, FNs dropped to 146 s, 197 s, and 62 s in 2014.

1.4.3. Starch granule morphology

Starch granules were isolated from flours and examined by SEM. Wheat starch consists of A and B type granules. A-type granules are large (10 – 35 μm in diameter) with a disk shape; B-type granules are small (2 – 10 μm in diameter) with a sphere or irregular shape. The granule morphology was similar between the three cultivars and no broken or severely damaged starch granules were observed. It was noticed that UI Stone grown in 2014 (Fig. 1.1-B) had slightly more B-type granules compared to the one harvested in 2013 (Fig. 1.1-A). This observation was later confirmed by particle size analysis.

After increasing the magnification, dents (circled areas in Fig. 1.2) and grooves (Fig. 1.2-E) were found on the surface of A-type wheat granules, indicating a minor enzymatic hydrolysis of starch granules in both 2013 and 2014; the extent of hydrolysis was similar in all three cultivars. B-type granules were intact in all three cultivars in both years (Fig. 1.3).

1.4.4. Starch granule size

The change in granule size distribution observed by SEM was confirmed by particle size analysis. All three cultivars had a significant increase in the proportion of B-type granules from 2013 to 2014 ($p < 0.05$, Table 1.1). The starch isolated from SA043 grown in 2013 had 92.6% of B-type granules, followed by Alturas (91.9%) and UI Stone (88.6%). In 2014, the percentage of B-type granules of UI Stone increased by 4.6%, whereas that of Alturas and SA043 increased by 1.2% and 1.8%, respectively, from 2013 to 2014.

1.4.5. Amylose content and molecular weight distribution

The apparent amylose content, which was defined as amylose content of starch without defatting (Morrison and Laignelet (1983), was measured by the blue value method and ranged from 22 to 27% (Table 1.2). The apparent amylose content of Alturas starch was significantly higher in 2014 (21.9% versus 26.9%, $p < 0.05$) compared to 2013, while UI stone and SA043 were the same in both years. Amylose content was also examined by HPSEC-RI. Amylose has few branches; thus, the amount of debranched amylose fraction was used to estimate the amount of amylose in the sample (Batey & Curtin, 1996). In 2014, all three cultivars had an increase in amylose amount compared to that in 2013. Moreover, UI Stone and SA043 from 2014 had a larger molecular weight of amylose compared to 2013.

1.4.6. Amylopectin chain-length distribution

Amylopectin was isolated and debranched, and its chain-length distribution was analyzed by HPSEC. The chromatograms were divided into three fractions (Fig. 1.4): Fraction I (DP > 31), Fraction II (DP 11 - 31), and Fraction III (DP < 11). UI Stone had a noticeable change in

the amounts of Fraction II (mass ratio increased by 1.2%) and III (mass ratio reduced by 3.2%) from 2013 to 2014, while Alturas and SA043 were the same between the two years.

1.4.7. Starch pasting properties

The pasting properties of starches were examined by an RVA. Each cultivar grown in 2013 had higher peak, trough, final, and setback viscosities than in 2014 ($p < 0.05$, Table 1.3). The extent of change in each cultivar was different. UI stone had a decrease in peak viscosity in 2014, from 402 RVU to 367 RVU, followed by SA043 (from 398 RVU to 364 RVU) and Alturas (from 444 RVU to 415 RVU). UI Stone also showed a larger drop in trough, final, and setback viscosities than Alturas and SA043. For peak time, Alturas had a significant decrease from 9.37 min (2013) to 9.03 min (2014, $p < 0.05$). Alturas and SA043 grown in 2014 had a significant increase ($p < 0.05$) in their pasting temperatures compared to that in 2013.

1.4.8. Starch thermal properties

The gelatinization temperature of wheat starches was 59 – 72 °C, and the change in enthalpy (ΔH) was 14 - 15 J/g (Table 1.4). Among the three cultivars, SA043 grown in both years displayed the lowest onset temperature, while Alturas had the lowest conclusion temperature. Both UI Stone and SA043 grown in 2014 had a lower onset, peak, and conclusion gelatinization temperature ($p < 0.05$) than those grown in 2013. The enthalpy change of SA043 grown in 2014 was also significantly lower than that grown in 2013 ($p < 0.05$). Alturas only showed a significant increase in conclusion gelatinization temperature from 2013 to 2014 ($p < 0.05$).

The DSC thermogram of all the samples also showed a melting peak of amylose-lipid complex at 92 - 104 °C. The three cultivars all had a significant increase in the enthaply change of amylose-lipid peak from 0.6 J/g in 2013 to 0.8 J/g in 2014 ($p < 0.05$), indicating more amylose-lipid complex presenting in the sample in 2014.

1.5. DISCUSSION

Many soft white wheat cultivars grown in Idaho and nearby regions had significant decreases in FN in 2014, when there were 12 raining days with a total of 5.2 cm of precipitation during the harvest season (Bureau of Reclamation, 2013, 2014). Thus, it was postulated that the low FN in this area in 2014 was due to pre-harvest rains that stimulated germination and raised α-amylase activity, leading to a high level of starch hydrolysis, low paste viscosity, and therefore low FN. Results of starch quantification and SEM examination of starch granule morphology demonstrated that starches in the low FN wheat were not severely damaged by α-amylase in the kernel. Moreover, we found the starch structure had developmental changes, which affected starch functionalities and influenced the paste viscosity.

Starch from wheat grown in 2014 had significant decreases in peak, trough, final, and setback viscosity (Table 1.3). The peak viscosity is measured when starch granules have the maximum swelling (Dang & Copeland, 2004). The peak viscosity of UI Stone, Alturas, and SA043 was decreased by 36, 29, and 33 RVU in 2014, respectively (calculated from the data in Table 1.3). The peak viscosity of flour was shown to have a strong correlation with FN in previous research (Hareland, 2003; León, Barrera, Pérez, Ribotta, & Rosell, 2006). Contrary to previous correlations, we did not observe a significant linear correlation between the starch peak viscosity and FN. α-Amylase and other constituents (e.g., protein) in flour can influence the measurement. However, the reduction of starch paste viscosity coincided with the decrease of FNs. Wheat starch from 2014 also had a decrease in final viscosity compared to 2013. For example, the final viscosity of UI Stone decreased from 516 RVU (year 2013) to 422 RVU (year 2014). Sasaki, Yasui, and Matsuki (2000) reported that final viscosity has a positive correlation with amylose content in wheat starch. We observed that starch isolated from wheat grown in 2014 had higher amylose content but presented lower final viscosity compared to that of the same cultivar grown in 2013. The decrease in final viscosity might be due to the presence of amylose-lipid complex or phospholipid that interferes amylose re-association (Shinde et al., 2003). Additionally, the low FN wheat was found to have a decreased setback (the difference between final and trough viscosity), which is associated with retrogradation and syneresis of the starch paste upon cooling (Vamadevan & Bertoft, 2015). Variation in setback is related to the amounts of apparent amylose, total amylose (Zeng,

Morris, Batey, & Wrigley, 1997), amylose-lipid complex, or phospholipid content in wheat starch. We observed an increase in amylose-lipid complex in all three cultivars in 2014, which corresponded to the decrease in final and setback viscosities.

FN measurement is a standard method to determine α-amylase activity; thus, a low FN wheat is thought to have quick starch liquefaction caused by high α-amylase activities (i.e., PHS or LMA). Usually, PHS affected wheat has numerous holes and cracks on starch granule surfaces due to high α-amylase activities, such as the starch granules shown by Simsek et al. (2014). However, the starch of low FN wheat (year 2014) examined in this study had minor dents on A-type granules and the B-type granules were almost intact (Fig. 1.1 – 1.3). Fannon, Hauber, and BeMiller (1992) explained that the pores found on the surface and along the equatorial groove of A-type wheat granules are not artifacts but the sites of initial enzyme attack. Thus, our findings demonstrated that starch granules of low FN wheat examined in this study were not much hydrolyzed by α-amylase. Instead, the change in the structure of substrate (starch) is an important factor contributing to the low FN wheat in this study.

There was a significant increase in the percentage of B-type granules in low FN wheat. Wheat starch is known to have a bimodal distribution in granule size. Both A- and B-type wheat starch granules are A-type polymorphs (Ao & Jane, 2007) but they are different in granule size, granule shape, chemical composition, molecular structure, and functionalities (e.g., starch paste viscosity). In normal wheat (non-waxy wheat), B-type wheat granules have a lower total amylose content (defined as amylose amount in defatted starch, 21%) and apparent amylose content (15%) than A-type wheat starch granules, 26% and 21%, respectively (Geera, Nelson, Souza, & Huber, 2006; Kim & Huber, 2010). B-type wheat granules have a higher lipid-complexed amylose (6%) than A-type wheat granules (5%) (Geera et al., 2006; Kim & Huber, 2010). Amylose-lipid complex can restrict granule swelling and change starch pasting behaviors; thus, it is associated with the decrease in peak, trough, final, breakdown, and total setback viscosities (Geera et al., 2006; Kim & Huber, 2010; Shinde et al., 2003). Ao and Jane (2007) compared the pasting profiles of reconstituted wheat starch using different proportions of wheat A- and B-granules. They showed that the paste viscosity of the starch decreased significantly as the B-type granule content increased from 0% to 30% in the reconstituted starch. Therefore, the increase in the population of B-type

granules observed in this study could explain the decrease in viscosity of starch isolated from low FN wheat.

B-type granules also showed lower onset, higher peak and conclusion temperatures, as well as smaller gelatinization enthalpy change (Geera et al., 2006; Kim & Huber, 2010). With higher population of B-type granules, the starch isolated from low FN wheat showed reductions in onset temperature and enthalpy changes, as well as increases in peak and conclusion temperatures, particularly SA043. However, it was noticed that UI Stone had lower peak and conclusion temperature in 2014, which was due to other starch structural changes (i.e., amylopectin structure and organization). Kim and Huber (2010) reported that B-type wheat granules had a higher quantity of short amylopectin branch chains (DP < 13), which was associated with a lower gelatinization onset temperature compared to A-type granules. As the proportion of B-type granule increased, the starch isolated from low FN wheat was anticipated having a higher quantity of short amylopectin chains. However, no significant change was found in the quantity of short amylopectin chains (DP < 11) in all three cultivars in both years (2013 and 2014). The increase in the number of B-type granules did not cause a significant change in the amylopectin chain-length distribution of whole starch (without separating A- and B-type granules).

More importantly, the increase in the proportion of B-type granules changes the interaction between the starch and α-amylase. B-type granules have a higher reactivity and enzyme susceptibility since their surface area (2.3 m^2/g) are larger than A-type granules (0.8 m^2/g) (Kim & Huber, 2010). Naguleswaran, Li, Vasanthan, Bressler, and Hoover (2012) showed that wheat B-type starch granules were hydrolyzed significantly faster than A-type granules by α-amylase. Similar results have also been found in barley starch (Naguleswaran, Vasanthan, Hoover, & Bressler, 2013; Vasanthan & Bhatty, 1996), which has a bimodal granule size distribution like wheat starch. Our findings suggest that the starch in low FN wheat is more susceptible to α-amylase, resulting in a faster liquefaction rate and diminished FN.

Although there were pre-harvest rainfalls in 2014 (Fig. 1.5), the starch in low FN wheat in 2014 did not have significant enzymatic damages, which are typical phenomena in PHS affected wheat. We hypothesized that there are other environmental stresses (i.e., temperature fluctuation) that altered starch structure during synthesis and resulted in different starch

properties, contributing to the decrease of FN. The three wheat lines were grown in the same location with the same agronomic management in 2013 and 2014. The weather data demonstrated that the daily minimum temperature was below 45 °F (7.2 °C; Fig. 1.6, Bureau of Reclamation 2013, 2014) between 1st and 6th day after anthesis in 2014. According to Bechtel, Zayas, Kaleikau, and Pomeranz (1990), this particular period is the time when starch granules started to generate. It is known that growing temperature has a strong influence on starch biosynthetic enzyme activities and the expression of those enzymes (Hurkman et al., 2003; Myllärinen, Schulman, Salovaara, & Poutanen, 1998; Yanagisawa, Kiribuchi-Otobe, & Fujita, 2004) as well as starch granule size distribution in wheat (Hurkman et al., 2003; Shi, Seib, & Bernardin, 1994). It is suspected that the cold temperature shock in 2014 triggered the changes in starch structure. An environment-controlled study is needed to confirm the hypothesis that the cold shock near starch granule initiation period could cause the change of starch synthesis and results in the reduction of FNs.

1.6. CONCLUSION

The previous research on low FN issue has mainly focused on the measurement of α-amylase activity and the damage of wheat kernels to evaluate wheat quality. In this thesis, we presented that the change of substrate (i.e., starch) directly changes starch pasting profiles and influence flour pasting property. The finding of the developmental changes of starch in the low FN wheat is novel; additionally, it was also found that the starch was not extensively damaged in the kernel in some low FN wheat. Although this work was not designed to identify which environmental stress(es) triggered the changes during the starch synthesis, the weather data indicated that the cold temperature shock during the starch granule initiation period is a potential cause. This research demonstrates the importance of starch in influencing wheat flour viscosity. Starch should be considered as a variable in FN, although α-amylase continues to have a dominant effect in this measurement. Additional environment-controlled research is needed to investigate the wheat flour viscosity related quality issue.

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1.8. TABLES AND FIGURES

Table 1.1 The proportion of A- and B-type starch granules of starch from UI Stone, Alturas, and SA043

Cultivar	Year	Number of A-and B-granules (%)	
		A-type	B-type
UI Stone	2013	11.4 ± 1.3	88.6 ± 1.3
	2014	6.8 ± 0.8*	93.2 ± 0.8*
Alturas	2013	8.1 ± 0.9	91.9 ± 0.9
	2014	6.9 ± 1.1*	93.1 ± 1.1*
SA043	2013	7.4 ± 0.7	92.6 ± 0.7
	2014	5.6 ± 0.5*	94.4 ± 0.5*

The cutoff of A- and B-type granules was 10 μm. All the percentages are expressed in mean ± standard deviation from six replicate measurements. The value with an asterisk (*) is significantly different from the same cultivar growing in the other year ($p < 0.05$, by two-sample t-test).

Table 1.2 Amylose content of starch from UI Stone, Alturas, and SA043

Cultivar	Year	% Apparent amylose (BV)	% Amylose (HPSEC-RI)	Weight average molecular weight	
				Debranched amylose	Debranched amylopectin
UI Stone	2013	23.1 ± 2.6%	16.8 ± 1.3%	1.5×10^5	2.1×10^3
	2014	25.1 ± 1.6%	17.4 ± 1.1%	2.0×10^5	2.2×10^3
Alturas	2013	21.9 ± 1.1%	14.1 ± 1.3%	2.2×10^5	2.1×10^3
	2014	26.3 ± 0.1%*	16.4 ± 0.5%	2.3×10^5	2.1×10^3
SA043	2013	27.3 ± 0.9%	15.0 ± 1.4 %	1.5×10^5	2.1×10^3
	2014	26.9 ± 1.0%	18.0 ± 0.7%	1.9×10^5	2.1×10^3

The % apparent amylose, measured by Blue value, is expressed in the mean ± standard deviation from triplicate measurements. The % amylose (HPSEC-RI) presents the ratio of amylose to the total mass, and the data are expressed as the mean from duplicate measurements. Debranched amylose and amylopectin were molecular distributions of the range of molecular weight $2.1\times 10^4 – 4.0 \times 10^5$ and $1.0 \times 10^3 – 2.1\times 10^5$ Da, respectively. The number with an asterisk (*) is significantly different from the same cultivar growing in the other year ($p < 0.05$, by two-sample t-test).

Table 1.3 Pasting properties of starches from UI Stone, Alturas, and SA043

Cultivar	Year	Peak	Trough	Breakdown	Final	Setback	Peak Time	Pasting Temp
UI Stone	2013	402 ± 0^{bc}	215 ± 4^{a}	187 ± 4^{b}	516 ± 2^{a}	300 ± 1^{a}	9.3 ± 0.0^{a}	75.3 ± 0.4^{b}
	2014	367 ± 5^{d}	176 ± 5^{b}	191 ± 0^{b}	422 ± 7^{c}	246 ± 2^{c}	9.3 ± 0.0^{a}	77.5 ± 0.5^{ab}
Alturas	2013	444 ± 7^{a}	190 ± 1^{b}	253 ± 5^{a}	386 ± 4^{d}	196 ± 2^{e}	9.4 ± 0.0^{a}	69.4 ± 1.4^{c}
	2014	415 ± 4^{b}	153 ± 0^{c}	262 ± 4^{a}	333 ± 1^{e}	180 ± 2^{f}	9.0 ± 0.0^{b}	75.7 ± 0.3^{b}
SA043	2013	398 ± 0^{c}	212 ± 8^{a}	185 ± 7^{b}	495 ± 7^{b}	282 ± 1^{b}	9.3 ± 0.0^{a}	76.4 ± 0.6^{b}
	2014	364 ± 1^{d}	179 ± 0^{b}	185 ± 1^{b}	414 ± 1^{c}	235 ± 0^{d}	9.4 ± 0.0^{a}	79.4 ± 0.5^{a}

The units of viscosity, peak time, and past temperature are RVU, min, and °C. The number is the mean of duplicate measurements. Means with the same letter are not significantly different from each other (one-way ANOVA and Tukey's test, $p < 0.05$) in the same column.

Table 1.4 Thermal properties of starches from UI Stone, Alturas, and SA043

Cultivar	Years	To (°C)	Tp (°C)	Tc (°C)	ΔH (J/g)	Amylose-lipid complex, ΔH (J/g)
UI Stone	2013	59.57 ± 0.13	64.10 ± 0.05	70.93 ± 0.15	14.61 ± 1.07	0.61 ± 0.08
	2014	59.35 ± 0.16*	63.64 ± 0.21*	70.36 ± 0.52*	14.21 ± 1.59	0.82 ± 0.06*
Alturas	2013	59.65 ± 0.10	64.15 ± 0.13	69.75 ± 0.23	13.90 ± 1.53	0.57 ± 0.09
	2014	59.66 ± 0.12	64.12 ± 0.20	70.67± 0.46*	14.89 ± 1.04	0.76 ± 0.05*
SA043	2013	58.97 ± 0.13	63.55 ± 0.21	70.99 ± 0.08	15.41 ± 0.21	0.63 ± 0.03
	2014	58.60 ± 0.04*	64.03 ± 0.14*	71.72 ± 0.12*	14.38 ± 0.92*	0.78 ± 0.14*

To: onset gelatinization temperature; Tp: peak gelatinization temperature; Tc: conclusion gelatinization temperature; ΔH: change in enthalpy. Mean and standard deviation was taken from triplicate measurements. The number marked with an asterisk (*) is significantly different from that of the same cultivar grown in the other year ($p < 0.05$, by two-sample t-test).

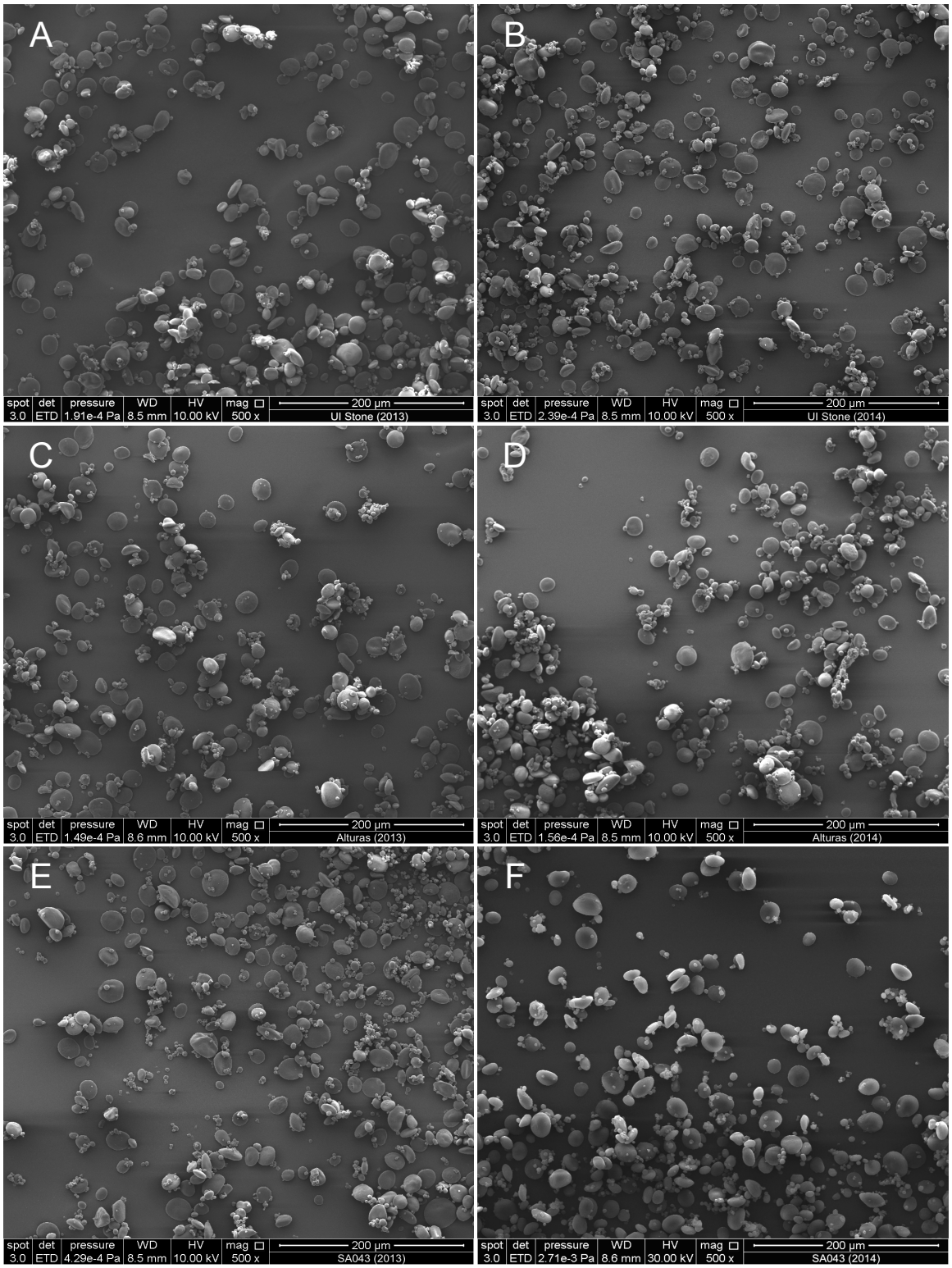

Fig. 1.1 SEM images of wheat starch granules. The magnification was set at 500 x. Starch granules were isolated from UI Stone (A, B), Alturas (C, D), and SA043 (E, F) grown in 2013 (A, C, E) and 2014 (B, D, F).

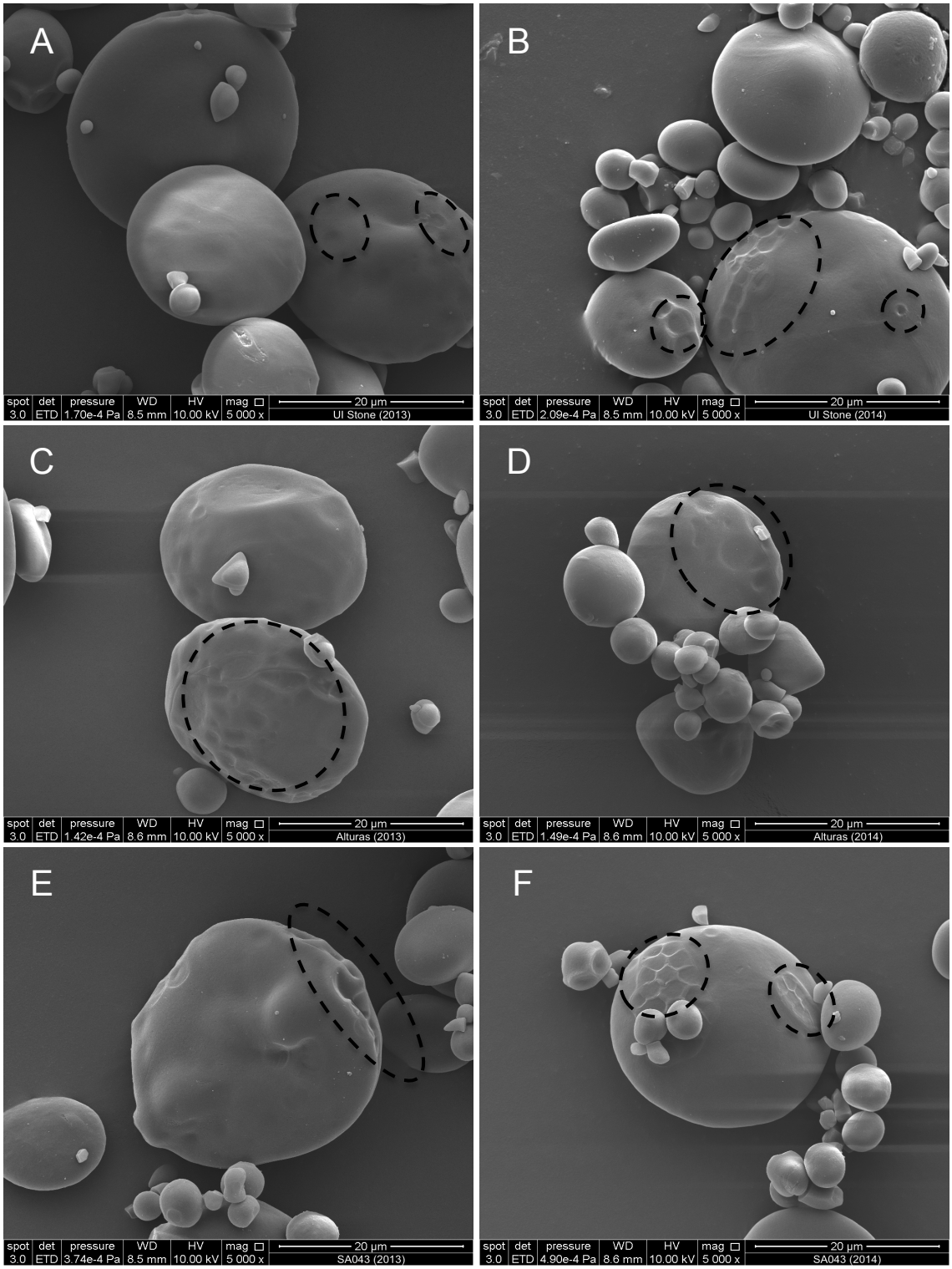

Fig. 1.2 SEM images of A-type wheat starch granules. The magnification was set at 5000 x. Starch granules were isolated from UI Stone (A, B), Alturas (C, D), and SA043 (E, F) grown in 2013 (A, C, E) and 2014 (B, D, F). Circled areas show the damage caused by minor enzymatic hydrolysis.

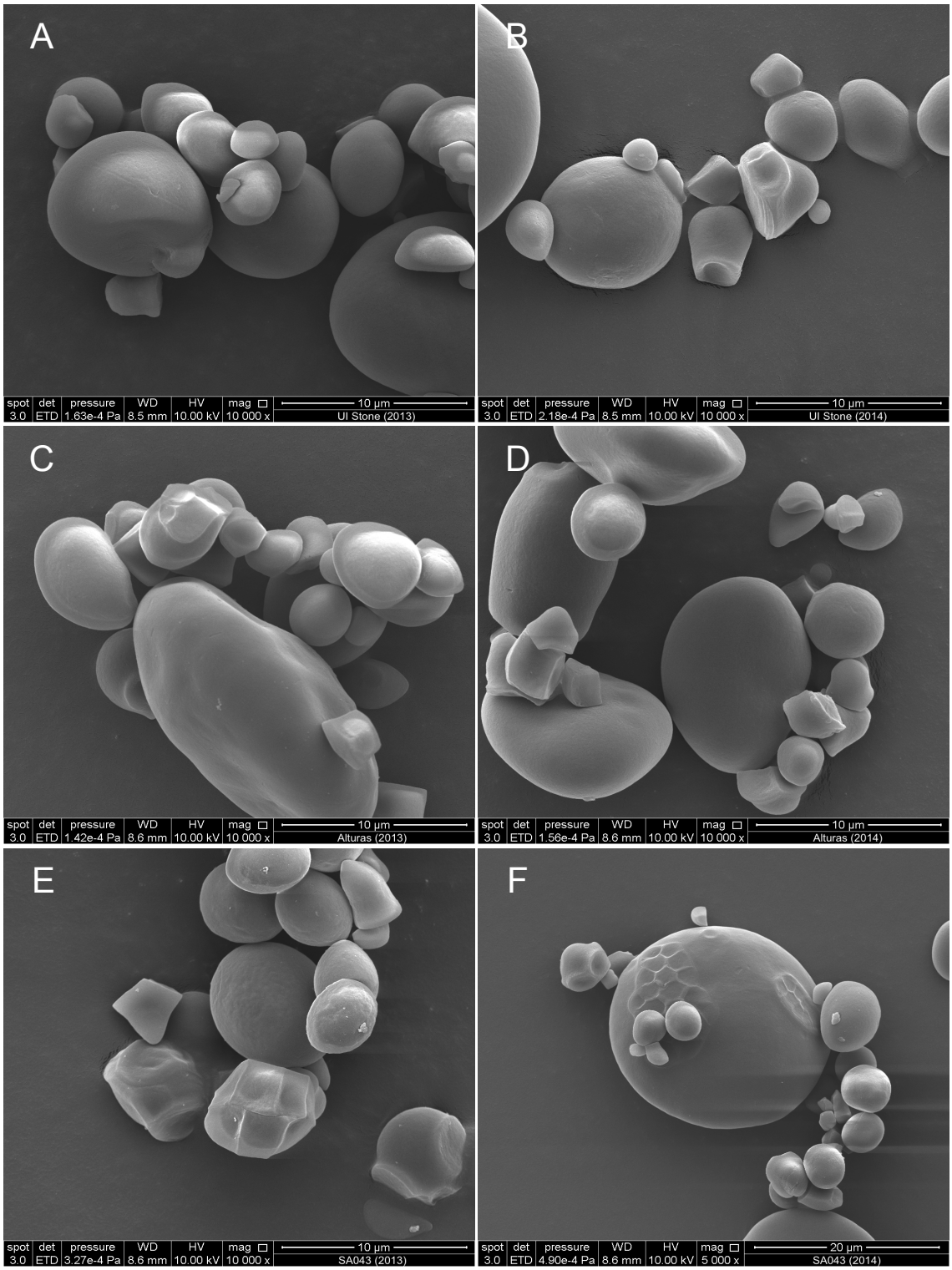

Fig. 1.3 SEM images of B-type wheat starch granules. The magnification was set at 10,000 x. Starch granules were isolated from UI Stone (A, B), Alturas (C, D), and SA043 (E, F) grown in 2013 (A, C, E) and 2014 (B, D, F).

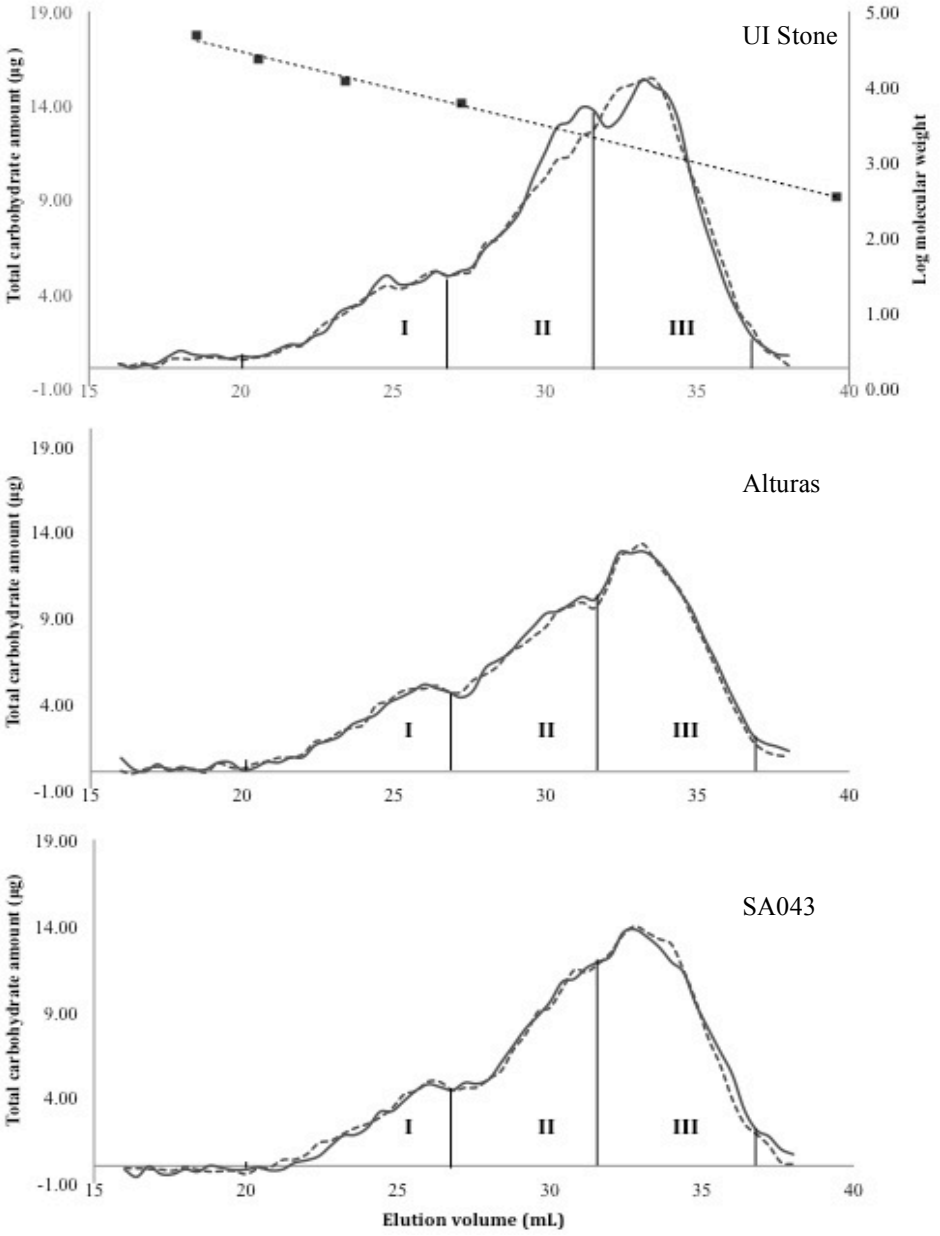

Fig. 1.4 HPSEC chromatograms of debranched amylopectin of UI Stone, Alturas, and SA043 in 2013 (dashed line) and 2014 (solid line). Fraction I (20 – 26.8 mL, DP > 31); Fraction II (27.2 – 31.6 mL, DP 11 - 31); Fraction III (32 – 36.8 mL, DP < 11). The calibration curve, shown in the top figure, of elution volume and log MW was made of a series of pullulan with MW of 342, 5,900, 11,800, 22,800, and 47,300 Da.

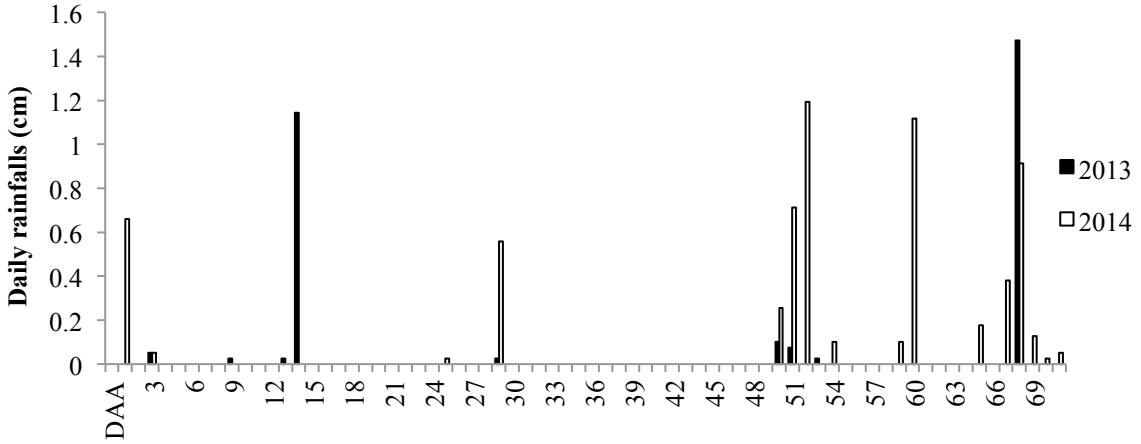

Fig. 1.5 Daily rainfalls after anthesis (DAA) in 2013 and 2014 in Aberdeen, ID. Reproduced from Bureau of Reclamation (2013, 2014).

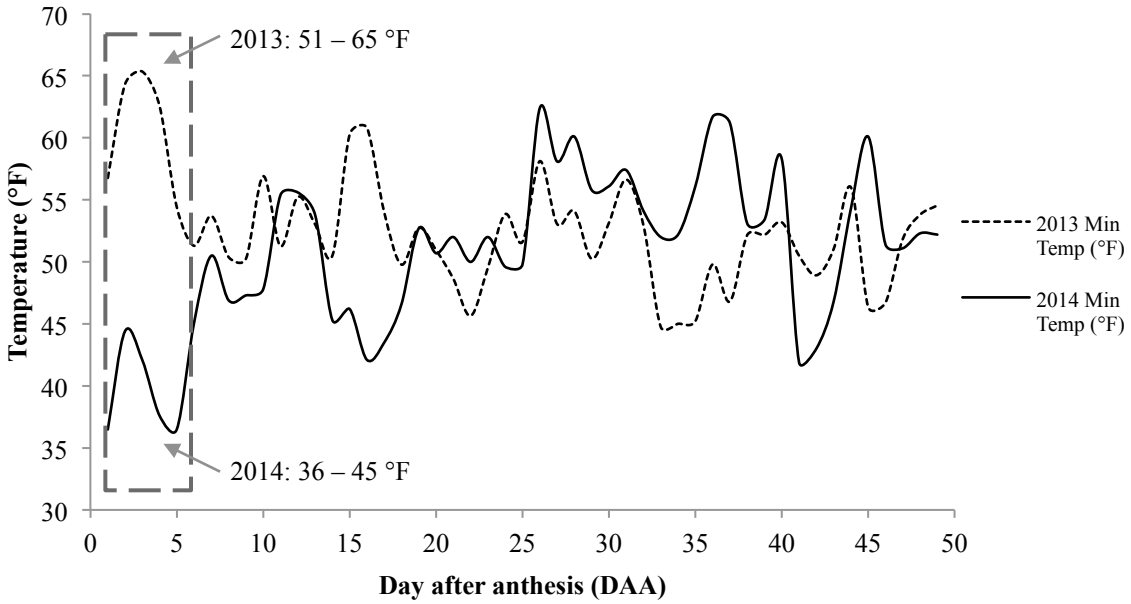

Fig. 1.6 Daily minimum temperature after anthesis (DAA) in 2013 and 2014 in Aberdeen, ID. Reproduced from Bureau of Reclamation (2013, 2014). The area highlighted shows that daily minimum temperatures between 1st and 6th DAA in 2013 was 51 – 65 °F (10.6 – 18.3 °C) whereas in 2014 they were all below 45 °F (7.2 °C).

CHAPTER 2 IMPROVEMENT IN THE QUANTIFICATION OF REDUCING SUGARS BY MINIATURIZING THE SOMOGYI-NELSON ASSAY USING A MICROTITER PLATE

Shao, Y., & Lin, A. H.-M. (2018). Improvement in the quantification of reducing sugars by miniaturizing the Somogyi-Nelson assay using a microtiter plate. *Food Chemistry, 240*, 898-903.

2.1. ABSTRACT

Measuring reducing sugar is a common practice in carbohydrate research, and the colorimetric assay developed by Somogyi and Nelson has a high sensitivity in a broad concentration range. However, the method is time-consuming when analyzing a large number of samples. In this study, a modified Somogyi-Nelson assay was developed using a 96-well microplate. This microassay greatly improves the analytic capacity and efficacy of the method while at the same time maintaining the accuracy and sensitivity of the traditional assay.

2.2. INTRODUCTION

Sugars containing a free hemiacetal or hemiketal group are referred to as reducing sugars. Measurement of reducing sugars is a common practice in carbohydrate research. A number of colorimetric methods have been developed to quantify reducing sugars, including those developed by Somogyi and Nelson (SN) (Nelson, 1944; Somogyi, 1952) and Park and Johnson (PJ) (Park & Johnson, 1949), as well as those based on the reduction of 3,5-dinitrosalicylic acid (Miller, 1959; Sumner, 1925; Sumner & Graham, 1921), also known as the dinitrosalicylate (DNS) reagent, and the formation of colored copper-bicinchoninate (CuBic) (Fox & Robyt, 1991; McFeeters, 1980; Mopper & Gindler, 1973).

All of these methods are based on the oxidation ability of reducing sugars. SN and CuBic assays measure the reduction of copper in an alkali solution by reducing sugars. The SN assay uses cuprous ions to reduce the arsenomolybdate complex, formed by the reaction of ammonium molybdate with sodum arsenate, to generate molybdenum blue. (Nelson, 1944; Woods & Mellon, 1941); The CuBic method reacts 2,2'-bicinchoninate with cuprous ions to

form a deep-blue complex, copper (I)-bicinchoninate (McFeeters, 1980; Mopper & Gindler, 1973). The PJ assay, same as SN and CuBic assays, uses alkaline-metal ions as the oxidizing agent and involves the reduction of ferricyanide ions in an alkaline solution to form Prussian blue, which is ferric ferrocyanide (Park & Johnson, 1949). The DNS assay involves the reduction of 3,5-dinitrosalicylic acid in an alkaline solution to produce colored 3-amino-5-nitrosalicylic acid (Miller, 1959; Sumner & Graham, 1921). A comparison of color formation mechanisms of these methods is shown in Table 2.1.

SN and DNS methods were first developed to measure glucose in urine and blood samples (Nelson, 1944; Sumner, 1925; Sumner & Graham, 1921). These methods were later adapted to measure the reducing power of samples containing various reducing sugars, but it was found that the DNS assay required separate calibration curves for each sugar and that sugars with more linkages were over-oxidized and generated higher absorbance values (McCleary & McGeough, 2015; McIntyre, Mukerjea, & Robyt, 2013; Robyt & Whelan, 1972; Saqib & Whitney, 2011; Sengupta, Jana, Sengupta, & Naskar, 2000). Since the reducing power is affected by the size of reducing sugars, it is usually overestimated when glucose is used as the standard (Miller, 1959; Sumner, 1925; Sumner & Graham, 1921). The accuracy of the PJ assay was also affected by the size of reducing sugar; this problem was later resolved by Hizukuri, Takeda, Yasuda, and Suzuki (1981). Therefore, SN, CuBic, and modified PJ assays are the recommended methods for measurement of reducing sugars because they yield the same results with equimolar of malto-oligosaccharides (maltodextrins) regardless of their size (Doner & Irwin, 1992; Hizukuri et al., 1981; McIntyre et al., 2013; Robyt & Whelan, 1972).

The sensitivity range of each assay is different. Among the four assays compared above, the DNS assay can measure up to 5.5 mM using glucose to generate a calibration curve (Sumner, 1925; Sumner & Graham, 1921). The SN assay has a detection range of 0.03 - 3.33 mM of glucose or equivalents (Hodge & Hofreiter, 1962). The CuBic and modified PJ assays are very sensitive at low concentrations. The CuBic assay can measure maltose in the range of 5 to 110 μM, and the modified PJ assay can measure glucose as low as 5 – 30 μM (Fox & Robyt, 1991; Hizukuri et al., 1981).

Another concern of these assays is the ease of measurement. The DNS assay is most popular because it is simple and fast. It requires only mixing and heating sugar solutions with

the 3,5-dinitrosalicylic acid reagent for 5 min, followed by cooling and absorbance reading (Sumner, 1925; Sumner & Graham, 1921). The other three methods require a longer heating time, and some require the addition of a color reagent and dilution. A comparison of their procedures is shown in Table 2.2. Heating a large number of tubes in a boiling water bath limits the analysis capability and efficiency of the methods. In this study, we modified the SN assay so that the method can quantify a mixture of reducing sugars in a broad concentration range with ease and high efficiency.

2.3. MATERIALS AND METHODS

2.3.1 Reducing sugar solutions

Reducing sugar solutions (0.01 mM – 0.6 mM) were serially diluted from stock solutions (1 mM). Reducing sugars used in this study included fructose (Sigma-Aldrich Ltd, St Louis, MO), glucose (Sigma-Aldrich Ltd, St Louis, MO), maltose (TCI America, Portland, OR), maltotriose (EMD Millipore, Billerica, MA), maltotetraose (Megazyme International Ireland Ltd., Ireland), and maltoheptaose (TCI America, Portland, OR).

2.3.2 Microplate SN assay

Preparation of reagents was the same as that reported by Somogyi and Nelson (Nelson, 1944; Somogyi, 1952). The copper-carbonate-tartrate reagent was prepared in two solutions. Sodium potassium tartrate tetrahydrate (12g), sodium carbonate (24 g), sodium bicarbonate (16 g), and sodium sulfate (144 g) were dissolved in deionized water and dilute to 800 mL to make stock Solution I. Copper sulfate pentahydrate (4 g) and sodium sulfate (36 g) were dissolved in deionized water and dilute to 200 mL to make stock Solution II. They were stored separately to prevent copper oxidation (Somogyi, 1952). Four parts of Solution I and one part of Solution II were freshly mixed to make the working reagent before analysis. The arsenomolybdate color reagent was prepared according to (Nelson, 1944). Ammonium molybdate (25 g) was dissolved in 450 mL of deionized water and mixed with 21 mL of concentrated sulfuric acid. In the mean time, sodium arsenate dibasic pentahydrate (3 g) was

dissolved in 25 mL deionized water and mixed with the ammonium molybadate solution. The reagent was incubated at 37°C for 24 – 48 h and stored in a brown bottle.

Detailed procedure is shown in Fig. 2.1. Each reducing sugar solution (45 µL) was placed in wells of a 96-well microplate (polypropylene, 360 µL well volume, flat-bottom, Corning Company, U.S.A.) in triplicate. The working reagent (45 µL) was then added. The plate was covered with a silicone microplate mat and taped with a layer of aluminum foil to avoid water vapor from entering the plate during heating (Fig. 2.1-A and -B). The sample and reagent were mixed by shaking the plate on a microplate reader for 10 s (Spectra Max 190 Microplate Reader, Molecular Devices, CA, U.S.A.) and then heated in a boiling water bath for 20 min (Fig. 2.1-C). The plate was placed on a rack in the water bath, and its bottom was immersed in the boiling water with a stir bar continuously stirring the water for even heating. After heating, the plate was placed in a zip-lock bag and cooled with running cold water for 5 min (Fig. 2.1-D). The arsenomolybdate color reagent (45 µL) was then pipetted into each well (Fig. 2.1-E). The plate was placed on the bench for 15 minutes to complete the color development. Before reading the plate, bubbles were removed by pipetting the entire solution up and down; the absorbance was recorded within 40 min after the addition of the arsenomolybdate color reagent.

2.3.3 Statistical analysis

Data were presented as means with standard deviations. Linear regression analysis was performed to determine how well the model (calibration curve) fits the data. The degree of correlation was indicated by the coefficient of determination, R^2. The measurement of different sugars in the modified SN assay was analyzed using one-way analysis of variance (ANOVA) and Tukey's multiple comparison tests (Minitab 17, Minitab, Inc., PA). The level of significance was set at 5%.

2.4. RESULTS AND DISCUSSION

Our modified SN assay had a linear relationship between concentration and absorbance with a coefficient of determination, R2, of 0.997 when maltose was used as the standard (Fig. 2.2). The original SN assay was highly recommended by Robyt and Whelan (1972) because it

gave the same absorbance value to equal molar quantity of maltodextrins. Robyt and Whelan (1972) explained that the reducing value of maltose, malto-oligosaccharides, and maltodextrins was proportional to the number of reducing groups and the over-oxidation did not occur in SN assay. The modified SN method reproduced this phenomenon, and therefore maltose, maltotriose, maltotetraose, and maltoheptaose can share the same calibration curve (Fig. 2.2). It is important to prepare sugar standards based on molar concentration because the assay is based on an oxidation-reduction reaction and standards that are prepared based on mass concentration (i.e., μg/mL) would not generate a universal calibration curve.

Our data showed that glucose had a higher absorbance than malto-oligomers with the same molar concentration but had the same absorbance as fructose (Fig. 2.2 and 2.3). A similar observation was first reported by Shaffer and Somogyi (1933) that the rate of reduction, measured by titrating reduced copper ions, of glucose and fructose was much faster than maltose. Paleg (1959) also found that glucose and fructose had higher absorbance than maltose (at 560 nm). Whelan, Bailey, and Roberts (1953) explained that the difference in the reducing power of glucose and maltodextrins was due to the difference of their hydroxyl group spatial configuration. In addition, glucose has free C4 while the C4 of maltodextrin is used to form a glycosidic linkage with another glucosyl unit (Robyt, Ackerman, & Keng, 1972). Thus, glucose has a higher reducing power than maltodextrins and is not recommended to be used as the standard to calibrate the absorbance and concentration of mixed reducing sugars. To obtain consistent results, it is critical to provide sufficient reaction (heating) time. In Somogyi's study (1937), it took 10 min for glucose to complete the oxidation, and additional 5 and 10 min for maltose and non-fermentable polysaccharides, respectively, to reach completion. Later, heating for 20 min was validated by other researchers (Marais, de Wit, & Quicke, 1966; Nelson, 1944). In our microplate method, a heating time of 20 min was sufficient and extending the heating from 20 to 40 min did not make a difference in the results (data not shown).

Although various wavelengths had been used in the literature, the optimal wavelength for the SN assay had never been definitely determined. When spectrophotometer was first used for the assay in 1944, Nelson chose 500 nm as a compromise between desired sensitivity and minimization of variation. Nelson (1944) suggested that the sensitivity of the assay is increased as the reading wavelength is increased. Thus, wavelengths such as 560 nm (Paleg,

1959) and 750 nm (Marais et al., 1966) were later applied to the SN assay. To determine the optimal wavelength for the modified microplate assay, we examined the absorption spectra of five reducing sugars (0.6 mM) from 500 to 750 nm. Our data showed absorbance ranged from 0.5 to 2.7 (Fig. 2.4). We recommend users to measure absorbance at 600 nm because the difference in the absorbance of sugars was less than 0.05, which was insignificant. This allows users to use one universal calibration curve to quantify the total amount of reducing sugars, which is not applicable at a longer wavelength. The lower wavelength also provided a broader detection range though the sensitivity would be higher at a longer wavelength. Measuring absorbance at 600 nm has the benefit to detect a broad range of concentrations with the acceptable sensitivity. In addition, it took less time (i.e., 20 min) to develop color for measuring at 600 nm but needed 1.5 h to develop full color for measuring at 750 nm (Marais et al., 1966)

The efficiency of the SN assay was greatly improved through the modifications in this study. The traditional SN procedure requires a large number of reaction tubes and transfer of the final solution to cuvettes for absorption reading (Table 2.1). The modified SN method is performed with a 96-well microplate, and both sugar standards (serially diluted maltose solution) and up to 25 samples can be measured together in triplicate in about an hour. The modified method provides a more homogeneous heating, and the standard deviation of triplicate measurements was smaller than 0.02. Another advantage of the modified microplate SN assay is that it requires a small volume (45 μL) of samples and reagents and produces very small amounts of chemical wastes.

A clear polypropylene microplate is recommended for the modified assay as it is heat resistant. Microplates made of polystyrene should not be used as they may be damaged during the heating process. For heating, a boiling water bath with a rack should be used, and the bottom part of the microplate should be immersed in the boiling water. The water bath should be covered with a lid to maintain sufficient heat for a complete reaction. The use of oven or heating block is not recommended as they may damage microplates. Sealing the microplate with a silicone mat and foil (Fig. 1-C) can avoid introducing water vapor to the samples. It is critical to remove bubbles generated during color development to avoid interference in absorbance reading. This can be done by pipetting the final solution up and down several

times. The procedure is demonstrated on the website: https://www.youtube.com/watch?v=f6fCBNxtOMA.

2.5. CONCLUSION

Measuring reducing power is a common practice in carbohydrate research but choosing a proper method can be challenging. We do not recommend using DNS-based method to analyze the reducing power of mixed sugars in a sample. The method developed by Somogyi and Nelson can accurately measure the reducing power, and we demonstrated a modification to conduct the reaction in a 96-well plate. Glucose behaves differently from maltose and maltodextrins. Thus, we suggest using maltose to generate a calibration curve and constructing the second calibration curve using glucose when measuring glucose or fructose. The miniaturized Somogyi - Nelson assay successfully reproduces the sensitivity and accuracy of the traditional SN assay with greatly improved analytical capacity and efficiency.

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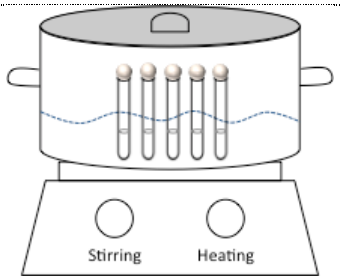
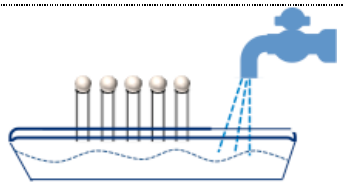
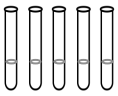
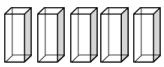
2.7. TABLES AND FIGURES

Table 2.1 Comparison of the mechanisms of SN, CuBic, PJ and DNS assays

Assay	Oxidizing agent	Final color compound	Reference
Somogyi-Nelson assay (SN)	Cupric Ion (Cu^{2+}) → Cuprous oxide (Cu_2O)	Arsenomolybdate complex → Molybdenum blue	Nelson (1944)
Copper-bicinchoninate (CuBic)	Cupric Ion (Cu^{2+}) → Cuprous ion (Cu^{+})	2,2'- Bicinchoninate → Copper (I)-bicinchoninate color complex	Mopper and Gindler (1973)
Park-Johnson assay (PJ)	Ferricyanide ion ($[Fe(CN)_6]^{3-}$) → Ferrocyanide ion ($[Fe(CN)_6]^{4-}$)	Ferric ion (Fe^{3+}) → Ferric ferrocyanide ($Fe_4[Fe(CN)_6]_3$)	Park and Johnson (1949)
Dinitrosalicylic acid assay (DNS)	3,5- Dinitrosalicylic acid →	3-Amino-5-nitrosalicylic acid	Miller (1959)

Table 2.2 Comparison of procedures of DNS, SN, and PJ assays

Steps		Dinitrosalicylic acid assay (DNS)	Somogyi-Nelson assay (SN)	Park-Johnson assay (PJ)
Add Reagent 1		✓	✓	✓
Heating		5 min	20 min	15 min
Cooling		✓	✓	✓
Add Reagent 2			✓	✓
Standing			✓ (≥ 15 min)	✓ (20 min)
Dilution		✓	✓	
Reading absorbance		✓ 	✓ 	✓ 

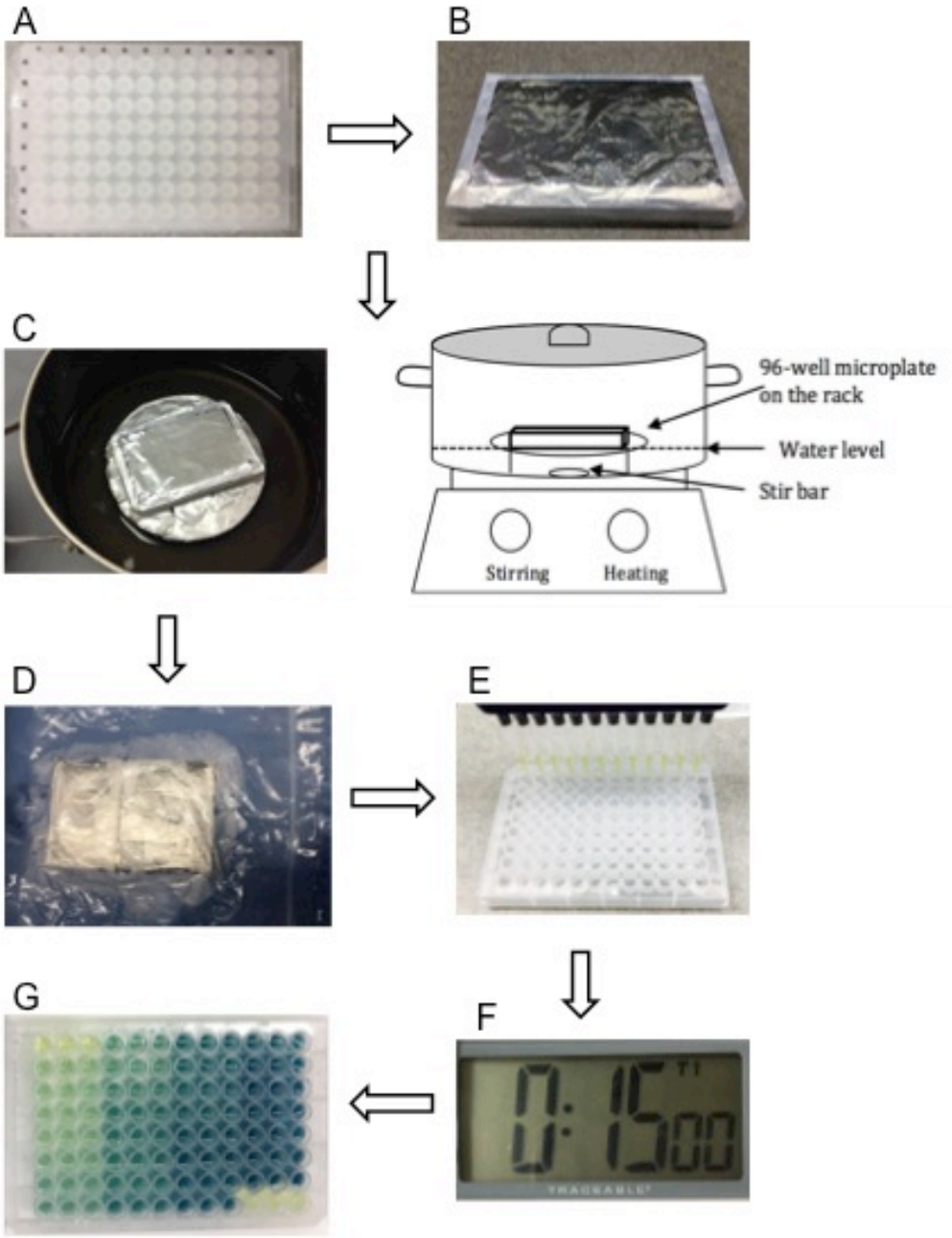


Fig. 2.1 Procedure of the modified SN assay. A. Cover the microplate with a silicone mat after adding samples and the working reagent; B. Tape a layer of aluminum foil; C. Heat the microplate in a boiling water bath for 20 min. Place the microplate on a rack and immerse the bottom of the plate in the water, and cover the water bath with a lid; D. Place the microplate in a zip-lock bag and cool it down with running cold water for 5 min; E. Add the color reagent; F. Wait for 15 min to fully develop the color and then remove bubbles; G. Read the absorbance.

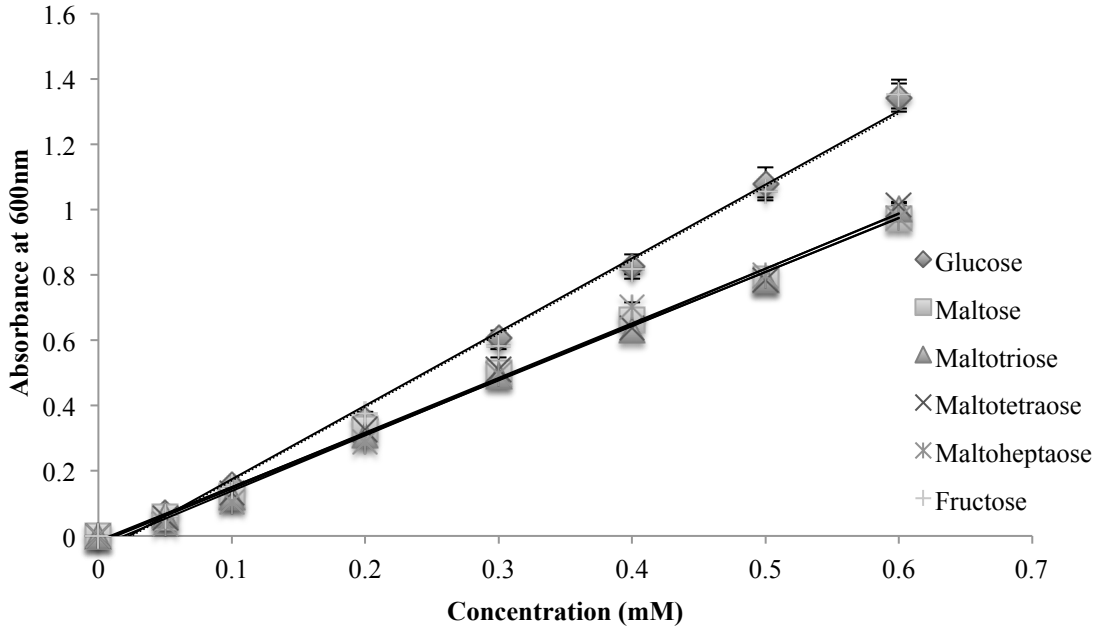


Fig. 2.2 Calibration curves of six reducing sugars using modified SN method. The absorbance was determined at 600 nm and averaged from triplicate measurements. Error bars present the standard deviation among triplicates. The R^2 of glucose, maltose, maltotriose, maltotetraose, maltoheptaose, and fructose are 0.996, 0.997, 0.997, 0.996, 0.993, and 0.994.

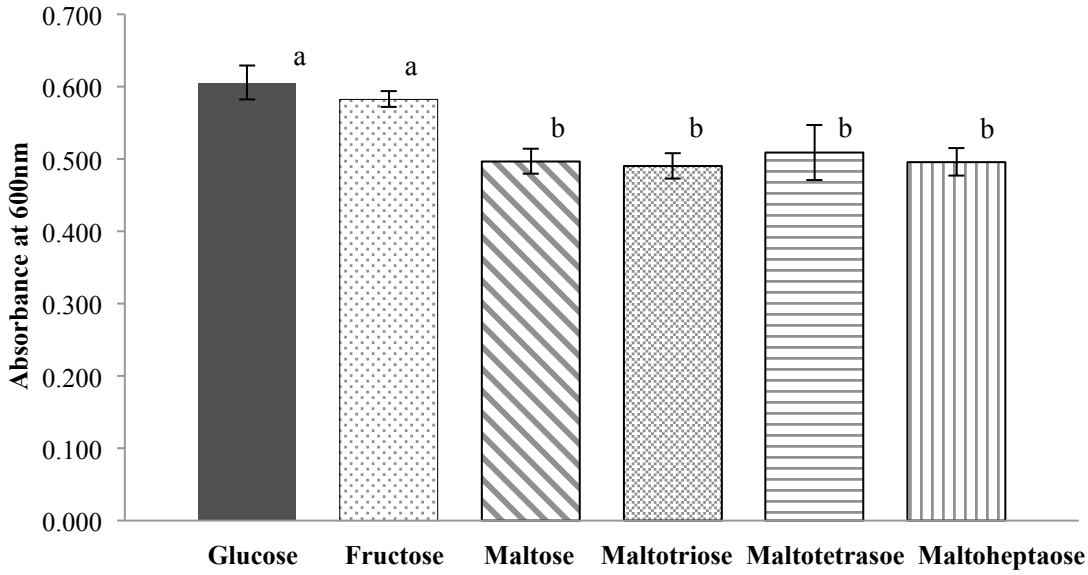


Fig. 2.3 Comparison of the absorbance of different reducing sugars at an equal molar concentration (0.3 mM) using modified SN method. Error bars present the standard deviation of triplicates. The values with the same letter are not significantly different from each other (one-way ANOVA and Tukey's test, $p < 0.05$).

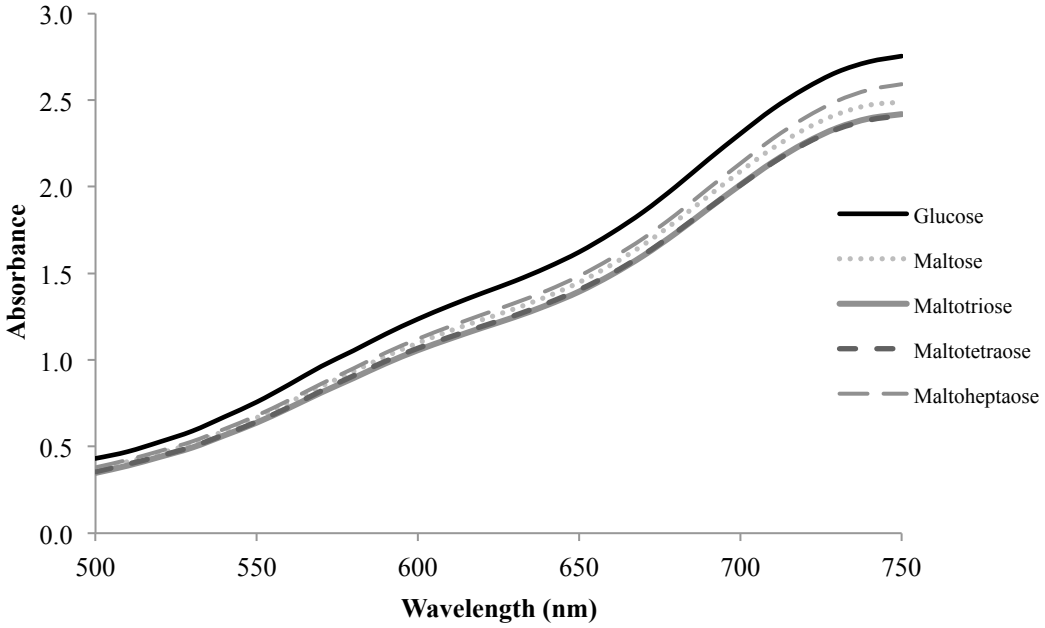


Fig. 2.4 Absorption spectra of five reducing sugars (0.6 mM) from 500 to 750 nm using modified SN method. The values were averaged from triplicate reactions.

APPENDIX A. PERMISSION FOR REUSING THE ARTICLE FROM ELSEVIER