Arabinoxylans (AX) of wheat (*Triticum aestivum* L.) play a critical role in processing, end-use quality, and human health and nutrition. Consequently, an efficient, accurate method of AX quantification is desirable. The objective of this work was to evaluate a standard phloroglucinol colorimetric method for quantification of wheat AX. The method is based on the formation and spectrophotometric quantification of a phloroglucide product that results from the reaction of furfural produced during the condensation of pentose sugars with phloroglucinol. Method parameters, including reaction reagents and reaction times, were varied to identify areas for improved accuracy and consistency. Phloroglucide formation at three xylose concentrations was examined over time. The optimal reaction reagents and reaction times were determined based upon improved consistency in xylose quantification. The optimized method was used on xylose and arabinose standards and on whole meal wheat samples for total and water-extractable AX content. Glucose was shown to be unnecessary in the reaction and was eliminated. A second-order polynomial equation provided a slightly better fit to the nearly linear standard xylose curve. A reduced concentration of phloroglucinol of 10% was found to give equivalent results to the standard 20%. Optimum reaction time was 25 min, and it required the inclusion of all reagents. The phloroglucide product decreased in absorbance over time such that, within the range of xylose concentration examined, about 40–50% of the colored product was lost over 100 min; however, the rate of loss was linear over time. Four operators performed the optimized method on whole wheat meal samples for total and water-extractable AX. Inter- and intraoperator variation was identified as an area requiring further study and improvement. However, all operators tended to rank the samples in a consistent manner. Compared with a gas chromatography–flame ionization detection method, the phloroglucinol method underestimated total AX by about 2.3% and water-extractable AX by about 0.08%.

The nonstarch carbohydrates of cereal grains are important for food processing, end-product quality, and human health and nutrition. In wheat (*Triticum aestivum* L.) grain, nonstarch carbohydrates are primarily associated with cell walls. Cell walls are composed of up to 75% nonstarch carbohydrates, which are mostly (85%) substituted pentose polymers referred to as arabinoxylans (AX) (Mares and Stone 1973). Other nonstarch carbohydrates include cellulose, lignin, glucomannans, and β-glucans (Lineback and Rasper 1988).

AX are composed of a β-1,4 linked β-d-xylopyranosyl backbone with substituted monomeric α-L-arabinofuranoside at the second and/or third carbon positions. The arabinose moiety can possess ferulic acid at the fifth carbon position (Courtin and Delcour 2002). The three-dimensional structure of AX is mainly determined by the length of the xylan backbone, the ratio of arabinose to xylose, the substitution pattern on the backbone, and the ferulic acid coupling to other AX molecules or the cell wall (Courtin and Delcour 2002). AX have a somewhat flexible structure (Dervilly et al 2000). These structural characteristics and the large molecular weight (65,000) (Andrewartha et al 1979) contribute to empirically derived subfractions based on extractability (generally in water at room temperature), namely, water-extractable (WEX) and water-unextractable AX (Izydorczyk and Biliaderis 1992; Courtin and Delcour 2002). AX also have the capacity to form oxidative cross-links and gel networks (Izydorczyk and Biliaderis 1992) and affect end-product quality (Betteg and Morris 2007; Ramseyer et al 2011a, 2011b), thus increasing their overall molecular weight and exerting different effects on food processing and human physiology.

AX exhibit nutritive properties as well as influencing end-use quality (Lu et al 2000). The postprandial glucose concentration was decreased after healthy individuals ate bread products with AX as an additive (Lu et al 2000). AX added to bread products also lowered the glucose and insulin levels of the blood 2 hr after consumption in individuals with type 2 diabetes (Lu et al 2004).

There are several techniques to quantify AX, including colorimetric assays (Wheeler and Tollens 1889; Dische and Borenfreund 1957; Douglas 1981; Ford 1981; Bell 1985) and gas chromatography–flame ionization detection (GC-FID) (Gebruers et al 2009). Some structural characteristics of AX can be determined by GC-FID (Gebruers et al 2009), enzyme mapping (Saulnier and Quemener 2009), molecular weight distribution (Andersson et al 2009), Fourier transform infrared spectroscopy (Toole et al 2009), Raman spectroscopy (Toole et al 2009), fluorescence microscopy (Toole et al 2009), and carbohydrate-binding molecules (Toole et al 2009). For quantification, phloroglucinol has been the most commonly used colorimetric assay since its first description in 1889 (Wheeler and Tollens 1889), as it can measure five-carbon monosaccharides. The method was later extended to quantify hydrolyzed pentosans (Kröber 1901; Kröber et al 1902). This general method was used with phloroglucinol as the reactant and with a distillation step to capture furfural until 1957 (Dische and Borenfreund 1957), when the distillation was eliminated, and the reactant concentrations and timing evolved into the widely used Douglas (1981) method. This method is among the quickest and most efficient methods for the quantification of total AX (TAX) and WEX. The biggest disadvantage observed in this method, however, was the loss of colored product over time, which decreased the accuracy of the test by roughly 20% in 60 min (Douglas 1981). Nevertheless, the method of Douglas (1981) has been particularly popular among cereal chemists. According to information provided by ISI Web of Science (Thomson Reuters, New York, NY), this method has been cited approximately 110 times. Rouau and Surget (1994) developed a semiautomated version of the assay.

The phloroglucinol method of Douglas (1981) has been used to determine the AX content of wheat flour (Hashimoto et al 1987;
Izydorczyk et al. 1991; Biliaderis et al. 1995; Bettge and Morris 2000, 2007; Finnie et al. 2006; Li et al. 2009; Ramseyer et al. 2011a) and barley flour (Izydorczyk et al. 2003; Izydorczyk and Dexter 2008). With this method, AX levels were shown to vary among wheat varieties (Finnie et al. 2006; Li et al. 2009) and flour mill streams (Ramseyer et al. 2011a) and were influenced by growing environment (Finnie et al. 2006; Li et al. 2009). AX levels were related to changes in dough development time and viscosity (Jelaca and Hlynka 1971) and were associated with soft wheat end-use quality, including variation in cookie diameter (Bettge and Morris 2000).

The objective of this research was to evaluate the Douglas (1981) phloroglucinol method and to determine ways in which it might be improved by varying components of the assay. Experiments were carried out to define the optimal reagents and timing of each step of the reaction to achieve more consistent and accurate results as well as to evaluate the repeatability of the assay. The method was applied to whole wheat meal samples, and results were compared with a standardized GC-FID method.

MATERIALS AND METHODS

Basic Method

The basic method of Douglas (1981) was conducted using the following procedure. Triplicate 2.0 mL aliquots each of a dilution series of xylose (Sigma-Aldrich, St. Louis, MO) were prepared to 0.0, 0.05, 0.10, 0.15, 0.20, 0.25, and 0.30 mg/mL and were added to 12 mL stoppered reaction tubes (Pyrex tube, 16 × 100 mm, screw cap with polytetrafluoroethylene liner). The assay can also be performed with arabinose as a standard with varying reaction times prior to the addition of phloroglucinol. As described in the basic method, reaction reagent containing acetic acid, hydrochloric acid, and water (substituted for glucose) were held constant, but the concentration of phloroglucinol in ethanol was varied at 10, 20, and 40% (w/v). Henceforth, all concentrations will be referred to by percentage of phloroglucinol. The reaction procedure was carried out as described for the basic method.

Phloroglucinol Concentration

The basic method was followed wherein the proportions of reaction reagents acetic acid, hydrochloric acid, and water (substituted for glucose) were held constant, but the concentration of phloroglucinol in ethanol was varied at 10, 20, and 40% (w/v). Henceforth, all concentrations will be referred to by percentage of phloroglucinol. The reaction procedure was carried out as described for the basic method.

Variation in Reaction Time

The basic method was used, but the phloroglucinol concentration was reduced to 10%. Once the reaction reagent was added to the standard xylose solutions, four different (reaction) times in the water bath were used: 15, 25, 35, and 45 min. The experiment was then continued as described for the basic method. A second experiment was performed to determine the effect of varying reaction times prior to the addition of phloroglucinol. As described in the basic method, reaction reagent containing acetic acid, hydrochloric acid, and water was added to the xylose standards. The tubes were placed in a boiling water bath for 15, 25, and 45 min. The test tubes were removed from the boiling water and immediately placed in an ice bath. Once they were cool, phloroglucinol was added to each tube, and the tubes were returned to a boiling water bath for 25 min. The tubes were then removed, cooled in an ice bath, and brought to room temperature in a room-temperature water bath. The colorimetric analysis was then carried out as previously described.

Reaction Product Absorbance Loss Over Time

Eleven tubes each of xylose at 0.10, 0.20, and 0.30 mg/mL and three tubes of a zero xylose standard were prepared. The experiment was carried out as previously described in the basic method, with water in place of glucose, 20% w/v concentration of phloroglucinol in ethanol, and a reaction time of 25 min in a boiling water bath. Immediately after removal of the tubes from the room-temperature water bath, the three tubes of the zero xylose standard were measured. At time zero (<2 min following removal from the water bath), the absorbance of one of each of the xylose concentrations (0.10, 0.20, and 0.30 mg/mL) was measured. Every 10 min following this, the absorbance of one tube each of the three concentrations was again measured until 100 min.

Comparison of Operators: Whole Wheat Meals

The whole wheat meal sample preparation and sampling were performed as described by Finnie et al. (2006). Twelve commercially grown, varietal wheat samples were provided by Dr. Laura Hansen (General Mills Inc.). Samples included two soft white spring, seven soft white winter, two club, and one hard red winter wheat varieties. With the exception of the hard red winter...
wheat, all the varieties were developed in and adapted to the Pacific Northwest states of Idaho, Oregon, and Washington. Sample origin was south-central Idaho. Samples were assayed in duplicate by adding 125 mg of meal to a 50 mL conical screw-cap polypropylene tube (Fischer Scientific, Pittsburgh, PA, or equivalent). To each tube, 25.0 mL of water was added, and the mixture was suspended by vortexing (≈7 sec). After mixing, duplicate 1.0 mL aliquots of the slurry were rapidly withdrawn (before settling) with a modified large-aperture (≈3 mm) pipette and added to 12 mL stopped reaction tubes. These samples were used to quantify the TAX content of the meals. The conical tubes containing hydrated meals were then placed on a laboratory rocker (AR-100, PGC Scientific, Gaithersburg, MD) for 30 min and then centrifuged for 10 min at 2,500 × g. Duplicate 1.0 mL aliquots of the supernatant were added to stopped reaction tubes, representing the WEAX content of the samples. These samples were then assayed following the optimized procedure (no glucose and 10% w/v phloroglucinol in ethanol in the reaction reagent). The assay was performed by four operators who were trained identically and were assessed to perform the assay proficiently. Each operator had at least five months of experience in performing the assay prior to undertaking the TAX and WEAX quantification with these 12 “standard” whole wheat meal samples.

**Analysis of Noncellulosic Sugar Content and Composition with Gas Chromatography**

Whole wheat meal samples were derivatized and analyzed by the method described by Englyst and Cummings (1984) with some slight modifications (Courtin et al 2000). Through this method, both TAX and WEAX were quantified. The samples were first hydrolyzed and subsequently derivatized to alditol acetates before injection into a capillary column with a split-splitless injector. The samples then entered the FID. The column used in the GC was a Supelco SP-2380 polar column (Supelco, Bellefonte, PA) in an Agilent 6890 series chromatograph (Wilmington, DE) with an autosampler and splitter injection port (injection volume, 1.0 µL; split ratio, 1:20). The FID used helium as the carrier gas and separation at 225°C; injection and detection were at 270°C. This procedure was performed at the Laboratory of Food Chemistry and Biochemistry, University of Leuven.

**Data Analysis**

All data were analyzed with SAS version 9.2 (SAS Institute, Cary, NC). The general analyses were done with PROC GLM. Three replicates of standard xylose (or arabinose) were used, and duplicates as well as replicates were used for whole wheat meal samples, resulting in four samples representing each whole wheat meal variety.

**RESULTS**

**Inclusion of Glucose**

The objective of this portion of the study was to determine the necessity of glucose as a reagent. Douglas (1981) provided no justification or explanation as to its inclusion in his method. We were unable to identify why glucose would be necessary for the reaction itself, for the development or stability of the colored product, or for the spectral analysis. Consequently, we performed the standard assay with and without glucose using a range of xylose concentrations from zero to 0.30 mg/mL.

The analysis of variance (ANOVA) of the results indicated that 98% of the variation could be explained among replicates, xylose concentration, and presence/absence of glucose (whole model $R^2$). Comparing the two treatments, namely, reaction with or without glucose, indicated no significant effect on absorbance (and therefore on ability to measure xylose content) (Table I). This result provided evidence that glucose was unnecessary in the reaction reagent and that the development of colored product (and absorbance) was the same when water was substituted. The concentration range of xylose was chosen to encompass a wide range of quantity and absorbance, commensurate with levels encountered in wheat samples. The ANOVA interaction observed between xylose concentration and presence or absence of glucose indicated some nonparallelism of response slopes. Upon closer examination, it was seen that the absorbance of the solutions containing water was greater than the absorbance of the solutions containing glucose at both the 0.25 and 0.30 mg/mL xylose concentrations, by 11 and 10%, respectively (Fig. 2).

**TABLE I**

**Analysis of Variance of a Phloroglucinol Colorimetric Assay for Pentoses Using Xylose at Seven Concentrations and With or Without Glucose in the Reaction (Treatment)**

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Mean Square</th>
<th>$F$ Value</th>
<th>$P$ Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole model</td>
<td>13</td>
<td>0.1382</td>
<td>179.16</td>
<td>&lt;0.00001</td>
</tr>
<tr>
<td>Xylose concentration</td>
<td>6</td>
<td>0.2977</td>
<td>386.12</td>
<td>&lt;0.00001</td>
</tr>
<tr>
<td>Treatment</td>
<td>1</td>
<td>0.0013</td>
<td>1.69</td>
<td>0.201</td>
</tr>
<tr>
<td>Concentration × treatment</td>
<td>6</td>
<td>0.0023</td>
<td>2.94</td>
<td>0.018</td>
</tr>
</tbody>
</table>

* Degrees of freedom.
Absorbance Response vs. Xylose Concentration

The curve-fit portion of the experiment was conducted because it was observed that the standard curves consistently deviated from linear at the highest xylose concentrations. The fit of the standard curve is of utmost importance when interpolating data. Linear and second-order polynomial curves were fit to the xylose concentration series (Fig. 3). The linear equation was \( y = 2.23x + 0.053 \), with an \( R^2 \) value of 0.984. The polynomial curve fit had an equation of \( y = 3.25x - 3.39x^2 + 0.011 \), with an \( R^2 \) value of 0.992. Although both equations described the standard curve quite well, the polynomial curve fit slightly better. It has been consistently observed that as the xylose concentration increased beyond 0.15 up to 0.30 mg/mL, the absorbance dropped off slightly, whereas at 0, 0.05, 0.10, and 0.15 mg/mL, the relationship was fairly linear (Fig. 3; additional data not shown). The present data were consistent with these past observations. A subsequent experiment compared the concentration–absorbance response of arabinose to xylose. The results (data not shown) indicated that the two monosaccharides produced identical absorbance response curves, such that either could be used as the standard.

Variation in Phloroglucinol Concentration

The phloroglucinol concentration was varied to determine whether it was a limiting reagent, especially at the higher concentrations of xylose. As shown in Figure 4, three concentrations of phloroglucinol, each a twofold change from the standard 20%, were used in the reaction reagent and compared using five xylose concentrations. Over the entire range of xylose, phloroglucinol concentrations of 10 and 20% were observed to produce similar absorbances, whereas the absorbance at 40% phloroglucinol was markedly lower. Based on these results, it can be concluded that at xylose concentrations from 0.0 to 0.30 mg/mL, a phloroglucinol concentration as low as 10% can be used without limiting the reaction, yielding similar results as the traditionally used concentration of 20% phloroglucinol (Douglas 1981). In addition, 40% phloroglucinol was clearly disadvantageous and significantly hindered the efficacy of the method (i.e., concentration response–color reaction).

Variation in Reaction Time

The objective of the reaction time variation experiment was to determine the optimal time that the reaction tubes should spend in the water bath, that is, sufficient time for the reaction to occur while limiting the time for the reaction products to degrade. The reaction occurs in two parts: hydrolysis of the AX to pentose and then into furfural, and condensation reaction of the furfural with phloroglucinol to form the pink-red phloroglucide precipitate (Leach 1905) (Fig. 1).

Fig. 4. Comparison of phloroglucinol concentrations. The concentration previously used (Douglas 1981) was 20% phloroglucinol (w/v). Three concentrations were compared: 10 ( ), 20 ( ), and 40% ( ) w/v.

Fig. 5. Comparison of four water-bath (hydrolysis) times at seven xylose concentrations. The times used were 15 ( ), 25 ( ), 35 ( ), and 45 min ( ).

Fig. 6. Loss of colored phloroglucide product over time at three xylose concentrations: 0.10 ( ), 0.20 ( ), and 0.30 mg/mL ( ). Each sample was an independent experimental unit, and absorbance was measured every 10 min for 100 min.

Fig. 7. Relationship of colored phloroglucide product loss over time as a function of beginning xylose concentration. Abscissa values are derived from the decay slopes of Figure 6; a 0.0 data point was added. The linear equation is \( y = 0.00859x + 0.000061 \), where \( y \) is the rate of color loss as absorbance units per minute per milligram of beginning xylose and \( x \) is the beginning xylose amount in milligrams per reaction.
Figure 5 depicts the four reaction times across a range of seven xylose concentrations. Overall, the absorbance values were consistently greatest at 25 min but decreased at 15 and 35 min and were lowest at 45 min. Based on these results, the optimal water bath time was selected as 25 min for the concentrations of xylose used, consistent with the method of Douglas (1981).

The reaction times were also varied at 15, 25, and 45 min prior to the addition of phloroglucinol. A standard curve was generated; however, the variation of this experiment was great, and no clear pattern was observed (data not shown). The coefficient of variation of the absorbance response observed throughout this portion of the experiment was 17%, whereas the coefficient of variation for the previous water bath experiment was only 4%, showing more than a fourfold difference in variation between experiments. We concluded that the phloroglucinol must be present and available for reaction with the generated furfural during the condensation reaction step.

**Reaction Product Absorbance Loss Over Time**

Douglas (1981) observed that there was a 20% decrease in absorbance over 60 min (loss of colored product). Because this method may be performed with a varying number of reaction tubes (often a greater number of assays is desired), it is important to determine the rate at which the colored reaction product decreases in absorbance over time.

When three concentrations of xylose (0.10, 0.20, and 0.30 mg/mL) were evaluated for loss of absorbance over 100 min, all three showed a significant decrease (Fig. 6). A linear rate function was fit to each xylose concentration over time and adequately modeled the phenomenon. At a xylose concentration of 0.30 mg/mL, the equation was $y = 0.613 - 0.00251x$, where $y$ is the absorbance and $x$ is the time in minutes. At 0.20 mg/mL, the color loss rate was $y = 0.447 - 0.00197x$, and at 0.10 mg/mL, the rate was $y = 0.1924 - 0.000914x$. These data showed the general pattern for decrease in absorption over time as well as highlighting the differences in rate of absorption loss, which was dependent on xylose (phloroglucide) concentration. In this regard, the highest concentration of xylose had the greatest absolute rate of color loss (negative slope), and conversely, the lowest xylose concentration lost color at the slowest absolute rate. When expressed as a proportionality, all xylose concentrations lost on the order of about 40–50% of their colored product over 100 min (48, 44, and 38% at xylose concentrations of 0.10, 0.20, and 0.30 mg/mL, respectively) (Fig. 6).

An equation can be developed to describe this decrease in absorbance. Figure 7 illustrates the combined rate loss function derived from the three xylose concentrations shown in Figure 6. By adding a 0.0 data point, the linear least squares fit produced the following equation: $y = 0.00859x + 0.000061$, where $y$ is the rate of absorbance loss per minute per milligram of xylose and $x$ is the original xylose concentration.

**Comparison of Operators**

Many experimental procedures are performed by more than one person, and the objective of this portion of the experiment was to determine how consistent this procedure was when comparing a set of samples across different operators following the exact same analytical procedure. As shown in Table II, four experienced laboratory technicians measured the TAX and WEAX contents of 12 whole wheat meal samples in the same laboratory using the same equipment and reagents. All were provided some training to follow the same procedure. The amount of variation attributable to the samples for each of the operators varied for the TAX measurement, as quantified by calculating the $R^2$ values, which ranged from 0.62 to 0.86. This result indicated that 14–38% of the variation could not be accounted for by the model (error variance). The within-operator range of TAX concentration for the 12 whole wheat meal samples (highest sample mean minus lowest sample mean) varied from 1.11% (operator 4) to 2.42% (operator 1). Operators 1 and 4 obtained a level of precision that declared different wheat samples to be significantly different ($P < 0.003$). The respective least significant differences ($P = 0.05$) were 0.82 and 0.40% TAX, respectively. Operator 2 was near the limit for declaring the highest and lowest means different, whereas the results of operator 3 returned no significant differences among the wheat samples.

The WEAX measurements also varied among operators, ranging from 0.33 to 0.50% (means across all 12 samples). Two of the four operators (operators 2 and 4) showed clear mean separation between the varieties ($P < 0.0004$). The least significant difference for both was 0.04% WEAX. Operators 1 and 3, however, were near the threshold of significance with $P$ values of 0.053 and 0.049, respectively. An analysis of variance was carried out, combining all four operators and all sample results, to determine the effects of operator, sample, and any interaction between sample and operator. When the operator and sample were analyzed for an interaction, the $P$ value for TAX was 0.20 and for WEAX 0.48. Overall, the rank order among samples was similar for all four operators.

**Comparison of Phloroglucinol vs. GC-FID Methods**

The AX content of the 12 whole wheat meal samples was also measured via GC-FID. The purpose of this portion of the experi-

### Table II

**Comparison of Four Operators Measuring the Total Arabinoxylan Content and Water-Extractable Arabinoxylan Content of 12 Whole Wheat Meal Samples Using an Optimized Phloroglucinol Colorimetric Method for Pentoses**

<table>
<thead>
<tr>
<th>Variety</th>
<th>Operator 1</th>
<th>Operator 2</th>
<th>Operator 3</th>
<th>Operator 4</th>
<th>Operator 1</th>
<th>Operator 2</th>
<th>Operator 3</th>
<th>Operator 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alpowa</td>
<td>5.92</td>
<td>4.54</td>
<td>5.50</td>
<td>4.20</td>
<td>0.51</td>
<td>0.59</td>
<td>0.58</td>
<td>0.39</td>
</tr>
<tr>
<td>ID0587</td>
<td>5.02</td>
<td>3.71</td>
<td>4.12</td>
<td>3.90</td>
<td>0.40</td>
<td>0.57</td>
<td>0.37</td>
<td>0.38</td>
</tr>
<tr>
<td>Madsen</td>
<td>4.97</td>
<td>4.96</td>
<td>4.64</td>
<td>3.86</td>
<td>0.39</td>
<td>0.53</td>
<td>0.41</td>
<td>0.32</td>
</tr>
<tr>
<td>Finch</td>
<td>4.68</td>
<td>4.44</td>
<td>5.22</td>
<td>3.68</td>
<td>0.33</td>
<td>0.44</td>
<td>0.28</td>
<td>0.27</td>
</tr>
<tr>
<td>Stephens</td>
<td>4.52</td>
<td>4.22</td>
<td>4.85</td>
<td>3.55</td>
<td>0.39</td>
<td>0.52</td>
<td>0.40</td>
<td>0.37</td>
</tr>
<tr>
<td>Alturas</td>
<td>4.51</td>
<td>3.85</td>
<td>4.94</td>
<td>3.90</td>
<td>0.36</td>
<td>0.44</td>
<td>0.38</td>
<td>0.32</td>
</tr>
<tr>
<td>Brundage</td>
<td>4.47</td>
<td>4.41</td>
<td>5.14</td>
<td>3.64</td>
<td>0.34</td>
<td>0.49</td>
<td>0.35</td>
<td>0.29</td>
</tr>
<tr>
<td>Jagger</td>
<td>4.40</td>
<td>4.29</td>
<td>4.93</td>
<td>3.35</td>
<td>0.38</td>
<td>0.49</td>
<td>0.36</td>
<td>0.30</td>
</tr>
<tr>
<td>Simon</td>
<td>4.04</td>
<td>3.49</td>
<td>4.65</td>
<td>3.16</td>
<td>0.34</td>
<td>0.42</td>
<td>0.34</td>
<td>0.30</td>
</tr>
<tr>
<td>Hiller</td>
<td>3.95</td>
<td>3.81</td>
<td>4.04</td>
<td>3.09</td>
<td>0.34</td>
<td>0.42</td>
<td>0.34</td>
<td>0.30</td>
</tr>
<tr>
<td>Coda</td>
<td>3.52</td>
<td>3.49</td>
<td>4.08</td>
<td>3.58</td>
<td>0.39</td>
<td>0.57</td>
<td>0.44</td>
<td>0.34</td>
</tr>
<tr>
<td>Brundage 96</td>
<td>3.49</td>
<td>3.88</td>
<td>3.78</td>
<td>3.52</td>
<td>0.35</td>
<td>0.48</td>
<td>0.31</td>
<td>0.31</td>
</tr>
<tr>
<td>R²</td>
<td>0.66</td>
<td>0.72</td>
<td>0.62</td>
<td>0.84</td>
<td>0.71</td>
<td>0.74</td>
<td>0.71</td>
<td>0.89</td>
</tr>
<tr>
<td>Overall mean</td>
<td>4.46</td>
<td>4.09</td>
<td>4.66</td>
<td>3.60</td>
<td>0.38</td>
<td>0.50</td>
<td>0.38</td>
<td>0.33</td>
</tr>
<tr>
<td>F value</td>
<td>6.48</td>
<td>2.74</td>
<td>1.80</td>
<td>5.80</td>
<td>2.67</td>
<td>17.95</td>
<td>2.73</td>
<td>8.72</td>
</tr>
<tr>
<td>P value</td>
<td>0.0016</td>
<td>0.0485</td>
<td>0.1624</td>
<td>0.0026</td>
<td>0.0529</td>
<td>&lt;0.0001</td>
<td>0.0491</td>
<td>0.0004</td>
</tr>
</tbody>
</table>
ment was to compare the two fundamentally different methods to determine how closely the measured values agreed. The percentage of TAX observed from the GC-FID method (6.48%) was markedly greater than that detected in the colorimetric phloroglucinol method (4.20%) (means across 12 samples and across four operators) (Table II and data not shown). The percentage of WEAX observed in the GC-FID and phloroglucinol colorimetric methods also varied considerably, with the GC-FID having an overall average of 0.48% as compared with 0.40% from the phloroglucinol method. The TAX content was also evaluated on a GC-FID at the Western Wheat Quality Laboratory using the same laboratory procedures and instrument settings as at the Laboratory of Food Chemistry and Biochemistry. Results were consistent between the two GC-FIDs, with means across the 12 samples differing by 0.12% TAX (data not shown). It should be noted that the GC-FID procedure extracted WEAX at 7°C, whereas the phloroglucinol method extracted it at room temperature. A side experiment with low (0.42%) and high (0.55%) WEAX whole wheat meal varietal samples indicated that room-temperature extraction increased WEAX by about 8–9 percentage points (data not shown).

In the TAX quantification, the difference between the GC-FID and phloroglucinol methods was twice (in magnitude) that of the differences between operators in the phloroglucinol method. In the WEAX quantification, the difference between the GC-FID and phloroglucinol methods was slightly over half of the difference between operators in the phloroglucinol method.

**DISCUSSION**

Analytical procedures are founded on accuracy and precision. In the present series of studies, we evaluated individual parameters of a popular phloroglucinol colorimetric assay (Douglas 1981) for pentose sugars with application to the measurement of AX in wheat and other cereals. The first parameter examined, glucose, was included in the original assay described by Douglas (1981) with no stated justification. On the basis of our results (Table I), glucose had no significant effect on the measurement of xylose. Consequently, we concluded that Douglas’s objective was to demonstrate that the subtraction of absorbance at 505 nm from that at 558 nm effectively removed any interference of hexoses, thereby verifying that the phloroglucinol method could accurately quantify pentoses in the presence of hydrolyzed starch. For wheat in particular, any method that hydrolyzes all grain or flour carbohydrates must accommodate large quantities of glucose (arising from starch). Rouau and Surget (1994) included glucose in their semiautomated phloroglucinol “Douglas” system and also showed that it had no effect on pentose measurements, but they made no suggestion to eliminate it from future analyses. In the present study, an equivalent volume of water was used in place of glucose solution to maintain consistent volumes and concentrations of the other reagents used in the reaction mixture.

The second feature of the phloroglucinol method involved the relationship between analyte concentration and product concentration (in this case, absorbance of phloroglucide). When absorbance was plotted against xylose concentration (Fig. 3), there was a slight but consistent deviation from linearity at the higher concentrations. Proportionally, there was a slightly lower absorbance response per unit of xylose at the highest concentrations. Douglas (1981) and Rouau and Surget (1994) also observed a drop in absorbance at higher pentose concentrations. As expected, a second-order polynomial, when fitted to our data, captured this slight curve with a small but appreciable increase in $R^2$ as compared with a linear fit.

The phloroglucinol concentration in the original assay (Douglas 1981) appeared to be excessive from a molar-based, theoretical basis (assuming two phloroglucinol molecules per furfural, as the chemical formulation and reaction would indicate). At the lowest concentration (10%), phloroglucinol was easily dissolved in ethanol, which was not the case at the higher concentrations. The reduced 10% concentration did not cause an absorbance response difference as compared with the original phloroglucinol concentration of 20% (Fig. 4). Reducing the phloroglucinol concentration by half saved cost and was easier to prepare. The highest phloroglucinol concentration examined (40%) was clearly detrimental to the assay, and the reagent was difficult to prepare.

Reaction time is important, as the AX has to be reduced to constituent sugars, and the pyranose ring of xylose and arabinose converted to furfural. The furfural molecule then donates electrons to two molecules of phloroglucinol, which condense to form phloroglucide. This phloroglucide precipitate is pink-red in color and is the molecule analyzed spectrophotometrically. As seen in Figure 5, the reaction time appeared to be optimum at 25 min. However, it was imperative that the hydrolysis and phloroglucinol–phloroglucide reaction occur together in the same reaction vessel, as opposed to hydrolyzing the sugars (or potentially the AX polymer) first and subsequently reacting the resulting furfural with phloroglucinol. The complete reaction appeared to proceed in a concerted chain, and it was imperative that there were no interruptions in any of the steps. We concluded that the phloroglucinol must be available to react with the furfural as it is generated to produce the phloroglucide product. Rouau and Surget (1994), on the other hand, hydrolyzed the TAX in a separate prehydrolysis step with sulfuric acid, and they indicated that it was the phloroglucinol mixed with acid that was unstable. In our preliminary studies, inclusion of this prehydrolysis step produced dramatically lower TAX levels. Conversely, Rouau and Surget (1994) stated that using the Douglas (1981) acid reagent “underestimated pentosan values.” The type of acid(s) and hydrolysis may deserve further study.

The loss of absorbance over time (Fig. 6) illustrated one of the primary limitations of the phloroglucinol method. Consequently, time and the number of samples must be considered when planning AX quantification with phloroglucinol. The colored product apparently broke down fairly rapidly but linearly and in predictable fashion over time. This result suggested that two possible approaches may be to limit the reaction to a smaller number of samples or to introduce a correction factor based on the rate of absorbance loss. The percentage (proportionality) of absorbance loss decreased slightly with increasing xylose concentrations, as is seen in Figure 6. Of interest, when this color rate loss was plotted, it produced a reasonably linear function (Fig. 7), suggesting that absorbance loss could potentially be accounted for.

With a standard laboratory assay, the need for consistency between operators is often crucial. Inter- and intraoperator consistency appears to be a significant issue with the Douglas (1981) method. Across all 12 whole wheat meal samples, mean TAX varied from 3.60 to 4.66%, with error variance ranging up to 38% (100 – model $R^2$). Variance rankings, however, were quite consistent across operators. Among operators 1–3, operator means varied by no more than 0.06% TAX. WEAX measurements overall showed greater whole-model $R^2$ values and more similar results between operators. This result may indicate that the soluble fraction of AX was more consistently and accurately captured by the phloroglucinol method. In the analysis of the WEAX results, the four operators tended to have greater levels of mean separation among varieties, but no consistent operator-dependent trend was evident (TAX vs. WEAX). As noted previously, in the combined analysis there was no significant interaction between operator and sample. It should be noted that WEAX concentrations were on the order of ten times less than TAX concentrations. Given the ANOVA and statistical power to separate means, the results suggest that WEAX was inherently much more reliably measured.

One feature of TAX that may reduce its consistent measurement is the highly heterogeneous and particulate nature of ground
whole wheat meals, which must be sampled from aqueous suspension. The samples were vigorously vortexed, and an aliquot was withdrawn as quickly as possible, always at the same height within the tube. In addition, the pipette tip was cut off to effect a larger orifice to improve speed. The error surrounding the TAX measurement was nevertheless seen as a notable weakness of the assay. Although there were efforts made to attain a homogeneous mixture in the reaction vessel, the TAX solution was, in fact, still a slurry (i.e., suspended particles). This heterogeneous solution was hypothesized to be the source of some portion of the underestimation of TAX content. However, considering the variation of the WEAX data as well, sampling did not appear to be the entire issue. As mentioned previously, a pre-hydrolysis step with sulfuric acid (Bell 1985) was performed, but it resulted in an even greater underestimation of TAX levels.

The quantification of pentoses and AX can be performed in multiple ways. When quantified with GC-FID, absolute values were greater than those obtained through the spectrophotometric assay. This difference between methods suggested that cross-comparison between assays may be an important consideration for interpreting published values and for planning future research. Differences between the two methods may be owing, in part, to the chemical reactions that were taking place in the two assays. The sample preparation and manner in which the samples were analyzed were markedly different, which could contribute to differences in the quantification of AX. Whereas the phloroglucinol method depends upon a chain-like series of chemical reactions and indirect measurement, the GC-FID analysis is a more direct analysis of a product that more closely resembles the starting chemical structure. Other possible sources of underestimating AX by phloroglucinol may have included incomplete hydrolysis of the AX molecule into pentose sugar constituents to complete the reaction as well as the immediate degradation of the colored phloroglucinol product upon completion of hydrolysis. There were conceptually several different issues to consider: the hydrolysis of liberated arabinose and xylose and their reaction with phloroglucinol, the liberation of bound AX from seed tissue, and the particle size, tissue composition, and general accessibility of AX for reaction. Sufice to say that the procedure was highly empirical, but we have identified areas of problems and improvement in the basic Douglas method. However, the GC-FID analysis was more demanding in terms of time and instrument cost.

CONCLUSIONS

The commonly used AX colorimetric quantification method of Douglas (1981) was successfully modified to increase accuracy by determining a closer relationship between xylose concentration and absorbance and by becoming more aware of sample product decomposition over time (loss of color). The original method (Douglas 1981) was validated in the hydrolysis time, stressing the importance of the specific time outlined. The assay was simplified by eliminating glucose and by decreasing the phloroglucinol concentration, thus allowing the solution to solubilize with greater ease. Instability of the colored phloroglucide product was confirmed as a significant source of variation. Operator-to-operator variation was observed to be a significant issue that must be considered at the outset before performing the assay. Apparently, there are as-yet-unidentified sources of technical variability that limit the absolute value and repeatability of AX measurement. Multiple operators did tend to rank wheat samples similarly, however. Therefore, within a study or lab, rankings and differences among samples are likely to be reliable. Increasing replication should provide greater statistical robustness. The phloroglucinol method apparently underestimated total and WEAX contents compared with the GC-FID method.

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