Research Article

Cell wall chemical characteristics of whole-crop cereal silages harvested at three maturity stages

Johanna Wallsten and Ronald Hatfield

Abstract

BACKGROUND: In cooler climates such as found in Scandinavian countries cereals are important feedstuffs for ruminants often ensiled as whole-crop cereal silages (WCCS) to preserve nutrients. Animal performance varies with the type of cereal forage and stage of cereal development being ensiled. Cell wall isolation and analysis was undertaken to determine differences among cereal silages harvested at different stages of maturity.

RESULTS: A set of 27 WCCS samples of barley, wheat and oats harvested at heading, early milk, and dough stages of maturity were analyzed for cell wall (CW) composition and compared to previous NDF analyses. Total CW concentrations of the WCCS were higher than the NDF concentration. The lignin concentration was higher ($P < 0.001$) in oats (111 g kg$^{-1}$ DM) than in barley (88 g kg$^{-1}$ DM) and wheat (91 g kg$^{-1}$ DM). Ferulates (ester and ether linked) ranged from 12.2 to 14.9 g kg$^{-1}$ across forage types and maturity stages. The correlation between total cell wall xylose and HC concentrations (NDF-ADF) was lower than expected in all forages ($R = 0.63$).

CONCLUSION: The more comprehensive analyses of cell walls provide detailed composition of the different WCCS that vary due to the maturity and type of cereal.

INTRODUCTION

High-producing dairy cows require diets that contain sufficient nutrients to support the desired levels of milk production and body maintenance. Producing forage crops that meet nutrient requirements is a challenge especially in regions of northern latitudes with restricted growing season limiting the range of cropping alternatives. Cereals such as wheat, barley, and oats are well suited for production in cooler shorter growing seasons found in Sweden.\(^1,2\) Production of cereals in cooler environments can be challenging since the goal is to maximize total nutritive value.\(^3\) Cell wall fractions (fiber) increase and generally have decreasing nutritive value due to decreased digestibility while continued development results in starch production that generally increases nutritive value.

With increasing maturity total biomass increases but digestibility decreases and is most often associated with increased levels of lignin.\(^4\) This relationship holds within a given species, but may not work so well across species even when comparing similar stages of development.\(^5\) In the Poaceae family (grass family) the hydroxycinnamates, $p$-coumaric acid ($p$CA) and ferulic acid (FA) influence digestibility. For $p$CA they are esterified primarily to sinapyl alcohol but also to coniferyl alcohol and become part of the lignin structure as the monolignols undergo radical mediated polymerization.\(^6\) The $p$CA does not become incorporated but remains simply esterified to the growing lignin polymer.\(^7\) To a lesser extent $p$CA can also be incorporated ester linked to arabinosyl (Ara) units of arabinoxylans.\(^8\) Ferulates are incorporated into the cell wall esterified to arabinosyl residues of arabinoxylans\(^9\) and form cross-links with other ferulates\(^10\) as well as incorporation into growing lignin polymers.\(^11\) In grasses the digestibility of cell walls is dependent not only on the amount of lignin but also the degree of cross-linking. Cross-linking can be between arabinoxylans as well as arabinoxylans and lignin polymers both having a negative impact upon digestibility.\(^12,13\)

The detergent system was developed as a rapid method of estimating nutritive value of forages and other feedstuffs and works well for this purpose.\(^14,15\) However, measuring lignin with the detergent system can be a challenge. It has been clearly demonstrated that the typical detergent method, acid detergent lignin

© 2016 The Authors. Journal of the Science of Food and Agriculture published by John Wiley & Sons Ltd on behalf of Society of Chemical Industry.

Keywords: cereal crops; silages; oat; barley; wheat; cell walls; digestibility; lignin; structural carbohydrates

* Correspondence to: R. Hatfield, USDA – Agricultural Research Service, U.S. Dairy Forage Research Center, 1925 Linden Drive, Madison, WI 53706, USA. E-mail: Ronald.Hatfield@ars.usda.gov

\(^a\) Department of Forest Biomaterials and Technology, Swedish University of Agricultural Sciences, SE-901 83 Umeå, Sweden

\(^b\) USDA-Agricultural Research Service, U.S. Dairy Forage Research Center, 1925 Linden Drive, Madison, WI 53706, USA

© 2016 The Authors. Journal of the Science of Food and Agriculture published by John Wiley & Sons Ltd on behalf of Society of Chemical Industry.

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.
(ADL), can lead to under-estimation of lignin in grass cell walls. Hot detergent solutions, especially acid detergent, solubilizes lignin from the cell wall matrix of grasses.16 17 The detergent system may not reveal sufficient detail about the chemical make up of certain types of forages to provide a clear picture of how chemical composition is related to animal performance. This study was undertaken to determine the chemical composition obtained from a complete cell wall analysis of three different whole cereal crop silages (WCCS).

**MATERIAL AND METHODS**

Silages

A total of 27 samples of nine different WCCS from two feeding experiments with dairy heifers were analyzed.12 18 The WCCS used were oats (Avena sativa L.), six-rowed barley (Hordeum vulgare L) and wheat (Triticum aestivum L.) (all Swedish varieties) harvested at the heading, early milk and early dough stages of maturity. The WCCSSs were ensiled in plastic wrapped big bales. Three bales were selected from each stage of development for each cereal. Multiple cored subsamples were taken from each bale and combined to create the replicate sample for analyses.1 18

Chemical analyses

**Cell wall extraction**

Approximately 1.5–1.7 g of sample was accurately weighed into 40-mL Oakridge tubes on a dry matter (DM) basis (55 °C). All samples were extracted as outlined in the cell wall extraction flow chart (Scheme 1). Final cell wall concentration was determined after drying at 55 °C for at least 24 h and was expressed on an ash-free basis (CWom). Determinations of cell wall components were based upon the isolated cell wall residue. Cell wall residues were oven-dried at 55 °C for 24 h before weighing subsamples for the different cell wall analytical procedures.

**Carbohydrate analysis**

Neutral sugar components were determined as alditol acetates of the released sugars following acid hydrolysis. Cell wall residues were hydrolyzed using the Saeman method20 as modified by Hatfield et al.17 Accurately weighed (~100mg) samples were subjected to a two-stage hydrolysis: stage 1, 12 mol L⁻¹ H₂SO₄, (2h, 22–24 °C); stage 2, acid was diluted to 1.5 mol L⁻¹ with dH₂O, capped tightly and placed in a 100 °C forced air oven for 3 h. After hydrolysis, samples were cooled in an ice waterbath, centrifuged (900 × g) for 10 min and 200 μL removed from each for total uronosyls determination and inositol was added as internal standard. Sub-samples were neutralized with barium carbonate, clarified by centrifuging (3200 × g, 15 min) and filtered through a glass fiber filter (0.2 μm, Acrodisc). Sub-samples were dried and sugars converted to alditol acetate derivatives using the procedure of Blakeney et al.21 and analyzed by FID-GLC (Supelco, Bellefonte, PA USA; SPB-225 column 30 m × 0.25 mm with 0.25 μm film thickness) using a temperature program of 215 °C initial for 2 min, 4 °C min⁻¹ to 230 °C and hold for 11.25 min.

Total uronosyls in the cell wall hydrolyzates were determined by colorimetric assay following the method of Blumenkrantz and Asboe-Hansen.22 The 200-μL aliquots removed from the cell wall hydrolyzate were individually diluted to 2 mL using dH₂O and this diluted sample was used in the assay.

**Lignin determination**

Acetyl bromide lignin (ABSL) was measured following the procedure of Morrison23 as modified by Hatfield et al.24 Dry cell wall samples of 20–25 mg were weighed into Pyrex tube (16 mm × 200 mm) fitted with a Teflon lined cap, suspended in 2.5 mL of a 25% acetic bromide in glacial acetic acid and heated for 2 h in a heating block at 50 °C. The samples were mixed every 20 min during heating. The absorbance maximum between 275 nm and 280 nm was determined by evaluating spectral scans (250–350 nm) for each sample.

**Cell wall phenolic determinations**

Ester and ether linked ferulic acid and p-coumaric acid, were analyzed using the sequential method.25 Phenolics were identified and quantified as trimethylsilane derivatives (40 μL TMS; Thermo Scientific, Rockford, IL, USA and 10 μL pyridine) using GLC-FID on a ZB-5 ms column (Zebron; 30 m × 0.25 mm, 0.25 μm film). The GLC conditions were injector 315 °C, detector 300 °C, and a temperature program of 150 °C for 5 min, 4 °C min⁻¹ to 200, 10 °C min⁻¹ to 240 °C, 30°C min⁻¹ to 300 °C and hold for 10 min.

**Nitrogen and ash**

All cell wall samples were analyzed for total N using a combustion assay (Leco FP-2000 Analyser; Leco Instruments, Inc., St. Joseph, MI, USA) and they were also analyzed for ash by combustion at 500 °C.

**Detergent analyses and permanganate lignin**

Detergent fiber fraction information (NDFom, ADF, ADL) was compiled from previous work on the same cereal silages.1 12 18

**Statistical analysis**

The statistical analysis was done with the mixed procedure in SAS version 9.2 (SAS Institute, Cary, NC, USA). The initial model for all chemical components was:

\[ Y_{ijk} = C_i + M_j + C \times M_j + e_{ijk} \]

where \( Y_{ijk} \) is the general mean, \( C_i \) is the fixed factor of cereal species, \( M_j \) is the fixed factor of maturity stage at harvest, \( C \times M_j \) is the crop \((C) \times \) maturity \((M) \) fixed interaction factor of cereal species and maturity stage at harvest, and \( e_{ijk} \) is the random error. If the \( C \times M \) interaction factor was significant \((P < 0.05)\), LS means for \( C \times M \) was tested with the PDDIFF statement in SAS, and the difference between the LS means was adjusted with Tukey's test. If the \( C \times M \) interaction effect was near significance \((0.10 > P > 0.05)\) it was kept in the model, but not evaluated and if it was not significant \((NS, P > 0.10)\) it was removed from the model. The LS means of significant single factors C and M were evaluated with the PDDIFF statement in SAS and the differences were corrected with Tukey's adjustment. In all situations differences between LS means was considered significant at \( P < 0.05 \). Linear regressions and correlations between chemical fractions were analyzed with the regression procedure and the CORR option in SAS version 9.2 and correlations were considered significant at \( P < 0.05 \).
**RESULTS**

### Cereal species

Table 1 provides the fermentation characteristics of the individual silages chemically characterized in this study. All silage samples were dried at 60 °C and ground in a hammer mill with 1-mm sieve. More detailed information about the experiment with the oat and six-rowed barley can be found in Wallsten et al., and about the experiment with the wheat silages in Rustas et al. The average detergent composition and lignin concentration for nine silages can be found in Table 2.

The CWom concentrations were higher in oats than in barley and wheat at all maturity stages (Table 3 and Table 4). The ash concentration was highest in barley silage. Total protein retained in the cell wall fraction (CP g kg⁻¹) was highest in barley, intermediate in oats and lowest in wheat (Table 4) corresponding to wheat having the lowest CP. Total lignin as ABlignin concentration was higher in oats than in barley or wheat. Permanganate lignin (PMlignin) performed on ADF residues resulted in lower total lignin values compared to ABlignin (Table 2 and Table 3). As a consequence PMlignin represented a fraction of the total lignin as measured by ABlignin in all the silages with oats having the lowest fraction of lignin (Tables 3 and 4).

Concentrations of pCA were nearly double in oats compared to wheat or barley at all maturity stages (Tables 3 and 4). The difference in total ester-linked FA (monomer and dimer) concentration among cereals varied with maturity stage, with highest concentration in barley at heading. Concentrations of total FA were higher in oats than barley at dough stage (Table 4). Wheat had lower concentrations of ester-linked FA at most stages of development. Ether-linked FA varied from 5.8 to 8.3 g kg⁻¹ CWom (monomer + dimer), but there were no significant differences among cereal species at any maturity stage (Table 4).

Cereals and grasses in general have low amounts of pectins (<2%) with the bulk of cell wall carbohydrates distributed between hemicelluloses and cellulose. Total uronic acids composed of both galacturonosyls and glucuronosyls were higher in wheat than in oats and tended (P<0.071) to be higher in wheat than in barley (Table 3) though these did not account for a major portion of the cell wall carbohydrates. Hemicellulosic sugars (primarily arabinoxylans) concentrations were higher in barley than in oats and wheat. Glucose concentrations in wheat tended (P<0.066) to be higher than barley but not significantly different from oat silage cell walls (Table 3).
Effect of maturity stages

CWom concentrations were higher at the heading stage for all three-cereal species (Tables 3 and 4). Differences between milk and dough stage CWom were not large enough to be significant. The higher cell wall residual crude protein (rCP) concentration at dough stage was only evident for barley and oats (Tables 3 and 4). For wheat there was a marginal decrease in rCP concentration at dough stage was only evident for barley and oats (Tables 3 and 4). The CP at dough stage was more difficult to remove during the washes and the percentage of rCP retained was higher at dough stage for all cereal species (Tables 3 and 4).

ABlignin concentration was lower at heading compared to at milk and dough stages. Moreover, the PMlignin at milk stage represented, and at dough stage tended to represent \( P_{\text{head}} = 0.069 \), a higher portion of the corresponding ABlignin, than PMlignin at heading. The pCA concentration decreased with maturity for barley and oats, but for wheat the concentrations were similar and the highest value was at milk stage (Tables 3 and 4). The concentration of ester-linked FA in barley decreased with maturity stage and a similar trend might be suggested for oats, though the numerical difference was small (7.2 to 6.7 g kg\(^{-1}\)). The HC sugars decreased in positive correlation for oats, but negative correlation of similar magnitude for barley. For barley the ABlignin was the same irrespective of PMlignin values (Fig. 1D). The PMlignin was not significantly correlated to the ABlignin, when looking at the whole dataset. Splitting it on cereal species, whereas splitting it on maturity stages increased the correlation only for the samples harvested at milk stage (Table 5). Generally, as CW increased, NDF increased across all types of silages (Fig. 1A). The correlation between HC NS sugars and HCNDF was significant, but the variation in HC NS values among the replicates was large for most of the silages (Fig. 1B; Table 5). The correlation improved only for wheat when splitting the dataset on cereal species. Glucose and cellulose was not significantly correlated for the whole dataset or for the cereal species (Table 5). However, when splitting the data on maturity stages the correlation was significant for heading and milk stage. There was a large variation in glucose concentration among replicates that was not evident for corresponding cellulose concentrations when considering the mean of replicates (Fig. 1C). The PMlignin was not significantly correlated to the ABlignin, when looking at the whole dataset. Splitting it on cereal species resulted in positive correlation for oats, but negative correlation of similar magnitude for barley. For barley the ABlignin was the same irrespective of PMlignin values (Fig. 1D).

Correlation of CWom to the detergent system and permanganate lignin

CWom values were generally higher than the corresponding NDFom values (Fig. 1A). Wheat at milk stage was the only silage where NDFom and CWom values were similar, and excluding it increased the positive correlation between the fiber fractions from 0.63 to 0.76. The correlation between NDFom and CWom increased when splitting the dataset on cereal species, whereas splitting it on maturity stages increased the correlation only for the samples harvested at milk stage (Table 5). Generally, as CW increased, NDF increased across all types of silages (Fig. 1A). The correlation between HC NS sugars and HCNDF was significant, but the variation in HC NS values among the replicates was large for most of the silages (Fig. 1B; Table 5). The correlation improved only for wheat when splitting the dataset on cereal species. Glucose and cellulose was not significantly correlated for the whole dataset or for the cereal species (Table 5). However, when splitting the data on maturity stages the correlation was significant for heading and milk stage. There was a large variation in glucose concentration among replicates that was not evident for corresponding cellulose concentrations when considering the mean of replicates (Fig. 1C). The PMlignin was not significantly correlated to the ABlignin, when looking at the whole dataset. Splitting it on cereal species resulted in positive correlation for oats, but negative correlation of similar magnitude for barley. For barley the ABlignin was the same irrespective of PMlignin values (Fig. 1D).

DISCUSSION

Based on the information compiled in Table 1 all the silages appeared to ferment reasonably well providing a good source of animal feed. It is also clear that fermentation at the later maturities

### Table 1. Fermentation characteristics of silages evaluated in this study

| Variable     | Barley           | Oats             | Wheat            |
|--------------|------------------|------------------|------------------|
|              | Heading  | Milk    | Early dough  | Heading  | Milk    | Early dough  | Heading  | Milk    | Early dough  |
| pH           | 4.6      | 4.4     | 4.5         | 4.5      | 4.1     | 4.6         | 4.1      | 4.6     | 5.5         |
| NH\(_3\)-N (g kg\(^{-1}\) N) | 71       | 70      | 67          | 131      | 72      | 58          | 72       | 72      | 54          |
| Lactic acid  | 43       | 31      | 29          | 61       | 73      | 38          | 63       | 22      | 12          |
| Acetic acid  | 8        | 11      | 8           | 20       | 14      | 8           | 14       | 13      | 5           |
| Propionic acid | 1.2  | 1.5     | 1.1         | 1.9      | 1.2     | 1.2         | <1       | <1      | <1          |
| Ethanol      | 5.3      | 2.9     | 2.6         | 4.5      | 2.8     | 3.4         | 5.5      | 3.8     | 6.9         |

Data compiled from previous work, with permission (Rustas et al.\(^{18}\) and Wallsten et al.\(^{1}\)).

### Table 2. Detergent composition and lignin content measured in nine different whole-crop cereal silages

| Component         | Barley       | Oats         | Wheat        |
|-------------------|--------------|--------------|--------------|
|                   | Head. | Milk | Early dough | Head. | Milk | Early dough | Head. | Milk | Early dough |
| NDFom (g kg\(^{-1}\) DM) | 500 | 433 | 411 | 527 | 530 | 442 | 539 | 487 | 466 | 448 | 500 | 497 | 522 | 483 | 440 |
| ADF (g kg\(^{-1}\) DM) | 307 | 277 | 263 | 346 | 346 | 289 | 347 | 325 | 311 | 282 | 327 | 328 | 333 | 316 | 288 |
| PMlignin (g kg\(^{-1}\) DM) | 44 | 53 | 51 | 56 | 60 | 52 | 51 | 57 | 61 | 49 | 56 | 56 | 50 | 57 | 55 |
| Hemicellulose (g kg\(^{-1}\) DM) | 193 | 156 | 148 | 181 | 184 | 153 | 191 | 162 | 156 | 166 | 173 | 170 | 188 | 167 | 152 |
| Cellulose (g kg\(^{-1}\) DM) | 263 | 224 | 212 | 290 | 286 | 237 | 296 | 269 | 249 | 233 | 271 | 272 | 283 | 260 | 233 |
| PMlignin (g kg\(^{-1}\) NDFom) | 88 | 122 | 124 | 106 | 113 | 118 | 95 | 116 | 132 | 110 | 112 | 113 | 96 | 117 | 125 |
| Hemicellulose (g kg\(^{-1}\) NDFom) | 386 | 360 | 360 | 343 | 347 | 346 | 355 | 332 | 334 | 370 | 346 | 341 | 361 | 346 | 346 |
| Cellulose (g kg\(^{-1}\) NDFom) | 526 | 517 | 516 | 550 | 540 | 536 | 550 | 552 | 535 | 520 | 542 | 546 | 542 | 537 | 529 |

Data are summarized from previously published work, with permission (Rustas et al.\(^{18}\) and Wallsten et al.\(^{1}\)).

\( \text{DM} = \text{dry matter}, \text{NDFom} = \text{ash-free neutral detergent fiber}, \text{ADF} = \text{ash-free acid detergent fiber}, \text{PMlignin} = \text{permanganate lignin}, \text{hemicellulose} = \text{NDFom-ADF}, \text{cellulose} = \text{ADF-PMlignin}. \)
Table 3. Ash-free cell wall (CWom) concentration (g kg\(^{-1}\) dry matter) and composition (g kg\(^{-1}\) CWom) of three cereals harvested and stored as whole-crop silage at three maturity stages (n = 9)

| Sample | Cereal (C) | Maturity (MS) | Significance |
|--------|------------|---------------|--------------|
|        | Barley     | Oat           | Wheat        | Heading | Milk | Dough | SEM | C | MS |
| CWom   |            |               |              |         |      |       |     |    |    |
|        | 534\(^a\)  | 588\(^b\)     | 534\(^a\)    | 581\(^b\) | 531\(^a\) | 543\(^a\) | 4 | *** | *** |
| Ash    | 104\(^b\)  | 80\(^ab\)     | 68\(^a\)     | 94      | 86   | 72    | 8.5 | *  | NS  |
| Ash (g kg\(^{-1}\) of total ash) | 440 | 400 | 420 | 430 | 420 | 420 | 26 | NS | NS |
| CP in CW | 73\(^c\)  | 62\(^b\)      | 43\(^a\)     | 56\(^a\) | 56\(^a\) | 66\(^b\) | 1.2 | *** | *** |
| CP (g kg\(^{-1}\) of total CP) | 340\(^b\) | 340\(^b\) | 210\(^a\) | 260\(^a\) | 260\(^a\) | 380\(^b\) | 7.0 | *** | *** |
| Abblignin | 166\(^a\) | 189\(^b\)     | 171\(^a\)    | 168\(^b\) | 183\(^b\) | 175\(^b\) | 3.0 | *  | *   |
| PMLignin (g kg\(^{-1}\) Abblignin) | 560\(^ab\) | 510\(^a\)     | 620\(^b\)    | 520\(^a\) | 590\(^b\) | 580\(^ab\) | 2.0 | **  | *   |
| pCA    | 4.8\(^a\)  | 10.1\(^b\)    | 5.1\(^b\)    | 7.6\(^a\) | 6.8\(^b\) | 5.6\(^a\) | 0.1 | *** | *** |
| FA ether | 6.9\(^b\) | 7.0\(^a\)     | 6.5\(^a\)    | 6.6\(^a\) | 6.6\(^a\) | 6.3\(^a\) | 0.1 | **  | *** |
| FA ether | 7.3 | 6.7 | 6.4 | 6.6 | 7.2 | 6.6 | 0.3 | NS | NS |
| UA     | 24.4\(^ab\) | 23.0\(^a\) | 28.5\(^b\) | 25.6 | 25 | 25.3 | 1.5 | NS | NS |
| HC sugars | 389\(^b\) | 368\(^a\)    | 358\(^a\)    | 393\(^c\) | 374\(^b\) | 349\(^a\) | 4.6 | *** | *** |
| Glucose | 453 | 460 | 473 | 455\(^a\) | 460\(^a\) | 473\(^b\) | 7.4 | NS | ** |

CP, crude protein; Abblignin, acetyl bromide lignin; pCA, p-coumeric acid; FA, ferulic acid; UA, uronic acid; HC sugars is the sum of xylose, arabinose, fucose, galactose, mannose and rhamnose.

Ash and CP are the amounts found in the CW isolates and the ash (g kg\(^{-1}\) of total ash) and CP (g kg\(^{-1}\) of the total CP) are the totals found in the DM that was retained in the CW isolation.

\(^{a-c}\)Values on the same row within cereal or maturity stage with different superscripts are significantly different (P\(_{\text{Tukey}}\) < 0.05).

NS, not significant; \(^*\)P < 0.05, \(^{**}\)P < 0.01, \(^{***}\)P < 0.001.

was not as robust as earlier stages most likely due to a decrease in readily available soluble sugars.\(^1\)\(^,\)\(^2\) The CW analysis system provides more detailed chemical information about the fiber fraction of plants. Neutral detergent fiber (NDF) method was developed to rapidly estimate the nutrient value of forages and other feedstuff. It is intended to represent the more slowly digested CW material in a forage sample. Typically for grasses there is a good correlation between NDF (Table 2) and the CWom (Tables 3 and 4). Grasses tend to produce NDF values aligned with CWom compared to legumes primarily due to the low levels of pectins in grasses.\(^27\) As forages mature there is an increase in the CWom as a proportion of the total DM. However, in the case of cereals advanced maturity results in the formation a grain head that contributes to a significant increase in overall DM content. For this study WCCS CWom and consequentially NDF decreases as a portion of the DM with increased maturity due to rapid accumulation of starch during grain head development boosting the overall DM content. Differences among the types of WCCS for CWom are due to compositional and structural changes during maturation.\(^28\) For this work the non-cellulosic sugars were combined into one group referred to as the hemicellulosic sugars. Although the detergent system creates an NDF fraction that does a reasonable job of representing the CW fractions there are significant differences between the two methods.

The CW isolation method (Scheme 1A) is designed to preserve all the CW components in the final insoluble residue while removing as much of the non-CW components as possible. With no detergent being used in the extraction protein removal is less efficient than with the NDF method (Scheme 1B). The CP recovery in the CWom in the present study ranged from 190 to 420 g kg\(^{-1}\) (Table 4) of the original CP in the DM. Acosta \(^{29}\) reported a 113 – 157 g kg\(^{-1}\) recovery in barley silage harvested at the boot and the dough stages. Cobelntz \(^{30}\) reported levels of 250 – 320 g kg\(^{-1}\) (in NDF) for wheat and oat whole crops harvested between the heading and the dough stages but not ensiled. Higher CP was recovered in the NDF fraction at later maturity stages as was seen for CP in CWom in this study (Table 4).\(^{29}\) Increases in cell wall associated protein might be related to a reduction in apparent CP digestibility at later maturity stages probably due to the slower degradation of mature CW.\(^1\)\(^,\)\(^8\)\(^,\)\(^30\)

Differences between NDF (Table 2) and total CWom (Table 4) may also reflect losses of cell wall material during the NDF procedure, due to solubility in the hot detergent\(^{16}\) and to particle losses during filtering.\(^{31}\) At early stages of development grass arabinoxylans may be highly branched\(^2\) and therefore more susceptible to solubilization in hot detergent solution. Arabinose side chains, which are typically in the furanose form, are susceptible to weak acid hydrolysis such as the low pH conditions produced during ensiling and could be sufficient to cleave some of these residues.\(^32\) Larger differences were seen in measuring the lignin fraction. Abblignin used in the CWom method accounts for all the lignin whereas the PMLignin method is measuring only a fraction of the total. Differences in the chemical/physical make-up of the cereal lignins could alter their solubility in hot detergent. Lignin, especially in grasses, can be soluble in both neutral\(^6\) and acid detergent\(^6\)\(^,\)\(^34\) resulting in much lower lignin values.\(^17\) Acetyl bromide lignin method was originally proposed as a rapid method for woody samples and adapted by Morrison for forages.\(^35\) The method effectively solubilizes the lignin from the cell wall matrix in an acidic medium leaving protein and complex carbohydrates insoluble. These insoluble materials are removed with centrifugation to prevent light scattering that would alter the true lignin value. Isolated and purified lignins can be used as standards to allow quantification of lignin in unknown samples.\(^36\) In this study all the WCCS had higher lignin values compared to the detergent PMLignin in the earlier studies (Table 2). Oat silages tended to have the highest Abblignin whereas wheat tends to be higher for PMLignin. The observation that cereals with the highest Abblignin did not also give the highest PMLignin values suggests there are structural and possibly compositional differences among the different lignins. These differences are due...
Table 4. Evaluation of the significant cereal *maturity stage interaction for nine different whole crop cereal silages (n = 3)

| Sample | Heading stage | Milk stage | Dough stage | SEM | C*M |  |
|--------|---------------|------------|-------------|-----|-----|---|
|        | Barley | Oat | Wheat | Barley | Oat | Wheat | Barley | Oat | Wheat |  |
| CWom   | 563<sup>a</sup> | 607<sup>b</sup> | 575<sup>ab</sup> | 507<sup>a</sup> | 587<sup>b</sup> | 497<sup>a</sup> | 530<sup>a</sup> | 569<sup>b</sup> | 530<sup>a</sup> | 6.9 | ** |
| Ash    | 111 | 107 | 63 | 113 | 75 | 70 | 86 | 59 | 71 | 15.5 | NS |
| Ash (g kg<sup>-1</sup> of DM ash) | 430 | 460 | 390 | 460 | 380 | 410 | 430 | 360 | 470 |  |
| CP     | 67<sup>b</sup> | 55<sup>a</sup> | 46<sup>a</sup> | 74<sup>b</sup> | 53<sup>b</sup> | 41<sup>a</sup> | 79<sup>b</sup> | 78<sup>b</sup> | 42<sup>b</sup> | 2.0 | *** |
| CP (g kg<sup>-1</sup> of DM CP) | 290<sup>b</sup> | 300<sup>b</sup> | 190<sup>a</sup> | 300<sup>b</sup> | 270<sup>b</sup> | 190<sup>a</sup> | 420<sup>b</sup> | 460<sup>b</sup> | 260<sup>a</sup> | 12.0 | *** |
| Ab lignin | 164 | 183 | 157 | 167 | 199 | 181 | 165 | 184 | 175 |  |
| PM lignin (g kg<sup>-1</sup> AB lignin) | 480 | 500 | 570 | 460 | 380 | 410 | 430 | 360 | 470 |  |
| pCA    | 6.1<sup>a</sup> | 11.6<sup>b</sup> | 5.0<sup>a</sup> | 4.6<sup>a</sup> | 10.7<sup>b</sup> | 5.2<sup>a</sup> | 3.8<sup>a</sup> | 8.0<sup>b</sup> | 5.0<sup>a</sup> | 0.2 | *** |
| FA ester | 8.6<sup>b</sup> | 7.2<sup>a</sup> | 6.9<sup>a</sup> | 6.5<sup>ab</sup> | 7.1<sup>b</sup> | 6.2<sup>a</sup> | 5.7<sup>a</sup> | 6.7<sup>b</sup> | 6.4<sup>ab</sup> | 0.2 | *** |
| FA ether | 6.3 | 6.5 | 6.9 | 8.3 | 7.0 | 6.4 | 7.3 | 6.6 | 5.8 | 0.5 | NS |
| UA     | 26.2 | 22.1 | 28.5 | 23.2 | 23.5 | 28.2 | 23.7 | 23.3 | 28.9 | 2.9 | NS |
| HC     | 420 | 383 | 374 | 384 | 378 | 360 | 364 | 344 | 338 | 7.9 | NS |
| Glucose | 450 | 440 | 475 | 431 | 463 | 458 | 476 | 487 | 486 | 13.3 | NS |

All values are in g kg<sup>-1</sup> DM. CP, crude protein; AB lignin, acetyl bromide lignin; pCA, p-coumeric acid; FA, ferulic acid; UA, uronic acid; HC sugars is the sum of xylose, arabinose, fucose, galactose, mannose and rhamnose. Ash and CP are the amounts found in the CW isolates and the ash (g kg<sup>-1</sup> of total ash) and CP (g kg<sup>-1</sup> of the total CP) are the % of the totals found in the DM that was retained in the CW isolation.

*–c Values on the same row within heading/milk/dough/barley/oats or wheat with different superscripts are significantly different (P<sub>Tukey</sub> < 0.05).

C*M type of cereal silage (C) by maturity (M).

Table 5. Correlation and linear regression statistics for different chemical fractions in nine different whole crop cereal silages

| Sample | CWom = NDFom | AB lignin = PM lignin | HC sugars = HC | Cellulose = glucose |
|--------|--------------|-----------------------|----------------|-------------------|
|        | Corr | RMSE | P value | Corr | RMSE | P value | Corr | RMSE | P value | Corr | RMSE | P value |
| All samples | 0.63 | 29.5 | <0.001 | 0.27 | 11.7 | 0.167 | 0.65 | 16.8 | <0.001 | 0.35 | 28.4 | 0.077 |
| Barley | 0.69 | 20.5 | 0.040 | 0.76 | 4.3 | 0.164 | 0.68 | 17.9 | 0.44 | 0.2 | 24.8 | 0.61 |
| Oats | 0.81 | 13.5 | 0.008 | 0.75 | 6.1 | 0.02 | 0.65 | 14.6 | 0.56 | 0.057 | 24.7 | 0.112 |
| Wheat | 0.73 | 24.7 | 0.26 | 0.55 | 1.8 | 0.125 | 0.9 | 8.6 | 0.001 | 0.41 | 20.2 | 0.277 |
| Heading | 0.43 | 21.7 | 0.253 | 0.50 | 10.2 | 0.171 | 0.053 | 14.1 | 0.893 | 0.67 | 14.4 | 0.049 |
| Milk | 0.74 | 30.8 | 0.022 | 0.49 | 14.6 | 0.18 | 0.505 | 17.7 | 0.166 | 0.74 | 20.0 | 0.023 |
| Dough | 0.26 | 24.1 | 0.51 | 0.09 | 10.4 | 0.823 | -0.455 | 8 | 0.219 | 0.13 | 18.9 | 0.735 |

Total observations N = 27, total silages three species x three maturities, n = 9.

NDFom, ash-free neutral detergent fiber; ADF, ash-free acid detergent fiber; AB lignin, acetyl bromide lignin; PM lignin, permanganate lignin; HC (hemicellulose) = NDFom-ADF; HC sugars = sum of xylose, arabinose, fucose, galactose, mannose and rhamnose; Cellulose = ADF-PM lignin.

to variable solubility in hot detergent solutions especially the AD treatment and could have implications on how cell wall materials can be degraded by microbes.

Lignin content does not appear to change significantly (Table 3) within individual silages during development when expressed on a CW basis. However, lignin on a total DM basis appears to decrease (Fig. 1B) due to the accumulation of starch as a significant portion of the DM. The most significant changes occur in the forage stems as they transition from vegetative to reproductive stages. Continued development of the grain head in cereals could account for increased accumulation of cell walls associated with the grain but contain low levels of lignin. This may be particularly true for oats and barley that accumulate large amounts of mixed linked β-glucans<sup>37</sup> that would remain as cell wall components during the CW extraction process. To clarify this relationship it would be necessary to evaluate the individual cereals as they stand in the field and separated into major tissue types (e.g. leaves, stems, sheaths, and reproductive parts) compared to what is available...
Correlations between chemical fractions in barley, oats, and wheat harvested at heading, milk stage, and dough stage of maturity. All comparisons are between results of the detergent analysis system and the cell wall isolation system on an ash free basis. (A) Total NDF versus total cell wall organic mater (CW); (B) detergent permanganate lignin (PMLignin) versus acetyl bromide lignin (ABLignin) determination in cell wall isolates; (C) NDF-ADF hemicellulose versus cell wall hemicellulose based on neutral sugar analysis (HC NS); (D) ADF-lignin cellulose versus total glucose from neutral sugar analysis of cell walls. All points are the mean of three separate samples. Error bars indicate the SD of the mean and all values are on a ash free dry matter basis. OH, oats at heading; OM, oats at milk stage; OD, oats at dough stage; BH, barley at heading; BM, barley at milk stage; BD, barley at dough stage; WH, wheat at heading; WM, wheat at milk stage; WD, wheat at dough stage.

After ensiling. However, this was beyond the scope of this work and is a subject for future research.

Grass pCA is primarily incorporated into CW as an ester linked conjugate with monolignols. In this study oats had the highest pCA and ABLignin, while wheat and barley lignin concentrations were less (90 and 120 g kg$^{-1}$, respectively). The pCA concentrations however, were 500 and 520 g kg$^{-1}$ lower. Previous studies indicated that across species there is not a good correlation between total lignin and pCA. It remains unclear as to the role of pCA in CW development. It has been suggested that the formation of p-coumaryl-sinapyl alcohol conjugates help in the formation of syringyl type lignins. Recently, the gene for the pCA-transferase was down regulated in two different plant systems, Brachypodium and corn. In both cases the proportion of syringyl units incorporated into lignin decreased, but the lignin content did not decrease. It may be the formation of pCA-sinapyl alcohol conjugates aids in the incorporation of syringyl units in lignin, but it does not control overall lignin formation.

Among the hydroxycinnamates, FA is most likely to influence CW digestibility, by cross-linking arabinoxylans forming FA-dimers. FA and CA-dimers can also be coupled with lignin forming cross-linked networks of arabinoxylans and lignin. For barley and oats ether linked FA increased or remained constant while wheat appeared to decline during maturation (Table 4). Linkage of ferulates, both monomers and dimers, to lignin is not always through an ether type linkage but only the ether linkages can be hydrolyzed and accounted for in this analysis. Therefore not all cross-links between lignin and xylans can be accounted for in the typical analysis procedure. As already discussed barley and oat cereals incorporate relatively high levels of mixed linked $\beta$-glucans during grain development. The $\beta$-glucans are a part of the grain CW but would not be retained during hot detergent extraction. Their rapid accumulation during grain development would contribute to an overall increase in the CW fraction without increasing FA content making it seem like it is decreasing during this stage of cereal development.

The hemicellulosic (HC) fraction is typically composed of all the non-glucose sugars, mainly arabinose and xylose with contributions from galactose, mannose, rhamnose and fucose. Glucose is primarily associated with cellulose. Exceptions are oat and barley at the grain filling stage where part of the glucose is from mixed linked $\beta$-glucans. The correlation between CWom HC and the NDF-ADF HC (Table 5) decreased due to a large variation between replicates. Replicates were from different bales and may be expected to have some difference in amounts. However, since the HC sugars did not differ in the same way it may instead have been a result of determining the NDF and the ADF on different sub-samples instead of doing a sequential NDF-ADF analysis. It is also possible for the hot detergent reagent for NDF to solubilize parts of the hemicellulosic fraction in these cereals resulting in variable recoveries. Although there is variability in the individual samples...
a plot of the means for WCCS samples by species and maturity stage shows a good relationship between CW HC-NS (based on a sum of the non-glucose sugars) and HC based NDF-ADF (Fig. 1C). When calculated on a DM basis there is a consistent decrease in hemicellulosic sugars as the cereals mature.

The cellulose concentration of replicates based on the detergent system was more tightly clustered. The use of permanganate to degrade the lignin (PMLignin) leaving a cellulose residue may actually help remove some of the variability as a result of ND analysis (Fig. 1D). Permanganate treatment can degrade some of the non-cellulosic carbohydrates in the CW that may remain in the ADF residue. The larger variation in the glucose content (based on CW isolation) is most likely due to the formation of mixed linked β-glucans in the developing grain in oat and barley. Depending upon the stage of grain fill the quantity of β-glucans could be variable as well as the solubility during the CW isolation procedure. 45 Wheat has low levels of β-glucans in the grain, but has arabinoxylans containing some ferulates. 46 Loss of lignin during the NDF and ADF analyses 16,47 could result in cellulose concentration being overestimated in these samples. The improved correlations (Table 5) when the dataset was split on maturity stages could be explained by the presence of β-glucans at dough stage, which is present in high amounts in the grains of barley and oats, but in lower amounts in wheat. 48 The presence of β-glucans might explain why the variation in glucose concentration among replicates was so much larger compared to both cellulose concentration and HC sugars.

CONCLUSIONS

• Differences between the CW and ND-AD method were due to solubilized CW fractions in hot detergents.
• Differences were observed in CW composition between cereals and among maturity stages that could explain differences in animal performance.
• More research is warranted in order to further elucidate details of CW composition related to maturity cereal type.

ACKNOWLEDGEMENTS

The authors wish to thank Jane Marita for assistance in overseeing the GC analysis of cell wall carbohydrate fractions from the silages. Thanks to the Swedish Research Council, FORMAS for providing financial support for JW. USDA is an equal opportunity provider and employer. Mention of a proprietary product does not constitute a recommendation or warranty of the product by USDA or Forest Biomaterials and Technology, Swedish University of Agricultural Sciences, Sweden, and does not imply approval to the exclusion of other suitable products.

REFERENCES

1 Wallsten J, Bertilsson J, Nadeau E and Martinsson K, Digestibility of whole-crop barley and oat silages in dairy heifers. Animal 4:432–438 (2010).
2 Wallsten J and Martinsson K, Effects of maturity stage and feeding strategy of whole crop barley silage on intake, digestibility and milk production in dairy cows. Livest Sci 121:155–161 (2009).
3 Nadeau E, Effects of plant species, stage of maturity and additive on the feeding value of whole-crop cereal silage. J Sci Food Agric 87:789–801 (2007).
4 Jung HG, Mertens DR and Payne AJ, Correlation of acid detergent lignin and Klosson lignin with digestibility of forage dry matter and neutral detergent fiber. J Dairy Sci 80:1622–1628 (1997).
5 Jung HG and Deetz DA, Cell wall lignification and degradability, in Forage Cell Wall Structure and Digestibility, ed. by Jung HG, Buxton DR, Hatfield RD and Ralph J. ASA-CSSA-SSSA, Madison WI, pp. 315–346 (1993).
6 Grabber JH, Quideau S and Ralph J, p-Coumaroylated syringyl units in maize lignin; implications for b-ether cleavage by thioacidolysis. Phytochemistry 43:119–1194 (1996).
7 Ralph J, Hatfield RD, Quideau S, Helm RF, Grabber JH and Jung H-JG, Pathway of p-coumaric acid incorporation into maize lignin as revealed by NMR. J Am Chem Soc 116:9498–9456 (1994).
8 Mueller-Harvey I, Hartley RD, Harris PJ and Curzon EH, Linkage of p-coumaryl and feruloyl groups to cell wall polysaccharides of barley straw. Carbohydr Res 148:71–85 (1986).
9 Kato Y and Nevis D, DJ, Isolation and identification of O-(S-O-feruloyl-a-L-arabinofuranosyl)–(1–3)-O-b-D-xlyopyranosyl–(1–4)-D-xylene as a component of Zea shoot cell-walls. Carbohydr Res 137:139–150 (1985).
10 Ralph J, Hatfield RD, Grabber JH, Jung HG, Quideau S and Helm RF, Cell wall cross-linking in grasses by ferulates and diferulates, in Lignin and Lignan Biosynthesis, ed. by Lewis NG and Sarkenah S. American Chemical Society, Washington, DC, pp. 209–236 (1998).
11 Grabber JH, Ralph J and Hatfield RD, Cross-linking of maize walls by ferulate dimerization and incorporation into lignin. J Agric Food Chem 48:6106–6110 (2000).
12 Grabber JH, Hatfield RD and Ralph J, Diferulate cross-links impede the enzymatic degradation of non lignified maize walls. J Sci Food Agric 77:193–200 (1998).
13 Grabber JH, Ralph J and Hatfield RD, Ferulate cross-links limit the enzymatic degradation of synthetically lignified primary walls of maize. J Agric Food Chem 46:2609–2614 (1998).
14 Van Soest PJ, A comprehensive system of feed analyses and its application to forages. J Anim Sci 26:119–128 (1967).
15 Goering HK and Van Soest PJ, Forage Fiber Analysis. (Approaches, Reagents, Procedures, and Some Applications) Agricultural Handbook No. 379, USDA-ARS, Madison, WI (1975).
16 Lowry JB, Conlan LL, Schlink AC and McSweeney CS, Acid detergent dispersible lignin in tropical grasses. J Sci Food Agric 65:41–49 (1994).
17 Hatfield RD, Jung HG, Ralph J, Buxton DR and Weimer PJ, A comparison of the insoluble residues produced by the klaslon lignin and acid detergent lignin procedures. J Sci Food Agric 65:51–58 (1994).
18 Rustas BO, Bertilsson J, Martinsson K, Elvestedt T and Nadeau E, Intake and digestion of whole-crop barley and wheat silages by dairy heifers. J Anim Sci 89:4134–4141 (2011).
19 Wallsten J, Nadeau E, Bertilsson J and Martinsson J, Voluntary intake and diet selection by dairy heifers fed ensiled whole-crop barley and oats harvested at different stages of maturity. Livest Sci 122:94–98 (2009).
20 Saeman JF, Moore WE and Millett MA, Sugar units present. Hydrolysis and quantitation of p-feruloyl chromotography, in Cellulose, ed. by Whitaker RL. Academic Press, New York, pp. 54–69 (1963).
21 Blakeney AB, Harris PJ, Henry RJ and Stone BA, A simple and rapid preparation of alditol acetates for monosaccharide analysis. Carbohydr Res 113:291–299 (1983).
22 Blumenkrantz N and Asboe-Hansen G, New method for quantitative determination of uronic acids. Anal Biochem 54:484–489 (1973).
23 Morrison IM, Improvements in the acetyl bromide technique to determine lignin and digestibility and its application to legumes. J Sci Food Agric 23:1463–1469 (1972).
24 Hatfield RD, Grabber JH, Ralph J and Brei K, Using the acetyl bromide assay to determine lignin concentrations in herbaceous plants: some cautionary notes. J Agric Food Chem 47:628–632 (1999).
25 Grabber JH, Hatfield RD, Ralph J, Zon J and Amrhein N, Ferulate cross-linking in cell walls isolated from maize cell suspensions. Phytochemistry 40:1077–1082 (1995).
26 Shea EM and Hatfield RD, Characterization of a pectic fraction from smooth bromegrass cell walls using an endopolygalacturonase. J Agric Food Chem 41:380–387 (1993).
27 Casler MD and Hatfield RD, Cell wall composition of smooth bromegrass plants selected for divergent fiber concentration. J Agric Food Chem 54:8206–8211 (2006).
28 Khorasani GR, Jedel PE, Helm JH and Kennelly JJ, Influence of stage of maturity on yield components and chemical composition of cereal grain silages. Can J Agric Sci 77:259–267 (1997).
29 Acosta YM, Stallings CC, Polan CE and Miller CN, Evaluation of barley silage harvested at boot and soft dough stages. J Dairy Sci 74:167–176 (1991).
30 Coblenz WK, Coffey KP, Turner JE, Scarbrough DA, Weyers JS, Harrison KF, et al., Ruminal nitrogen disappearance from sod-seeded cereal grain forages in Northern Arkansas. *Anim Feed Sci Technol* **89**:17 – 32 (2001).

31 Uden P, Recovery of insoluble fibre fractions by filtration and centrifugation. *Anim Feed Sci Technol* **129**:316 – 328 (2006).

32 Carpita N, Hemicellulosic polymers of cell walls of *Zea* coleoptiles. *Plant Physiol* **72**:515 – 521 (1983).

33 Jones BA, Hatfield RD and Muck RE, Effect of fermentation and bacterial inoculation on lucerne cell walls. *J Sci Food Agric* **60**:147 – 153 (1992).

34 Kondo T, Mizuno K and Kato T, Variation in solubilities of lignin in acid detergent and in alkali. *Jpn Grassl Sci* **33**:296 – 299 (1987).

35 Morris IM, Semimicro method for the determination of lignin and its use in predicting the digestibility of forage crops. *J Sci Food Agric* **23**:455 – 463 (1972).

36 Fukushima RS and Hatfield RD, Extraction and Isolation of lignin for utilization as a standard to determine lignin concentration using the acetyl bromide spectrophotometric method. *J Agric Food Chem* **49**:3133 – 3139 (2001).

37 Aman P and Graham H, Mixed-linked beta-(1–3), (1–4)-D-glucans in the cell-walls of barley and oats – chemistry and nutrition. *Scand J Gastroenterol* **22**:42 – 51 (1987).

38 Hatfield RD, Marita JM, Frost K, Grabber J, Ralph J, Lu FC, et al., Grass lignin acylation: p-coumaroyl transferase activity and cell wall characteristics of C3 and C4 grasses. *Planta* **229**:1253 – 1267 (2009).

39 Lu F and Ralph J, Detection and determination of p-coumaroylated units in lignins. *J Agric Food Chem* **47**:1988 – 1992 (1999).

40 Hatfield R, Ralph J and Grabber JH, A potential role for sinapyl p-coumarate as a radical transfer mechanism in grass lignin formation. *Planta* **228**:919 – 928 (2008).

41 Petrik DL, Karlen SD, Cass CL, Padmakshan D, Lu FC, Liu S, et al., p-Coumaroyl-CoA:monolignol transferase (PMT) acts specifically in the lignin biosynthetic pathway in brachypodium distachyon. *Plant J* **77**:713 – 726 (2014).

42 Marita JM, Hatfield RD, Rancour DM and Frost KE, Identification and suppression of the p-coumaroyl CoA:hydroxycinnamyl alcohol transferase in *Zea mays* L. *Plant J* **78**:850 – 864 (2014).

43 Ralph J, Grabber JH and Hatfield RD, Lignin–ferulate crosslinks in grasses: active incorporation of ferulate polysaccharide esters into ryegrass lignins. *Carbohydr Res* **275**:167 – 178 (1995).

44 Iiyama K, Lam TBT and Stone BA, Phenolic acid bridges between polysaccharides and lignin in wheat internodes. *Phytochemistry* **29**:733 – 737 (1990).

45 Amann P and Graham H, Analysis of total and insoluble mixed-linked (1–3),(1–4)-beta-D-glucans in barley and oats. *J Agric Food Chem* **35**:704 – 709 (1987).

46 Kent NL and Evers AD, *Kent’s Technology of Cereals*. Elsevier Science Inc., New York (1994).

47 Fukushima RS and Hatfield R, Comparison of the acetyl bromide spectrophotometric method with other analytical lignin methods for determining lignin concentration in forage samples. *J Agric Food Chem* **52**:3713 – 3720 (2004).

48 Henry RJ, Pentosan and (1–3),(1–4)-beta-glucan concentrations in endosperm and wholegrain of wheat, barley, oats and rye. *J Cereal Sci* **6**:253 – 258 (1987).