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Abstract

To identify differences in seed lignin content, 520 randomly chosen independent lines were screened in mutagenized oat population and lines with the seed lignin levels ranging from 20-63 gkg^{-1} were identified. In commercial variety Belinda, from which the mutated population was developed, seed lignin level was determined to be 41 gkg^{-1}. In Assiniboia, a Canadian low lignin variety, it was found to be 21 gkg^{-1}. To investigate if extracted lignin from the mutated lines were also qualitatively different from Belinda, two lines with the lowest and highest lignin levels were selected for structural analyses using XRD, UV and FT-IR spectroscopy. Results showed that there were significant qualitative differences in seed lignin levels in the mutated lines and in Belinda, and based on this, we predict that lignin from the mutated seeds will be more digestible in ruminant animals than Belinda seeds. This prediction was confirmed in preliminary *in-vitro* digestion experiments.

Keywords: Oat, Lignin, Belinda, Assiniboia, Dioxane, UV, FT-IR spectroscopy

1. Introduction

Oat (*Avena sativa*) is grown in significant areas in the USA, Canada, Europe, China, Brazil and Australia (Zwer, 2004). Oat is mostly consumed as grain with a yearly world grain production of approx. 25 million tons. Oat grains have high oil content and the oil is rich in unsaturated fatty acids. It also contains unique galacto-lipids. Oat proteins have the highest proportion of globular proteins amongst any cereal starch. In addition, oats are rich in essential dietary minerals and health benefitting 1-3, 1-4 β-D-glucans (Ripsin et al., 1992).

Although, oat provides excellent health benefits for humans, it is mostly used as feed for cattle. Oat hulls, which make up 25% or more of the total oat seed, have high fibre content and low digestibility (Thompson et al., 2000). In order to maximize its utilization for the oat milling industry and cattle producers, an economical method to improve fibre digestibility of hulls needs to be developed. The major factor limiting fibre digestion is lignin (Sewalt et al., 1997). Lignin content may be treated as an excellent indicator of quality for oat marketing and in feed formulations for animals. If core lignin and free phenolic acid barriers are removed from the fibers, microbial and enzymatic attachment to hemicellulose and cellulose will increase, which in turn will lead to an increased fibre digestion (Titgemeyer et al., 1996). It has been estimated that a more easily digestible oat kernel containing a low lignin hull would increase the energy value of the feed by approx. 15% (Casler and Jung, 2006).

In 2002, a Canadian variety with a low hull lignin content, AC Assiniboia, was released (Thompson et al., 2002). It was shown by biochemical analysis that the hulls from AC Assiniboia contained 1.3% ADL (acid detergent lignin), while other market

varieties had between 5.4-7.7%. In addition, experiments using cow rumen liquid indicated that the digestibility of Assiniboia hulls was almost twice as that of other varieties (Thompson et al., 2002)**.** Thus, an altered lignin level could indeed lead to change in digestibility and an increased digestibility would substantially improve the feed value. No differences in yield, kernel quality, and disease resistance could be detected between Assiniboia and other Canadian market varieties. Assiniboia was tested in field trials for several years in Sweden, but due to lower yield and poor straw strength it is not considered suitable for commercial growth.

In this work, by starting from a good commercial variety, SW Belinda, that grows well in Scandinavia, and by using a recently developed TILLING-population for oat (Chawade et al., 2010) we identified several low lignin oat lines. By qualitative characterization of lignin from two high- and two low lignin lines using X-ray diffraction, UV and FT-IR spectroscopy, we show that there are compositional and structural differences between the lignins and preliminary digestion analysis supported that the differences could lead to differences in digestibility. We also determined the lignin content in leaf and stem tissue in the two low lignin mutants as well as in Belinda and Assiniboia.

2. Materials and Methods

2.1 Plant material and cultivation

SW Belinda is a Swedish spring oat variety originally developed by Lantmännen SW Seeds AB, Landskrona, Sweden. AC Assiniboia is a Canadian low lignin oat developed by Crop Development Center, Saskatoon, Canada. All plants were grown in a greenhouse with halogen lamps, with photon flux density of 240

µmol/m^2/sec and photo period of 18 h and day/night temperature of 25 ^{0}C/16 ^{0}C. Plants were grown in five litre pots in standard soil as described (Chawade et al., 2010).

2.2 Lignin quantification

Based on the qualitative analysis of lignin in oat as reported earlier (Chawade et al., 2010) the lignin content in 520 mutant lines from an oat TILLING-population was determined by a modified acetyl bromide procedure (Iiyama and Wallis, 1988). Briefly, from each line, ten seeds (hull+groat) were individually weighed, crushed and transferred to separate glass test tubes (16 x 150 mm) fitted with PTFE-coated silicone screw cap. In each tube, perchloric acid (70%, 0.08 ml) was added followed by the addition of 2 ml of acetyl bromide-glacial acetic acid (1:3, v/v) and incubated at 70 ^{0}C for 15-20 min with intermittent gentle shaking to promote complete dissolution. The solution was then transferred with the aid of acetic acid to 100 ml volumetric flasks containing NaOH (2M, 5 ml) and acetic acid (12 ml). The final volume was adjusted to 50 ml with acetic acid. Blank sample, without any seed was also run in conjugation with other samples. The absorbance was measured at 280 nm (UV-2401PC, Shimadzu, Japan) and lignin content was determined as described (Morrison, 1972)**.** Means were measured from the ten seed samples and standard error calculated.

2.3 Lignin extraction

An extractive-free and dry oat powder was obtained by a 2h treatment ethanol/toluene (1:1, v/v). The powder was then utilized for lignin extraction by the acidic-dioxane method. Ten g of dry oat powder was placed in a 250-ml round-bottom flask, and 200 ml of acidic dioxane (dioxane/water, 9:1, v/v and 0.2 M HCl

106 solution) was added slowly from the funnel; the flask was connected to a reflux
107 condenser and N_2 gas was blown onto the liquid surface for 20-30 s. The reaction
108 mixture was heated and refluxed (80-95 ^{0}C, 40 min). The mixture was allowed to cool
109 to around 40-50 ^{0}C, filtered and the filtrate was saved. The solid residue was
110 subjected to a second extraction with 200 ml of the acidic dioxane/water solution for a
111 period of 30 min as described above. Two more extractions were performed in the
112 same fashion except that no hydrochloric acid was added to the dioxane/water mixture
113 in the last (fourth) extraction. Each portion of dioxane filtrate was concentrated
114 separately on a rotatory evaporator (200 ml to around 40 ml) and finally all
115 concentrates were pooled and lignin was precipitated by adding the concentrate into
116 cold distilled water (1600 ml) under stirring. The precipitate formed was pelleted by
117 centrifugation (9000g, 20 min) and the supernatant was removed. The pellet was
118 partially dried in a forced air oven (60 ^{0}C, 15 min). Lignin residues were dissolved in
119 4-5 ml of dioxane (100%), filtered through a 0.45µm nylon membrane, and added
120 dropwise to 50 ml of anhydrous diethyl ether under rapid stirring. The precipitate was
121 separated by centrifugation (9000g, 15 min, 0 ^{0}C) and the entire solubilization in
122 dioxane and ether wash steps were repeated to remove hydrophobic non-lignin
123 contaminants. Following the diethyl ether removal, 50 ml of petroleum ether was
124 added under stirring to thoroughly wash the lignin residue. The residue was then
125 allowed to settle and the solvent was removed. The final lignin residue dioxane lignin
126 (DL) was freeze-dried for 8 h and stored at -20 ^{0}C until further use.

127 **2.4 X-ray diffraction**

128 Powdered lignin samples were used for obtaining X-ray diffraction patterns. X-
129 ray diffractograms with 2θ, ranging from 10^0 to 40^0 were collected with a Siemens

D5000 X-ray diffractometer (Germany) using Brag-Brentano geometry with a secondary monochromator (CuKα radiation, 40kV/40mA, step 0.05 in 2θ, 6 sec./step).

2.5 UV scanning

Isolated lignin (5 mg) was dissolved in 10 ml of 95% dioxane:water (v/v) and an 1 ml aliquot was diluted to 10 ml with dioxane:water (50:50, v/v). The scanning was performed in the range of 250 – 400 nm (UV-2401PC, Shimadzu, Japan).

2.6 Fourier transform infrared (FT-IR) spectroscopy

Isolated lignin was analysed by FT-IR spectroscopy using a 2000 FT-IR spectrophotometer (Perkin Elmer, Beaconfield Bucks, England) to examine the functional groups as well as differences in chemical structures. Lignin sample and KBr were mixed in a ratio 1:100 and then pressed into transparent thin pellets for obtaining the spectra at room temperature. FT-IR spectra (40 scans) of each sample were obtained in the range of 400 - 4000 cm^{-1}. Transmittance mode as a function of wavenumber (cm^{-1}) was recorded. The spectra were converted to absorbance, automatically baseline corrected, and normalized using the associated software. The assignments of absorbance peaks were as per the FTIR database for lignin (Faix, 1991).

2.7 *In-vitro* digestion

Triplicate samples of 0.5 gram of seeds were individually grinded, transferred to 50 ml Falcon tubes and 30 ml of a pepsin-hydrochloric acid (HCl) solution was added (Weisbjerg, 2004). The tubes were sealed and incubated in a water bath at 40°C for 24 hours. The tubes were shaken twice during the incubation period and were then moved to 80°C and incubated for another 45 minutes. The samples were then

transferred to pre-weighed filter crucibles and washed twice with 100 ml water to neutralize the samples. After sealing the bottom of the filter, 30 ml of an enzyme-acetate buffer solution was added (Weisbjerg, 2004). The filters were sealed and the samples incubated in a water bath at 40°C for 24 hours followed by 60°C for 19 hours. The samples were washed with 2 x 100 ml boiling water and 2 x 20 ml acetone and dried at 103°C over-night in an oven. The crucibles were then transferred to a desiccator, cooled to room temperature and weighed. Finally the crucible were placed in an ash oven at 500°C and incubated over night, after which they were cooled down and weighed again. The moisture content in all seed material was determined in a Denver instrument (Germany BR35).

Reproducibility of results

All experiments were performed in triplicates and the results represent the mean of three identical experimental setups with ± SE.

3. Results and discussion

3.1 Screening of the oat TILLING-population

Lignin content was determined by the quantitative acetyl bromide method. Compared to other lignin determination methods the acetyl bromide method is relatively non-laborious and appropriate for small sample sizes. In this procedure, lignin is almost entirely dissolved and hence precise absorbance values for total lignin content are provided with little interference from non-lignin products. When analysing lignin content in seeds (hull and groat combined) of the oat cultivars Belinda and Assiniboia, we found it to be 41 gkg^{-1} and 21 gkg^{-1}, respectively (Table 1). This is in good agreement with previous reports on lignin measurements for Assiniboia and Belinda (Thompson et al., 2000, Chawade et al., 2010). We then

screened 520 random lines from the oat TILLING-population and found the lignin contents to be in a range from 20 - 63 gkg^{-1} (Fig. 1). Mutant lines #836 (denoted L3) and #1960 (denoted L4) were found to be the lowest with 20 gkg^{-1} and 24 gkg^{-1} of lignin content respectively, which is in the same range as in Assiniboia. The highest values were found in mutant line #1849 (denoted H5), (63 gkg^{-1}) and #827 (denoted H6), (62 gkg^{-1}), with approx. three times higher levels compared to L3 (Table 1).

In addition, lignin contents in leaves and stem of the Belinda, Assiniboia, L3 and L4 were also determined but in these tissues no significant differences were observed between the different cultivars (Fig. 2). Apparently, the reduced lignin content in the mutated lines was confined to seeds, which indicate that they could be useful as breeding lines. In addition, the phenotype of greenhouse grown mutant plants remained similar to non-mutated Belinda with no visible differences in height, strength and stability of plants (data not shown).

3.2 X-ray diffraction

All the X-ray diffractograms from the different lignins isolated from Belinda, Assiniboia, L3, L4, H5 and H6, respectively showed a broad diffraction of amorphous halo with a maximum at about $2\theta = 22\,^{0}C$ (Fig. 3). Such a diffused pattern, lacking intense and sharper peak, is typical of resin compounds and are expected for lignin and its derivatives, as they are non-crystalline polymers. Thus, the X-ray diffractograms indicates that the lignin samples were composed of amorphous polymers and that they were cellulose free (Rohella et al., 1996). The conclusion from the X-ray diffraction measurements is thus that the lignin preparations are of good quality and lack cellulose.

3.3 UV scanning

The obtained UV spectra of all the extracted lignins depicted a typical absorption band for annual plants, in agreement with previous reports (Fig. 4), (Seca et al., 2000). In addition, a shoulder at 310-315 nm, which is typical for grass lignin, was seen in the spectra and indicates the presence of esters of hydroxycinnamic acid such as p-coumaric or ferulic acid (H) (Lybeer and Koch, 2005). Another shoulder was observed at 280-284 nm originating from non-conjugated phenolic groups in lignin, such as sinapyl alcohol (S), coniferyl alcohol (G) and even p-coumaryl alcohol.

The Belinda spectrum had relatively stronger absorbance at 280-284 nm than at 310-315 nm, indicating relatively high content of guaiacyl (G) units, which is similar to that of other monocotyledons and is consistent with a guaiacyl rich lignin (Lybeer and Koch, 2005). In addition, the Belinda spectrum showed a relatively weaker shoulder at 310–320 nm compared to the other 5 studied lines, indicating that it also contained lower amounts of esters of hydroxycinnamic acid units.

The spectra obtained for Assiniboia and all mutant lines (L3, L4, H5 and H6) were almost opposite to the spectra from Belinda, as they exhibited a weaker shoulder at 280 nm and a stronger and higher peak at 310-320 nm (Fig. 4). This suggests that lignin from Assiniboia and the mutants were similar with lower levels of G units and higher levels of H units, compared to Belinda. However, the intensity of the shoulders was higher in the mutants than in Assiniboia, indicating the presence of the same type of phenolic structures, albeit in different quantities.

One possible reason for the decreased G unit content could be mutations in the COMT gene, since a down-regulation of COMT reduces lignin by decreasing the G units in

switchgrass (Fu et al., 2011). However, all of the analysed lignin samples are still of the HGS type (Xu et al., 2008), as further confirmed by FT-IR.

3.4 FT-IR analysis

Lignin is a highly branched phenolic polymer providing many active regions for chemical and biological interactions with a wide variety of additional functional groups like hydroxyl-, methoxy-, carbonyl-, and carboxylic groups. Since this can change the chemical and biophysical properties of the lignin, an analysis of various active groups attached to lignin is of importance in a functional classification of lignins. Such analysis can be performed by Fourier Transformed Infrared analysis (FT-IR) (El Mansouri and Salvado, 2007).

Here, we generated several independent FT-IR spectra for Belinda, Assiniboia and the different mutated lines, analysed the spectra, indicated possible functional groups (Fig. 5; a & b, Table *2*) and classified the spectra guided by the paper by Faix (1991). Belinda and the mutants displayed spectra in the fingerprinting region having many characteristics typical for HGS lignin, such as high absorbance in the 1710-1665 cm^{-1} range, similar absorbance around 1600 and 1510 cm^{-1}, relatively more intense peaks around 1328 cm^{-1}, similar absorbance for the peaks around 1267, 1229, the characteristic HGS peak around 1166 cm^{-1}, a tall peak around 1127 cm^{-1}, much smaller absorbance around 1031 than around 1229 cm^{-1} and finally a characteristic tall peak around 834 cm^{-1}. However, the spectra also showed a clear peak around 1085 cm^{-1}, commonly seen in spectra from G and some GS lignin types, indicating some structural differences from idealized HGS lignin. The spectra for Assiniboia also showed a similar HGS character, especially if neglecting the region between 1700-

1500 cm^{-1}, which probably is influenced by associated proteins. There is a clear peak around 1328 cm^{-1}, similar absorbance around 1267 and 1229 cm^{-1}, the characteristic HGS peak around 1166 cm^{-1}, much smaller absorbance around 1031 than around 1229 cm^{-1} and finally a characteristic tall peak around 834 cm^{-1}. A striking difference from ideal HGS spectra, and from the spectra of the samples derived from Belinda, is a relatively low absorbance around 1127 cm^{-1}.

Thus, all investigated samples were classified as HGS lignin. However, some differences from ideal spectra could be seen and a comparative analysis of the spectrum of different samples revealed slight differences in several characteristic bands. This indicates that there are structural differences between the lignin from the different lines, like differences in the ratio of HGS units, different subunit composition, and differences in associated carbohydrates and proteins. Therefore, in screening for different seed lignin levels in the oat TILLING population it hypothesize that the lines were selected that carry mutations in genes encoding key enzymes in the particular part of the biosynthetic pathway of lignin synthesis that control monolignol biosynthesis and quality.

From the FT-IR analysis, it is difficult to draw conclusions on the exact differences between the samples due to the complexity and inherent variability of lignin. However, some of the differences are highly pronounced and located to a specific region, and hence were analysed in detail. At large wavenumbers, a prominent peak at ~3,400 cm^{-1}, caused by the presence of OH groups in aliphatic and phenolic structures (Faix, 1992), appeared in all samples (Fig. 5a). In addition, peaks at ~2,928 and ~2853 cm^{-1} were also detected, that have been attributed to the presence of methyl/methylene (C-H) groups in the samples (El Mansouri and Salvado, 2007; Faix,

1991). In this region, Assiniboia displayed a shift towards smaller wavenumbers of the peak around 3400 cm^{-1}, as well as opposite ratio between the peaks at 3400 and 2900 cm^{-1}, compared to Belinda. The same reversed peak ratio was seen for the L4 line, most likely reflecting additional methyl substitution, making this line more similar to of Assiniboia than the other mutants. Another similarity between L4 and Assiniboia was a shoulder in both spectra at 1739 cm^{-1}. In the region 1750-1600 cm^{-1}, all samples display complicated peak-patterns associated with C=O groups (see Table *2* for details) and around 1509 cm^{-1} a peak assigned to aromatic skeletal vibrations plus C=O stretch is present. At 1634 and 1539 cm^{-1} non-lignin specific absorbance was seen, where the latter was visible only in spectra of Assiniboia and Belinda. Those peaks were most likely caused by amides in associated proteins, as seen from FT-IR analysis of protein by Kong and Yu (2007), and it can be concluded that the presence of such proteins were high in Assiniboia, slightly lower in Belinda and even lower in the mutants. The presence of those non-lignin derived bands complicates interpretation of the spectra in the region. However, without doubt, the L3 and L4 mutants displayed increased absorbance at 1603 cm^{-1}. For the peak at 1463 cm^{-1} an increased absorbance was detected for the L4 mutant. For the peaks at 1366 and 1329 cm^{-1} increased absorbance was detected for the L3 mutant. Thus, it seems as both the low lignin mutants contained increased fractions of S or condensed G units, based on the peak assignment given in table *2*. For the double peaks around 1265 and 1240 cm^{-1} all the mutant spectra as well as that of Assiniboia displayed significantly higher absorbance at 1240 than at 1265 cm^{-1}, while for Belinda, the relationship was opposite. This could indicate an increased fraction of condensed G units in Belinda compared to the mutants and Assiniboia, or alternatively a reduced amount of G units

297 for the mutants and Assiniboia compared to Belinda. When comparing the ratio of the
298 HGS characteristic peak at 1167 cm^{-1} with the peak at 1128 cm^{-1} (I_{1167}/I_{1128}), it is clear
299 that there are differences in ratios of the two peaks in a way that Assiniboia > L4 ≈ L3
300 > H6 ≈ H5 >Belinda. This indicates that there is a higher proportion of
301 hydroxycinnamic acid (H) units in lignin from Assiniboia and the mutants compared
302 to Belinda. This result is in good agreement with the results of UV spectra.

303 In summary, the FT-IR analysis confirmed the conclusions from the UV-spectra
304 analysis, i.e. lignin from the reported mutant lines were structurally different from the
305 lignin isolated from the original Belinda line. Lignins from mutants were more similar
306 to Assiniboia lignin, having high hydroxycinnamic acid (H units) content and either
307 lower guaiacyl (G units) content or higher fraction of condensed G units. These
308 differences may turn out to be beneficial in terms of nutrition and digestibility, as
309 discussed below.

310 **3.5 Lignin digestion studies**

311 In preliminary experiments, we tested seed lignin digestibility in 836 (L3) and
312 compared these to Belinda and Assiniboia. This showed that the NDF (Non Digestible
313 Fiber) values were higher in Assiniboia but lower than in Belinda (Table 3). Thus, this
314 was in agreement from the predictions made from the lignin structural analysis as
315 Assiniboia has higher levels of hydroxycinnamic acid. The digestibility was also in
316 agreement to what was previously found for Assiniboia (Thompson et al., 2000). A
317 visual inspection of insoluble remaining fibres showed that there were structural
318 differences between Assiniboia, which were short and finely dispersed, 836 L3 and
319 Belinda fibres, which were cruder and longer. Such differences will probably affect
320 both digestibility and rate of decomposition.

Plant cell walls are almost entirely constituted of ligno-cellulose, which limits digestion of the wall polysaccharides in the rumen. Although it is not possible to attribute cell wall digestibility to a single effect or factor, the exact composition and structure of the lignin will influence the digestibility. Furthermore, the guaiacyl and p-coumaric acid content of lignin appear to be good predictors of digestibility of maize silage in sheep (Novo-Uzal et al., 2011). One reason for the correlation between lignin quality and digestibility is that some lignin structures will sterically hinder enzymatic hydrolysis of cell-wall polysaccharides by shielding otherwise digestible chemical bonds. This negative effect of lignin on digestibility is greater in grasses than in legumes (Buxton and Casler, 1993). The main factors influencing the energy availability in grass and thus its appropriateness as feed are the composition and concentration of the lignin itself and the presence of hydroxycinnamic acid (H) in the cell wall (Grabber et al., 1998).

Assiniboia, is more digestible, and thus is more nutritious than oats with higher lignin contents like e.g in Belinda, which was used in this study. By using various analytical techniques, we show that Assiniboia hull lignin is rich in esters of hydroxycinnamic acid i.e. p-coumaric acid (PCA) and ferulic acid (FA). Interestingly, similar structural features were also detected in all four mutants (L3, L4, H5 and H6) studied here. In grass cell walls, phenolic acids (PCA) and (FA) are bound to lignin through both ester and ether linkages although the major part of PCA in the grass hay lignins are esterified to lignin components, while FA is predominantly ether-linked (Iiyama et al., 1990). During the sheep digestion of grass lignins, the proportion of the lignin that disappears i.e. dissolves is dependent on the degree of ester-linked PCA and ether-linked FA. Consequently, this indicates that the low lignin H unit rich mutants

345 described here may be more digestible. The FA esterified to cell walls is more
346 digestible/degradable than cell wall esterified PCA. This can partly be explained by
347 the findings that FA is mainly bound to the cell wall polysaccharides, whereas PCA
348 are exclusively bound to lignin components (Kato et al., 1984). Ruminal bacteria have
349 the ability to metabolise FA and PCA and the forage phenolic acid esters are cleaved
350 extensively during in vivo ruminal fermentation. There are several studies that show
351 that lignin content and digestibility of feeds follow an inverse relationship (Jung et al.,
352 1997). In addition, on the other hand by comparing the digestibility in sheep for two
353 oat samples that differed in their lignin content, a higher digestible energy was
354 recorded in the low-lignin sample (Rowe and Crosbie, 1988). Apparently lower lignin
355 levels may in some cases improve the digestibility of oat in ruminants. Jung and
356 Deetz (Jung and Deetz, 1993) reported that the improved digestibility of cell walls is a
357 result of both the reduced lignin content and its composition. There are soluble
358 hydroxycinnamic acid esters (e.g. FA) that also contribute to the energy release.

359 The reported mutants and Assiniboia may be more digestible, and therefore also more
360 nutritious, as they show lower G and higher H content compared to Belinda both in
361 the UV- and FTIR spectra. Fu et al., (Fu et al., 2011) observed the same trend in the
362 switch grass. A down-regulation of the caffeic acid o-methyl transferase (*COMT*)
363 gene in the switch grass lowered the lignin content, reduced the G and S units in
364 lignin and improved forage quality. Digestibility was tested in vitro by both true dry
365 matter digestibility (IVTDMD) and neutral detergent fiber digestibility (NDFD).
366 These values increased 9 and 11%, respectively in the transgenic low-COMT line
367 compared to wild type. Furthermore, COMT activity was also more directly tested by
368 a reaction to the specific COMT substrates 5-OH coniferaldehyde and caffeyl

aldehyde, and this showed that the transgenic line had a significant reduction in COMT enzyme activity (Fu et al., 2011). Reddy et al. (2005) also observed a strong negative relationship between lignin content and rumen digestibility, but no relationship was found between lignin composition and digestibility in down regulated COMT and caffeoyl CoA 3-O-methyltransferase (CCoAOMT) transgenic lines of alfalfa. Various other reports are also available (Chen et al., 2004) where COMT has been down regulated in alfalfa and other species leading to lignin modifications.

To confirm the structural lignin assays we tested the digestibility of lignins from chosen lines in vitro. There are a number of different methods in the literature for doing this (Cherney and Cherney, 2003) including *in situ* determinations of feed digestibility in fistulated cows. However, such methods are complicated, expensive and difficult to standardise (Spanghero et al., 2003). A number of alternative methods have therefore been developed based on the incubation of the sample in rumen fluid (Tilley and Terry, 1963). However, the quality of the rumen fluid varies, which makes standardisation difficult (Spanghero et al., 2003). More recently, a method where the rumen liquid was replaced by a defined enzymatic mixture was developed in Denmark. It is denoted EFOS (EnzymFordøjeligt Organisk Stof = Enzyme digestible organic matter) (Weisbjerg and Hvelplund, 1993). The EFOS method is easy to standardize and gives a significant correlation between the rate of breakdown of not digestible fibres (NDF) *in situ* and the digestibility of organic matter (Weisbjerg, 2004) and the EFOS method is now the recommended procedure in the Nordic feed evaluation system (NorFor) (Åkerlind et al., 2011). In this work, we therefore chose the EFOS method to estimated lignin digestibility in the different lines and cultivars.

Our results confirmed the predictions from the structural measurements in that the Assiniboia lignin displayed the highest digestibility followed by one low-lignin CT-lines, while the Belinda variety showed the least digestibility (Table 3).

4. Conclusions

Our analysis showed that samples were composed mostly of lignin components, indicating satisfactory lignin purification protocol. Spectroscopic analysis showed presence of comparatively higher ratios of hydroxycinnamic acid units in Assiniboia and mutant lines in contrast to Belinda. These mutant lines will be more digestible *in-vivo* than Belinda variety since grass lignin higher in H content has previously been reported to be more digestible. This was confirmed in preliminary *in-vitro* digestion experiments using EFOS method. Further *in-vitro* rumen digestion experiments to get a deeper insight how the lignin structural differences influence degradation of cell walls by rumen microflora are in progress.

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6. Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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Figure Caption:

Figure. 1 Screening of TILLING population of oat for lignin mutants (gkg^{-1}).

Figure. 2 Lignin content (gkg^{-1}) in stem and leaf.

Figure. 3 X-ray diffractogram of lignin from Belinda, Assiniboia and mutant lines (L3, L4, H5, H6)

Figure. 4 UV spectra of lignin from Belinda, Assiniboia and mutants lines (L3, L4, H5, H6)

Figure. 5 Fourier Transform Infrared spectra of lignin from Belinda, Assiniboia and mutant lines (L3, L4, H5, H6), relevant wavenumbers are indicated in the figure. (a) 3800-2200 cm^{-1}, (b) 2000-600 cm^{-1}.