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Effect of Lignin Content and Subunit Composition on Digestibility in Clones of Timothy (*Phleum pratense* L.)

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ABSTRACT: Lignin amount and subunit composition were analyzed from stems and leaf sheaths of timothy (*Phleum pratense* L.) clones of different in vitro digestibility. Lignin concentration in stems and leaf sheaths was higher in clones of low digestibility than those of high digestibility. No change in lignin concentration occurred in stems as digestibility decreased. Intriguingly, the lignin concentration was lower and the syringyl/guaiacyl (S/G) ratio was higher in stems compared to leaf sheaths at all developmental stages studied. The developmental-associated decrease in digestibility correlated with the increase in S units in lignin in stems and leaf sheaths and in the amounts of *p*-coumaric acid and ferulic acid residues in the cell wall of stems. Yields of copper oxidation products increased in stems during maturation indicating qualitative changes in the lignin structure. This correlated strongly with the developmentally linked decrease in digestibility. The information obtained is valuable for breeding and for DNA marker development.

KEYWORDS: *cell wall, digestibility, ferulic acid, lignin amount, lignin subunit composition, lignin linkage structure, timothy*

INTRODUCTION

Grass silage is an important source of metabolizable energy for milk and beef production in many countries, particularly in the temperate and cool zones of Europe and North America.[1] The main goal in forage production is to obtain high herbage mass that is of high nutritive value and digestibility. Plant cell wall polysaccharides cellulose and hemicellulose are of great importance for the energy content of silage. In grasses, lignin, a phenolic component in the cell wall, increases with increasing maturity, as the need for structural strength increases during stem elongation. This is accompanied by a decrease in digestibility.[2] Lignin provides rigidity and structural support to the cell wall polysaccharides, and it also excludes water from the wall. In grasses and cereals, cell walls of several parenchymatous cell layers in the cortex thicken and lignify during stem development, i.e., a sclerenchyma ring develops.[3−5]

In grasses and cereals lignin is composed of guaiacyl (G), syringyl (S), and *p*-hydroxyphenyl (H) units originating from coniferyl alcohol, sinapyl alcohol, and *p*-coumaryl alcohol, respectively.[6] In addition, cell walls of grasses contain hydroxycinnamic acids (*p*-coumaric and ferulic acid).[7−10] The monolignol biosynthetic pathway leading to lignin precursors is well-known.[11,12] Each gene of the pathway has been individually down-regulated, and the effects on plant growth, lignin amount, and composition have been assayed. Down-regulation of certain genes in the pathway has led to reduced lignin content and/or change in lignin composition and improved digestibility, for example, in cultivars of maize (*Zea mays*) and alfalfa (*Medicago sativa*).[2,11]

Accumulation of lignin reduces digestibility of cell wall polysaccharides as lignin makes them inaccessible to rumenal enzymes that would normally digest them. Di- and oligoferulate cross-links between ferulic acid units ester-linked to arabinoxylans and linkages between ferulates/di- and oligoferulates and lignin further impede enzymatic degradation of cell wall polysaccharides.[2,6,7,13,14] Several studies have shown that there is a negative relationship between the amount of lignin and digestion.[4,15−17] The effect of lignin subunit composition on digestibility, however, remains unclear. In many forage crops both the lignin content and its S/G ratio increase during development.[4,16] In the case of alfalfa, no clear relationship was shown between in vitro digestibility and the S/G ratio.[16] In addition, in maize, artificially produced lignins had similar inhibitory effects on cell wall degradation independently of their monolignol composition.[18] In maize plants the lignin interunit linkage pattern (with uncondensed *β*-O-4 units correlating with low cell wall digestibility) and S units acylated with *p*-coumaric acid had a greater impact on cell wall degradability than lignin subunit composition.[19]

Whereas much is known about the relationship between lignification and digestibility in other major forage grass species,[4,15,17] in the case of timothy (*Phleum pratense* L.) there is relatively little information about this relationship. Timothy is the most important perennial forage grass grown for silage and hay production in Scandinavia[20] and is also widely grown in other cool and humid regions in Europe, Asia, and North America.[21] Earlier studies have shown that both total and

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Table 1. Sampling Dates and Corresponding Weather Parameters in 2008−2012

	2008	2011	2012
	expt I	expt II	expt II
plant material	Tammisto II	clonal material, Tammisto II	clonal material
analysis	comparison of different quantitative methods	lignin analyses, microscopy, IVOMD; Tammisto II for Klason lignin determination and for AcBr assay as a standard	determination of leaf sheath−stem proportions and IVOMD of stems and leaf sheaths separately, microscopy
fertilizer application date	May 8th for the first cut	May 17th for the first cut; July 7th for the second cut	May 8th for the first cut
sampling date	June 30th	June 11th−13th	June 18th
days from onset of growth period	64	48−50	43
GDD,[a] °C	381.0	308.9−322.7	302.7

[a]GDD = growing degree days, °C (base temperature, 5 °C).

acid-insoluble lignin increase during the development of timothy tillers.[15] Increase in acid-insoluble lignin, expressed as a percentage of total lignin, was more closely related to decreasing in vitro digestibility than that in total lignin. The increased portion of acid-insoluble lignin in total lignin suggests that the quality of lignin changed during plant maturation.[15] In addition, by using histochemical stains and biochemical tests, Stafford observed that the lignin in xylem vessels of timothy is different from that in the subepidermal sclerenchyma which develops later.[3] It is known that S units become more abundant in lignin at later developmental stages,[4,18] a phenomenon also detected by Quicke and Bentley as an increase in methoxyl groups in total lignin.[15]

Because the digestibility of grass is crucial in ruminant production, the biological processes controlling the digestibility of harvested forage are therefore of great importance. Changes of digestibility in the stem fraction (true stem and leaf sheath) of cool-season grasses during plant development are much larger than in leaf blades.[22−24] Furthermore, the contribution of the stem fraction to yield formation of harvested grass is high.[24,25] Therefore, in this study we focused on lignification in the stem fraction.

The aims of the present study were to compare different analytical methods to quantitate lignin in timothy and to evaluate how the lignin amount and the subunit composition affect the organic matter (OM) digestibility in timothy clones selected for either high or low OM digestibility. It is well-known that the developmental stage of the tiller and the leaf to stem (which includes leaf sheath) ratio affect both the lignin component and the digestibility of timothy.[26,27] Therefore, these factors were taken into account in the sampling procedure. In addition we carried out the lignin analyses of leaf sheaths and stems separately, as the leaf sheaths are botanically part of the leaf though typically analyzed within the stem fraction.[22,24,26,27]

MATERIALS AND METHODS

Plant Material. For comparing several methods for quantitative lignin analyses cultivar (cv.) Tammisto II was used (expt I; collected in 2008). In order to explore the role of lignin in determining the in vitro organic matter digestibility (IVOMD) of different genotypes, timothy clones that were characterized as having either low or high digestibility were used (expt II; years 2011−2012). The clones originated from the breeding material of Boreal Plant Breeding Ltd. (Jokioinen, Finland). Based on the results of a previous experiment, six clones were selected for each of the low- and the high-digestibility groups. The selection was done by taking account of the stem proportion of each clone in order to avoid selection for leafiness only.[23] Three clones were selected from each of the two groups for this study. Clones 50, 51, and 85 represented the high-IVOMD group, whereas clones 41 and 84, together with cv. Grindstad, represented the low-IVOMD group. The cultivar Grindstad was chosen as a control because it is a high-yielding variety that has a relatively low IVOMD value when cut at the same date as the other cultivars based on the Finnish cultivar testing system.[28] The sampling dates and corresponding weather parameters are presented in Table 1.

The plant material was cultivated on a coarse loamy soil (Aquic Cryorthent) at the Maaninka Research Station of MTT Agrifood Research Finland, Maaninka, Finland (63°08′ N, 27°19′ E). The clones were propagated vegetatively, and cv. Grindstad and cv. Tammisto II were propagated from seeds. The clonal tillers and cv. Grindstad were grown individually in cardboard boxes (3 cm × 3 cm) in commercial garden peat (Kekkilä White 630 W; Kekkilä Ltd., Vantaa, Finland) in an unheated greenhouse and planted on Sept. 27 and 28, 2010 in 10 cm × 12.5 cm grids on the field in microplots of 50 cm × 100 cm. The plots were fertilized with N 100, P 15, and K 25 kg ha^{-1} for the first cut and N 100, P 0, and K 35 kg ha^{-1} for the second cut. Cultivar Tammisto II was sown on May 26, 2005 in plots of 12 m^2 in the field, and the plots were fertilized for the first cut in year 2008 with N 90, P 13.5, and K 22.5 kg ha^{-1}. In 2011 fertilization was at the same rates as applied to the clone microplots. The dates of fertilizer application are given in Table 1. Both 2008 and 2011 were the third grass year for cv. Tammisto II plots.

The experimental layout of the clonal plots was a randomized block design with four replicates. Due to the limited amount of plant material, the tillers collected from blocks 1 and 2 were used for lignin analyses, and the tillers from the blocks 3 and 4 were used for IVOMD analysis. There were no blocks for cv. Tammisto II samples.

The tillers were collected at three developmental stages according to Simon and Park[29] (stage 45, boot swollen; stage 56, three-fourths of the inflorescence emerged; stage 58, base of the inflorescence just visible). The sampling time corresponded to the time of spring harvesting, except in 2008 when fully mature generative plants were collected for lignin quantitative analyses. Tillers were harvested by cutting with a razor blade immediately above the soil, and the height to the uppermost ligule was measured after which the leaves, leaf sheaths, stems, and heads were separated. For chemical analyses, leaf sheaths and stems of six tillers collected from the same block were separately pooled and stored at −20 °C until dried. Two sets of pooled samples (i.e., biological replicates) were collected from blocks 1 and 2.

At the same time, 18−25 tillers of each clone at developmental stages 45 and 58 (see preceding descriptions) were collected for IVOMD analysis from blocks 3 and 4. These tillers were pooled due to a limited amount of plant material. Leaves and heads were removed, and stems and leaf sheaths were kept together and dried in a forced draft oven at 60 °C (approximately 40 h). Dry weights were determined and the proportions of the dry matter per tiller calculated. In order to determine the organ portions in each clone at the developmental stages studied, leaves, leaf sheaths, stems, and heads were separated from 10 (stages 45 and 56) or 30 tillers (stage 58) in year 2012. Fractions were dried at 60 °C (approximately 40 h) and

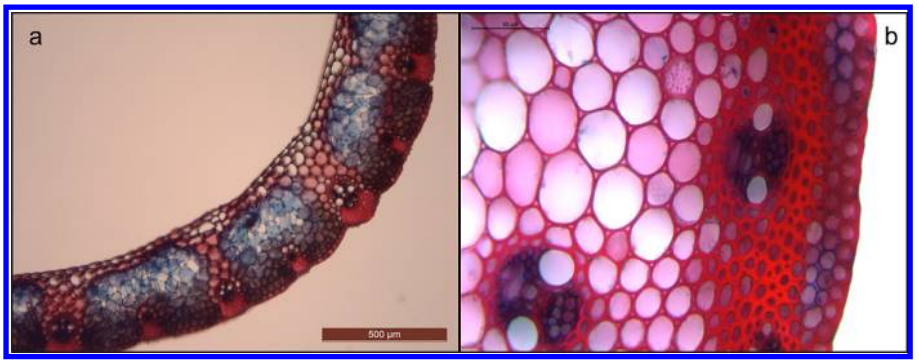

Figure 1. Cross-section of a timothy (a) leaf sheath and (b) stem stained with Alcian blue-Safranin O staining.

weighed. The proportions of leaf sheath and stem were determined by using dry weights.

Microscopy. The lignification stage of the tillers at each developmental stage was studied by microscopy. A sample (1 cm) was taken from the middle of the second lowest internode and fixed in a formalin (2%, v/v)−acetic acid (5%, v/v)−ethanol (63%, v/v) mixture (FAA). Hand-made or paraffin-embedded sections were made, and the developmental stage was observed after Alcian blue−Safranin O staining.[5]

Chemical Analyses. For lignin analyses plant material was dried in an oven at 60 °C and milled to a fine powder with a Retch ball mill (Retsch MM400, Haan, Germany; 50 mL grinding jars with one ball; stems, 4 × 30 s; leaf sheaths, (1−)2 × 30 s; frequency, 30 s^{-1}). To prevent warming of the samples during the milling, the grinding jars were cooled on carbon dioxide ice between samples. The extractive-free alcohol-insoluble residue (AIR) was obtained by extracting the powder for 8 times with 70% (v/v) ethanol in a water bath sonicator (25−30 °C; 30 min incubation in each extraction, with regular mixing) followed by 3 times with acetone. The tubes were centrifuged (4000 rpm, 10 min, at room temperature) before the solvent change. AIR was dried overnight at 60 °C and stored in firmly closed tubes in the dark at room temperature.

Since different quantitative methods for lignin determination are known to give different results,[30] we compared several methods by using AIR of cv. Tammisto II as a material (stems only, leaf sheaths removed). Permanganate lignin[31] was assayed in the MTT laboratory, and acid detergent lignin (ADL) by using the Foss Tecator Fibertec System M 1020 hot extractor and Fibertec System 1021 cold extractor, as described in Ordior Application Note AN 304, Ordior Application Sud Note ASN 3430 (AOAC-973.18) and USDA Forage Analyses Procedures, July 1993. Acid dioxane lignin was prepared as described.[32] Klason lignin was analyzed with a method commonly utilized by the Finnish Forest Research Institute.[33] For Klason lignin, the plant powder was sequentially extracted in a Soxhlet apparatus with acetone, ethanol, and water, 6 h each. The total nitrogen content of the Klason lignin was determined using a Leco CHN-1000 analyzer (St. Joseph, MI, USA), and the protein content calculated using the equation 6.25 × N%.[34]

Lignin content of timothy clones (stems and leaf sheaths separately; two pooled biological replicates) was analyzed with an acetyl bromide (AcBr) method[35] with modifications. Corresponding cv. Tammisto II samples of known Klason lignin content were used as standards. Briefly, 5 mg of AIR was transferred into a glass tube with a Teflon-coated lid. A 5 mL aliquot of AcBr reagent (20% AcBr (v/v) in glacial acetic acid without perchloric acid)[35] was added and the sample incubated at 50 °C for 3 h 15 min with regular mixing. The reaction was stopped in a −20 °C freezer for 15 min. An aliquot of 1.0 mL was transferred into a 10 mL volumetric flask containing 1 mL of 2 M NaOH and 2.4 mL of glacial acetic acid. After careful mixing, 0.1 mL of 7.5 M hydroxylamine−HCl was added. The volume was filled to 10 mL with acetic acid, and the absorbance at 280 nm was read with a Shimadzu UV-2401 spectrophotometer (Shimadzu Corp., Kyoto, Japan) against a blank that had been treated similarly to the samples.

Lignin subunit composition and *p*-hydroxycinnamic acid content in the cell wall of stems and leaf sheaths were studied by an alkaline cupric (II) oxidation method (CuO)[36] with modifications using AIR as a starting material. Oxidations were performed using a microwave digestion system MSD-2000 and a liquid phase hydrolysis accessory set (CEM Corp., Matthews, NC, USA). The gas chromatography−mass spectroscopy (GC-MS) detection of trimethylsilyl derivatives of CuO oxidation products was made using a HP 6890/HP 5972 (Hewlett-Packard, Palo Alto, CA, USA) equipped with a HP-5MS column (30 m; 0.25 mm i.d.; film thickness, 0.25 µm). The concentrations of individual compounds were calculated using response factors of commercially available standards. The internal standard, cinnamic acid, was added into the reaction vessel before microwave-assisted CuO treatment of samples. The method is time-consuming, and therefore the analysis was restricted to samples of one pooled biological replicate. Lignin subunit content was calculated as the percent of the corresponding total yield of CuO oxidation products, whereas *p*-coumaric acid and ferulic acid content was calculated as µmol g^{-1} AIR.

Digestibility Values. IVOMD (g kg^{-1} OM (cellulase))[37] with calculation of the results as described in Huhtanen et al.[38]), ash (g kg^{-1} dry matter (DM)), and neutral detergent fiber (NDF, g kg^{-1} DM) were assayed from the same clonal material (stems and leaf sheaths together) for the developmental stages 45 and 58 in the MTT laboratory. IVOMD was also determined separately for stems and leaf sheaths of clones at developmental stage 58 collected in 2012. Digestibility value (*D* value; g kg^{-1} DM) was calculated by (1000 − ash) × (IVOMD × 1000^{-1}).

Statistical Analyses. In order to study the relationship between measured IVOMD and the parameters obtained from lignin analyses, the lignin parameters were first calculated to correspond the same plant organs as the IVOMD analyses; i.e., we calculated weighted means for the whole stem (which includes leaf sheath; subsequently referred to as stem + leaf sheath) using original values of true stems and leaf sheaths separately, and their corresponding organic proportions that were determined in 2012 from the same plots. The generated weighted means for stem + leaf sheath were subjected to analysis of variance together with the original separate values for leaf sheath and stems.

Analysis of variance was performed using a mixed model with a residual maximum likelihood (REML) estimation method. In the model, the phenological stage, digestibility group, and their interaction were fixed effects, while clones were considered as a random effect. In the case of AcBr analyses with two biological replicates, the replicate was a random effect and the fixed effects were phenological stage and clone. Stage × clone interaction was not significant for any of the AcBr parameters, so it was not included in the model. The difference between digestibility groups was determined using contrast. The same model without a random effect was also used for the proportion of leaf sheaths and stems as there were no replicates. Pairwise comparisons between phenological stages were assessed by Tukey's test in all models. The MIXED procedure of SAS software (version 9.2; SAS Institute Inc., Cary, NC, USA).[39] The statistical dependences between IVOMD and other parameters were determined by Spearman's rank

correlation coefficient (r_s) using the CORR procedure of SAS software. Usage of r_s does not require the assumption of normality. Statistical significance was postulated at the 0.05 level of probability.

RESULTS AND DISCUSSION

Timothy clones of different in vitro digestibility were analyzed for lignin amount and subunit composition to evaluate their effects on digestibility. We separated leaf sheaths from stems before the analyses to assay the lignin in both organs separately. Timothy has a regular veining pattern in the leaf sheath that is an important support-giving structure for developing grass stems with intercalary meristems. Each vascular strand in the leaf sheath is accompanied by highly lignified sclerenchyma cells toward the adaxial epidermis (Figure 1a). The central area of the leaf sheath contains large, thin-walled cells that probably store water and are important for turgor-driven adjustment of the leaf sheath around the stem. In stems, xylem vessels in the vascular strands lignify first, followed by the sclerenchyma cells in and around the vascular strands (data not shown). The cell walls of the outer cortex cell layers also thicken and lignify during further development (Figure 1b).

Lignin Quantitative Analyses. Results obtained with various lignin quantitative methods differed (Figure 2), which is

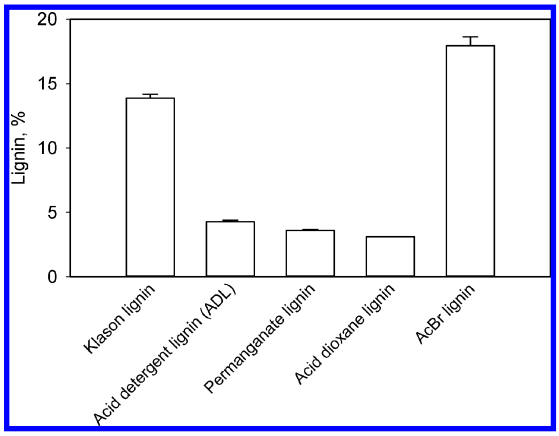

Figure 2. Lignin concentration (%) in stems of cv. Tammisto II as assayed by using several quantitative methods. AcBr = acetyl bromide. Error bars are standard errors of the means.

in accordance with a previous report.[30] Klason lignin and AcBr lignin (with acid dioxane standard) methods gave 3.5−6 times higher lignin concentrations than ADL, permanganate lignin, and acid dioxane lignin methods. Of the methods used, the ADL and Klason methods are gravimetric methods where cell wall carbohydrates are hydrolyzed with concentrated sulfuric acid, after which the residue that represents nonsoluble lignin is weighed. The acid-soluble part of the lignin is assayed from the solution and taken into account in the Klason estimate. The ADL procedure, which is based on the preparation of acid detergent fiber (ADF), however, underestimates the lignin content in grasses as a considerable amount of lignin (50% or more) is acid-soluble.[30] The S lignin, especially, is more soluble than other types of lignin during sulfuric acid hydrolysis.[32]

Timothy leaf sheaths were observed to contain more lignin than was present in stems. This occurred at all developmental stages studied, as calculated per mass unit of AIR, and was the case with AcBr lignin (Table 2) and Klason lignin (data not shown). The organ proportions (Table 2) were used to calculate the lignin concentration in stem + leaf sheath. Surprisingly, the phenological stage did not have any effect on the lignin concentration in AIR of stems, whereas that in AIR of leaf sheaths increased during development (Table 2). As the proportion of leaf sheaths decreased during development, however, the phenological stage had no effect on the calculated lignin concentration in AIR of stem + leaf sheath. The digestibility groups did not differ in the proportion of leaf sheaths but clones of low digestibility had higher lignin concentration both in AIR of stems and in AIR of leaf sheaths, and thus, also in the AIR of stem + leaf sheath (Table 2). This correlated negatively with IVOMD (Table 3). There were also some differences between individual clones in the lignin concentrations and also in the proportions of leaf sheaths (Table 2). The proportion of leaf sheaths did not, however, correlate with the IVOMD of the whole stem (stem that includes the leaf sheath; Table 3). The fact that the organ proportions were not determined in the same year as the samples for chemical analyses were collected may, however, weaken the relationship between plant organ proportions and chemical analyses.

Lignin Subunit Composition and Hydroxycinnamic Acids. Relatively mild oxidation with CuO induces cleavage of β-O-4 ether bonds in lignin;[36] thus, the subunit composition observed with this method originates from these noncondensed linkages. In timothy the abundance of S units in lignin increased during tiller development in both digestibility groups (Tables 4−6), correlating with the decrease in digestibility (Figure 3a; Table 3). In stems the S/G ratio was higher than in leaf sheaths at all developmental stages studied, suggesting that, in the case of timothy, the lignin subunit composition differs between stems and leaf sheaths (Tables 4 and 5). A similar difference in S/G ratio has been observed in leaf sheaths and stems of maize.[40] Interestingly, the relative increase in S units during development was higher in leaf sheaths than in stems of timothy. S units are able to form C−C bonds at the 8-position (β−β pinoresinol type structures), but they are unable to form C−C bonds in the 5-position due to methoxylation. Thus, an increase in S units observed during development suggests that the linkage structure in lignin changed. This conclusion is supported by the increased yields of CuO oxidation products obtained in stems at later developmental stages (Table 4) indicative of an increase in noncondensed β-O-4 bonds. There was, however, no developmental-related change in the yields of CuO oxidation products in leaf sheaths (Table 5) when both the lignin concentration (Table 2) and the proportion of S units increased. This indicates qualitative changes in lignin structure and possible cross-linking with carbohydrates.[40] The lower yields of CuO oxidation products obtained at earlier developmental stages from stems that contain higher relative proportions of S units than leaf sheaths, however, suggest that early in true-stem development the amount of β-O-4 linkages in stem lignin is less than in leaf-sheath lignin, but that during stem development S units are preferentially incorporated in these noncondensed linkages in stem lignin (Tables 4 and 5). Clones of low digestibility resulted in higher yields of CuO oxidation products in stem + leaf sheath than those of high digestibility (Table 6); furthermore, the increase in the yields CuO oxidation products strongly correlated with the decrease in digestibility during development (Figure 3b; Table 3).

Gramineous plants contain *p*-hydroxycinnamic acid residues esterified to arabinoxylan in the cell wall.[7,9,40] Ferulates can also be ether-linked to lignin. The ester-linked units can be released by saponification at room temperature whereas those residues

Table 2. Acetyl Bromide (AcBr) Lignin (Klason Standard) Concentration in Leaf Sheaths, Stems, and Stems + Leaf Sheaths (Calculated by Using the Proportions of These Organs in a Tiller As Determined from the Dry Weight) of Timothy Clones of High and Low Digestibility at Three Phenological Stages (Stage 45, Boot Swollen; Stage 56, Three-fourths of the Inflorescence Emerged; Stage 58, Base of the Inflorescence Just Visible)

digestibility group[a]	clone	lignin in leaf sheaths, g kg^{-1} AIR[b]	lignin in stems, g kg^{-1} AIR	lignin in stem + leaf sheath, g kg^{-1} AIR	proportion of leaf sheaths vs stems, %
high	clone 50	134	99	113	38
high	clone 51	124	95	108	44
high	clone 85	127	104	112	35
low	clone 41	140	111	123	42
low	clone 84	141	112	123	38
low	Grindstad	133	116	121	34
	SEM[c]	2.0	3.3	2.4	1.2
phenological stage					
45		130 a	104	114	40 b
56		132 a	110	119	39 ab
58		138 b	104	117	37 a
	SEM	1.4	2.4	1.9	0.9
high		128	100	111	39
low		138	113	122	38
P values	clone	<0.001	<0.001	<0.001	0.002
	phenological stage	<0.001	0.090	0.14	0.037
contrast	low−high	9.7	13.1	11.6	−0.7
P value		<0.001	<0.001	<0.001	0.47

[a]In relation to stem proportion. [b]Alcohol-insoluble residue. [c]Standard error of the mean. Values marked with the same letter do not differ (Tukey's test).

Table 3. Correlations between in Vitro Organic Matter Digestibility (IVOMD) and Other Parameters in Stems + Leaf Sheaths of Timothy Clones of Different Digestibility

	IVOMD, g kg^{-1} OM	
	r_s[a]	*P* value
proportion of leaf sheaths vs stems, %	0.48	0.11
stem height, cm	−0.90	<0.001
NDF, g kg^{-1} DM	−0.94	<0.001
AcBr lignin, g kg^{-1} AIR[b]	−0.59	0.042
total S units, %	−0.72	0.008
total G units, %	0.68	0.015
total P units, %	0.76	0.004
S/G	−0.58	0.048
p-coumaric acid, μmol g^{-1} AIR	−0.65	0.022
ferulic acid, μmol g^{-1} AIR	−0.77	0.003
total CuO oxidation products, mg g^{-1} AIR	−0.94	<0.001

[a]Spearman's rank correlation coefficient. [b]Alcohol-insoluble residue.

that are etherified to lignin can be released by high-temperature alkali treatment.[7] While being esterified to carbohydrates and etherified to lignin, ferulates make bridges between carbohydrates and lignin; these enhance the mechanical properties of the lignified cell walls and also contribute to digestibility decrease.[9,14] Ferulic and *p*-coumaric acid contents were higher in timothy stems than in leaf sheaths (Tables 4 and 5). The amount of ferulic acid in timothy cell walls increased during development in stems, and thus in stem + leaf sheath (Tables 4−6), correlating negatively with digestibility (Figure 3d; Table 3). This observation suggests that ferulates were continuously incorporated into the cell wall of stems leading to formation of di- and oligoferulate bridges as well as carbohydrate−lignin complexes.[14] Also, *p*-coumarate is associated with lignins, mainly esterified to S units.[14,41] In timothy, the level of cell wall-located *p*-coumaric acid rose slightly (ca. 1.1 times) in stems during development when S units increased (Table 4), suggesting that *p*-coumarates were at least partly coupled to S units. In leaf sheaths, however, no change in *p*-coumarate or ferulate concentration occurred during maturation (Table 5). This observation is interesting as ferulate cross-linking often correlates with reduced digestibility.[9,14] In maize, the concentrations of both ferulate esters and ferulate ethers increase between immature and mature (silage stage maturity) samples in both stems and leaf sheaths.[40] We do not know whether there are differences in the CuO procedure in the effectiveness of releasing of the hydroxycinnamic acids from the AIR of leaf sheaths and stems. However, none of the methods presently used in lignin analyses gives the absolute amounts of lignin subunits. The observation that the CuO method used in the present study gave repeatable results with different plant organs (leaf sheaths and stems, respectively) justifies making comparisons between these organs and the developmental stages. The results indicate that the ferulic acid content alone, and the cross-linking to arabinoxylans do not explain the differences observed in the digestibility of stems and leaf sheaths. A pronounced difference exists in cell wall-located *p*-coumarates in stems of timothy, a C3 plant, and maize, a C4 plant, because in maize stem the amount of *p*-coumarate esters increase by 2.6-fold during maturation,[40] whereas in maize leaf sheaths the situation is similar to that of timothy (1.2-fold increase in maize; no change in timothy). *p*-Coumarates in lignin are known to exist as terminal pendant groups; thus, the growth of the lignin polymer is restricted after incorporation of *p*-coumarylated S units.[6]

Table 4. Lignin Subunit Composition and the Concentration of Hydroxycinnamic Acids in Stems of Timothy Clones of High and Low Digestibility at Three Phenological Stages (Stage 45, Boot Swollen; Stage 56, Three-fourths of the Inflorescence Emerged; Stage 58, Base of the Inflorescence Just Visible) As Determined by the CuO Method

digestibility group[a]	phenological stage	total S units, %	total G units, %	total P units, %	S/G	*p*-coumaric acid, μmol g^{-1} AIR[b]	ferulic acid, μmol g^{-1} AIR	CuO oxidation products, mg g^{-1} AIR
	45	58 a	37	4.8 b	1.5	48 a	57 a	17 a
	56	60 ab	36	4.6 b	1.7	46 a	52 a	21 b
	58	62 b	35	3.2 a	1.8	54 a	89 b	26 c
SEM[c]		0.9	0.8	0.28	*d*	2.5	3.3	1.1
high		60	35	4.5	1.7	47	65	19
low		59	37	3.9	1.6	52	67	23
SEM		0.8	0.8	0.26	–	2.8	2.7	1.3
P values	phenological stage	0.040	0.108	0.005	0.086	0.049	<0.001	<0.001
	digestibility group	0.32	0.22	0.18	0.26	0.27	0.55	0.064
	stage × group	0.50	0.44	0.79	0.46	1.00	0.53	0.51

[a]In relation to stem proportion. [b]Alcohol-insoluble residue. [c]Standard error of the mean. [d]Logarithmic tranformation used; SEM not presented. Values marked with the same letter do not differ (Tukey's test).

Table 5. Lignin Subunit Composition and the Concentration of Hydroxycinnamic Acids in Leaf Sheaths of Timothy Clones of High and Low Digestibility at Three Phenological Stages (Stage 45, Boot Swollen; Stage 56, Three-fourths of the Inflorescence Emerged; Stage 58, Base of the Inflorescence Just Visible) As Determined by the CuO Method

digestibility group[a]	phenological stage	total S units, %	total G units, %	total P units, %	S/G	*p*-coumaric acid, μmol g^{-1} AIR[b]	ferulic acid, μmol g^{-1} AIR	CuO oxidation products, mg g^{-1} AIR
	45	43 a	52 b	4.5	0.8 a	35	43	23
	56	50 b	45 a	4.7	1.1 b	31	33	24
	58	54 b	42 a	3.8	1.3 b	34	43	25
SEM[c]		1.3	1.5	0.37	0.05	2.0	3.6	1.0
high		50	46	4.7	1.1	36	42	23
low		49	47	4.1	1.0	31	38	26
SEM		1.1	1.2	0.40	0.04	2.3	3.5	1.0
P values	phenological stage	<0.001	0.001	0.14	<0.001	0.22	0.099	0.21
	digestibility group	0.45	0.32	0.35	0.20	0.18	0.48	0.098
	stage × group	0.57	0.65	0.97	0.45	0.87	0.93	0.90

[a]In relation to stem proportion. [b]Alcohol-insoluble residue. [c]Standard error of the mean. Values marked with the same letter do not differ (Tukey's test).

Digestibility Values. The effect of the phenological stage was evident for tiller dry weight, stem height, NDF, IVOMD, and *D* value of the stem (including leaf sheath) in the clonal material (Table 7). The clones of the high-digestibility group had lower NDF and higher IVOMD and *D* values. The differences between digestibility groups in IVOMD and *D* value were more evident at the later development stage 58 than at the earlier stage 45. Surprisingly, similar interaction (stage × group) was not found in the lignin subunit composition analysis (Table 6). In general the measured NDF values of the stem fraction were similar to those of early and late maturing timothy cultivars[22] and logically higher than determined for the total DM yield of timothy.[27] Furthermore, the observed IVOMD in stems including leaf sheaths were markedly higher than in the results given by Nissinen and co-workers[24] even for stage 58 tillers of the low-digestibility group. The NDF concentration and stem height correlated strongly with the decrease in IVOMD (Table 3) referring to the role of fiber fraction (NDF) as mechanical support needed for higher stems.

There were clear differences in the lignin concentration and its subunit composition in stems and leaf sheaths of timothy clones. Unfortunately, in the IVOMD analyses these plant parts were not separated. When IVOMD was analyzed separately in stems and leaf sheaths for the clonal material collected in the following year (2012) from the same plots at stage 58, the leaf sheaths had lower digestibility (663 vs 650 g kg^{-1} DM, P = 0.020) and higher ADL lignin concentration (53 vs 62 g kg^{-1} DM, P = 0.020) than stems. Similarly, in cv. Tammisto II collected in 2011, the leaf sheaths had lower digestibility than stems (data not shown). This is probably due to higher lignin concentration in leaf sheaths than in stems. However, the yields of CuO oxidation products obtained from leaf sheaths at all developmental stages studied were approximately at the same level as in stems at stage 58, when digestibility had already decreased (Tables 4, 5, and 7). This finding suggests that lignin linkage structure in leaf sheaths is not optimal for digestion even at an early development stage.

Table 6. Lignin Subunit Composition and the Concentration of Hydroxycinnamic Acids in Stems + Leaf Sheaths of Timothy Clones of High and Low Digestibility at Three Phenological Stages (Stage 45, Boot Swollen; Stage 56, Three-fourths of the Inflorescence Emerged; Stage 58, Base of the Inflorescence Just Visible) As Determined by the CuO Method

digestibility group[a]	phenological stage	total S units, %	total G units, %	total P units, %	S/G	*p*-coumaric acid, μmol g^{-1} AIR[b]	ferulic acid, μmol g^{-1} AIR	CuO oxidation products, mg g^{-1} AIR
	45	52 a	44 b	4.6 b	1.3 a	43 ab	52 a	19 a
	56	56 b	39 a	4.6 b	1.5 b	41 a	45 a	22 b
	58	59 b	38 a	3.4 a	1.6 b	46 b	72 b	26 c
SEM[c]		0.8	0.8	0.22	0.05	1.6	2.3	0.8
high		56	39	4.5	1.5	43	56	20
low		55	41	3.9	1.4	44	57	24
SEM		0.7	0.7	0.23	0.04	1.7	2.1	0.8
P values	phenological stage	<0.001	<0.001	0.003	<0.001	0.042	<0.001	<0.001
	digestibility group	0.30	0.099	0.13	0.090	0.59	0.85	0.021
	stage × group	0.47	0.52	0.76	0.32	0.92	0.50	0.76

[a]In relation to stem proportion. [b]Alcohol-insoluble residue. [c]Standard error of the mean. Values marked with the same letter do not differ (Tukey's test).

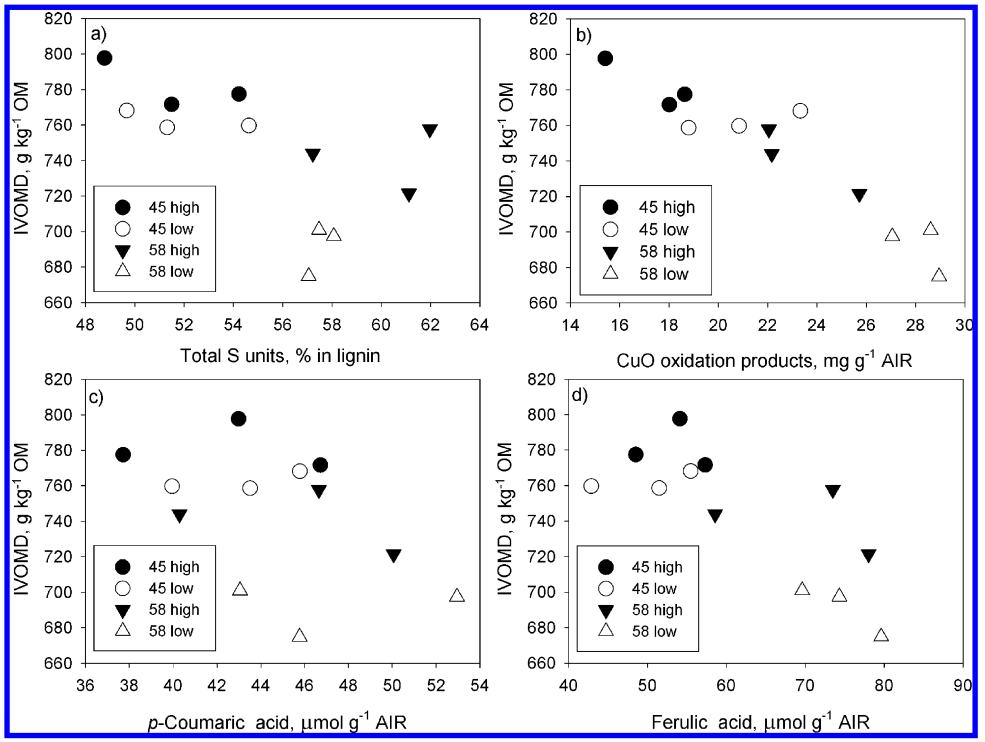

Figure 3. Relations between IVOMD (g kg^{-1} OM) and (a) amount of S units in lignin, (b) total yield of CuO oxidation products, (c) *p*-coumaric acid concentration, and (d) ferulic acid concentration in stems + leaf sheaths at different phenological stages (stage 45, boot swollen; stage 58, base of the inflorescence just visible) in high- and low-digestibility groups of timothy.

Summary. Lignin concentration in cell walls of timothy is the factor that explains the digestibility difference between clones of low and high digestibility. The developmental-associated decrease in digestibility correlates with the change in lignin subunit composition, and especially with the change in lignin linkage structure as shown by the increase in the yields of CuO oxidation products indicative of noncondensed β-O-4 linkages. In addition, the increase in the content of cell wall-located hydroxycinnamates contributes to the digestibility decrease during maturation. The difference in the lignin amount and the subunit composition between stems and leaf sheaths is interesting, as leaf sheaths have lower digestibility than stems. This is probably due to the fact that leaf sheaths contain more lignin than stems, but the lignin linkage structure may also contribute to this. Interestingly, there are less cell wall-bound hydroxycinnamic acids in leaf sheaths compared with stems. Considering that leaf sheaths, which consist of ca. one-third of the whole stem (stem including leaf sheath), are less digestible than stems, some goals in the future breeding efforts of timothy could include a reduction of the leaf-sheath proportion in the yield or a targeted lowering of the lignin concentration in leaf sheaths to the level of that in stems. It should be noted, however, that cell wall composition (lignin among other factors) in leaf sheaths is important in resistance

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Table 7. Tiller Dry Weight, Stem Height and Contents of Neutral Detergent Fiber (NDF), in Vitro Organic Matter Digestibility (IVOMD), and Digestibility (*D*) Value of Timothy Clones (Stem Including Leaf Sheath) of High and Low Digestibility at Two Phenological Stages (Stage 45, Boot Swollen; Stage 58, Base of the Inflorescence Just Visible)

digestibility group[a]	phenological stage	tiller dry weight, mg tiller^{-1}	stem height, cm	NDF, g kg^{-1} DM	IVOMD, g kg^{-1} OM	*D* value, g kg^{-1} DM
high	45	382	35	641	782	724
low	45	296	36	660	762	703
high	58	606	47	670	741	686
low	58	652	52	695	691	640
SEM[b]		38.1	1.8	3.6	7.9	7.4
P values	phenological stage	<0.001	<0.001	<0.001	<0.001	<0.001
	digestibility group	0.67	0.22	<0.001	0.026	0.024
	stage × group	0.107	0.17	0.41	0.031	0.045

[a]In relation to stem proportion. [b]Standard error of the mean.

toward insects and diseases,[42] and against lodging, so the effects of the lignin decrease need to be evaluated in ways that also consider these aspects.

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ABBREVIATIONS USED

AcBr, acetyl bromide; ADL, acid detergent lignin; AIR, alcohol-insoluble residue; CuO, alkaline cupric (II) oxidation method; *D* value, digestibility value; DM, dry matter; G, guaiacyl unit; H, *p*-hydroxyphenyl unit; IVOMD, in vitro organic matter digestibility; NDF, neutral detergent fiber; OM, organic matter; S, syringyl unit; *S*/*G*, syringyl/guaiacyl ratio

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