African Journal of Agricultural Research Vol. 7(2), pp. 257-264, 12 January, 2012
Available online at http://www.academicjournals.org/AJAR
DOI: 10.5897/AJAR11.1485
ISSN 1991-637X ©2012 Academic Journals

Full Length Research Paper

Changes in lignin structure with maturation of alfalfa leaf and stem in relation to ruminants nutrition

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Accepted 16 December, 2011

In the present investigation, the major chemical constituents in leaf and stem of alfalfa (*Medicago sativa* L.) *cv* Elena at three different stages of maturity were studied. The experiment was designed as a randomized block replicated three times. During growth and development, crude protein content decreased and cell wall constituents (Neutral Detergent Fiber-NDF, Acid Detergent Fiber-ADF and ADL-Acid Detergent Lignin) increased in both plant anatomical fraction of alfalfa. Lignin is a poorly characterized polymer and its exact properties vary depending on both the species of the plant and its location within the plant. Three stages of maturity taken from alfalfa leaf and stem were examined. The investigation was concentrated on the determination of chemical changes in the lignin during growth and development by the Attenuated Total Reflectance-Furior Transform Infrared (ATR-FTIR) spectrometric technique. The predominant component of lignin from alfalfa leaf was guaiacyl-type lignin. Despite the leaf, the predominant component of lignin from alfalfa stem was guaiacyl-syringyl-type lignin. A comparison between the signals from lignin in different development stages revealed the appearance of new peaks, which are indications of new bonds and changes in the structure of the lignin.

Key words: Alfalfa, cell-wall, lignin, attenuated total reflectance-furior transform infrared (ATR-FTIR) spectroscopy.

INTRODUCTION

Forages represent the major feed source for cattle production systems, and improvement in cell wall degradability is an important goal of many plant-ruminant animal research programs (Morrison et al., 1998). Alfalfa (*Medicago sativa* L.) is a valuable forage crop for ruminant livestock in all temperate regions of the world and is generally regarded as one of the best forage crop for feeding ruminant animals because of its perceived high nutritive value (Schnurr et al., 2007). Alfalfa leaves are protein-rich and low in cell wall concentration and therefore, highly digestible. In contrast to leaves, stems exhibit low digestibility as a result of high concentrations of cell wall polysaccharides and lignin (Jung and Lamb, 2006). Lignin is an important chemical component of forage cell walls; however, it is essentially undigestible and inhibits rumen fermentation of forage cell wall polysaccharides. Lignification of cell walls during plant development has been identified as the major factor limiting digestibility-degradability of forage crops (Kondo et al., 1998). Lignin in forages is composed of guaiacyl (G) units derived from coniferyl alcohol, syringyl (S) units derived from sinapyl alcohol, and p-hydroxyphenyl (H) units derived from p-coumaryl alcohol (Jung, 1989),

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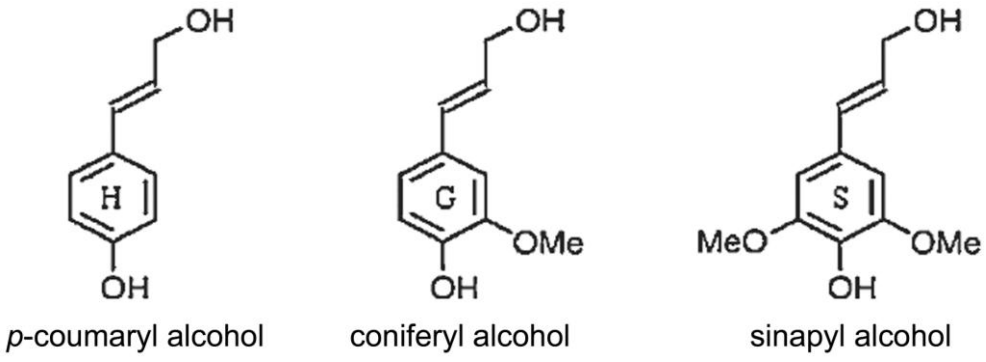

Figure 1. The monolignols.

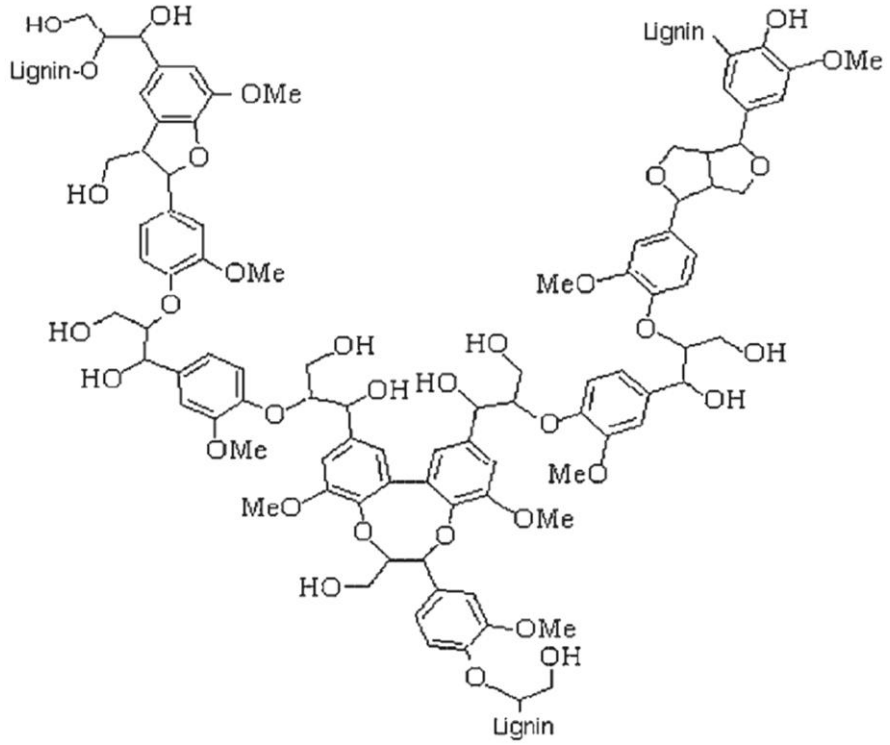

Figure 2. Fragment of the hypothetic structural formula of lignin.

(Figure 1). In addition to lignin content (or concentration), the composition of lignin is an important factor that influences cell wall degradability of forages (Chen et al., 2002). It has also been suggested that the anatomical structure of cells and tissues in legumes may be more important than wall chemistry in determining the rate and extent of fiber digestion *in vivo*, because anatomical structure significantly influences wall accessibility to rumen microorganisms (Wilson and Hatfield, 1997). These aromatic building units are linked with a variety of ether and carbon-carbon bonds. The predominant linkage is the so-called β-O-4 linkage. About 40 to 60% of all inter-unit linkages in lignin is via this ether bond. Different types of linkages between phenyl propane units from three-dimensional structure and makes it difficult to completely degrade non-regular lignin macromolecules (Figure 2).

Because of structural complexity there are no uniformic knowledges about chemical and physical structure of this polymer and it is possible to consider only chemical lignin models. These models are based on analysis of degradation products from lignins. Such structural models with different amount of phenyl propane units having good agreement with the various analytical data in the lignin chemistry were proposed (Sakakibara, 1980). Recent investigations (Åkerholm and Salmén, 2001)

Table 1. Sampling of alfalfa (*Medicago sativa* L.) for evaluation.

Sample	Stage	Average monthly temperature/rainfall	Botanical phenophases
1 (June 17)	I		Young growth; standing height 35-40 cm, formation of flower buds
2 (June 24)	II	21.4°C/37 mm	Standing height 50-55 cm, beginning of blooming up to full blooming
3 (July 01)	III		Standing height 80-85 cm, full blooming

pointed out the existence of some organization of lignin macromolecules in cell wall. Raman microprobe studies of secondary walls in black spruce revealed that phenyl rings of lignin are aligned preferentially in the plane of the cell walls and this observation was supported by dynamic FTIR spectroscopy investigations (Derkacheva and Sukhov, 2008). We assume that ATR-FTIR spectroscopy is non-destructive and the most informative method of lignin investigation. The method opens perspective to find structural discrepancies in lignins isolated by different methods and from different plant material. Molecular spectra see differences in chemical structure and physical organization of different lignins that it is invisible for other analytical methods. There are many articles devoted to ATR-FTIR spectra of lignins but full band interpretations are under discussion (Besle et al., 1994; Chen et al., 2002; Grabber et al., 1997; Martinez et al., 1999).

Characterization of lignin is an extremely difficult task because of their diversity in respect both to source and extraction method. The heterogeneity of lignin is due to the changes in polymer composition, shape and size of the morphological units, crosslinking, nature of the functional groups, linkage types between various units such as phenylpropanoic, p-hydroxyphenyl, guaiacyl, syringyl, etc. FTIR spectroscopy has been used to identify lignins of different origin, to estimate the lignin / hemicellulose and guaiacyl / syringyl ratio, to study changes in lignin during maturation. In the present study, ATR-FTIR spectrometry was used as a structural, non-destructive, and simple tool to qualitatively analyze the chemical structure of lignins isolated from leaf and stem of alfalfa in different stages of plant development.

MATERIALS AND METHODS

The cultivar of alfalfa was grown at the Institute for forage crops in Kruševac (21°19`35``E, 43°34`58``N, elevation 166 m), central region of Serbia, as three field replicates in a randomized complete block design. Soil type was with an organic matter content of approximately 3.5% and a pH of 6.5. Alfalfa (*M. sativa* L., Elena variety) forage was evaluated at three different stages in 2008. The first regrowth (II) included samplings on June 17, June 24 and July 01, respectively. A description of sampling times and conditions is indicated in Table 1.

Plants from a pure stand were cut manually with scissors about 5 to 7 cm above the soil surface, and separated into leaves and stems. Samples were dried to constant weight at 65°C for 48 h and dried samples were ground through a screen size of 1 mm. All samples were analyzed for crude protein (CP), neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL). All analysis were done in duplicate and data were corrected to the dry matter basis by determining 105°C dry matter, overnight. Crude protein content were calculated by multiplying N contents by a coefficient of 6.25. NDF and ADF were determined applying the method of Van Soest et al. (1991). Briefly, NDF was measured by extraction of 0.5 g sample with 100 ml of boiling neutral detergent solution for 60 min. The residue was collected by filtration through a coarse porosity Gooch crucible, washed with hot water, rinsed with acetone and dried at 100°C overnight. ADF was measured by extraction of 1.0 g sample with 100 ml of boiling acid detergent solution for 60 min. The ADF was then collected by filtration through the crucible. The ADF, after washing with hot water and rinsing with acetone, was dried overnight at 100°C.

ADL was determined as lignin insoluble in 72% sulfuric acid, applying the method of Van Soest et al. (1991). Briefly, ADL was isolated by filling the crucible containing the ADF with 12 M sulfuric acid and allowing it to drain from the crucible. Additional acid was added periodically over the 3 h room-temperature hydrolysis. The acid insoluble residue was collected by filtration and extensively washed with hot water. A final acetone rinse was used prior to drying the sample overnight at 100°C. The ADL content was determined as the difference in weight of the residue before and after ashing at 450°C. The experiment was designed as a randomized block arrangement of treatments replicated three times. Effects were considered different based on significant ($P< 0.05$) F-ratio. The significance of differences between arithmetic means was tested by LSD test. FTIR-ATR spectra were recorded of lignin with the FTIR spectrometer NIKOLET, 6700 FT-IR, Crystal Diamond at a resolution of 4 cm^{-1} for 32 scans in the range from 600 to 4000 cm^{-1}. The samples were pressed against the diamond crystal of the ATR device. Spectral data were evaluated using OMNIC, Macros / Basic Version 7.3 SP1 software in the fingerprint region of 1800 to 600 cm^{-1}.

RESULTS

Cell wall composition of alfalfa leaf and stem

The cell-wall composition of different botanical fractions of alfalfa harvested at three different development stages is presented in Table 2. The results of this investigation indicated that the crude protein concentration declined in leaves and stem with plant maturation. Alfalfa leaf contained more crude protein than stem at all sampling dates. Variables differed ($P<0.05$) between treatments. With growth and development crude protein concentration decreased after the first stage by 5.75% in leaves and 3.73% in stems, and after the second stage by 14.61% in leaves and 3.16% in stems.

Table 2. Chemical composition of leaves and stems of alfalfa in different stages of maturity (g kg^{-1} of dry matter).

Sample	Leaf			Stem		
	(June 17)	(June 24)	(July 01)	(June 17)	(June 24)	(July 01)
Stage	**I**	**II**	**III**	**I**	**II**	**III**
CP	351.4^{a}	331.2^{b}	282.8^{c}	147.6^{a}	142.1^{b}	137.6^{b}
NDF	207.9^{b}	216.1^{ab}	222.3^{a}	520.0^{c}	577.2^{b}	616.0^{a}
ADF	150.0^{c}	164.5^{b}	201.7^{a}	449.5^{b}	459.9^{b}	552.5^{a}
ADL	28.5^{c}	41.5^{b}	52.5^{a}	84.4^{b}	110.7^{a}	115.5^{a}

The values are arithmetic means (n = 2) on a dry matter basis; CP-Crude Protein, NDF-neutral detergent Fiber, ADF-acid detergent fiber, ADL-acid detergent lignin. Different letters within a row denote significantly different means ($P<0.05$).

The results of this study show that contents of cell walls increased differently in plant organs of this legume species as plant age. During maturation NDF increased from 207.9 to 222.3 g kg^{-1} of DM in leaves and from 520.0 to 616.0 g kg^{-1} of DM in stems ($P<0.05$). The highest content of NDF was recorded in stems in the third stage of development (Table 2). Concentrations of ADF increased as the plant matured in leaves ($P<0.05$) and stems. Stems contained 3 times more ADF than leaves at the first development stage and 2.74 times more than leaves at the third development stage. The results of this study show that ADF increased from 150.0 to 201.7 g kg^{-1} of DM in alfalfa leaf and from 449.5 to 552.5 g kg^{-1} in alfalfa stem. The highest increase of ADF content was recorded in stems (92.6 g kg^{-1} of DM after the second development stage). Overall, as alfalfa matured, lignin increased in both investigated anatomical fractions. Much higher concentration of ADL was recorded in alfalfa stem (from 84.4 to 115.5 g kg^{-1} of DM) than in leaves (from 28.5 to 52.5 g kg^{-1} of DM).

Interpretation and characterization of lignin spectra

All spectra show typical lignin patterns, although some differences in the intensities and widths of the absorption bands are observed. All spectra have a strong width band between 3500 to 3100 cm^{-1} assigned to OH stretching vibrations. The band is caused by presence of alcoholic and phenolic hydroxyl groups involved in hydrogen bonds. The absorption bands located between 3100 to 2800 cm^{-1} are caused by vibrations of CH_2- and CH_3- groups. Spectra of lignin show no absorption bands in the 2800 to 1800 cm^{-1} wavenumber range (Hergert, 1971).

In the spectrum of ADL from the first development stage of alfalfa leaf (Figure 3a) there are: peak at 1728.5 cm^{-1} which shows carbonyl stretching in lignins and carboxyl acid esters; peak at 1462.9 cm^{-1} represents substituted CH_2- and CH_3- aromatic skeletal vibration, guaiacyl type; peak at 1165.2 cm^{-1} shows CH- in plane deformation, guaiacyl type; peak at 1031.8 cm^{-1} also represents aromatic CH-deformation, guaiacyl type and peaks at 870.5 and 722.0 cm^{-1} show aromatic skeletal CH- deformation. In the spectrum of ADL from the second development stage of alfalfa leaf (Figure 3b) there are: peak at 1728.2 cm^{-1} represents C=O stretch in unconjugated ketones, carbonyls and in ester groups, frequently of carbohydrate origin; peak at 1031.2 cm^{-1} shows aromatic skeletal vibrations in plane deformation-guaiacyl type; peak at 785.7 cm^{-1} shows aromatic C-H in plane deformation, guaiacyl type.

The spectrum of ADL from alfalfa leaf (the third stage of development) shows similar lignin structure. An intensive signal at 1729.8 cm^{-1} represents unconjugated C=O stretching; peak at 1463.0 cm^{-1} shows C-H deformation, asymmetric in CH_3- and CH_2-, guaiacyl type; peak at 1161.3 cm^{-1} shows aliphatic COC deformation and C-H in plane deformation, guaiacyl type; peak at 1035.8 cm^{-1} shows aromatic C-H in plane deformation, guaiacyl type and peak at 721.5 cm^{-1} also shows aromatic C-H in plane deformation. In the spectrum of ADL from the first development stage of alfalfa stem (Figure 4a) there are: the peak representing aromatic skeletal vibrations at 1602.8 cm^{-1} is relatively weak; the signal at 1455.4 cm^{-1} represents asymetric C-H vibrations, guaiacyl-syringyl type; the signal at 1727.7 cm^{-1} indicates existence of unconjugated C-O-C vibration in xylan, and peak at 1031.3 cm^{-1} is from C-O-C vibration in cellulose and hemicellulose, guaiacyl type. Signal at 860.0 cm^{-1} shows aromatic C-H deformation out of plane, syringyl type. Peak at 1166.7 cm^{-1} represents p-coumaryl units in lignin and signal at 1493.7 cm^{-1} shows aromatic skeletal vibration, guaiacyl-syringyl type. The spectrum of ADL from alfalfa stem (the second stage of development) shows similar lignin structure (Figure 4b). Signal at 1455.4 cm^{-1} represents asymmetric C-H vibration, guaiacyl-syringyl type. The signal at 1600.8 cm^{-1} indicates existence of aromatic skeletal vibration, guaiacyl-syringyl type. Peak at 1160.0 cm^{-1} is from plane C-H deformation, syringyl type. Peak at 1727.4 cm^{-1} represents unconjugated C=O vibrations in xylan and peak at 1032.1 cm^{-1} indicates existence of C-O-C deformation in cellulose and hemicellulose, guaiacyl type. Peak at 1032.1 cm^{-1} shows aromatic C-H out of plane

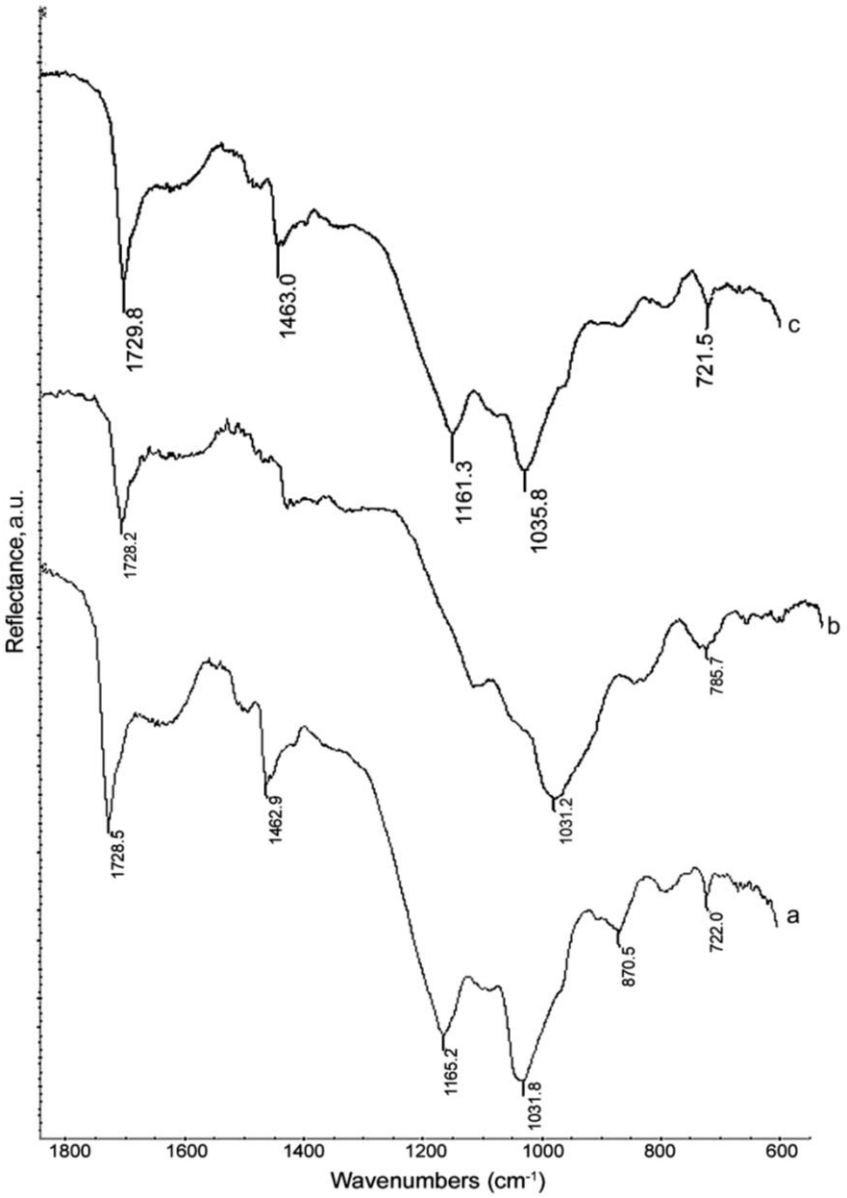

Figure 3. ATR-FTIR spectra of lignin from alfalfa leaf, a) the first stage of development, b) the second stage of development and c) the third stage of development.

deformation, guaiacyl-syringyl type.

In the spectrum of ADL from the third development stage of alfalfa stem (Figure 4c) there are: peak at 1730.9 cm^{-1} which represents unconjugated C=O vibration in lignin and carboxylic acid esters. The signal at 1463.4 cm^{-1} indicates existence of asymetric C-H deformation, syringyl type. Signal at 1167.3 cm^{-1} shows p-coumaryl units in lignin. Relatively strong signal at 1037.6 cm^{-1} represents plane aromatic skeletal vibrations and primary hydroxyl group in β-hydroxy coniferyl alcohol. Signal at 863.8 cm^{-1} shows aromatic C-H out of plane deformation, syringyl type and peak at 722.4 cm^{-1} also shows aromatic C-H out of plane deformation, syringyl type.

DISCUSSION

Cell wall components

Plant cell walls are the major source of dietary fiber for animals. Polysacharides in cell walls cannot be degraded by mammalian enzymes. Instead, animals depend on microbial fermentation and ruminants are especially well-adapted for using plant fiber for energy. Stems of most plant species have a greater fiber concentration and less crude protein content than do leaf blades. In the investigation by Stanisavljević et al. (2008) a high positive correlation (r=0.962) was detected between crude protein

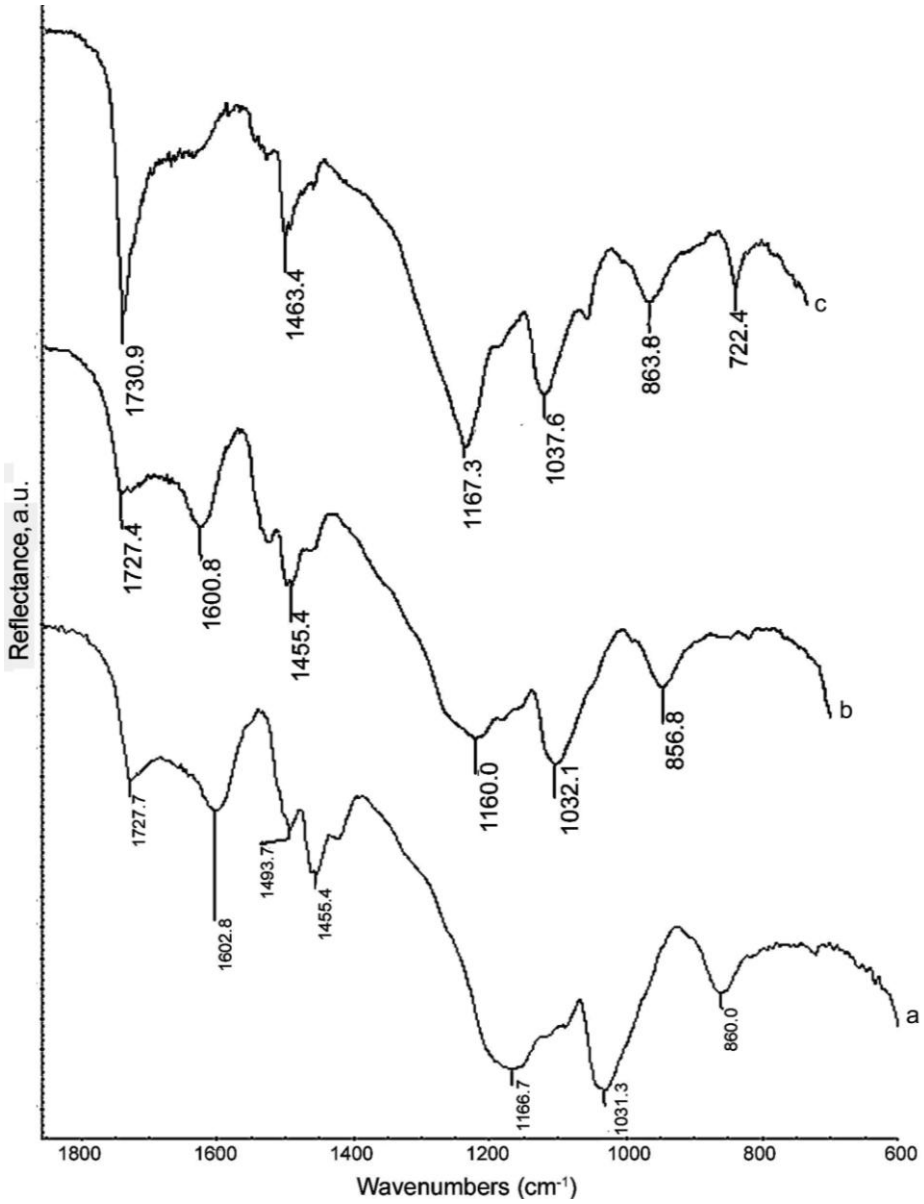

Figure 4. ATR-FTIR spectra of lignin from alfalfa stem, a) the first stage of development, b) the second stage of development and c) the third stage of development.

content and leaf proportion. Fiber concentration also increases as plants mature, which is the most important factor affecting dry matter digestibility. Neutral detergent fiber accounts for about 25% of legume leaf-blade mass in alfalfa and red clover when these species are at the mid flowering stage of maturity (Buxton and Redfearn, 1997). This compares with 40 to 55% NDF in stems of these species.

ATR-FTIR spectroscopy of lignin

FTIR spectroscopy is a further method to characterize lignin and lignin derivatives. Absorption bands, which arise in the lignin spectra can be assigned to different structural groups (Fengel and Wegener, 2003). However, careful assignments of these bands are a prerequisite for useful results (Hergert, 1971). In ligno-cellulose assessments of the lignin, concentration as well as information on lignin molecular composition and inter-unit linkages are possible employing FTIR spectroscopy (Faix, 1988; Martinez et al., 1999). Stem tissue has been considered as the preferred material for studying lignin and degradability in forage grasses and legumes (Vailhe et al., 2000). Structural changes in stems during development were associated with the increase in lignin content and the decrease of degradability. The effects of plant maturity on lignin and forage digestibility have been

reported in different grass species (Besle et al., 1994). A dramatic increase in lignin content in developing tall fescue stems was observed, and this change was closely related to the decrease in degradability. Research on lignin structures in grasses has been largely on the effects of ferulic acid and cross-linking (Casler and Jung, 1999; Besle et al., 1994; Hatfield et al., 1999). Little information is available on developmental changes of H, G, and S lignin components in forage grasses. Chemical analysis of developing tall fescue stems revealed that H lignin remained at low levels at different developmental stages; thus, it probably does not have much effet on degradability. S lignin and S / G ratio were negatively correlated with degradability.

However, this does not necessarily mean that G lignin is more digestible than S lignin. Grabber et al. (1997) showed that H, G, and S lignin have similar inhibitory effects on wall degradability in maize. Because forage quality is mainly influenced by lignification at the later developmental stage, it would be wise to design strategies to reduce S lignin biosynthesis to improve forage quality. The spectra of lignin from alfalfa leaf were similar. In the spectrum of lignin from alfalfa leaf, a guaiacyl ring breathing signal was observed. Despite the leaf, in the spectra of lignin from alfalfa stem, guaiacyl and syringyl ring breathing signals were registered. The spectra of lignin showed syringyl and condensed guaiacyl units from the first to the third stage of alfalfa stem development. All spectra of ADL from leaf and ADL from stem of each stage are similar in frequency of absorption bands, but different in their intensities. However, quantity of lignin types in leaf and stem of each stage is different which is observed from different intensities of the same absorption bands.

Stems differ from leaf blades in that their tissue characteristics change greatly with age. This change is reflected in a change in digestibility, which can be as high or higher than that of leaves when young, but declines at a faster rate than blade or sheath to be much lower when old. In legumes, the proportion of lignified tissue continues to increase over time as the stem develops in size. Stems and leaves of immature legume are of similar digestibility, but with advancing maturity, stem digestibility decreases markedly, while that of leaf changes a little. He and Terashima (1991) reported that the phenolic units (hydroxyphenyl-, guaiacyl- and syringyl- propane) varied in the different regions and differed with maturity. The secondary wall which is adjacent to the lumen and therefore, most susceptible to microbial colonization, has a relatively high amount of syringyl units. Syringyl units are less degraded by microorganisms than are the other lignin units.

Conclusions

The spectral data of lignin from alfalfa leaf and stem indicate changes of peaks in fingerprint region at different stages of growth. Comparison between the peaks from different development stages revealed the appearance of new spectral signals. Syringyl lignin content and syringyl / guaiacyl lignin ratio increased in lignin from alfalfa stem when plants matured.

This investigation showed that C-H deformations in plane and out of plane – guaiacyl type were dominated in alfalfa leaves, whereas C-H deformations in plane and out of plane – guaiacyl / syringyl and syringyl type were dominated in alfalfa stems. Guaiacyl lignin was deposited at the early stage of plant growth and syringyl lignin was preferentially deposited at the later developmental stages and in the stem.

ACKNOWLEDGEMENTS

The authors are grateful to other colleagues from the Institute for Forage Crops, Kruševac and other members of the Department of Chemistry of the Institute of Chemistry, Technology and Metalurgy, University of Belgrade for facilities and assistance. This work was in part supported by the Ministry of Science of the Republic of Serbia by project 20048.

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