Size Exclusion Chromatography of Cotton Stalk Lignins Isolated from Rumen Digesta and Feces of Sheep

Daniel Ben-Ghedalia,*,† Edith Yosef,† Ran Solomon,† Joshua Miron,† Aloys Huttermann,‡ Andrzej Majcherczyk,‡ and Oleg Milstein‡

The Metabolic Unit, Institute of Animal Science, ARO, The Volcani Center, Bet Dagan 50250, Israel, and Forstbotanisches Institut, Universitat Gottingen, D-3400 Busgenweg 2, Germany

Sheep were fed two rations based on cotton stalks (CS) and ozonated cotton stalks (OCS). Lignins from these materials and the appropriate rumen liquor and feces were isolated and examined by high-performance size exclusion chromatography (HPSEC). Dioxane lignins (DL) isolated from ball-milled plus cellulase-pretreated neutral detergent fiber of OCS were more dispersive and had a higher weight-average molecular weight (MWt) than the DL isolated from CS. The carbohydrate content of both CS and OCS DL was about 5%; xylose, uronic acid, and glucose were the major sugars. The patterns of molecular weight distribution of DL in CS and its respective fecal material were similar. This was not the case for OCS, in which the fecal DL was lower in MWt and consisted of a larger proportion of low molecular weight lignins. Water-soluble lignins (WSL) were isolated from the water extract of CS and OCS and from the respective rumen liquor and fecal materials. On both rations, rumen WSL were remarkably more dispersive and had a much higher MWt than the WSL isolated from the respective CS and OCS materials. The proportion of the high molecular weight fractions of WSL was markedly higher for rumen liquor than for feces on both rations. Cell wall degradation in the rumen probably is due to the removal into solution of matrix polymers, rather than monomer degradation.

INTRODUCTION

The distribution of molecular weight has been determined for straw lignins (Scalbert and Monties, 1986; Kondo et al., 1992) and in various wood lignins (Faix et al., 1981; Milstein et al., 1990). Little information is available on lignins isolated from forages and sheep digesta and feces and almost none on cotton stalk lignins. Cotton stalks (CS) may contain more than 20% lignin and are considered a poor forage for ruminants (Shefet and Ben-Ghedalia, 1982). However, treating CS with ozone increased its digestibility (Ben-Ghedalia and Shefet, 1983) and its conversion into productive feed (Solomon et al., 1992). About 50% of the lignin in CS is degraded during ozonation, mostly to organic acids (Ben-Ghedalia et al., 1982). Although very few monomeric phenolics are released following ozonation of CS (Yosef et al., 1994), the E_{280} of the water extract of ozonated cotton stalks (OCS) is increased (Ben-Ghedalia et al., 1980), probably due to the release of oligophenolic materials.

Because lignins and lignin–carbohydrate complexes frequently are mentioned as factors related to cell wall degradation in the rumen (Wallace et al., 1991; Cherney et al., 1992), we studied the effect of ozonation and sheep digestive processes on the size exclusion chromatography features of these materials.

The objectives of this study were (i) to determine the impact of digestion by sheep on the distribution of molecular weight of lignins isolated from neutral detergent fiber (NDF) of CS and OCS and (ii) to assess the high-performance size exclusion chromatography (HPSEC) features of water-soluble lignins (WSL) extracted from the CS and OCS and from rumen liquor and feces of sheep fed CS and OCS rations.

* Author to whom correspondence should be addressed.
† The Volcani Center.
‡ Universitat Gottingen.

MATERIALS AND METHODS

Cotton Stalks and the Feeding Trial. Cotton stalks chopped to pass through a 6-mm screen were moistened to 50%, placed in a 80 × 8 cm perspex column, and flushed with a stream of ozone gas generated from air by a Fischer ozonator (Bon-Bad Godesberg, Germany) until completely bleached, as described by Ben-Ghedalia et al. (1982). The final pH of the ozonated material was 2. The pH of the OCS was raised to 6.5 by adding a solution of ammonium hydroxide; this enriched the total crude protein of the stalks and made it acceptable to the sheep. This pH level was set to avoid losses of ammonia from the OCS and to prevent any alkali effect on the lignocellulose.

Two isonitrogenous rations (2.8% N) were formulated, consisting on a dry matter (DM) basis of 30% cotton stalks, either untreated or ozonated and ammoniated, plus a corn-based concentrate mixture including the required minerals and vitamins. Thus, ammonia was the major source of N in OCS and soybean in the CS ration.

Four Merino rams, cannulated in the rumen, were allocated randomly to the two rations in a 2 × 2 changeover design. The rams were kept in metablism cages in an air-conditioned animal house (23 °C) to allow sampling of rumen liquor and total feces collection. The daily ration, 800 g DM, was divided into 12 portions and delivered by automatic feeders at 2-h intervals to create steady-state conditions. Sampling of rumen contents (4 days) and total collection of feces (10 days) were started after 15 days of adaptation to the rations. Rumen digesta were sampled four times per day at different intervals, 25 mL per sampling, composited to one sample per ram per period, and stored at −20 °C until further processing. Samples of feces, taken from the daily total collection, were composited proportionately to one sample per ram per period and stored at −20 °C. Samples of feces were freeze-dried, ground to pass a 1-mm screen, and stored at −20 °C under nitrogen. One composite sample of rumen digesta and feces per treatment, made of samples from two rams of period I plus two rams of period II, were used for the isolation and characterization of WSL and lignin fractions.

Analytical Procedures. *Isolation of Water-Soluble Lignins (WSL).* CS and OCS ground to 1 mm were added to distilled water at 5 g/100 mL, boiled for 10 min, and filtered through Whatman No. 41 filter paper. Freeze-dried fecal material was used for water extraction of WSL. Ten grams of DM freeze-

0021-8561/94/1442-1160$04.50/0

dried fecal material was added to 100 mL of boiling distilled water and stirred for 10 min. The diluted fecal material and the rumen liquor were centrifuged at 30000*g* for 30 min. The straw filtrates and the fecal and rumen liquor supernatant fluids were used for the isolation of WSL; 3 mL of the filtrate or supernatant fluid was transferred to a 10-mL screw-capped test tube with 3 mL of 2 M NaOH and 2 mL of a 50 mM solution of *N*-methyltridecylammonium chloride (*N*-MTAC) in ethyl acetate. The tube was closed with a Teflon-sealed screw cap, shaken for 10 min, and recentrifuged for 10 min at 2000*g* to ensure completion of phase separation. One milliliter of the organic phase was transferred to a new tube and washed twice with an equal volume of 1% NaCl solution. Aliquots (0.4 mL) of the ethyl acetate phase containing the *N*-MTAC–WSL complexes were transferred to sample vials and evaporated overnight under vacuum at room temperature. The samples were dissolved in 1.10 mL of tetrahydrofuran, and 40 μL of the solution was injected into the HPLC column for determination of the molecular weight distribution.

Isolation of Dioxane Lignins (DL). Ground (1 mm) CS and OCS and the respective fecal samples were treated with neutral detergent according to the procedure of Van-Soest et al. (1991). The neutral detergent fiber (NDF) samples were ball-milled according to the method of Polcin and Bezuch (1978) for 14 days in a rotary porcelain ball-mill at 60 rpm, using 20-mm porcelain balls at a balls/NDF weight ratio of 50:1. The ball-milled NDF samples were subjected to an exhaustive cellulase (Novo) hydrolysis in citrate buffer, pH 4.8, at 50 °C for 96 h (Miron and Ben-Ghedalia, 1981). The residues were washed, centrifuged, and freeze-dried. Lignin was extracted from the freeze-dried residue by dioxane/water mixtures, using two consecutive 24-h extractions with a 95:5 mixture, followed by two consecutive 24-h extractions with a 50:50 mixture (Lai and Sarkanen, 1971; Chang et al., 1975). The extractions were performed using 1 g of residue to 15 mL of solvent, at room temperature, in darkness, and under N_2. The four extractions were combined into one composite extraction per sample, and the dioxane was removed by a rotary vacuum evaporator.

The dry extract was dissolved in 90% acetic acid in the proportion of 1 g of extract to 20 mL of acid; the acetic acid solution was added dropwise to water with stirring at a ratio of 1 mL of extract to 10 mL of water. The lignin precipitated in water was centrifuged, washed, recentrifuged, and freeze-dried. Yield of DL was determined as its proportional weight of permanganate lignin (Van Soest et al., 1991).

For the size exclusion chromatography determinations, the freeze-dried lignin was dissolved in N-NaOH and subjected to the procedures of *N*-MTAC complexation and ethyl acetate extraction as with the water-soluble lignins. The HPSEC system used for the determination of molecular weight distribution of WSL and dioxane lignins was developed by Majcheczyk as described by Milstein et al. (1990). The column system consisted of two Zorbax PSM 60S columns and one Zorbax PSM 1000S column (DuPont, Wilmington, DE) packed with silica and coupled in order of increasing pore size. The chromatography was run in an isocratic mode; the mobile phase was 0.02 M solution of *N*-methyltridecylammonium chloride in freshly distilled tetrahydrofuran at a flow rate of 1.2 mL/min. The HPLC system consisted of an HP 1090 with diode array detector and Pascal work station (Hewlett-Packard, Palo Alto, CA).

The phenolic component of the WSL and DL was used as an indicator for detecting the elution of the lignin at 280 nm. The number-average molecular weight (MN), the weight-average molecular weight (MWt), and the dispersity (MWt/MN) were calculated by the data processing system from area/time and MWt of each segment, as described by Faix et al. (1981); polystyrene standards in the range 800–2 500 000 (Waters) were used for calibration.

Monosaccharide composition of the DL isolates was determined after hydrolysis with 24 N H_2SO_4 for 1 h at 21 °C followed by 1 N H_2SO_4 for 5 h at 100 °C, as described by Miron and Ben-Ghedalia (1992). The free sugars were converted to alditol acetates and determined by gas–liquid chromatography (Blakeney et al., 1983). Uronic acids in the hydrolysates were determined colorimetrically (Blumenkrantz and Asboe-Hansen, 1973).

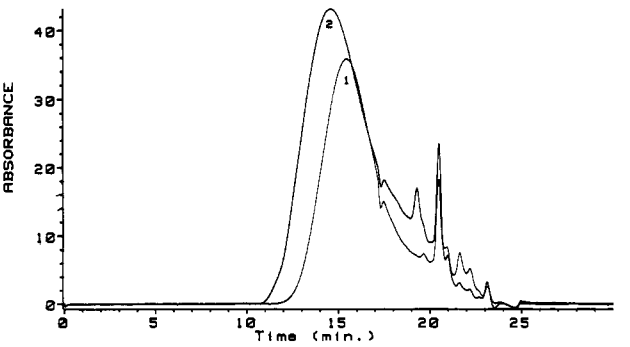

Figure 1. High-performance size exclusion chromatograms of dioxane lignins extracted from ball-milled plus cellulase-pretreated NDF of CS (1) and OCS (2); absorbance at 280 nm.

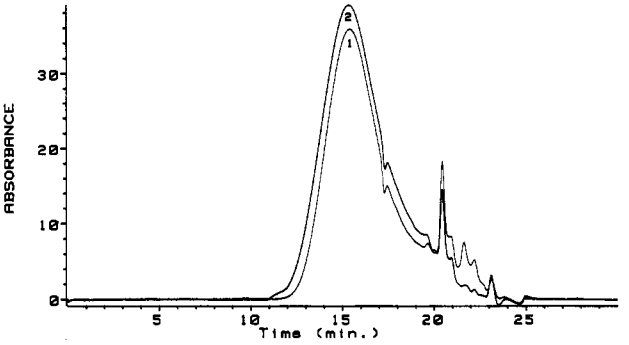

Figure 2. High-performance size exclusion chromatograms of dioxane lignins extracted from ball-milled plus cellulase-pretreated NDF of CS (1) and feces (2) of sheep fed the CS ration; absorbance at 280 nm.

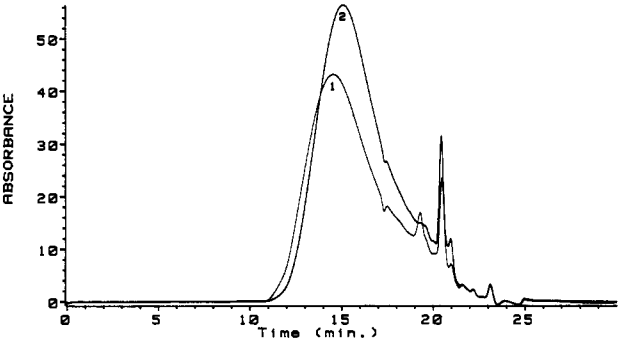

Figure 3. High-performance size exclusion chromatograms of dioxane lignins extracted from ball-milled plus cellulase-pretreated NDF of OCS (1) and feces (2) of sheep fed the OCS ration; absorbance at 280 nm.

RESULTS AND DISCUSSION

The focus in this study was on the determination of the molecular weight distribution of (i), dioxane lignins isolated from cotton stalks and from fecal NDF which had been pretreated with ball-milling (BM) plus cellulase and (ii) water-soluble lignins isolated from the water extract of CS and OCS and from the respective liquid phases of rumen liquor and feces. The yield of DL was around 20% of the actual permanganate lignin.

The size exclusion chromatograms of DL are shown in Figures 1–3, and the molecular weight data are given in Table 1. Figure 1 and Table 1 show that OCS-DL have a higher weight-average molecular weight (MWt) and are more dispersive than the CS-DL. A similar trend was noticed with DL isolated from wheat straw and SO_2-treated wheat straw (Ben-Ghedalia et al., 1994). Thus, we suggest that the CS-DL and OCS-DL originate from different regions of the cell walls. In OCS some 50% of the lignin had been oxidized by ozone, thus increasing the proportion

Table 1. Molecular Weight Distribution (Percent of Total Peak Area), Weight-Average Molecular Weight (MWt), Number-Average Molecular Weight (MN), and Dispersity (MWt/MN) of Dioxane Lignins Extracted from Ball-Milled plus Cellulase-Pretreated NDF of Cotton Stalks (CS) and Ozone-Treated Cotton Stalks (OCS) and from Feces of Sheep Fed the CS and OCS Rations

	MW ranges, $\times 10^3$						
source of lignins	0.8–1.6	1.6–10.1	10.1–23.1	23.1–128	MWt	MN	MWt/MN
CS	5.40 ± 0.20	45.7 ± 0.85	42.6 ± 0.80	6.30 ± 0.28	11224 ± 172	5784 ± 108	1.94 ± 0.01
feces of CS	5.10 ± 0.11	45.2 ± 1.20	40.9 ± 0.35	8.80 ± 0.90	12799 ± 696	5888 ± 121	2.18 ± 0.08
OCS	5.60 ± 0.20	34.8 ± 0.45	39.9 ± 0.82	19.7 ± 0.85	17888 ± 274	6369 ± 33	2.81 ± 0.06
feces of OCS	5.60 ± 0.03	42.3 ± 0.65	41.2 ± 0.10	10.9 ± 0.65	13186 ± 313	5851 ± 67	2.26 ± 0.03

Table 2. Carbohydrate Content [Grams (100 g of DL)$^{-1}$] and Profile [Grams (100 g of Sugars)$^{-1}$] of Dioxane Lignins (DL) Extracted from Ball-Milled plus Cellulase-Pretreated NDF of Cotton Stalks (CS) and Ozone-Treated Cotton Stalks (OCS)

carbohydrate	CS	OCS
total content	5.40 ± 0.03	5.38 ± 0.08
profile		
xylose	35.1	38.7
arabinose	3.40	2.40
galactose	5.80	5.39
mannose	3.40	3.72
rhamnose	3.40	3.72
glucuronic acid	35.0	29.9
glucose	13.9	16.2
total	100	100

of the oxidation-resistant lignin, suggested to be the highly condensed lignin residing in the middle lamella (Mulder et al., 1992). We assume, therefore, that the CS-DL with the lower MWt originated from the secondary walls, whereas more of the OCS-DL came from the middle lamella.

Table 2 shows that the carbohydrate level in the DL preparations of CS and OCS was very low. Xylose was the major monosaccharide component; however, the high proportion of uronic acid and glucose hints that these sugars may interlink the heteroxylan to lignin and cellulose. Figures 2 and 3 and Table 1 show the distribution of molecular weights in DL isolated from the CS and OCS and from the respective fecal material. Very few such comparisons have been published. The pattern found in CS, namely a similar distribution and MWt in the lignocellulose and in its fecal material, was found also in wheat straw (Ben-Ghedalia et al., 1994). However, the DL from fecal OCS contained more low molecular weight fractions and had a lower MWt than the DL of the ingested OCS. Thus, it appears that in the OCS treatment a larger proportion of high molecular weight fractions have been removed from the walls in the course of rumen digestion. This view is supported by the data of Table 3 and Figures 4 and 5 showing the patterns of molecular weight distribution in the water-soluble lignin. Jung et al. (1983) have shown with a dicotyledonous forage that lignin is modified and not completely recovered following digestive processes in the ruminants. On the basis of our data, matrix particles are released from the walls into solution during cell wall degradation in the rumen. Table 3 shows a remarkable increase in the dispersity of rumen liquor WSL, as compared with the water extract or the DL of the respective lignocelluloses. The high proportion of WSL molecules residing over and in the range 23 700–128 000 may imply that large matrix particles are being released from the cell walls into solution during cell wall degradation in the rumen. The proportion of those high molecular weight fractions is much lower in the liquid phase of the fecal material from sheep fed both rations. The most likely explanation of this phenomenon could be insolubilization during passage along the gastrointestine; the least likely possibility is continual breakdown and fragmentation in the lower gut. The major processes resulting in cell wall degradation in the rumen probably are based on the removal into solution of matrix polymers, rather than on on-site monomer by monomer degradation. CS lignin is extensively degraded by ozone, mostly to organic acids (Shefet and Ben-Ghedalia, 1982). However, the absorbance at 280 nm is increased in the water extract of OCS (Ben-Ghedalia et al., 1980). Table 3 shows that the low molecular weight lignin fragments in the range 1500–10 000 are the major water-soluble E_{280}-absorbing materials in OCS.

In this study WSL should represent lignin–carbohydrate complexes (LCC). However, due to the use of NaOH plus *N*-MTAC in our isolation procedure, most of the carbohydrate component was cleaved, leaving only traces of monosaccharides attached to the WSL. Morrison (1973) was the first to address the properties of LCC in a forage-oriented study on a monocot, suggesting the linkage between lignin and heteroxylan. Gaillard, and Richards (1975) and Neilson and Richards (1982) studied solubilized LCC recovered from the bovine rumen. Conchie et al. (1988) presented data on rumen-solubilized LCC of a monocotyledonous forage. We are unaware of similar HPSEC data on dicot forages or lignocelluloses. Both Figures 4 and 5 and Table 3 show that the rumen WSL are highly polydisperse, with a large group of molecules smaller than 10 000 and a second large group in the range 24 000–128 000.

Exploring the molecular weight distribution of LCC in

Table 3. Molecular Weight Distribution (Percent of Total Peak Area), Weight-Average Molecular Weight (MWt), Number-Average Molecular Weight (MN), and Dispersity (MWt/MN) of Water-Soluble Lignins (WSL) Isolated from CS and OCS and from the Rumen Liquor and Feces of Sheep Fed the CS and OCS Rations

	MW ranges, $\times 10^3$							
source of WSL	0.8–1.5	1.5–10.4	10.4–23.7	23.7–128	>128	MWt	MN	MWt/MN
				CS Treatment				
CS	10.0 ± 0.19	42.0 ± 1.10	35.8 ± 0.30	12.0 ± 0.90	0.20 ± 0.08	13298 ± 923	4353 ± 79	3.06 ± 0.21
rumen liquor	22.5 ± 0.40	32.4 ± 1.35	16.7 ± 0.25	26.1 ± 0.60	2.30 ± 1.20	21705 ± 1349	2777 ± 107	7.82 ± 1.56
feces	19.0 ± 0.25	57.0 ± 0.15	17.6 ± 0.15	6.08 ± 0.08	0.32 ± 0.02	9562 ± 1105	2818 ± 116	3.42 ± 0.62
				OCS Treatment				
OCS	28.0 ± 0.05	63.6 ± 0.68	6.99 ± 0.09	1.41 ± 0.02		4383 ± 40	2080 ± 6	2.11 ± 0.02
rumen liquor	17.8 ± 0.90	31.7 ± 0.25	17.3 ± 0.95	29.1 ± 0.20	4.10 ± 0.01	28989 ± 682	3465 ± 16	8.38 ± 0.28
feces	20.0 ± 0.10	59.4 ± 0.25	13.3 ± 0.10	6.88 ± 0.18	0.42 ± 0.09	9476 ± 885	2518 ± 7	3.76 ± 0.42

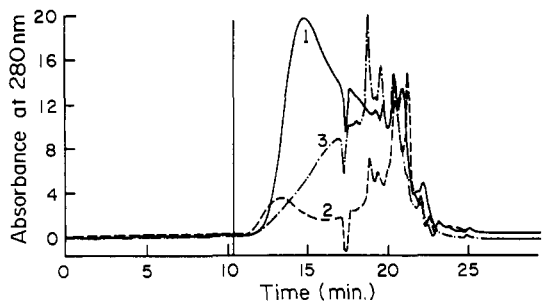

Figure 4. High-performance size exclusion chromatograms of water-soluble lignins isolated from CS (1), rumen liquor (2), and feces (3) of sheep fed the CS ration.

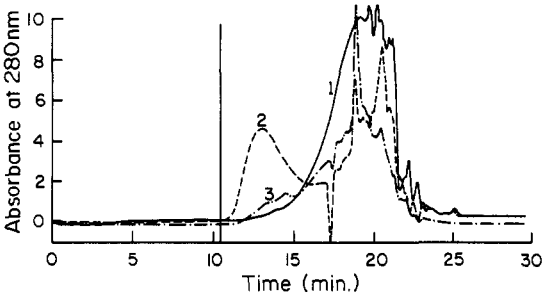

Figure 5. High-performance size exclusion chromatograms of water-soluble lignins isolated from OCS (1), rumen liquor (2), and feces (3) of sheep fed the OCS ration.

the rumen could lead to a better understanding of the mechanisms of cell wall biodegradation by rumen microorganisms.

ACKNOWLEDGMENT

This research was supported by a Grant from the GIF, the German–Israeli Foundation for Scientific Research and Development.

LITERATURE CITED

Ben-Ghedalia, D.; Shefet, G. Chemical Treatments for Increasing the Digestibility of Cotton Straw. 2. Effect of Ozone and Sodium Hydroxide Treatments on the Digestibility of Cell Wall Monosaccharides. *J. Agric. Sci.* **1983**, *100*, 401–406.

Ben-Ghedalia, D.; Shefet, G.; Miron, J. Effect of Ozone and Ammonium Hydroxide Treatments on the Composition and In Vitro Digestibility of Cotton Straw. *J. Sci. Food Agric.* **1980**, *31*, 1337–1342.

Ben-Ghedalia, D.; Shefet, G.; Miron, J.; Dror, Y. Effect of Ozone and Sodium Hydroxide Treatments on Some Chemical Characteristics of Cotton Straw. *J. Sci. Food Agric.* **1982**, *33*, 1213–1218.

Ben-Ghedalia, D.; Yosef, E.; Miron, J.; Huttermann, A.; Majcherczyk, A.; Milstein, O. Characterization of Lignins in Straw, Rumen Liquor and Faeces of Sheep Fed Untreated and SO_2-Treated Wheat Straw. *Anim. Feed Sci. Technol.* **1994**, in press.

Blakeney, A. B.; Harris, P. J.; Henry, R. J.; Stone, B. A. A Simple And Rapid Preparation of Alditol Acetates for Monosaccharides Analysis. *Carbohydr. Res.* **1983**, *113*, 291–299.

Blumenkrantz, N.; Asboe-Hansen, G. New Method for Quantitative Determination of Uronic Acids. *Anal. Biochem.* **1973**, *54*, 484–489.

Chang, H. M.; Cowling, E. B.; Brown, W. Comparative Studies on Cellulolytic Enzyme Lignin and Milled Wood Lignin of Sweetgum and Spruce. *Holzforschung* **1975**, *29*, 153–159.

Cherney, D. J. R.; Cherney, J. H.; Patterson, J. A.; Axtell, J. D. In Vitro Ruminal Fiber Digestion as Influenced by Phenolic-Carbohydrate Complexes Released from Sorghum Cell Walls. *Anim. Feed Sci. Technol.* **1992**, *39*, 79–93.

Conchie, J.; Hay, A. J.; Lomax, J. A. Soluble Lignin-Carbohydrate Complexes from Sheep Rumen Fluid; Their Composition and Structural Features. *Carbohydr. Res.* **1988**, *177*, 127–151.

Faix, O.; Lange, W.; Salud, E. C. The Use of HPLC for the Determination of Average Molecular Weights and Molecular Weight Distributions of Milled Wood Lignins from Shorea polysperma (Blco.). *Holzforschung* **1981**, *35*, 3–9.

Gaillard, B. D. E.; Richards, G. N. Presence of Soluble Lignin-Carbohydrate Complexes in the Bovine Rumen. *Carbohydr. Res.* **1975**, *42*, 135–145.

Jung, H. G.; Fahey, G. C.; Merchen, N. R. Effects of Ruminant Digestion and Metabolism on Phenolic Monomers of Forages. *Br. J. Nutr.* **1983**, *50*, 637–651.

Kondo, T.; Oshita, T.; Kyuma, T. Comparison of Characteristics of Soluble Lignins from Untreated and Ammonia-Treated Wheat Straw. *Anim. Feed Sci. Technol.* **1992**, *39*, 253–263.

Lai, Y. Z.; Sarkanen, K. V. Isolation and Structural Studies. In *Lignins*; Sarkanen, K. V., Ludwig, C. H., Eds.; Wiley-Interscience: New York, 1971; pp 165–240.

Milstein, O.; Huttermann, A.; Ludemann, H. D.; Majcherczyk, A.; Nicklas, B. Enzymatic Modification of Lignin in Organic Solvents. In *Biotechnology in the Pulp and Paper Manufacture*; Kirk, T. K., Chang, H. M., Eds.; Toscano: Sommerville, MA, 1990; pp 363–375.

Miron, J.; Ben-Ghedalia, D. Effect of Chemical Treatments on the Degradability of Cotton Straw by Rumen Microorganisms and by Fungal Cellulases. *Biotechnol. Bioeng.* **1981**, *23*, 2863–2873.

Miron, J.; Ben-Ghedalia, D. The Degradation and Utilization of Wheat Straw Cell Wall Monosaccharide Components by Defined Ruminal Cellulolytic Bacteria. *Appl. Microbiol. Biotechnol.* **1992**, *38*, 432–437.

Morrison, I. M. Isolation and Analysis of Lignin-Carbohydrate Complexes from Lolium multiflorum. *Phytochemistry* **1973**, *12*, 2979–2984.

Mulder, M. M.; Engels, F. M.; Schurmans, J. L. L.; Boon, J. J. In Vitro Digested and Potassium Permanganate Delignified Maize Internode Sections Studied by Histochemistry and Analytical Pyrolysis Mass Spectrometry. *Anim. Feed Sci. Technol.* **1992**, *39*, 335–346.

Neilson, M. J.; Richards, G. N. Chemical Structures in a Lignin-Carbohydrate Complex Isolated from the Bovine Rumen. *Carbohydr. Res.* **1982**, *104*, 121–138.

Polcin, J.; Bezuch, B. Enzymic Isolation of Lignin from Wood and Pulps. *Wood Sci. Technol.* **1978**, *12*, 149–158.

Scalbert, A.; Monties, B. Comparison of Wheat Straw Lignin Preparations. *Holzforschung* **1986**, *40*, 249–254.

Shefet, G.; Ben-Ghedalia, D. Effect of Ozone and Sodium Hydroxide Treatments on the Degradability of Cotton Straw Monosaccharides by Rumen Microorganisms. *Eur. J. Appl. Microbiol. Biotechnol.* **1982**, *15*, 47–51.

Solomon, R.; Miron, J.; Rubinstein, A.; Ben-Ghedalia, D. Ozone-Treated Cotton Stalks as a Component of a Ration for Growing Lambs. *Anim. Feed Sci. Technol.* **1992**, *37*, 185–192.

Van Soest, P. J.; Robertson, J. B.; Lewis, B. A. Methods for Dietary Fiber, Neutral Detergent Fiber, and Nonstarch Polysaccharides in Relation to Animal Nutrition. *J. Dairy Sci.* **1991**, *74*, 3583–3597.

Wallace, G.; Chesson, A.; Lomax, J. A.; Jarvis, M. C. Lignin-Carbohydrate Complexes in Graminaceous Cell Walls in Relation to Digestibility. *Anim. Feed Sci. Technol.* **1991**, *32*, 193–199.

Yosef, E.; Ben-Ghedalia, D.; Miron, J.; Huttermann, A.; Majcherczyk, A.; Milstein, O.; Ludemann, H. D.; Frund, R. Characterization of Some Cell Wall Components of Untreated and Ozone-Treated Cotton Stalks. *J. Agric. Food Chem.* **1994**, *42*, 86–90.

Received for review September 2, 1993. Revised manuscript received November 17, 1993. Accepted February 22, 1994.[⊛]

[⊛] Abstract published in *Advance ACS Abstracts*, April 1, 1994.