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Generation of soluble lignin-derived compounds during degradation of barley straw in an artificial rumen reactor

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Summary. The supernatants of effluents from an artificial rumen reactor degrading barley straw have been shown to contain lignin-derived compounds by UV spectral characteristics and pyrolysis mass spectrometry (PYMS). Most of these compounds were shown to be released by the action of rumen microorganisms. The compounds were quantified by measuring absorbance at 280 nm using bamboo-milled wood lignin as a standard. The concentration of the compounds rose from 0.5 $mg \cdot ml^{-1}$ at solid and liquid retention times (SRT and HRT) of 60 and 12 h, respectively, and a loading rate (LR) of 25 g total solids (TS)$\cdot l^{-1}$ per day to 3.5 $mg \cdot ml^{-1}$ at a SRT of 144 h, an HRT of 20 days and an LR of 15 g TS$\cdot l^{-1}$ per day. The highest concentration was below the level known to be toxic to rumen microorganisms in vitro. No indications were found for anaerobic lignin degradation in the rumen reactor.

Introduction

Until recently, the lignin component of plant materials was believed to be inert to anaerobic biodegradation (Hackett et al. 1977; Odier and Monties 1983). A review of the fate of lignin and lignin-derived compounds by Young and Frazer (1987) has now made the following points clear: (1) lignin monomers can be readily metabolized and completely mineralized anaerobically; (2) lignin oligomers can undergo anaerobic depolymerization and metabolism; (3) lignin monomer substituents (e.g. – OCH_3) can serve as a C_1 substrate for acetogens; (4) complex lignin polymers can undergo a small but significant rate of degradation in several anaerobic environments. At environmental temperatures (21°–30° C), 1.5%–17% of lignin was reported to be anaerobically mineralized to CO_2 and CH_4 after incubations of about 9 months (Benner et al. 1984).

In the rumen, soluble lignin-carbohydrate complexes were found to be released from grass and were suggested to account for the apparent digestion of 50% of the total lignin observed (Gaillard and Richards 1975). More recently, a rumen isolate was reported to be capable of delignifying Bermuda grass (Akin 1980). Based on these observations, it has been suggested that rumen microbes may mediate a partial degradation of plant lignin (Young and Frazer 1987).

During anaerobic degradation of various lignocellulosic materials by rumen microorganisms in batch cultures, an apparent loss of lignin was always observed together with a decrease in cellulose/hemicellulose content (Op den Camp et al. 1988). Earlier, similar findings were obtained with similar substrates in an artificial rumen reactor (Gijzen et al. 1987). Considering the solid and liquid residence times applied, the loss of lignin was suggested to be due to solubilization (Gijzen et al. 1987; Op den Camp et al. 1988).

In order to confirm the solubilization of lignin in the artificial rumen reactor degrading barley straw, the presence of soluble lignin-derived compounds was determined. Furthermore, attempts were made to quantify the soluble lignin compounds in the reactor(s) and to check for lignin modifications.

Materials and methods

Materials. Barley straw *(Hordeum vulgare)* obtained from a farmer (Nijmegen, The Netherlands), dried (16 h, 70° C) and milled to pass through a 2-mm mesh sieve was used as a substrate for anaerobic digestion. Its chemical composition is shown in Table 1. Bamboo milled wood lignin was a gift from Dr. O. Faix, Bundesforschungsanstalt für Forst- und Holzwirtschaft, Hamburg, FRG. This dry preparation was solved in acidified water and the solution was neutralized to pH 7 with 0.1 *M* NaOH.

Source of samples and preparation. Effluent samples were taken from an artificial rumen reactor (one-phase) and from an artificial rumen reactor coupled to an upflow anaerobic sludge blanket (UASB) reactor (two-phase system), both described previously

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Table 1. Composition of native milled barley straw

Determination		
Total solids (TS)	(%)	89.0±0.5[a]
Ash	(% of TS)	6.0±0.8
Volatile solids	(% of TS)	94.0±0.4
NDF	(% of TS)	80.0±0.1
ADF	(% of TS)	60.0±2.0
Hemicellulose	(% of TS)	20.0±1.0
Cellulose	(% of TS)	47.0±2.0
Lignin	(% of TS)	15.8±1.0
Cell solubles[b]	(% of TS)	14.0±0.5

NDF = neutral detergent fibre; ADF = acid detergent fibre
[a] Values are means ±SD ($n=6$)
[b] Volatile solids minus NDF

(Gijzen et al. 1986, 1988). The latter system operated with a semi-closed fluid circuit where the volume of the rumen reactor was maintained at 1500 ml. Daily, a certain volume of homogenous reactor content, according to the solid retention time (SRT) applied, was removed before feeding and filtered through nylon gauze (30 μm). A volume of 150 ml filtrate was discarded and the remainder poured back into the reactor. After feeding, the volume was adjusted to 1500 ml by adding rumen buffer solution prepared according to Rufener et al. (1963). The two fermentation systems were fed with barley straw and operated at various SRT, liquid retention times (HRT) and loading rates (LR). The samples were centrifuged (10000 g, 10 min) and both the residue and supernatant fractions were used for various analyses. For comparison 0.5 g milled barley straw (dry weight) was incubated during 24 h at 39° C with 50 ml rumen buffer and centrifuged.

Analytical determinations. The presence of soluble lignin-derived compounds was determined by UV-spectral characteristics and pyrolysis mass spectrometry (PYMS). The latter method has been successfully used to evaluate differences between various types of barley straw cell walls (Boon 1989; Hartley and Haverkamp 1984; van der Valk et al. 1985). Curie-point PYMS was carried out on the FOMautoPYMS® (Institute for Atomic and Molecular Physics, Amsterdam, The Netherlands). The pyrolysis chamber and the expansion chamber were heated to 160° C and 200° C, respectively. To prevent extensive condensation of pyrolysate the waiting time before pyrolysis was 10 s. The pyrolysis temperature was 510° C and the total pyrolysis time 0.8 s. Low voltage electron impact ionization (EI) at about 15 eV was employed to obtain molecular weight distributions of the pyrolysate with a minimum of fragmentation due to the ionization process. The mass range of the mass spectrometer used was mass number (m/z) 20–250. The scan speed was 10 scans·s^{-1} with a total data acquisition time of 20 s. The PYMS spectra shown are summarized average spectra for the total pyrolysis period calculated from three samples analysed.

Ultraviolet (UV) absorption spectra were recorded with a Hitachi (Tokyo, Japan) D2200 spectrophotometer.

Protein content of the sample was determined according to Sedmak and Grossberg (1977) with bovine serum albumin as a standard.

Neutral detergent fibre, acid detergent fibre, cellulose, hemicellulose and lignin content of the barley straw were determined according to the method of Goering and van Soest (1970).

Estimation of the concentration of soluble lignin-derived compounds. Starting with a 1% solution of bamboo milled wood lignin (the standard), several dilutions containing from 5 to 120 mg·l^{-1} were prepared. Absorption at 280 nm of the standards and appropriately diluted samples was measured versus the blank (rumen buffer). Absorbances of the standards were plotted versus their concentrations. The concentrations of soluble lignin compounds in the samples were extrapolated from the standard curve and corrected for dilution.

Results

UV-spectral characteristics

In order to check for the presence of soluble lignin-derived compounds in the reactor effluents, the UV-spectral characteristics were determined. Absorption curves of the samples in the UV range are shown in Fig. 1. For comparison an absorption curve of the bamboo lignin standard is included. All spectra showed maximum absorption in the lower UV range with a characteristic plateau occurring in the range 260–280 nm, which is typical for phenol-containing compounds (Sarkanen and Ludwig 1971; Gaillard and Richards 1975). The absorbance due to protein in the samples was negligible and, therefore, did not interfere with the measurements. Barley straw incubated in rumen buffer was used as a control to test solubilization in the absence of microorganisms. The sample from the one-phase reactor operating at an SRT and HRT of 60 and 12 h, respectively, and at an LR of 20 g total solids (TS)·l^{-1} per day showed a low absorbance whereas the effluent sample from the two-phase system fed with a slightly lower load (15 g TS·l^{-1} per day) of substrate and operating at an SRT and HRT of 144 h and 20 days, respectively, had the highest absorbance.

PYMS fingerprinting

In order to confirm the presence of soluble lignin compounds in the reactors, PYMS fingerprinting was per-

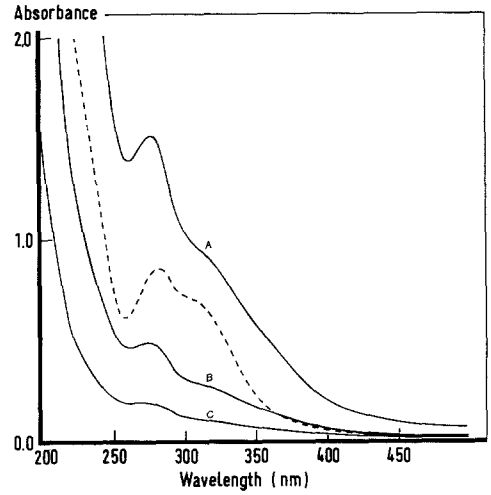

Fig. 1. Ultraviolet absorbance spectra of solubilized lignin fractions: *A*, effluent two-phase system rumen reactor (solid retention time (SRT) 144 h, liquid retention time (HRT) 20 days); *B*, effluent one-phase system rumen reactor (SRT 60 h, HRT 12 h); *C*, supernatant after incubation of barley straw in rumen buffer. Samples A, B and C were diluted 1:30 in rumen buffer. The *dotted line* indicates the spectrum of the bamboo-milled wood lignin standard (60 mg·l^{-1})

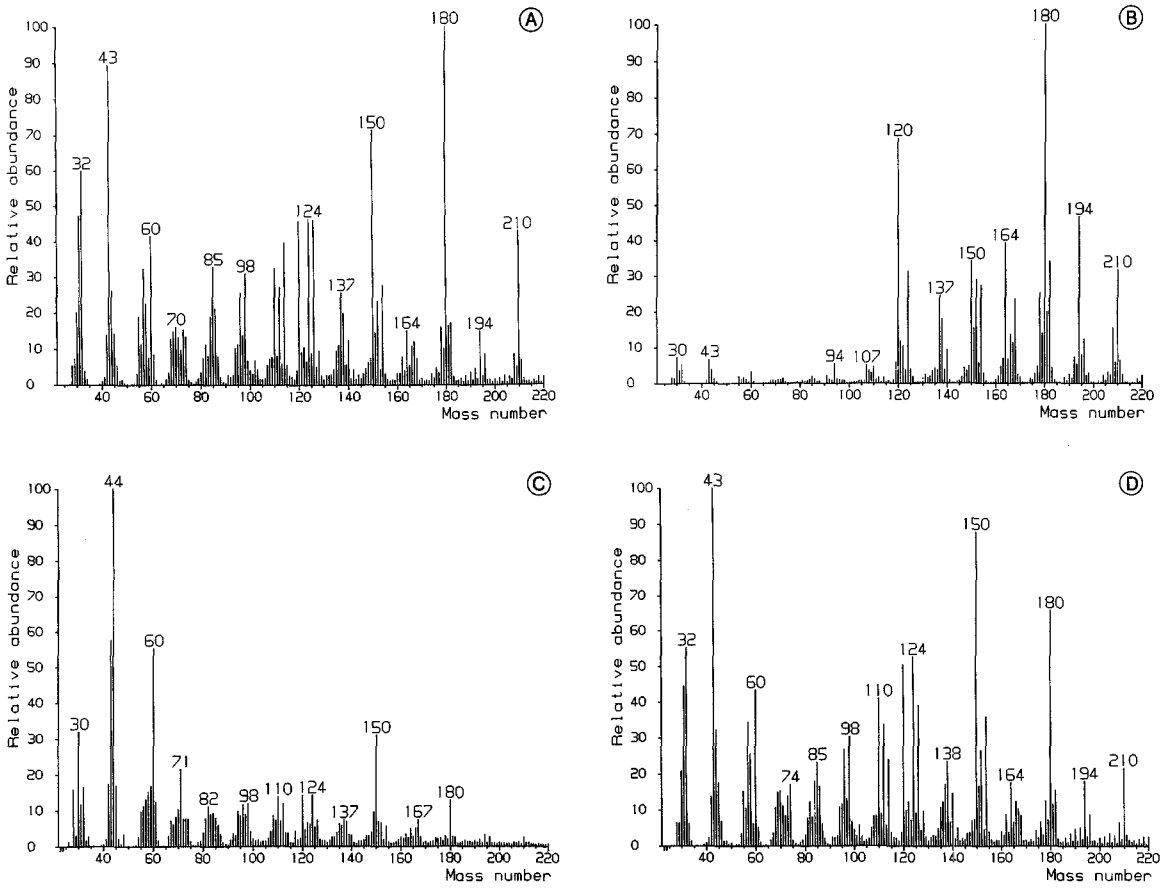

Fig. 2A–D Pyrolysis mass spectrometry (PYMS) spectra of different lignin fractions. **A** Milled native barley straw. **B** Bamboo milled wood lignin. **C** Supernatant after treatment of barley straw with rumen buffer (24 h, 39° C). **D** Residue after this treatment with rumen buffer

formed. For reference, PYMS spectra of milled native barley straw and bamboo milled wood lignin are included (Fig. 2A and B). According to Boon (1989) and Pouwels et al. (1987), the mass peak information below m/z 118 in low voltage EI spectra of lignocellulosic material is mainly from the pyrolysis products of polysaccharides, whereas many of the mass peaks above m/z 118, except m/z 126, 128 and 144, are from lignin-derived phenolic compounds.

The PYMS spectra of the supernatant and residue fraction of milled barley straw extracted with rumen buffer (24 h, 37° C) are shown in Fig. 2C and D, respectively. Upon incubation with rumen buffer, carbohydrate and lignin compounds were solubilized. The presence of carbohydrate compounds is indicated by the mass peaks at m/z 32, 43, 55, 58, 60, 61, 68, 72, 74, 82, 84, 85, 86, 96, 98, 110, 112, 114, 126 and 144, while the presence of soluble lignin compounds is shown clearly by the mass groups containing m/z 120, 124, 138, 150, 152, 154, 164, 178, 180, 182, 194, 196, 208 and 210. These groups have been identified as derivatives of coumaryl, coniferyl, and sinapyl alcohols (Boon 1989).

The nature of the soluble lignin fraction (Fig. 2C) differs from lignin in the solid phase. The PYMS fingerprint of the residue fraction after extraction with rumen buffer (Fig. 2D) is somewhat different compared to native barley (e.g. lower intensity of m/z 180 and 210), also indicating solubilization. The PYMS fingerprint obtained from the supernatant after centrifugation of homogeneous one-phase reactor content is shown in Fig. 3A. As expected the major products of rumen digestion, namely acetic and propionic acids were present. These acids are indicated by m/z 60 and 74, respectively. The same typical lignin marker peak profile is present compared to the rumen buffer supernatant fraction (Fig. 2C) but the relative abundance of the carbohydrate marker peaks is much lower. With centrifuged reactor effluent the same results were obtained (data not shown). The PYMS fingerprint of the residue obtained after centrifugation of reactor content (Fig. 3B) is almost identical to the buffer extracted barley straw (Fig. 2D).

Enrichment of soluble lignin-derived compounds in the semi-closed fluid circuit reactors

In the two-phase system fed with barley straw and operating at an HRT of 20 days (semi-closed system) a gradual intensification of a brownish colour in the fluid was observed. The colour was thought to be due to an

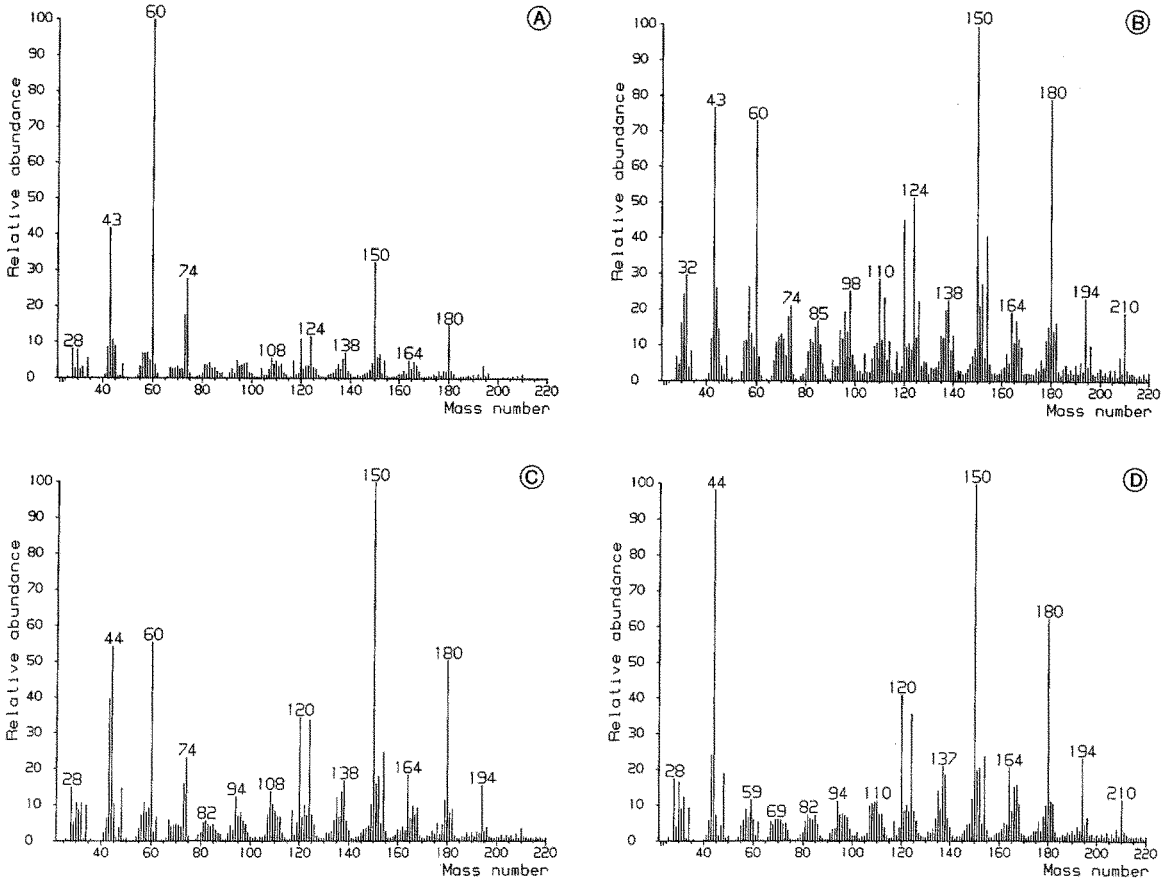

Fig. 3A–D. PYMS spectra of samples from the rumen reactor systems. Supernatant **(A)** and residue **(B)** after centrifugation of one-phase system rumen reactor content (SRT 60 h, HRT 12 h). Effluent of the rumen reactor **(C)** and the upflow anaerobic sludge blanket reactor before recycling to the rumen reactor **(D)** in the two-phase system (SRT 144 h, HRT 20 days)

accumulation of the soluble lignin-derived compounds as a consequence of the long HRT. In order to check for enrichment of the compounds in the reactor their concentration in the effluent was estimated.

Figure 4A shows the concentration of the soluble lignin-derived compounds in the system operating with an HRT of 20 days and an SRT of 60 h. The level of the soluble compounds appeared to rise sharply after coupling (day 0). The coupling resulted in an increase of the HRT from 12 h to 20 days. After the second week of operation, the concentration of soluble compounds stabilized at about 2 $mg \cdot ml^{-1}$ effluent. On the other hand, the level of the compounds in the effluent from the system operating at an HRT of 20 days and varying SRT appeared to increase with increasing SRT and reached about 3.5 $mg \cdot ml^{-1}$ effluent at an SRT of 144 h (Fig. 4B).

Since the above estimation of the concentration of the compounds in the reactors is based on a calibration curve, the PYMS information on the samples with the highest absorption levels was used for comparison on a molecular level. Figure 3C and D shows the PYMS spectra of the reactor effluents from the two-phase system operating at an HRT of 20 days and an SRT of 144 h.

Effluent samples were taken from the rumen reactor and the UASB reactor after 8 weeks of operation. The lignin enrichment can clearly be seen when comparing the relative abundances of the peaks in the PYMS spectra of the two-phase reactor with those of the one-phase

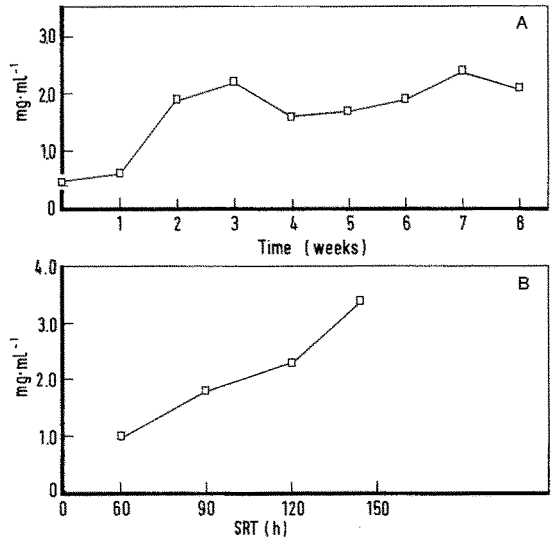

Fig. 4. Concentration of lignin-derived compounds in the effluent of the two-phase system rumen reactor after coupling (day 0) at SRT and HRT of 60 h and 20 days, respectively **(A)** and at steady state for different SRT and an HRT of 20 days **(B)**

rumen reactor, operating at an HRT of 12 h (Fig. 3A). No alteration in the carbohydrate marker region was observed indicating that the solubilized carbohydrate compounds were metabolized by the rumen microorganisms. Figure 3C also shows the acetic and propionic acid marker peaks (m/z 60 and 74). The removal of these acids in the UASB reactor is evident in Fig. 3D, as shown by the sharp decrease in the relative abundance of these marker mass peaks. No modifications in the soluble lignin PYMS fingerprint are observed when the mass spectra of native barley (Fig. 2A and C) are compared with those of barley residues and supernatant fractions from the artificial rumen reactor (Fig. 3).

Discussion

It has been suggested that lignin-related compounds are released from plant cell walls, lignin and lignin precursors in the rumen (Borneman et al. 1986; Gaillard and Richards 1975). The results of this study show strong evidence for the generation of soluble lignin-related compounds from barley straw in the artificial rumen reactor. Previously, it was believed that any lignin-containing material can only be liberated upon enzymic scission of the chains of the associated polysaccharides, namely cellulose and hemicellulose, and that it was not extractable by water (Gaillard and Richards 1975). However, this study has demonstrated that spontaneous solubilization of carbohydrate and lignin-derived compounds does occur by incubation in rumen buffer.

Even though relatively very little lignin appears to be extracted by the buffer, it could not account for all of the soluble lignin observed. It can therefore be concluded that lignin-carbohydrate complexes go into solution both spontaneously and through the action of rumen microorganisms. After solubilization the carbohydrate part of the complex is largely metabolized by the rumen microorganisms, while the lignin part appears to remain unaffected. This is in agreement with the results on rumen liquor samples obtained by Conchie et al. (1988) on the basis of permethylation analysis of the sugars.

At species level little is known about the microorganisms that degrade lignin carbohydrates. The major cellulolytic bacteria in the rumen, *Ruminococcus albus, R. flavefaciens,* and *Bacteroides succinogenes,* are presumably involved. Chesson and Forsberg (1988) recently reviewed polysaccharide degradation by rumen microorganisms also discussing the role of the anaerobic fungi and protozoa.

The concentration of the soluble lignin compounds in the reactors appear to be affected by the SRT and HRT applied. It was unexpected that longer residence times would result in an enrichment of the soluble lignin compounds in the reactors rather than to their decrease, since under long residence times the degradation of lignin monomers to volatile fatty acids and gaseous products by rumen microorganisms is possible (Chen et al. 1985; Stack and Cotta 1986; Varel and Jung 1986). However, the PYMS information on the effluent samples (Fig. 3C and D) gives no indication of any lignin modification.

Previously we reported an apparent lignin loss of about 40% (measured using the Goering and van Soest (1970) method) during degradation of barley straw in the one-phase artificial rumen reactor (Kivaisi et al. 1988). Assuming an LR of 15 g $TS \cdot l^{-1}$ per day (lignin content 15.8% of TS), an average lignin loss of 40% and an HRT of 20 days, a lignin removal of 1.4 g $lignin \cdot day^{-1}$ can be calculated. On the basis of the lignin determination using bamboo milled wood lignin as a standard, we measured 20%–40% of this amount. A reason for this discrepancy may be a difference in chemical composition between the bamboo and barley straw lignin (Fig. 2A and B) or alternatively to an overestimation of the lignin loss as measured by the Goering and van Soest (1970) method.

Some of the lignin monomers have been shown to be toxic to rumen microorganisms (Akin 1980; Borneman et al. 1986). It is possible that they could have a toxic effect on the microorganisms in the absence of their degradation in the reactors. However, this effect is unlikely since the concentration of the soluble lignin in the reactors, either calculated or measured (see above), was well below the toxic level for rumen microorganisms observed for Kraft pine lignin, ferulic acid, *p*-coumaric acid and vanillic acid (Op den Camp et al. 1988).

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References

Akin AD (1980) Attack on lignified grass cell walls by a facultatively anaerobic bacterium. Appl Environ Microbiol 40:809–820

Benner RA, Maccubin AE, Hodson RE (1984) Anaerobic biodegradation of the lignin and polysaccharide components of lignocellulosic and synthetic lignin by sediment microflora. Appl Environ Microbiol 47:998–1004

Boon JJ (1989) An introduction to the pyrolysis mass spectrometry of lignocellulosic material: case studies on barley straw, corn stem and *Agropyron*. In: Chesson A, Ørskov ER (eds) Physicochemical characterisation of plant residues for industrial and feed use. Elsevier Applied Science, London, pp 25–50

Borneman WS, Akin DE, Eseltine WP van (1986) Effects of phenolic monomers on ruminal bacteria. Appl Environ Microbiol 52:1331–1339

Chen W, Supanwong K, Ohmiya K, Shimuzu S, Kawakami H (1985) Anaerobic degradation of veratrylglycerol-β-guaiacyl ether and guiacoxy-acetic acid by mixed rumen bacteria. Appl Environ Microbiol 50:1451–1456

Chesson A, Forsberg CW (1988) Polysaccharide degradation by rumen microorganisms. In: Hobson PN (ed) The rumen microbial ecosystem. Elsevier Applied Science, London, pp 251–284

Conchie J, Hay AJ, Lomax JA (1988) Soluble lignin-carbohydrate complexes from sheep rumen fluid: their composition and structural function. Carbohydr Res 177:127–151

Gaillard BDE, Richards GN (1975) Presence of soluble lignin carbohydrate complexes in the bovine rumen. Carbohydr Res 42:135–145

Gijzen HJ, Zwart KB, Gelder PT van, Vogels GD (1986) Continuous cultivation of rumen microorganisms, a system with possible application to the anaerobic degradation of lignocellulosic waste materials. Appl Microbiol Biotechnol 25:155–162

Gijzen HJ, Lubberding HJ, Verhagen FJ, Zwart KB, Vogels GD (1987) Application of rumen microorganisms for an enhanced anaerobic degradation of solid organic waste materials. Biol Wastes 22:81–95

Gijzen HJ, Zwart KB, Verhagen FJ, Vogels GD (1988) High-rate two-phase process for the anaerobic degradation of cellulose, employing rumen microorganisms for an efficient acidogenesis. Biotechnol Bioeng 31:418–425

Goering HK, Soest PJ van (1970) Forage fiber analysis. United States Department of Agriculture, Agricultural Research Service, Agricultural Handbook no. 379

Hackett WF, Connors WJ, Kirk TK, Zeikus JH (1977) Microbial decomposition of synthetic ^{14}C-labeled lignins in nature: lignin degradation in a variety of natural materials. Appl Environ Microbiol 33:43–51

Hartley RD, Haverkamp J (1984) Pyrolysis-mass spectrometry of the phenolic constituents of plant cell walls. J Sci Food Agric 35:14–20

Kivaisi AK, Lubberding HJ, Op den Camp HJM, Robben AJPM, Vogels GD (1988) Application of rumen microorganisms to degrade lignin-rich waste materials. Med Fac Landbouww Rijksuniv Gent 53:1863–1872

Odier E, Monties B (1983) Absence of microbial mineralization of lignin in anaerobic enrichment cultures. Appl Environ Microbiol 46:661–665

Op den Camp HJM, Verhagen FJ, Kivaisi AK, Windt F de, Lubberding HJ, Gijzen HJ, Vogels GD (1988) Effects of lignin on the anaerobic degradation of (ligno)cellulosic wastes by rumen microorganisms. Appl Microbiol Biotechnol 29:408–412

Pouwels AD, Tom A, Eijkel GB, Boon JJ (1987) Characterization of beech wood and its holocellulose and xylan fractions by pyrolysis-gas chromatography-mass spectrometry. J Anal Appl Pyrol 11:417–436

Rufener WH Jr, Nelson WO, Wolin MJ (1963) Maintenance of the rumen microbial population in continuous culture. Appl Microbiol 11:196–201

Sarkanen KV, Ludwig CH (eds) (1971) Lignins: occurrence, formation, structure and reactions. Wiley-Interscience, New York

Sedmak JJ, Grossberg SE (1977) A rapid, sensitive, and versatile assay for protein using Coomassie brilliant blue G250. Anal Biochem 79:532–544

Stack RJ, Cotta MA (1986) Effect of 3-phenylpropanoic acid on growth and cellulose utilization by cellulolytic ruminal bacteria. Appl Environ Microbiol 52:209–210

Valk F van der, Boon JJ, Hartley RD (1985) Curie-point pyrolysis-MS, -GC/MS, and -MS/MS of native steam exploded and ammonia treated OECD barley straw. In: Todd JFJ (ed) Advances in Mass Spectrometry, Wiley & Sons, New York, pp 655–656

Varel VH, Jung H-JG (1986) Influence of forage phenolics on ruminal fibrolytic bacteria and in vitro fiber degradation. Appl Environ Microbiol 52:275–280

Young LY, Frazer AC (1987) The fate of lignin and lignin-derived compounds in anaerobic environments. Geomicrobiol J 5:261–293