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D6: Comparison of traditional analytical procedure for assessing lignin and polysaccharides, van Soest, with TAPPI and ^{13}C CPMAS NMR

1 Introduction

Plant material consists of lignin, cellulose and hemicellulose fractions, which quantitatively vary according to the plant material. Hard and softwood consists almost exclusively of lignocellulose (89 to 98%) in which 63 to 78% are cellulose and hemicellulose and 15 to 38% is lignin (Colberg, 1988). On the other hand, plant material show a lignin content of 5%, hemicellulose of 19% and cellulose of 27% (Pabon et al., unpublished). Cellulose, which is the most abundant natural organic compound, is located in the primary and secondary cell walls of plants (Colberg, 1988). Cellulose is hydrolysed to cellobiose and finally to glucose while hemicellulose is hydrolysed to pentoses, hexoses and uronic acids (Colberg, 1988). Lignin is a cross linked network hydrophobic polymer (Hatfield and Fukushima, 2005) that is fairly resistant to anaerobic degradation. When cellulose is in the crystalline form the hydrolysis proceeds quite fast, however when encrusted in lignin the cellulases will be prevented from accessing the cellulose, retarding or even preventing hydrolysis (Gallert and Winter, 2005). The hydrolysis of hemicellulose proceeds ten times faster than lignin-encrusted cellulose(Buchholz et al., 1988).

Cellulose, Hemi-cellulose and lignin content can be determined using different methods, which can be divided into two basic categories: invasive and non-invasive (Hatfield and Fukushima, 2005). The van Soest method, which is extensively used for forage analysis (Jung et al., 1997), is an invasive gravimetric method based on sequential extractions (Van Soest et al., 1991). Wet chemical methods, like the van Soest, involve the formation of derivates through methylation or acetylation (Stevenson, 1994). Therefore, this type of method has been reported to change the structure of the molecules, inducing in error the exact quantities of lignin, cellulose and hemi-cellulose (Dien et al., 2006; Hatfield and Fukushima, 2005; Jung et al., 1997; Veeken et al., 2001; Wolters et al., 1992).

The combined method for determination of contents of extractives, lignin, polysaccharides and uronic acids in fibrous materials, named is this report as TAPPI, since most of the method is based on TAPPI Standard Test Methods, which are standardized and are mostly used in the paper and pulp industry, is also an invasive method. The TAPPI methods were developed for wood species, or wood derivates, and have been constantly adapted to be used for herbaceous species (Hatfield and Fukushima, 2005). Therefore, it has been suggested that when the lignin concentrations are low, this method is not sufficiently accurate since, for example for soluble lignin determination at lower wavelengths than 250nm, the abundance of released sugars may overestimate the potential lignin fraction (Hatfield and Fukushima, 2005).

Solid state 13C cross polarization and magic angle spinning (CPMAS) nuclear magnetic resonance (NMR) spectroscopy has become an important technique in the study of intact plant biopolymers (Lopes et al., 2000a), since it has enabled the non-invasive characterization of complex systems such as wood and other plant material (Lopes et al., 2000b). In a heterogeneous system, differences in proton relaxation times of distinct domains or components may be exploited to obtain 13C CPMAS NMR spectra of each component without requiring their physical separation. Appropriate linear combination of those spectra can give a sub-spectrum of the sample components that share similar relaxation properties, thus enabling the characterization of each component through the spectra obtained for the all complex system (Lopes et al., 2000b).

The objective of deliverable D6 was to describe the usefulness and possible shortcomings of the van Soest method for the polymer characterisation of plant material. Therefore the van Soest method was compared with the TAPPI and ^{13}C CPMAS NMR methods to determine if more accurate results could be achieved.

2 Material and methods

Three plant species, bracken, straw and hay, were ground to particles of approximately 1 cm (Retsch Muhle; type sm1) and air-dried at 40°C. Part of the homogenized samples was used for the determination of fibres by the TAPPI useful method. The remaining part of the homogenized samples was further ground to particles smaller than 1mm by a cutting mill (Krups KM75) and freeze dried (Lyovat GT2) to be analysed by ^{13}C CPMAS NMR and by forage fibre method (Van Soest et al., 1991).

After anaerobically digesting the three plant species up to maximum biodegradability was achieved, at 35±5˚C, the remaining digested material, i.e. digestate, was freeze dried and ground to particles smaller than 1mm by a cutting mill (Krups KM75). Due to a lack of digestate quantity, approximately 3 g per specie, the samples were not analysed by the TAPPI useful methods and for all fractions of the van Soest method. Additionally and unfortunately, due to technical problems the analysed fractions, for the van Soest method, were ADL and ADF. This resulted in the impossibility of comparing the polysaccharide results of the digestate by ^{13}C CPMAS NMR and by forage fibre method.

Figure 1. Ground and freeze dried bracken, straw and hay before anaerobic digestion.

2.1 Fibre analysis

Forage fibre analyses were performed in duplicate for acid detergent lignin (ADL), acid detergent fibre (ADF) and neutral detergent fibre (NDF) according to van Soest et al. (1991).In the Neutral Detergent Fiber (NDF), the sample is boiled with neutral detergents (ND), which dissolves the cell content. Enzymatic incubation with ND treatment breaks down protein and starch. The residue is filtered, dried and incinerated. The weight reduction by incineration is the sample content of hemi-cellulose, cellulose and lignin. In the Acid Detergent Lignin (ADL) procedure the sample is boiled with acid detergents (AD), which dissolves the cell content and hemi-cellulose. The residue is filtered and soaked with 72% sulphuric acid, which dissolves the hemi-cellulose. The residue is then filtered, dried and incinerated. The weight reduction by incineration is the sample content of cellulose and lignin. In the Neutral Detergent - Acid Detergent Fibre (ADF) the sample is boiled with ND, which dissolves the cell content. Enzymatic incubation with ND treatment breaks down protein and starch. After washing the sample with AD, which dissolves the hemi-cellulose, the

residue is filtered, dried and incinerated. The weight reduction by incineration is the sample content of cellulose and lignin (Van Soest et al., 1991). Conclusively, hemicellulose was calculated as NDF – ADF, cellulose as ADF – ADL and lignin was directly determined by the results of ADL (Van Soest et al., 1991).

Solid-state ^{13}C NMR spectra were recorded using CPMAS on a wide-bore AMX 300 spectrometer (Brucker, Karlsruhe, Germany) operating at 75.47 MHz of ^{13}C frequency. Samples were packed into a 4 mm rotor and placed inside the magnet. The spinning speed was 4.5 kHz, the acquisition time was 33 ms, the 13C 90° pulse length was 4 μs, the contact time was 0.8 ms, the recycle delay was 1 s and the line broadening was 50 Hz (Veeken et al., 2001). The resulting spectra were analysed quantitatively according to (Veeken et al., 2001), where the NMR spectrum is divided into four types of carbon: alkyl (0-50 ppm), O-Alkyl (50-110 ppm), aromatic (110-160 ppm) and Carbonyl (160-220 ppm). From the four types of carbon relative areas, four biomacromolecules were calculated: Aliphatics (lipids, biopolymers and fatty acids), polysaccharides (cellulose and hemicellulose), proteins and lignins. The results were presented in g biomacromolecule per kg of volatile solids (VS). These four biomacromolecules represent most of the organic matter produced by plant material (Nelson and Baldock, 2005)

Lignin and neutral sugars was determined in duplicate according to the modified TAPPI methods UM250 (1983), T222 om-83 (1983) and T249 cm-85 (1985), Teunissen et al. (1993) and (Stolle-Smits et al. 1997). While the uronic acids were measured according to Blumenkrantz et al. (1973). The milled samples were extracted with soxtec-extraction using ethanol: toluene 2:1, 96% (v/v) ethanol and hot water (1 hour) at boiling temperature. The extracted samples were dried at 60 °C for 16 hours. The content of neutral sugars and lignin of the ethanol-extracted material was determined after a two-step hydrolysis with sulphuric acid (12 M for 1 hour at 30 °C; 1M for 3 hours at 100 °C) according to modified TAPPI methods (TAPPI T 222 om-83, 1983 and TAPPI T 249 cm-85, 1985; Teunissen et al., 1993). Neutral sugars were determined by HPAEC with pulsed amperometric detection on a CarboPac PA1 column (Dionex) with a water-sodium hydroxide gradient (Stolle-Smits T. et al., 1997). The insoluble lignin (AIL) in the hydrolysate was measured by weight as Klason lignin, whereas the soluble lignin (ASL) content was determined by spectrophotometric determination at 205 nm (TAPPI UM250, 1983). Uronic acids in the sulfuric acid hydrolysate were spectrophotometrically determined at a wavelength of 520 nm (Blumenkrantz et al., 1973). All samples were analyzed in triplicate.

3 Results and discussion

The results are presented firstly as the outcome of each fibre analysis method, van Soest, ^{13}C CPMAS NMR and TAPPI, followed by a comparison between the methods before and after digestion. No comparison on lignin and polysaccharides degradation in time was evaluated, due to indistinguishable data from the samples mixed with sludge, after digestion, possibly resulting in an unclear conclusion.

3.1 Van Soest method

The ADL, ADF and NDF results obtained before and after anaerobic digestion of bracken, straw, hay and maize (sludge) are presented in Table 1. The results in Table 1 were recalculated according to the measured VS concentrations of each plant material before digestion. After digestion, NDF, TS and VS could not be determined due to a lack of sample. Therefore the VS concentrations were recalculated based on the remaining VS of each bottle, thus subtracting from the added VS, in the substrate and inoculum, the VS that was converted to biogas. The ADL, ADF and NDF results for

the sludge, after digestion, were not subtracted from the results of bracken, straw and hay as it can not be guaranteed that the remaining ADL, ADF and NDF would be exclusive from the plant material.

The ADF, ADL and NDF, calculated according to van Soest et al. (1991), into lignin, cellulose and hemicellulose, is presented in Table 2.

Table 1. ADL, ADF and NDF results of bracken, straw and hay before and after digestion. N.D. = not determined. Data expressed as mean ± standard deviation.

Plant material	Before digestion (g.kgVS^{-1})			After digestion (g.kgVS^{-1})		
	ADL	ADF	NDF	ADL	ADF	NDF
Bracken	204.1 ± 3.21	529.31 ± 43.89	648.51 ± 17,52	158,45 ± 29,78	599,80 ± 73,20	N.D.
Straw	62.85 ± 7.92	632.17 ± 205.19	866.08 ± 104,93	95,06 ± 6,74	443,01 ± 38,28	N.D.
Hay	28.01 ± 2.84	360.46 ± 5.10	653.76 ± 1,91	90,16 ± 24,08	386,46 ± 75,81	N.D.
Sludge (maize)	N.D.	N.D.	N.D.	114,68 ± 49,63	379,08	N.D.

Table 2. Lignin, cellulose and hemicellulose results of bracken, straw and hay before and after digestion. N.D. = not determined. Data expressed as mean ± standard deviation.

Plant material	Before digestion (g.kgVS^{-1})			After digestion (g.kgVS^{-1})		
	Lignin	cellulose	hemicellulose	Lignin	cellulose	hemicellulose
Bracken	204.1 ± 3.21	325.25 ± 40.68	119.20 ± 26.36	158,45 ± 29,78	441.35 ± 102.97	N.D.
Straw	62.85 ± 7.92	569.31 ± 213.11	233.91 ± 100.26	95,06 ± 6,74	347.95 ± 45.03	N.D.
Hay	28.01 ± 2.84	332.45 ± 2.26	293.30 ± 3.19	90,16 ± 24,08	296.30 ± 99.89	N.D.
Sludge (maize)	N.D.	N.D.	N.D.	114,68 ± 49,63	229.31	N.D.

Hemicellulose could not be calculated due to lack of sample to perform the NDF analysis. Therefore, the comparison between van Soest and ^{13}C CPMAS NMR was only performed with the cellulose results.

3.2 ^{13}C CPMAS NMR method

The results shown by ^{13}C CPMAS NMR give information on approximately 94% of the organic matter present in the samples, however for the methods comparison only lignin and polysaccharides will be discussed. Figure 2 shows the ^{13}C CPMAS NMR spectra of the raw plant material, which is, of bracken, straw and hay before anaerobic digestion. The spectra had a similar carbon distribution between the three plant materials analysed. The peak at 174 ppm is assigned to carboxylic acids, amides and ester groups (Veeken et al., 2001). The chemical shift region from 160 to 110 ppm, attributed to aromatic carbons, i.e., lignin and lignin derivates (Trinsoutrot et al., 2001; Veeken et al., 2001) showed a higher intensity for bracken. Especially in the region between 160 and 140 ppm, which is due to oxygen substituted aromatic ring carbons (Haw et al., 1984). The chemical shift region from 110 to 60 ppm is mainly attributed to polysaccharides and proteins (Veeken et al., 2001), which, as expected, was the region with the highest intensity for all plant samples analysed, since plants are comprised mainly by cellulose and hemicellulose. The peak at 56 ppm, visible for Bracken as a shoulder of the C_2, C_3 and C_5 cellulose peak (72-74 ppm) is assigned as a methoxyl in lignin and in hemicellulose (Veeken et al., 2001).

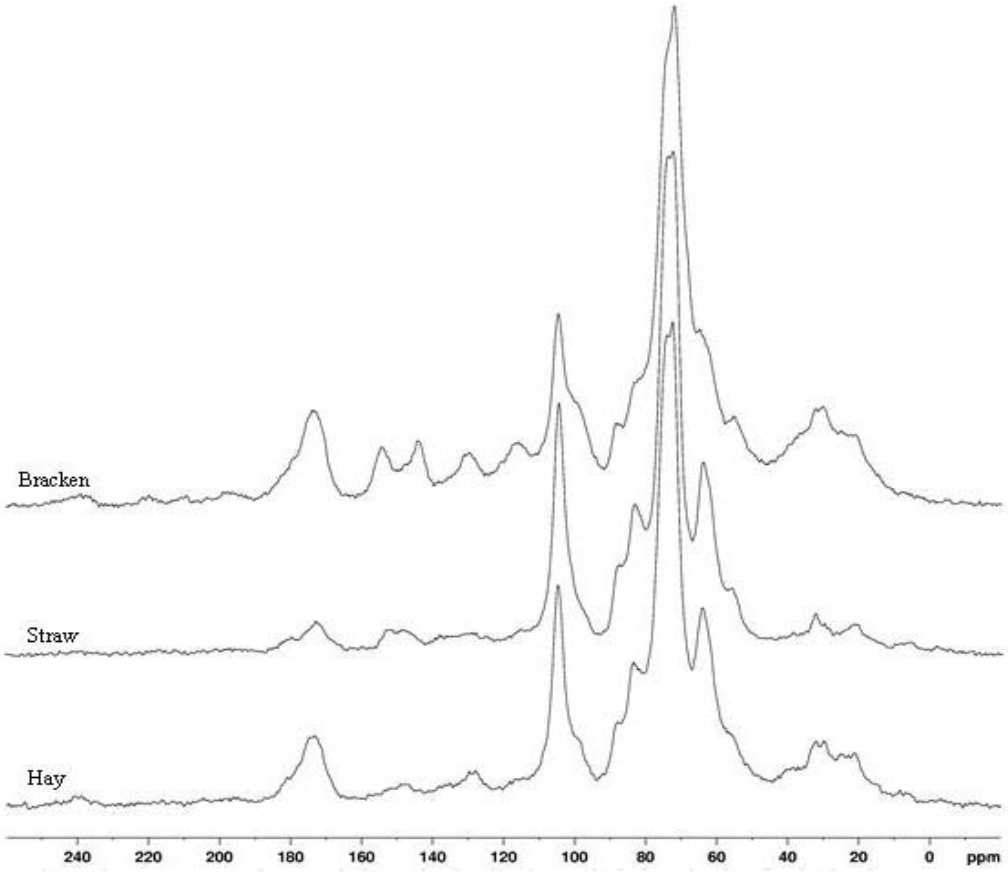

Figure 2. ^{13}C CPMAS NMR spectra of bracken, straw and hay before anaerobic digestion.

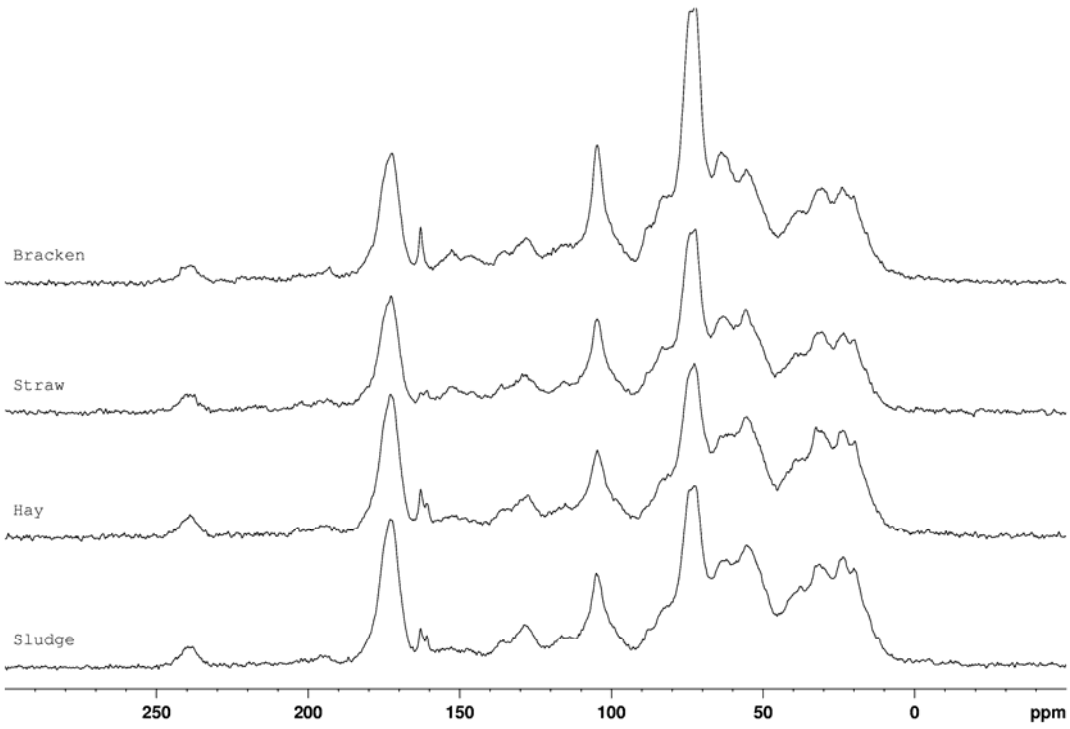

Figure 3. ^{13}C CPMAS NMR spectra of bracken, straw, hay and sludge (maize) after anaerobic digestion.

Figure 3 shows the ^{13}C CPMAS NMR spectra of bracken, straw and hay after anaerobic digestion. Once again, the spectra had a similar carbon distribution between the three plant materials analysed. Moreover, the spectrum of the inoculum used, digested maize, had also identical carbon distribution. When comparing the spectra before and after digestion it can be seen that all plant materials had higher intensities, of all forms of carbon, before digestion. Anaerobic digestion of the plant materials resulted in an increase of peak at 174 ppm, which is assigned to carboxylic acids, amides and ester groups. Between 160 and 165 ppm, for bracken, hay and maize (sludge) a single or double peak was found. However it is not clear which type of carbon it might correspond to, since it is in an area of the spectra where there might be overlap of carbon types, explicitly o-aromatic carbons, carboxylic amides and ester groups (Nelson and Baldock, 2005; Veeken et al., 2001). Table 3 shows the quantitative results of the ^{13}C CPMAS NMR spectra, before and after digestion, of bracken, straw, hay and maize (sludge), calculated according to Veeken et al. (2001).

Table 3. Lignin, polysaccharide, protein and aliphatic results of bracken, straw and hay before and after digestion by ^{13}C CPMAS NMR. N.D. = not determined.

Plant material	Before digestion (g.kgVS^{-1})				After digestion (g.kgVS^{-1})			
	Lignin	Polysacc.	Protein	Aliphatic	Lignin	Polysacc.	Protein	Aliphatic
Bracken	186.1	550.3	258.5	5.0	132.1	431.6	436.3	0.0
Straw	109.4	751.2	139.4	0.0	129.9	376.2	493.9	0.0
Hay	92.0	647.8	260.2	0.0	109.5	345.0	521.8	23.7
Sludge (maize)	N.D.	N.D.	N.D.	N.D.	94.7	358.4	522.0	25.0

The concentrations of bracken, straw and hay after digestion include the biomacromolecules concentrations originated from the sludge, since the substrate could not be separated from the sludge after digestion. The biomacromolecules present in the sludge were not subtracted from the bracken, straw and hay as it can not be guaranteed that the remaining biomacromolecules are exclusive from the plant material.

3.3 TAPPI method

The results of lignin and polysaccharides by the TAPPI method are presented in Table 4. As mentioned before, due to the limited amount of sample after digestion, the results are only of the raw plant material. In addition the straw results from Table 4 are from previous analysis and not from the same sample that was added into the batch bottles for anaerobic digestion. Even though the straw used for our tests came from the same stack as the straw previously analysed, it can not be guaranteed that the results would be identical. Moreover, the straw triplicate results were not available up to the time of delivery of this report.

Table 4. AIL, ASL, Uronic acid, polysaccharide and extractive results of bracken, straw and hay before digestion by TAPPI. Data of bracken and hay expressed as mean ± standard deviation.

Analysis	Plant material (g.kgVS^{-1})		
	Bracken	Straw	Hay
AIL	20.29 ± 1.98	19.30	7.80 ± 0.66
ASL	0.03 ± 0.01	0.74	0.08 ± 0.02
Uronic acid	4.74 ± 0.30	2.76	1.80 ± 0.17
Arabinose	0.80 ± 0.07	2.02	1.47 ± 0.12
Xylose	2.84 ± 0.31	18.19	11.48 ± 0.22
Mannose	2.90 ± 0.14	1.38	0.57 ± 0.10
Galactose	2.19 ± 0.19	1.01	0.94 ± 0.09
Glucose	17.35 ± 0.54	30.60	22.19 ± 0.18
Rhamnose	0.22 ± 0.06	0.28	0.07 ± 0.02

3.4 Van Soest, ^{13}C CPMAS NMR and TAPPI comparison before digestion

Table 5 shows the results of lignin and polysaccharides before digestion by van Soest, ^{13}C CPMAS NMR and TAPPI methods.

Table 5. Lignin and polysaccharide results of bracken, straw and hay before digestion by van Soest, ^{13}C CPMAS NMR and TAPPI. N.D. = not determined. * Only cellulose.

Plant material	Lignin (g.kgVS^{-1})			Polysaccharides (g.kgVS^{-1})		
	NMR	Van Soest	TAPPI	NMR	Van Soest	TAPPI
Bracken	186.1	204.1	203.2	550.3	444.5	310.3
Straw	109.4	62.9	200.3	751.2	803.2	562.4
Hay	92.0	28.0	78.8	647.8	625.8	385.2
Sludge (maize)	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

The results of fibre composition of the undigested plant material diverged between the applied methods, as shown in Table 5 and illustrated in Figure 4.

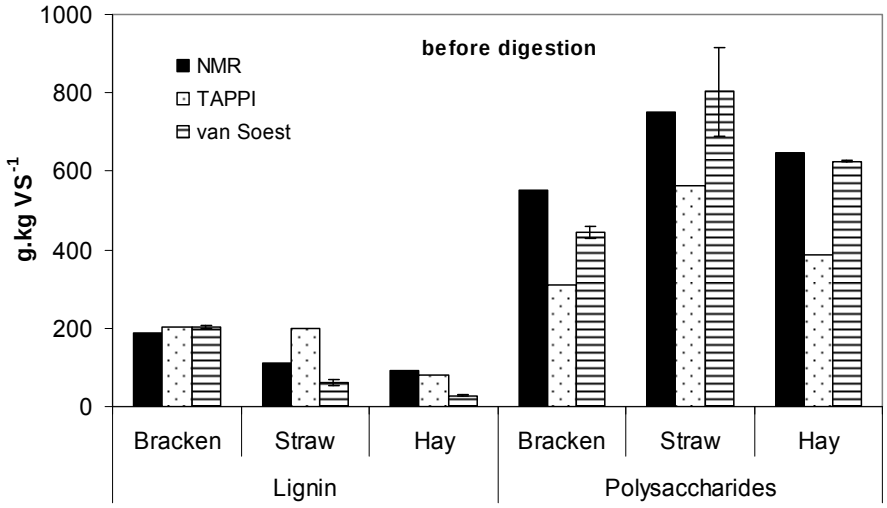

Figure 4. Lignin and polysaccharide concentrations of bracken, straw and hay obtained with ^{13}C CPMAS NMR, TAPPI and van Soest before anaerobic digestion.

The lignin concentration of bracken was the highest according to all methods, followed by straw and finally hay. The lignin concentration of the bracken was identical with all tested methods, however this was not the case for the straw and hay. The straw concentration measured by NMR was half as measured by TAPPI and almost twice as much as measured by van Soest. The TAPPI acid soluble lignin and insoluble lignin, also known as Klason lignin, of the straw was most likely overestimated, due to, the already mentioned, complications related to analysis storage time. Hay lignin concentration was three times higher for NMR than van Soest, but similar for NMR and TAPPI. The clear differences verified by NMR and van Soest in relation to straw and hay are probably due to solubilisation of lignin during the acid detergent procedure resulting in lower lignin content than in reality, as been already reported by (Fukushima and Hatfield, 2004). Moreover, also the acid insoluble lignin has been reported by Jung et al. (1997) to give higher concentrations than the ADL by van Soest. This difference is however not verified in the bracken lignin concentrations probably because bracken had neglectable acid soluble lignin, contrary to what can be verified specially in straw. Moreover, probably the lignin structure of bracken is more resistant to chemical solubilisation, resulting in more accurate results.

The polysaccharides (cellulose + hemicellulose) concentrations determined by NMR were always higher than the ones determined by TAPPI and van Soest, except on one of the van Soest straw samples, were the samples were not totally homogenized resulting in a difference of 160 g.kg VS^{-1} between the two duplicates. The polysaccharides by TAPPI, shown in Figure 4, included, besides the neutral sugars determined by the TAPPI method, also the uronic acids measured by Blumenkrantz et al. (1973), since uronic acids are the hydrolysis products of hemicellulose (Colberg, 1988). The polysaccharides measured by NMR were approximately 40% lower for bracken and hay, and 25% lower for straw, than measured by TAPPI. Once again, this difference between the straw results and the bracken and hay may be due to complications related to analysis storage time. When comparing the polysaccharide results by NMR and van Soest the differences were lower, even though for the bracken there was still a 20% difference. This is probably due to the nonselective nature of the extraction procedure by van Soest (Veeken et al., 2001), resulting in losses of polysaccharides during chemical extraction, secondary reactions, or from incomplete release of degradation products (Kogel-Knabner, 1997)

The correlation between the methods, presented in Figure 5, was good for the van Soest and NMR, with an R^2 of 0.9998 for lignin and 0.9995 for polysaccharides. The correlation between van Soest and TAPPI and NMR and TAPPI for polysaccharides was good, with an R^2 of 0.9551 and 0.9452. However, the correlation between van Soest and TAPPI and NMR and TAPPI for lignin was poor (R^2 = 0.4), due to the high lignin content measured by TAPPI. In all cases one should keep in mind that such correlations can be used as an indication only, since they were performed with only three experimental points.

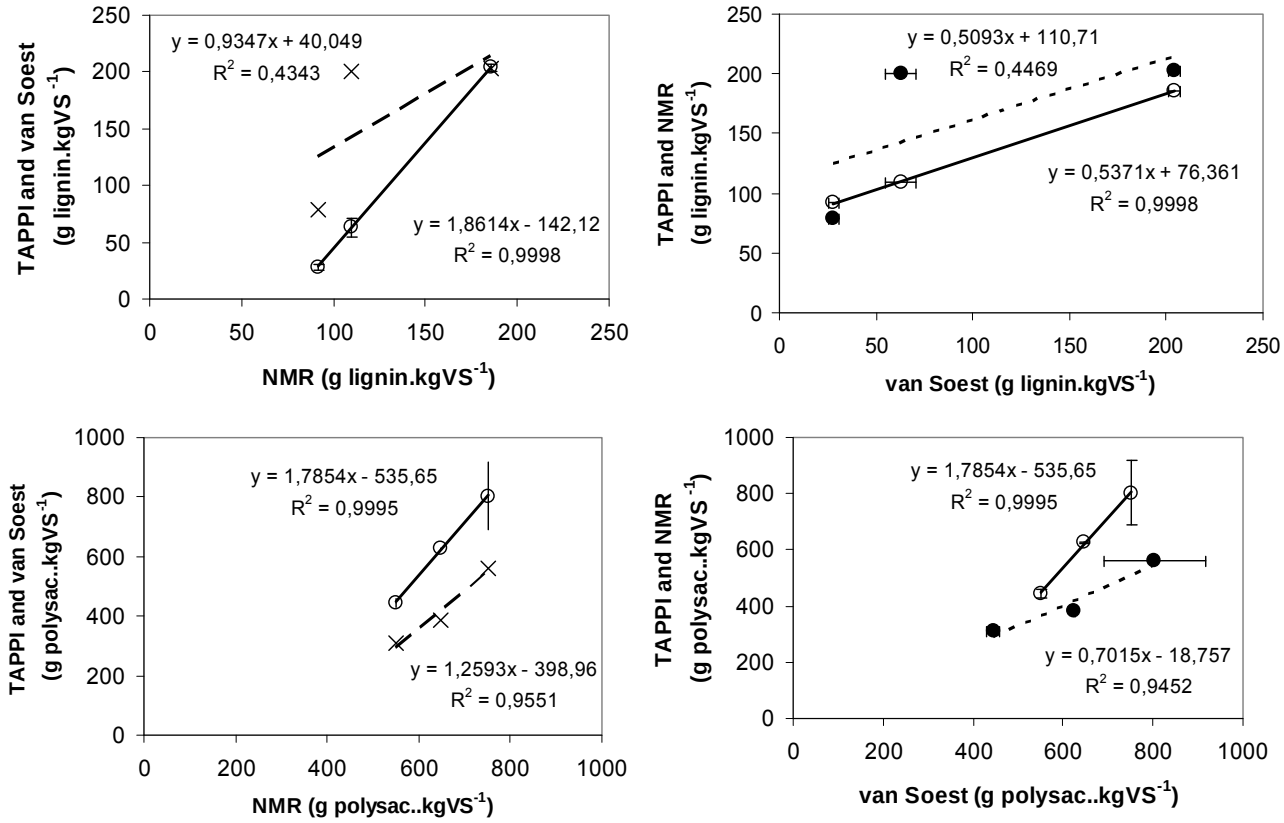

Figure 5. Correlation between lignin and polysaccharide methods before anaerobic digestion for bracken, straw and hay. NMR with van Soest (○); NMR with TAPPI (●); van Soest with TAPPI (×).

3.5 Van Soest and ^{13}C CPMAS NMR comparison after digestion

Table 6 shows the results of lignin and polysaccharides before and after digestion by van Soest, ^{13}C CPMAS NMR and TAPPI methods.

Table 6. Lignin and polysaccharide results of bracken, straw and hay after digestion by van Soest, ^{13}C CPMAS NMR and TAPPI. N.D. = not determined. * Only cellulose.

Plant material	Lignin (g.kgVS^{-1})		Polysaccharides (g.kgVS^{-1})	
	NMR	Van Soest	NMR	Van Soest
Bracken	132.1	158.5	431.6	441.4*
Straw	129.9	95.1	376.2	348.0*
Hay	109.5	90.2	345.0	296.3*
Sludge (maize)	94.7	114.7	358.4	229.3*

The lignin and polysaccharides results present in the sludge were not subtracted from the bracken, straw and hay as it can not be guaranteed that the remaining lignin and polysaccharides are exclusive from the plant material. Therefore, the results will be compared in terms of the different methods applied and not in terms of degradation of lignin and polysaccharides achieved.

When comparing the results of the digested material, shown in Figure 6, similar conclusions, to what as been mentioned in the discussion of the undigested plant material, can be attained. The lignin and polysaccharides concentrations obtained with van Soest and ^{13}C CPMAS NMR after digestion were closer than before digestion. However, due to difficulties in analysing the resulting small quantities of samples, the van Soest results after digestion had higher standard deviations between duplicates, indicating that the differences between the methods could in fact be greater. Moreover, the polysaccharides results by van Soest, shown in Figure 6, only include cellulose. Therefore higher concentrations of polysaccharides by van Soest could be expected, resulting in an overestimation of polysaccharides by the van Soest method as reported by (Dien et al., 2006).

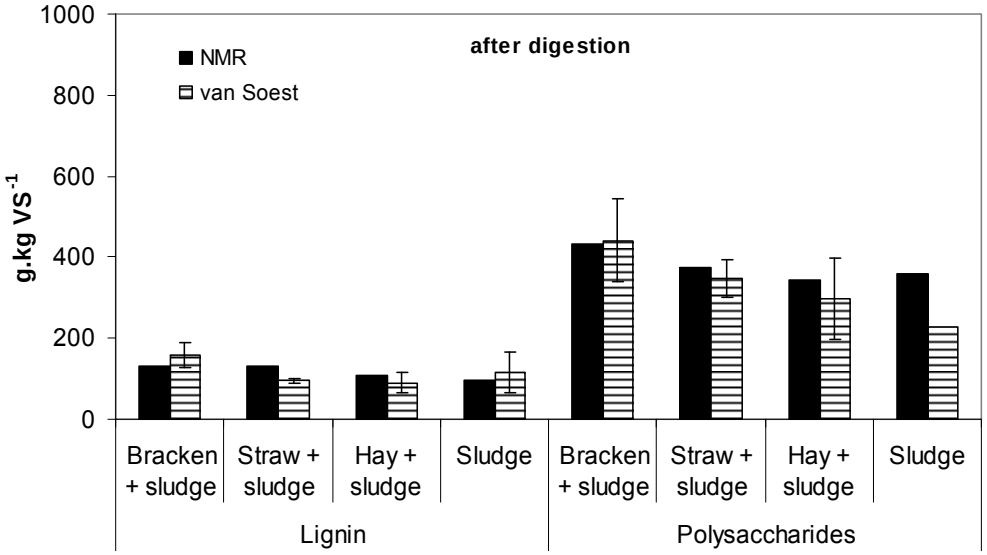

Figure 6. Lignin and polysaccharide concentrations of bracken, straw and hay obtained with ^{13}C CPMAS NMR and van Soest after anaerobic digestion.

Looking at the lignin correlation between the van Soest and the NMR methods, the results were unsatisfactory, with an R^2 of 0.1276. Since the polysaccharides results by van Soest, only include cellulose data and, from the polysaccharides results by NMR it is not possible to quantitatively

separate the data of cellulose and hemicellulose, no correlation is presented. The lignin correlation was performed with the addition of sludge, therefore with four experimental points.

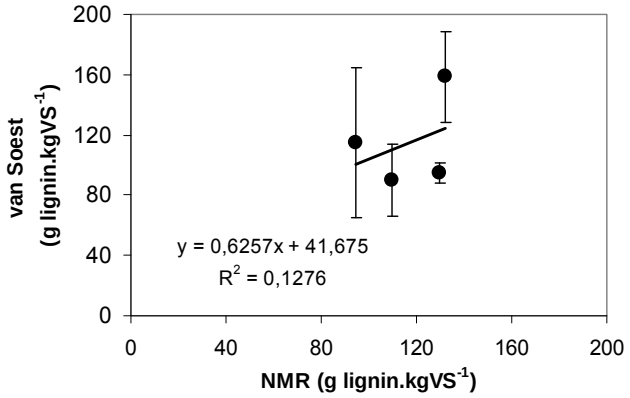

Figure 7. Correlation between lignin methods after anaerobic digestion for bracken, straw and hay.

3.6 Final objective discussion

The initial objective of this deliverable was to evaluate the degradation process of lignin and polysaccharides during anaerobic digestion in terms of hydrolysis kinetics, since hydrolysis is the rate limiting step for this type of substrate (Pavlostathis and Giraldo-Gomez, 1991). Consequently, determining how fast a certain lignocellulosic substrate with certain lignin and polysaccharide contents, could be hydrolysed during anaerobic digestion, could give insight on the hydrolysis rate constants, therefore enabling a prediction of degradation time according to the fibre content of the substrate. However, since the degradation rate is also dependent on the availability of the substrate (Gallert and Winter, 2005), it makes it extremely difficult to predict degradation rates based only on the fibre content of the lignocellulosic substrate. Meaning that two substrates with identical lignin and polysaccharide concentrations could have different degradation rates according to the way the cellulose and hemicellulose are accessible to the enzymes. Of course, this does not imply that one should not thoroughly study the relations between degradation rates and fibre content of lignocellulosic substrates, however additional studies, with innovative techniques, should also provide information on the accessibility of easier degradable polymers, like cellulose and hemicellulose.

According to (Hatfield and Fukushima, 2005), up to present date there is no single method that is, non-invasive, inexpensive, fast, with the possibility of handling several samples and supplies accurate lignin content. Therefore, one should choose the best suitable method to analyse the lignocellulose substrate and always use the same method, allowing a relative comparison between the samples (Hatfield and Fukushima, 2005). Due to its non-invasive characteristics, ^{13}C CPMAS NMR is a reliable method and a promising technique for fibre analysis, since it allows more detailed information in relation to the carbon types and quantities and may also give further information on the carbons structure using other NMR techniques, like two-dimensional NMR.

4 Conclusion

- All three methods, van Soest, ^{13}C CPMAS NMR and TAPPI, indicated that bracken had the highest lignin content and hay the lowest, before and after anaerobic digestion.
- Van Soest and ^{13}C CPMAS NMR methods had better correlation for lignin and polysaccharides than either of these two methods with the TAPPI method.

- Lignin quantification, of the undigested plant material, was identical by ^{13}C CPMAS NMR, TAPPI and van Soest when the total lignin concentration was high (bracken), however, underestimated by TAPPI and van Soest when the lignin concentration was low (hay).
- Due to its non-invasive characteristics, ^{13}C CPMAS NMR is a reliable and promising method for fibre analysis, however, still unavailable to many laboratories due to its high costs.

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