Detection and quantification of residual lignin in cellulose extracted by the diglyme method from Pinus spp. wood samples by infrared spectroscopy and analytical pyrolysis

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5   Running title: Detection and quantification of residual lignin in cellulose diglyme

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14   Keywords: Cellulose diglyme; *Pinus halepensis*; *Pinus sylvestris*, *Pinus pinaster*; ATR-FTIR

15   Analytical pyrolysis

16

17   **Abstract (150 a 250)**

18   The diglyme method is a popular method for the quantification of cellulose in hardwoods for

19   genetic analysis as well as for the development of near infrared (NIR) models. It is also useful for

20   the extraction of cellulose for stable-isotope analysis. It was already known that when applied to

21   softwoods a residual lignin content remained in the extract. The infrared spectra obtained with an

22   attenuated total reflectance accessory (ATR-FTIR) allowed not only the detection of the residual

23   lignin but also the variation among cellulose extracted from among trees and *Pinus* species.

24   Analytical pyrolysis allowed the quantification of the residual lignin content and the correction of

25   cellulose yield allowing the obtention of a good correlation with the NIR spectral information. The

26   particle size of the wood between 10 to 60 mesh did not influence noticeably the cellulose yield or

27   the residual lignin content, however ball milling did cause a decrease in the cellulose yield of at

28   least 30 %.

Introduction

29

30   The determination of the wood polysaccharide fraction in wood is long recognized as a challenging

31   task. The majority of cellulose methods require a two-step procedure starting with the isolation of

32   holocellulose followed by isolation of alpha-cellulose (Browning 1967; Fengel and Wegener 1984).

33   A simple method for cellulose determination is the diglyme method (Wallis et al. 1997). Not

34   only it is a one-step method but the procedure is quite simple and easily scaled up for large scale

35   screening as require for the assessment of the heritability of the cellulose content in *Eucalyptus*

36   *nitens* (Kube et al. 2001) and *Eucalyptus urophyla* (Kien et al. 2009) clonal trials.

37   But in terms of simplicity and throughput the diglyme method is not comparable to NIR

38   spectroscopy coupled with multivariate analysis, even though it is a proven effective method for the

39   establishment of partial least squares regression (PLS-R) models based on near infrared (NIR)

40   spectroscopy for cellulose content of Eucalyptus species (Schimleck et al. 2000; Downes et al.

41   2010; Downes et al. 2011; Downes et al. 2012).

42   The cellulose diglyme method is also a popular method for cellulose extraction for stable-

43   isotope analysis, used already for different species as for instance *Eucalyptus globulus*, *Pinus*

44   *radiata*, and *Pinus pinaster* wood (Macfarlane et al. 1999), although later Cullen and MacFarlane

45   (Cullen and MacFarlane 2005) recognized that the cellulose prepared with the diglyme method

46   contained what they called "a small lignin residue" at least for pines, although no attempt was made

47   for the quantification of the residual lignin, also difficult due to the low sample amount and even

48   more the low amount of lignin in the sample.

49   Infrared spectroscopy, particularly in transmission mode, is long recognized as a valuable

50   technique for the characterization of lignocellulosics in solid state (Hergert 1971; Faix 1992), used

51   for lignin classification based on infrared spectral features (Faix 1991) and also used as a fast and

52   reliable method for quantitative work (Rodrigues et al. 1998; Rodrigues et al. 2001). The attenuated

53   total reflectance (ATR) technique, allows a more convenient and fast acquisition of the spectra

54 (Pizzo et al. 2013; Pizzo et al. 2015), in the right circumstances can rival in throughput with near

55 infrared spectroscopy (Costa et al. 2013).

56   Analytical pyrolysis was long recognized as a suitable tool for lignin characterization as well

57 as for the analysis of residual lignin in pulp (Meier and Faix 1992; Choi et al. 2001; Rodrigues et al.

58 2001; Meier et al. 2005), credited with a simple sample preparation and a low sample amount

59 required for the analysis (less than 1 mg) (Meier and Faix 1992). The quantification of the lignin

60 content directly from the pyrogram (Py-lignin) was found to be well correlated to the Klason

61 reference method for *Pinus pinaster*, *Picea abies* and *Larix* sp. Samples (Alves et al. 2006; Alves et

62 al. 2008). Analytical pyrolysis was also a proven reference method for the development of a NIR

63 model to assess the H/G ratio in *Pinus pinaster* (Alves et al. 2006b), as well as, in combination with

64 principal component analysis, for the separation of species, tissues, and sites (Alves et al. 2009).

65   This work used FTIR-ATR as a fast, non-destructive way to detect and analytical pyrolysis to

66 quantify, the residual lignin in cellulose extracted by the diglyme method from *Pinus halepensis*,

67 *Pinus sylvestris* and *Pinus pinaster* wood samples. The influence of wood particle size and

68 vibratory ball milling on the yield of cellulose was also assessed.

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70

71 **Materials and Methods**

72 *Material*

73 A total of 27 cellulose samples extracted by the diglyme method from extractive-free wood meals

74 of *Pinus pinaster* (4, Pnb), Pinus *silvestrys* (9, Psyl) and *Pinus halepensis* (14, Phal) were

75 characterized by infrared spectroscopy (FTIR) and analytical pyrolysis. The chemical composition

76 of the wood samples used to extract the cellulose in terms of lignin amount and composition as well

77 as cellulose diglyme varied more among tress than species (Table 1). Despite the range of cellulose

78 (40 % to 50 %) the statistics (R^2, RMSECV and RPD), of the correlation with the NIR spectral

79 information, were not as good as expected considering the 10 % range (0.74, 1.9, 2.0 respectively).

80 *Particle size*

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81 Four additional Pnb samples with similar lignin content and composition but differing in the

82 particle size (10-18, 18-40 and 40-60 mesh) were selected for cellulose extraction by the diglyme

83 method to assess the influence of the particle size on the performance of the cellulose extraction.

84 *Vibrational ball milling*

85 Two aliquots of the coarser samples (10-18 mesh and 18-40 mesh) were further ball milled and

86 subsequently the cellulose extracted by the diglyme method, in an attempt to improve the lignin

87 solubility.

88 *Diglyme method*

89 The cellulose was extracted from approximately 250 mg of each sample placed in tared 15 mL glass

90 vials with ground glass joints, after the dry weight determination (overnight at 60 ºC followed by 2

91 hours at 100 ºC), 2,5 ml de diethylene glycol dimethyl ether anhydrous (99.5%), 0,5 mL

92 hydrochloric acid 10 M and a small magnetic stirrer were added to the sample and the vial caped

93 and fitted with stainless steel joint clips. Extraction was performed at 90 ºC for one hour in a stirred

94 water bath. The residue was filtered through G3 crucibles and washed with methanol (20 ml)

95 followed by hot water (200ml). The cellulose was gently removed from the crucible after dry

96 weight determination (overnight at 60 ºC followed by 2 hours at 100 ºC) for further analysis.

97 *Infrared spectroscopy*

98 The infrared spectra of the cellulose diglyme residues were acquired with a Bruker FT-IR

99 spectrometer (Alpha) using a diamond single reflection attenuated total reflectance (ATR)

100 accessory. The ATR-FTIR spectra were acquired in the wavenumber range from 4000 to 400 cm^{-1}

101 with 24 scans per spectrum, a spectral resolution of 4 cm^{-1} and a zero filling of 2. NIR spectra of the

102 exactly same samples used for cellulose diglyme (250 mg) were previously acquired in 22 mm flat

103 bottom vials (Bruker IN440-005) placed directly above the integration sphere window of a Bruker

104 MPA spectrometer (Bruker, Ettlingen, Germany). The spectra were recorded from 12000 – 4000

105 cm^{-1} with 100 scans per spectrum at 8 cm^{-1} spectral resolution and a zero filling of 2. At least two

106 spectra were acquired per sample and averaged. Post spectral manipulation were restricted to min

107 max normalization between the minimum at 1800 cm-1 and the maximum below 900 cm^{-1}.

5

108 *Analytical pyrolysis*

109 Analytical pyrolysis was performed using a CDS Pyroprobe 1000 with a coil filament probe

110 connected to a GC (HP 7820 FID) by a heated interface (270°C). The pyrolysis was carried out at

111 650°C for 10 s, using between 75 and 78 μg of the of the cellulose diglyme. Details of the

112 quantification procedure was already published (Alves et al. 2006; Alves et al. 2006b).

113

114 **Results and Discussion**

115 *ATR-FTIR spectroscopy*

116 The spectra of the cellulose diglyme between 1800 cm^{-1} and 800 cm^{-1}, of two samples with

117 contrasting residual lignin content (13, shows the characteristics bands of cellulose with the

118 exception of the band with a maximum at 1500 cm^{-1} (Figure 1). The presence of this band,

119 attributed to the aromatic skeleton vibration of lignin, is an unequivocal sign for the presence of

120 lignin (Faix 1992) also used for the identification of the lignin type (Faix 1991). The clear

121 difference in the relative intensity of this band in the two spectra, normalized between the minimum

122 at 1800 cm^{-1} and the maximum above 900 cm^{-1}, is in line with the residual lignin content as

123 determined by analytical pyrolysis (Figure 1). No absorbance scale is given in the figure, because

124 the spectra were normalised.

125 The ATR-FTIR spectra of two cellulose diglyme preparations with contrasting residual

126 [i]lignin content (13.6 dashed line, 6.1 solid line). The spectra were converted to absorbance and

127 normalized between the minimum at 1800 cm^{-1} and the maximum above 900 cm^{-1}.

128 *Analytical pyrolysis*

129 The residual lignin in the cellulose diglyme for all samples ranged from 5.9 % to 13.6 % (Table 2).

130 The variation among trees within species was larger than among species both for residual lignin

131 content as well as for composition (H/G), even considering the restricted number of Pnb samples

132 that, by the way, do not cover the range of variation in terms of lignin amount and composition for

133 this species (Alves et al. 2006; Alves et al. 2006b).

134   The H/G of the residual lignin was about twice that of the original woods with an overall

135   correlation between the two for all samples of 0.87.   This correlation is higher than the correlation

136   between the lignin content of the wood and that of the residual lignin content in the cellulose

137   diglyme (0.76), that is also similar to the correlation between Klason lignin content and composition

138   (H/G) of the wood. The correlation within species, only presented for Psyl and Phal due to the

139   restricted number of Pnb samples, is similar despite the fact that the ranges are lower and smaller

140   for Psyl, but with a systematic difference between the two, for the same lignin composition in the

141   wood the residual lignin of Psyl will have a higher ratio. This in principle shows that it is not only

142   the ratio that plays a role but also differences in the lignin composition as already revealed by the

143   combination of analytical pyrolysis and principal component analysis for other species (Alves et al.

144   2009). The Phal sample marked with an x in Figure 2, with the higher ratio H/G, did not fit well the

145   tendency line, its original wood had the higher lignin content as well as the higher H/G consistent

146   with the presence of compression wood (Alves et al. 2009). So it seems likely that samples with

147   compression wood will have a residual lignin with a disproportionate H/G ratio compared to the

148   amount.

149   *Particle size*

150   The cellulose extraction of the Pnb samples with similar lignin content and composition and

151   variable particle size (10-18, 18-40, and 40-60 mesh) resulted in a slightly decrease in the crude

152   cellulose yield as well as in the residual lignin content with the particle size (Table 3). The ball

153   milling resulted in a slightly increase in the residual lignin between 0.6 % to 1.0 %, but the main

154   effect was an important loss of the cellulose at least 32% (Table 3). This could in principle be

155   attributed to a decreases in the degree of crystallinity and/or in the degree of polymerization of

156   cellulose due to ball-milling (Schwanninger et al. 2004) hence improving its solubility by the

157   diglyme treatment. In order to assess the true effect of the ball milling on the solubilisation of lignin

158   one should consider the residual lignin based on the original wood or reported to the lignin content

159   of the wood. These calculations did show that ball milling also improved the removal of lignin

160 between 1.2 % to 1.4 %, or 4% to 5% if reported on the wood or lignin basis. This effect was

161 masked by the concomitant loss of cellulose (Table 3).

162 *PLS-R model*

163 The cellulose yield corrected for the Py-lignin show an improved correlation with the spectral

164 information (Figure 3). Although the number of samples so far analysed did not allow a proper

165 external validation, the improvement of the statistics ($R^2 = 0.82$, RMSECV = 1.53 and RPD = 2.35)

166 are encouraging and point to at least an improved model for corrected cellulose content. The

167 improvement of the model corroborates the findings that the residual lignin varied among trees, and

168 it is not a simple scale factor due to a systematic error. However, the error is still higher than the

169 reported error for hardwoods close or below 1 % (Raymond and Schimleck 2002; Schimleck et al.

170 2005; Schimleck et al. 2005; Poke and Raymond 2006).

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173 **Conclusions**

174 The cellulose extracted by the diglyme method contains residual lignin, which content and

175 composition varies more among trees within species as well as among the studied species. The

176 residual lignin content and its composition are well correlated with the lignin content and

177 composition of the original wood samples. ATR-FTIR is a fast, non-destructive technique well

178 suited to detect the residual lignin in cellulose. Analytical pyrolysis is well suited for the

179 quantification of the residual lignin content as well as the lignin composition (H/G). Vibratory ball

180 milling had a strong influence on the cellulose yield (above 37 % loss) and a small impact on the

181 lignin. The cellulose content corrected by Py-lignin is more suitable for obtaining better NIR based

182 PLS-R models then the uncorrected cellulose content.

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275   Caption of figures

276   Figure 1 ATR-FTIR normalized spectra between 1800 cm^{-1} to 800 cm^{-1}, of two cellulose diglyme

277   samples with contrasting residual lignin content (13.6 dashed line, 6.1 solid line)

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279   Figure 2 Correlation between the H/G ratio of the residual lignin and the H/G ratio of the wood of
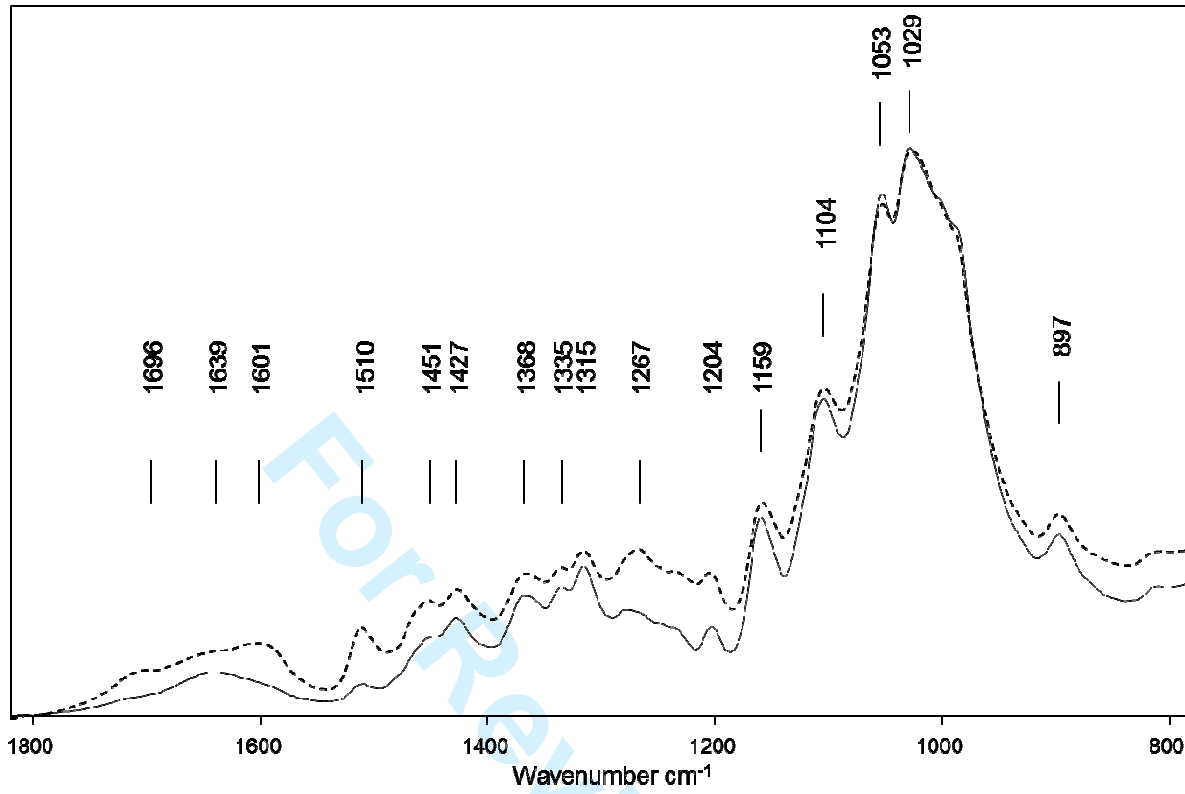
280   *Pinus halepensis* and *Pinus sylvestris*

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282   Figure 3 Line of best fit for the predicted versus determined of the corrected cellulose diglyme yield

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285   FIG. 1

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288   FIG. 2
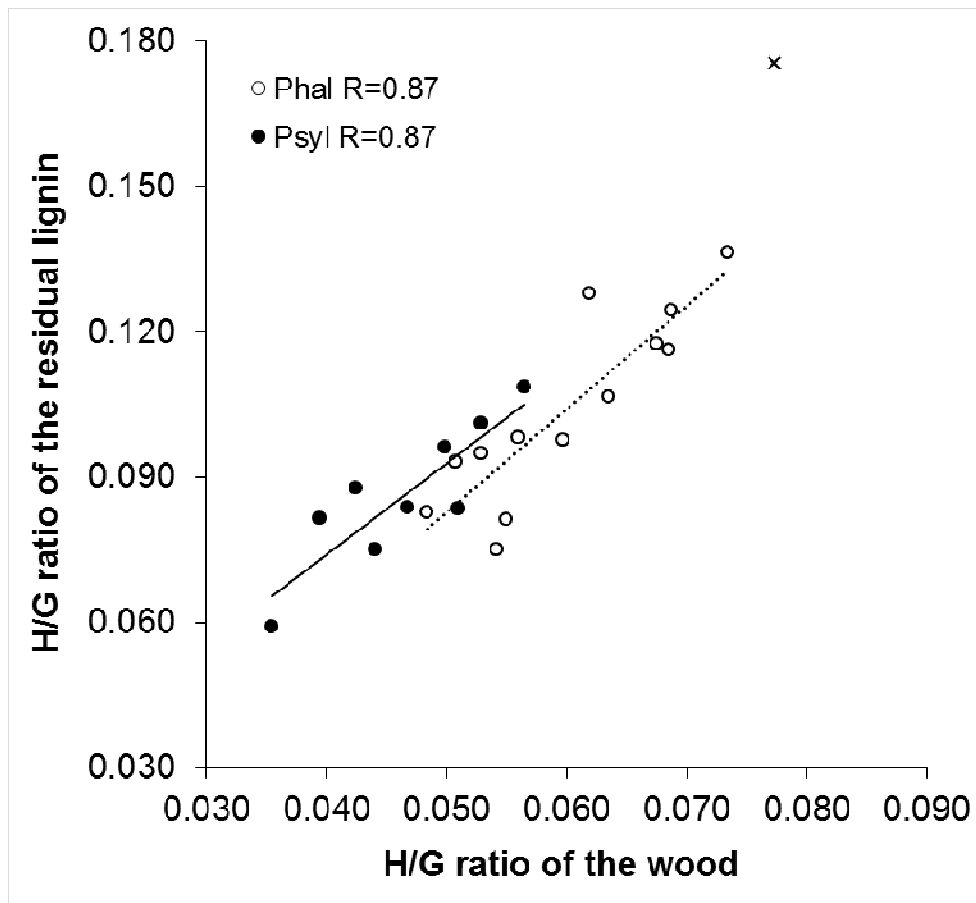
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302   FIG. 3
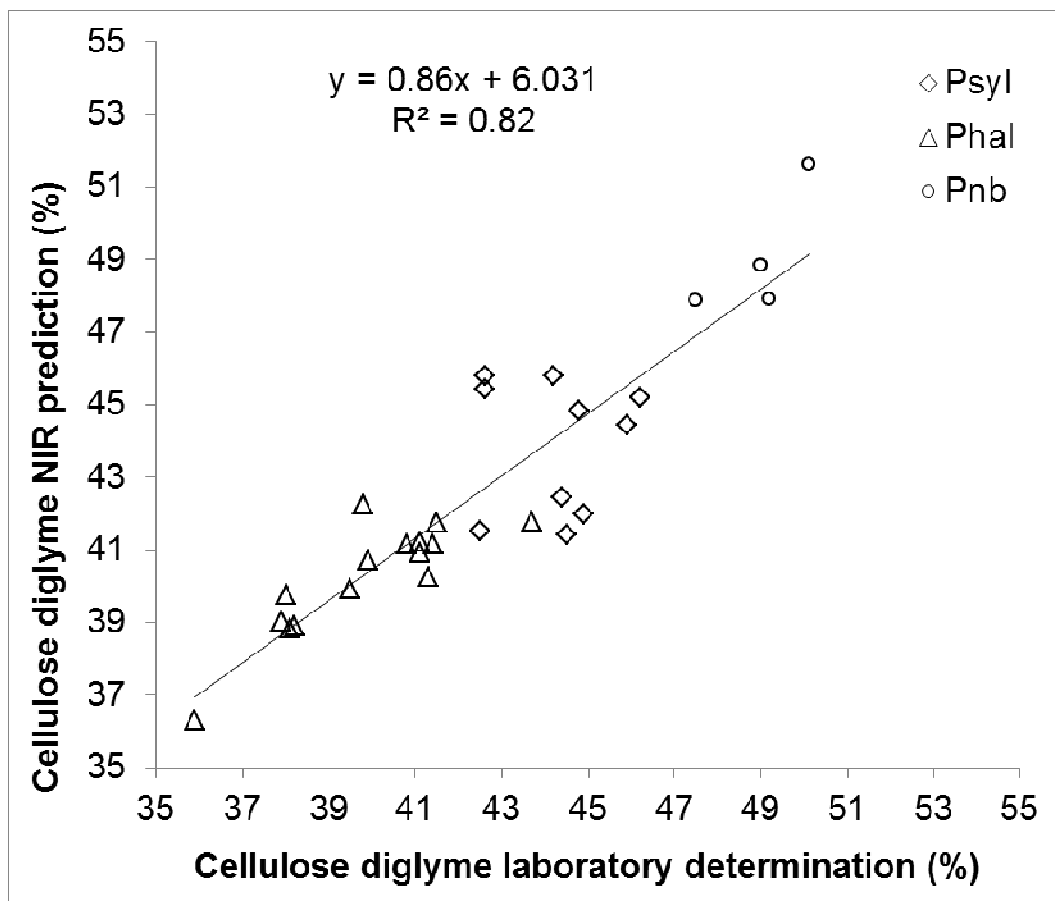
316   Caption for Tables

317   Table 1 Chemical composition of the wood samples used to obtain the cellulose diglyme

318

319   Table 2 Characterization of the residual lignin of the extracted cellulose diglyme

320

321   Table 3 Chemical characterization of the Pnb samples with similar lignin content and composition

322   and different particle size distribution (10- 18, 18-40 and 40-60 mesh) and ball milled (MM)

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343 TAB. 1

		Phal			Psyl			Pnb	
	H/G	Klason	Celldig	H/G	Klason	Celldig	H/G	Klason	Celldig
Av	0.061	27.4	43.4	0.047	27.5	48.2	0.038	26.3	52.6
Stdev	0.009	2.1	1.9	0.007	1.3	1.8	0.004	0.56	1.4
Max	0.077	31.1	48.0	0.057	29.4	50.8	0.041	27.0	54.4
Min	0.048	24.4	41.1	0.035	25.5	45.5	0.033	25.8	50.9

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367 TAB. 2

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	Phal		Psyl		Pnb	
	Res lignin	H/G	Res lignin	H/G	Res lignin	H/G
Av	8.3	0.109	7.8	0.086	6.9	0.081
Stdev	1.9	0.027	1.5	0.015	0.9	0.014
Max	13.6	0.175	9.6	0.108	7.9	0.098
Min	6.1	0.075	5.4	0.059	5.9	0.063

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391 TAB. 3

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Particle size (mesh)	Cellulose diglyme (%)	Cellulose corrected (%)	Residual lignin (%)	Residual lignin based in wood (%)	Residual lignin based on Klason (%)	Cellulose loss (%)
10-18	54.4	50.1	7.9	4.3	16.1	
10-18 MM	34.2	31.3	8.5	2.9	10.9	40
18-40	52.8	49.1	7.1	3.8	14.0	
18-40 MM	31.8	29.2	8.1	2.6	9.6	32
40-60	52.3	49.2	5.9	3.1	9.6	
40-60	50.9	47.5	6.7	3.4	10.6	

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