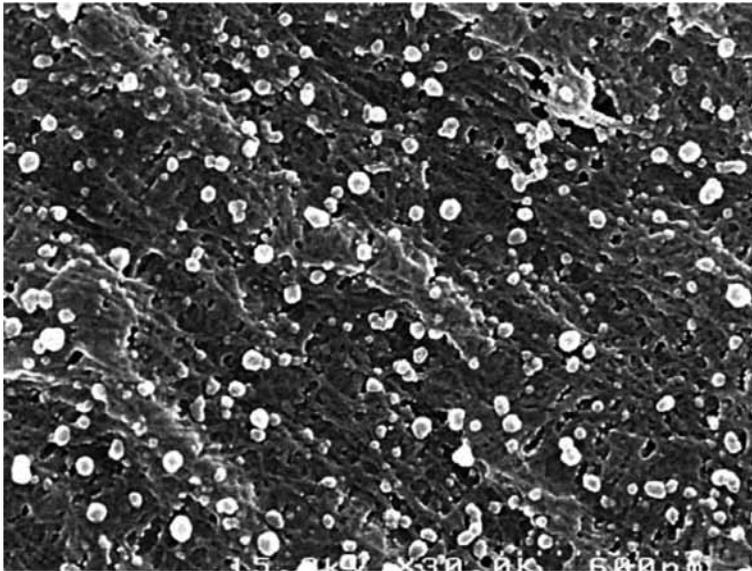


Chemical Pulping

The influence of cooking with high xylan liquor concentration on xylan fiber wall distribution

Geoffrey Daniel, *SLU*; **Anne-Mari Olsson**, *Innventia*; **Paul Ander**, *SLU*;
Lennart Salmén, *Innventia*; **Andrea Kaňuchová**, **Lada Filonova**, **Dinesh**
Fernando and **Jong Sik Kim**, *SLU*; **Elisabet Brännvall**, *KTH*

Karin Sjöström, *Södra*; **Leif Boussard**, *Stora Enso*; **Staffan Berg**, *Iggesund*
Paperboard, *Holmen*; **Anette Heijnesson-Hultén**, *Akzo Nobel*;
Curt Lindström, *Smurfit Kappa*



Intern rapport nr 8 (begränsad spridning)

CRUW
Centre for Research on Ultrastructure of Wood fibres
Centrum för forskning om Vedfibers Ultrastruktur
Sveriges lantbruksuniversitet
Institutionen för skogens produkter
Uppsala 2012



Chemical Pulping

The influence of cooking with high xylan liquor concentration on xylan fiber wall distribution

Geoffrey Daniel, *SLU*; **Anne-Mari Olsson**, *Innventia*; **Paul Ander**, *SLU*;
Lennart Salmén, *Innventia*; **Andrea Kaňuchová**, **Lada Filonova**, **Dinesh**
Fernando and **Jong Sik Kim**, *SLU*; **Elisabet Brännvall**, *KTH*

Karin Sjöström, *Södra*; **Leif Boussard**, *Stora Enso*; **Staffan Berg**, *Iggesund*
Paperboard, *Holmen*; **Anette Heijnesson-Hultén**, *Akzo Nobel*;
Curt Lindström, *Smurfit Kappa*

Intern rapport nr 8 (begränsad spridning)

CRUW

Centre for Research on Ultrastructure of Wood fibres

Centrum för forskning om Vedfibers Ultrastruktur

Sveriges lantbruksuniversitet

Institutionen för skogens produkter

Uppsala 2012



Summary

The aim of this study was to investigate if a very high concentration of xylan in the cooking liquor would lead to diminished dissolution of native xylan from the fiber walls and thereby preserve xylan to a greater extent in the fiber walls. Another aim was to investigate if the amount of native xylan in the fiber wall contributes to the sensitivity towards mechanical damage in the cook, affecting the strength properties.

Cooks have been performed with beech- and wheat xylan added in the impregnation stage of a softwood kraft cook. The beech- respectively wheat xylan concentration used was high (i.e. *ca* 30 g/L). The cooks with added (extra) xylan have been compared with a reference cook, performed without addition of external xylan. The three cook-variants, i.e. the reference cook, the cook with beech xylan addition and the cook with wheat xylan addition was performed with- and without the introduction of mechanical damage in the cook, thereby simulating a laboratory cook respectively an industrial cook.

The pulps produced with beech xylan addition had about 6% higher xylan content compared to the Ref-pulps. The pulp produced with wheat xylan addition had only about 1% higher xylan content.

That the higher amount of xylan in the cooking liquor contributed to a lower dissolution of the native spruce xylan could not be confirmed in the study. The structure for spruce and beech/wheat xylan is similar and it was therefore not possible to differentiate spruce xylan from that of beech or wheat with any of the analysis techniques used. Instead the amount of spruce respectively beech/wheat xylan in the pulp was calculated from cooking liquor analysis. Cooking with added beech xylan did not lead to less dissolution of native xylan compared to the reference cook. But the readsorption of the dissolved spruce xylan was lower in the cook with added beech xylan. For the cook with added wheat xylan, possibly more spruce xylan has been readsorbed compared to the reference cook.

No significant differences in sensitivity towards mechanical treatment could be noted between the differently produced pulps.

Contents

Summary

Contents	3
1 Background	5
2 Experimental	6
2.1 ANALYSIS.....	6
3 Results and discussion	8
3.1 INVESTIGATED PULPS.....	8
3.2 CARBOHYDRATE COMPOSITION AND FIBER CHARGES.....	8
3.3 QUANTIFICATION AND DIFFERENTIATION OF SPRUCE RESPECTIVELY BEECH/WHEAT XYLAN.....	9
3.3.1 <i>Xylan distribution within the fiber wall; quantification and visualization</i>	9
3.3.2 <i>Carbohydrate composition in cooking liquors</i>	11
3.4 INTRODUCTION OF FIBER DAMAGE AND INFLUENCE ON STRENGTH PROPERTIES..	11
4 Conclusions	18
5 Recommendation	19
References	20
Appendices	21

APPENDIX 1.

APPENDIX 2. IMMUNOFLUORESCENCE MICROSCOPY AND ELISA EVALUATION OF XYLANS ON THE SURFACE OF FIBRES DERIVED FROM SPRUCE KRAFT PULPS DURING WHICH BEECH AND WHEAT XYLANS WERE ADDED AT THE COOKING STAGE

Geoffrey Daniel, Andrea Kaňuchová, Lada Filonova, SLU, Uppsala

APPENDIX 3. SEMI-QUANTITATIVE IMMUNOFLUORESCENCE STUDIES ON THE PRESENCE OF NATIVE AND ADDED BEECH AND WHEAT XYLANS DETECTED ON CROSS-SECTIONS OF KRAFT SPRUCE PULP FIBRES USING SPECIFIC XYLAN PROBES

Geoffrey Daniel, Lada Filonova, SLU, Uppsala

APPENDIX 4. TRANSMISSION ELECTRON MICROSCOPY (TEM) ON BEECH AND WHEAT XYLAN TREATED KRAFT SPRUCE FIBRES

Jong Sik Kim, Geoffrey Daniel, SLU, Uppsala

APPENDIX 5. SEM OBSERVATIONS ON BEECH AND WHEAT XYLAN TREATED KRAFT SPRUCE PULP FIBERS

Geoffrey Daniel, SLU, Uppsala

APPENDIX 6. USE OF SIMONS STAINING FOR CHARACTERIZING XYLAN TREATED CHEMICAL PULPS

Dinesh Fernando, Geoffrey Daniel, SLU, Uppsala

APPENDIX 7. XYLAN ABSORPTION BY POLARISED FTIR MICROSCOPY

Anne-Mari Olsson, Lennart Salmén, Innventia

APPENDIX 8. INFLUENCE OF WHEAT AND BEECH XYLANS ON SHEARING AND HCL SENSITIVITY OF SPRUCE KRAFT PULPS

Paul Ander, SLU, Uppsala

1 Background

Previously the influence of the redistribution of xylan in the cook has been studied, i.e. the xylan-containing cooking liquor was removed early in the cook and replaced with a xylan free-liquor. The removed xylan-rich liquor was then added later in the cook (Daniel *et al.* 2010). The investigated pulp-variant was compared to a reference pulp produced without redistribution of the cooking liquors. Both pulps were produced with- and without introduced mechanical damage in the cook. The cook performed with reduced xylan concentration in the cooking liquor early in the cook resulted in a pulp with impaired strength properties. The removal of xylan from the cooking liquor seemed to have drained the fiber wall of the native xylan to a higher extent making the fibers more sensitive towards the mechanical damage introduced during the cook.

In this study softwood pulps were produced with- or without mechanical treatment in the cook and with a very high xylan concentration in the cooking liquor early in the cook. The high xylan concentration was achieved by adding extra xylan from an external source. Beech- or wheat xylan was added with a concentration of 30 g/L.

The aim was to investigate if the high xylan concentration could diminish the dissolution of native xylan in the fiber wall and if the dissolution was reduced, investigate whether this would lead to improved resistance towards mechanical damage introduced during the cook, measured as improved strength properties.

2 Experimental

The pulps were produced from industrial produced chips from round wood spruce. The chips were laboratory screened according to SCAN 40:01 using a Chip Classifier model JWIII A with fractions 2, 3 and 4 used in the cooking experiments.

Three different pulp variants were produced. Two variants with extra xylan added in the impregnation stage, one with beech- and one with wheat xylan added with a concentration of 30 g/L, as well as one reference pulp without any extra xylan added. For each variant, 2 pulps were produced, one with- and one without mechanical treatment at the end of the cook. The two pulps were produced using exactly the same conditions during the cook, i.e. half of the pulp in the digester was subjected to mechanical forces and half was not. Pulps were produced at Innventia using a digester equipped with a device that can introduce mechanical forces, i.e. shear and compressive forces on the pulp. The digester was described earlier by Salmén and Lundqvist (2011). To obtain enough pulp, more than one cook was performed and merged. Primary data for all cooks are shown in *Appendix 1a*. All pulps were cooked to about the same kappa number of *ca* 30. The cooking conditions used are shown in *Table 1* and *Appendix 1a*.

The beech xylan was obtained from Sigma (X4252) and the wheat arabinoxylan from Megazyme. Data on carbohydrate composition, molecule weight and degree of substitution for the beech- and wheat xylan can be found in *Appendix 1b*.

Table 1. Cooking conditions

	Shear temp. °C	Compr, %	OH ⁻ charge g/L	H-factor	Cooking time ¹ min	Residual OH- g/L	Kappa no N ² , S ³
Ref	165	32.9	44	1300	120	12.8	29.7, 29.2
Beech	165	32.1	45	1300	120	9.2	31.0, 29.3
Wheat	165	32.1	45	1300	120	8.8	31.7, 31.3

¹at 165 °C

²N cooks without mechanical treatment, i.e. only compression

³S cooks with compression and shearing forces applied during 2 minutes, 15 minutes before the end of cook

2.1 ANALYSIS

The chemical composition of pulps was analyzed after acidic hydrolysis and HPLC-analysis using electrochemical detection. Analyses were performed by Stora Enso. Bulk and surface charge analysis were also performed by Stora Enso.

Quantification of beech- and wheat-xylan on fiber surfaces was done using ELISA. More details can be found in *Appendix 2*.

Fiber properties and strength properties were evaluated after PFI-refining. The analyses were performed by Akzo Nobel, except analysis of rewetted zero-span which was performed by Södra on sheets prepared at Akzo Nobel.

Visualization of adsorbed xylan on whole fibers and on cross sections was analyzed using immunofluorescence microscopy. More details can be found in *Appendix 3*.

Visualization of xylan in the pulp fiber cell walls at high magnification was achieved using correlated immunofluorescence and Transmission Electron Microscopy. More details are shown in *Appendix 4*.

Visualization of adsorbed xylan on whole fibers was also done with SEM. More details can be found in *Appendix 5*.

Studies on changes in porosity of whole fibers with xylan treatment using Simons stain are given in *Appendix 6*.

Distribution of xylan on fiber surfaces and overall composition was analyzed with FTIR microscopy. More details can be found in *Appendix 7*.

Measurements on the number of weak points in fibers were analyzed using the HCl-method. More details are found in *Appendix 8*.

3 Results and discussion

Three different pulp variants were produced; one Ref-pulp, one with beech xylan addition and one with wheat xylan addition in the impregnation stage; the xylan retained for the rest of the cook. The beech- and wheat xylan concentration in the cooking liquor was very high (30 g/L). All three variants were produced with- and without mechanical treatment in the cook. The cooked pulps were evaluated regarding xylan distribution, introduced amount of damage and strength properties.

The aim was to investigate if the high concentration of xylan early in the cook would diminish the dissolution of native xylan from the fiber wall and if the dissolution was reduced, investigate if the higher amount of native xylan in the fiber wall would lead to improved resistance towards mechanical damage introduced during the cook, measured as improved strength properties.

3.1 INVESTIGATED PULPS

The cooking results for the six pulps included in the study are shown in *Table 2*. The pulps had about the same kappa number after cook (i.e. *ca* 30). The pulp produced with mechanical treatment at the end of the cook (S) had a higher dry content compared to the pulp produced without mechanical treatment (N), as analyzed after standardized centrifugation as previously observed (Daniel *et al.* 2010; Daniel *et al.* 2011a; Daniel *et al.* 2011b).

Table 2. Cooking results

	Residual OH ⁻ g/L	Dry content % N ¹ , S ²	Kappa no N ¹ , S ²	Shives %	Total yield %
Ref	12.8	25.7, 30.6	29.7, 29.2	0.89	47.7
Beech	9.2	26.6, 31.4	31.0, 29.3	3.4	51.6
Wheat	8.8	25.4, 30.8	31.7, 31.3	1.7	48.7

¹cooks without mechanical treatment, i.e. only compression

²cooks with compression and shearing forces applied during 2 minutes, 15 minutes before the end of cook

3.2 CARBOHYDRATE COMPOSITION AND FIBER CHARGES

The pulps produced with beech-xylan addition contained a much higher xylan content (6%) compared to the Ref-pulps (*Table 3*). The pulp produced with wheat addition only had about 1% higher content, although the charge of xylan was the same for the beech- and wheat-xylan produced cooks. It is likely that the wheat xylan with its much higher content of substituted arabinose units had more difficulty to absorb onto the fibers. The arabinose units probably act as steric hindrance for the absorption.

Table 3. Carbohydrate content and fiber charges in the investigated pulps

		Ref-N	Ref-S	Beech-N	Beech-S	Wheat-N	Wheat-S
Galactose	Rel. %	0.4	0.4	0.4	0.4	0.4	0.4
Glucose	Rel. %	84.2	84.4	79.2	78.9	83.0	83.3
Mannose	Rel. %	7.1	7.1	6.8	7.0	6.9	6.9
Arabinose	Rel. %	0.6	0.6	0.5	0.5	0.8	0.8
Xylose	Rel. %	7.7	7.5	13.1	13.2	9.0	8.7
Galactose anhydrose	%	0.4	0.4	0.4	0.4	0.4	0.4
Glucose anhydrose	%	75.8	75.3	70.3	69.1	74.5	76.3
Mannose	%	6.4	6.4	6.1	6.1	6.2	6.3
Arabinose anhydrose	%	0.5	0.5	0.4	0.4	0.7	0.7
Xylose anhydrose	%	6.8	6.6	11.4	11.3	7.9	7.7
Tot anhydrose sugar	%	89.8	89.1	88.5	87.4	89.6	91.3
Hydrorest.	%	4.5	4.0	4.7	5.0	5.1	5.7
Tot acid groups	mmole/kg	75	72	95	89	80	74
Surf. charge (neg)	meqv/kg	8	7	10	9	8	8

3.3 QUANTIFICATION AND DIFFERENTIATION OF SPRUCE RESPECTIVELY BEECH/WHEAT XYLAN

The aim of using high xylan concentration in the cooking liquor was to diminish the loss of native spruce xylan from the fiber wall and thus obtain fibers less sensitive towards mechanical damages. To be able to draw suitable conclusions on whether the dissolution of spruce xylan had diminished or not, different analysis techniques were used to investigate and visualize the xylan composition in the pulp. Also the cooking liquors were used for analysis.

The influence of added xylan on the dissolution of native xylan was analyzed using a number of different analysis methods. Results are discussed below. A more detailed description of the analysis methods and results will be found in *Appendices 2-8*. However, it was not possible with any of the advanced techniques used to truly distinguish between the native spruce xylan from that of beech- and wheat xylan.

3.3.1 Xylan distribution within the fiber wall; quantification and visualization

From the semi-quantitative ELISA studies, highest values of xylans were found with the Beech-N and Beech-S followed by Wheat-N/S and the Ref. N and S pulps. Reduced levels were noted with the reaction of the LM10 probe for epitopes of unsubstituted and poorly substituted xylans with the wheat xylans using ELISA and most of reaction here can be assumed to be similar to that of the Ref. pulps. Quantitative ELISA results confirmed the much higher level of xylans on Beech-N (OD_{405} 1,309) and Beech-S (OD_{405} 1,222) pulp fibres than the Ref.-N (OD_{405} 0,792) and S (OD_{405} 0,843) pulps using LM10. Immunofluorescence studies of whole fibres showed variable results of labeling even within a pulp type but overall the beech and wheat normal pulps showed the strongest fluorescence at least with LM11. A novel method allowing for the semi-quantitative analysis of larger

populations of fibre cross-sections of the pulps was developed using image analysis and imageJ. Studies using this approach further confirmed the strong fluorescence for the beech and wheat xylan treated pulps with LM11. In some cases more intense fluorescence was noted on the exposed edges of the fibres (i.e. primary wall/S1; lumen wall) consistent with the sorption of the beech and wheat xylans on the surfaces. However, even the reference pulps showed in some case strong fluorescence indicating presence of xylans adsorbed during processing. Using TEM immunogold labeling of cross-sections of early- and latewood which allows better definition at higher magnification, a fairly even spatial distribution of xylans was recorded across the fibre walls. Possibly here, a weaker labeling pattern was recorded with the LM10 for the Ref. and wheat N/S treated pulps. SEM observations showed the fibre surface of both Beech- N/S and Wheat-N/S treated pulps to have abundant aggregates presumed to be composed of xylans with differences in size, shape and morphology. In addition, encapsulation of the microfibrillar structure with was presumed to be xylan was also evident. Simons stain was used as an attempt to determine whether a change in porosity had occurred with the fibres with sorption of beech/wheat xylans. However, very little difference was noted either between the pulps or between the normal and treated pulps.

Taken as a whole the results indicate that by including 30 g/L of beech or wheat xylans in the cooks that most of the xylan retained by the fibres is associated with the fibre surfaces – both outer (probably significant because of area) and inner surfaces (lumen wall); results similar to previously observations (Daniel *et al.* 2010; Daniel *et al.* 2011a; 2011b). Differences were noted between the sorption of the beech (best) and wheat xylans but little difference was noted between the normal and sheared pulps of each pulp type. Presumably the xylan was sorbed as several molecular layers onto the fibre cellulose microfibrillar surface structure or formed aggregates that precipitated out on the fibres. It should be remembered however, that surface detection techniques such as ELIZA/immunofluorescence will only be able label the exposed xylan –i.e. extreme outer layer available for reaction with the epitopes so the results are probably an underestimate of the total xylan present.

Finally, it was not possible with the methods used to distinguish between spruce (native) and beech or wheat xylan since the xylans are structurally very similar. Using specific probes to only spruce xylan or specifically marked added xylan could be a way to distinguish between different types of xylan. This was not however, explored in this study.

3.3.2 Carbohydrate composition in cooking liquors

Attempts to calculate the amount of spruce respectively beech/wheat xylan in the pulp from analysis of the cooking liquor removed at the end of the cook and the carbohydrate composition in the pulp were done. In *Appendix 1c* the primary data for cooking liquor analysis from the different cook variants is outlined.

The content of xylan in the cooking liquor and the ratio of arabinose and xylose for the different cooks are shown in *Tables 4 and 5*, respectively. From the cooking liquor data, no evidence is given that the higher amount of xylan in the cooking liquor has contributed to a lower dissolution of the native spruce xylan. From the cook with added beech xylan the calculation of the spruce xylan indicate that this has not led to less dissolution but less reabsorption compared to the reference cook. For the cook with added wheat xylan, possibly more spruce xylan has been reabsorbed compared to the reference cook.

Table 4. Amount of xylan in cooking liquor

	Dissolved xylan, g	Added xylan, g	Amount in cooking liquor, g
Ref.	14.8	0	6.1
Beech	3.4	90	36.2
Wheat	11.7	90	43.4

Table 5. Arabinose/xylan quota

	Wood	Added xylan	Pulp	Pulp calculated	Cooking liquor
Ref.	0.15-0.25	-	0.075	-	0.12
Beech	0.15-0.25	0.0	0.035	0.044	0.043
Wheat	0.15-0.25	0.61	0.090	0.150	0.29

3.4 INTRODUCTION OF FIBER DAMAGE AND INFLUENCE ON STRENGTH PROPERTIES

The hypothesis was that having a higher content of native xylan in the fiber wall would influence the sensitivity of the fibers to mechanical damage. However, a reduced dissolution of native xylan could not be confirmed thus an improved resistance towards mechanical damages should not be expected and thus improvements on strength properties.

The tear index vs tensile index is shown in *Figure 3*. The Ref-pulp with the lowest xylan content had the highest tear-strength at a certain tensile strength and the Beech-pulp had the lowest tear-strength. The pulps produced without mechanical damage in the cook had significantly higher tear strength compared with those

produced with shearing, the reduction in strength due to mechanical action being about the same for all three pulp variants.

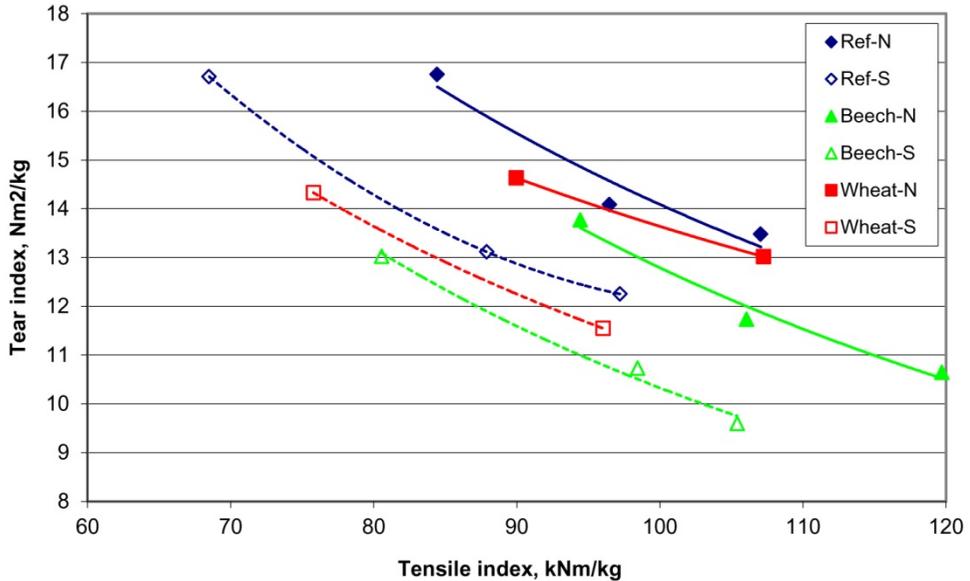


Figure 3. Tear index vs tensile index.

The zero-span levels were similar for all pulps produced with- and without mechanical treatment (Figure 4). The pulps produced without mechanical treatment had a higher strength level. The stretch vs PFI beating revolution is shown in Figure 5. No clear conclusions can be drawn regarding the influence of xylan content or mechanical treatment on the stretch. Possibly the pulps with highest xylan content had the straightest fibers. The Beech-pulps showed a lower stretch compared to the other pulps.

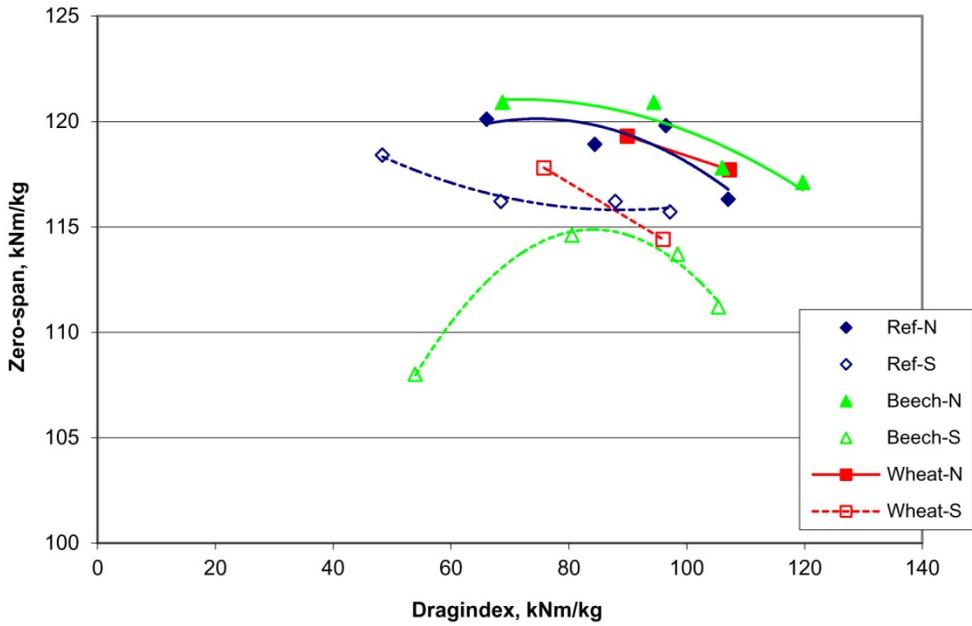


Figure 4. Zero-span vs PFI beating revolutions.

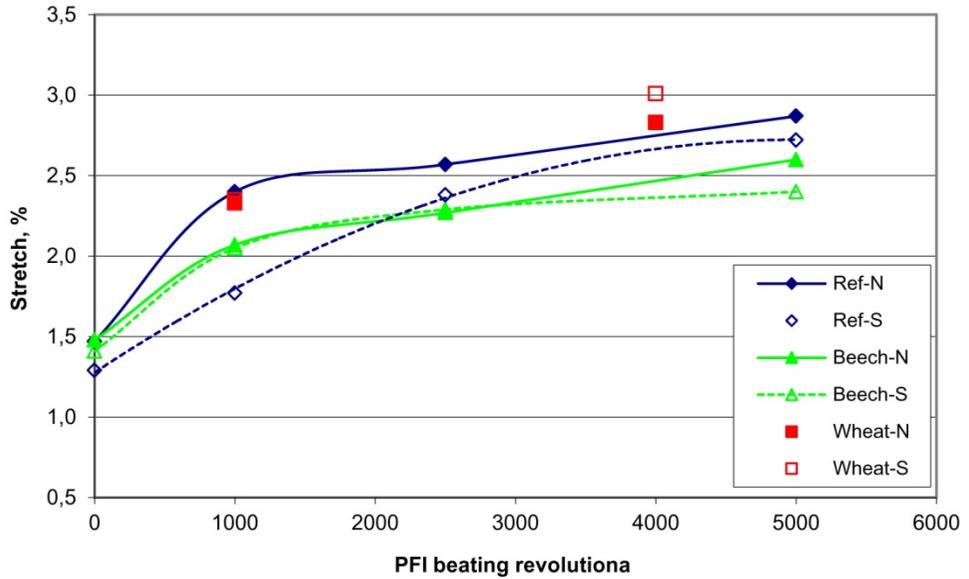


Figure 5. Stretch vs PFI beating revolutions.

Tensile development as a function of PFI beating revolutions was significantly higher for the Beech-N pulp compared to the Ref-N-pulp (*Figure 6*). Also the tensile-density relationship was improved for the Beech-N pulp compared to the reference (*Figure 7*). However, the influence from the mechanical damage seems to be about the same for all pulp variants. All strength data are presented in *Appendix 1d*.

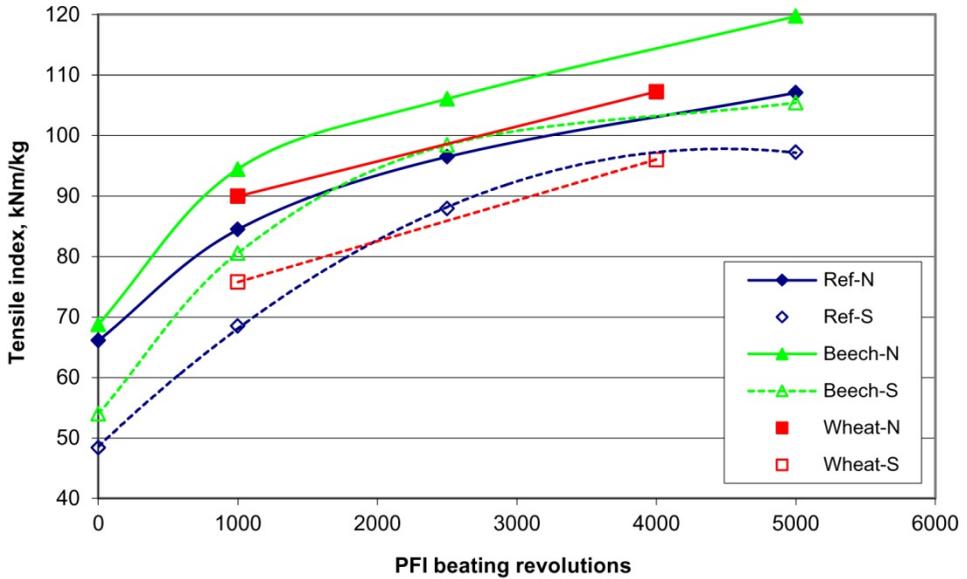


Figure 6. Tensile index vs PFI beating.

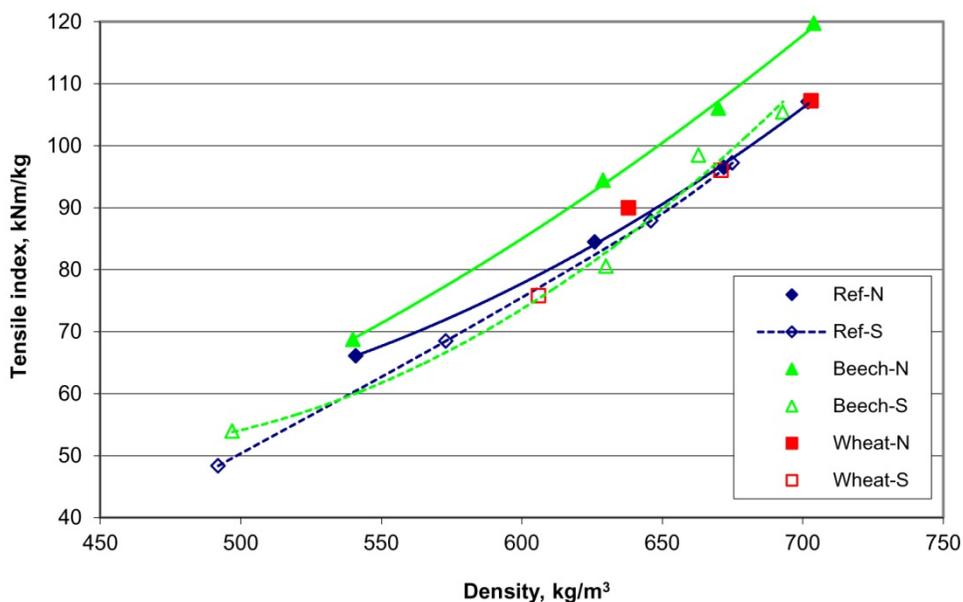


Figure 7. Tensile index vs density.

The pulps exposed to mechanical damage in the cook and with extra xylan contained a lower number of damaged areas compared to pulps without added xylan analyzed as number of cleavage/fiber by the HCl-method (Table 6). The difference is also quite large between the two variants with extra xylan added in the cook. The Beech-pulp with significantly higher xylan content compared to the Wheat-pulp showed a higher resistance towards HCl-degradation. Pulps produced without mechanical treatment in the cook contained as expected a significant lower amount of damaged areas compared to pulps produced with mechanical treatment, i.e. showed a significantly lower number of cleavages/fiber. The analyzed differences in cleavage number were not however reflected by strength properties.

Table 6. Length weighted fiber lengths (LWFL) and cleavage per fiber by HCl (standard method: 4h at 80 °C + 30 min final cleavage)

		Ref-N	Ref-S	Beech-N	Beech-S	Wheat-N	Wheat-S
LWFL H2O	mm	2.16	2.27	2.28	2.30	2.26	2.31
LWFL after HCl	mm	1.11	0.74	1.40	1.04	1.20	0.86
Cleavage/fiber		0.95	2.08	0.63	1.22	0.88	1.69

The pulps produced with added beech-xylan, i.e. Beech-N and Beech-S had straighter fibers compared to the respective Ref-pulp (Figure 8). A correlation between higher xylan content, straighter fibers and improved tensile strength development has previously been observed in several studies (Daniel *et al.* 2010; Daniel *et al.* 2011a; 2011b). The pulps produced without mechanical treatment in

the cook being straighter than those produced with treatment. However, all pulps are on a very high shape factor-level. The difference between the Wheat-N and S-pulp and respectively Ref-pulp was insignificant; i.e. the difference in xylan content was not that large either. The fiber length of the pulps is show in *Figure 9*. An explanation for the high fiber cutting for the Wheat-N-pulp and resulting measured increase in shape factor could not be found. All fiber data for the investigated pulps are tabled in *Appendix 1e*.

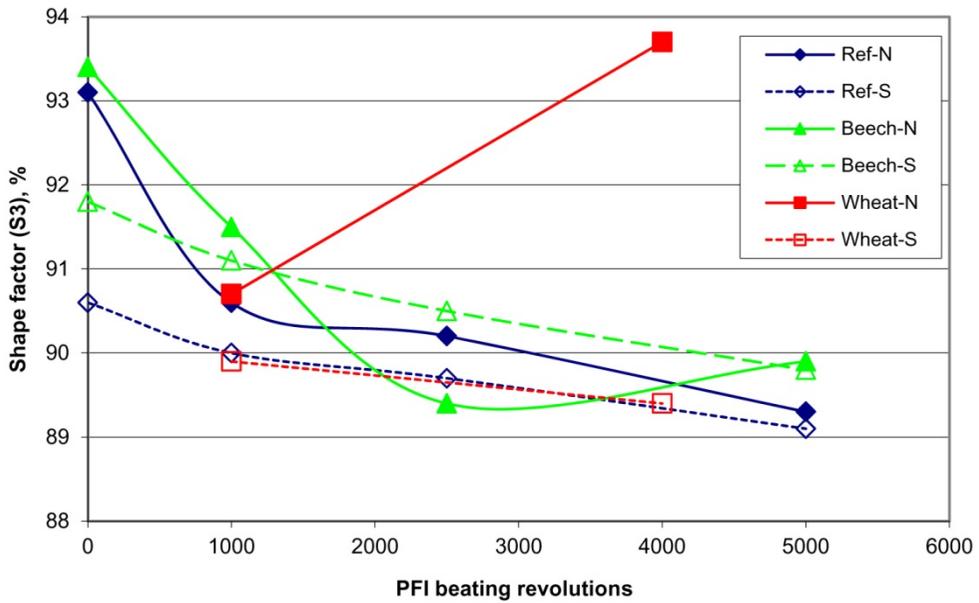


Figure 8. Shape factor vs PFI beating revolutions.

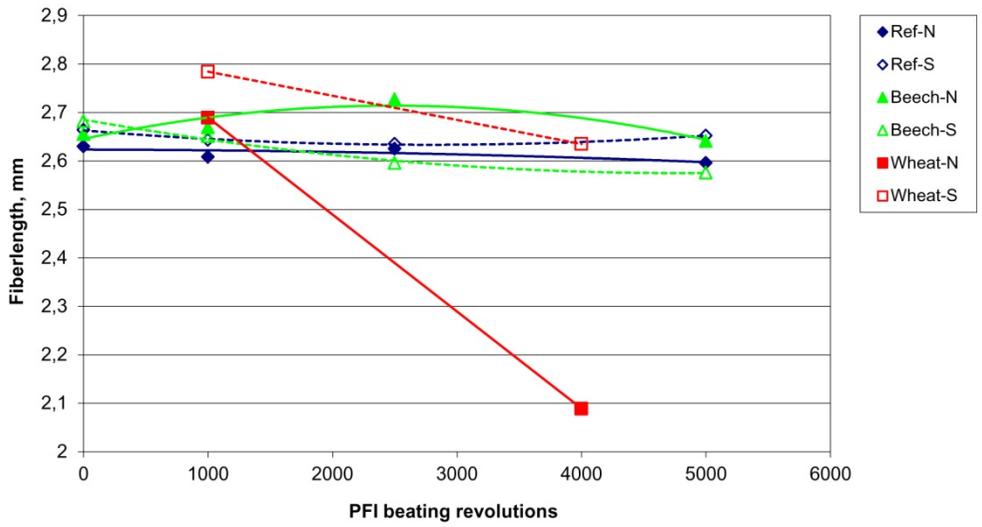


Figure 9. Fiber length vs PFI beating revolutions.

4 Conclusions

That a very high concentration of xylan in the cooking liquor contributes to diminished dissolution of the native spruce xylan from the fiber walls could not be confirmed in the present study.

With the methods and techniques used, it was not possible to distinguish between spruce (native) and added xylan (beech/wheat). The xylans are structurally very similar and a specific probe to only spruce xylan or specifically labeled xylan could be a way to distinguish between different types of xylan. That was not explored in this study. The amount of spruce respectively beech/wheat xylan in the pulp was instead calculated from cooking liquor analysis. The results could be interpreted that cooking with added beech xylan did not lead to less dissolution of native xylan compared to the reference cook, but that the readsorption of the dissolved native xylan was lower. For the cook with added wheat xylan, possibly more spruce xylan was readsorbed compared to the reference cook.

No significant differences in sensitivity towards mechanical treatment could be noted between the differently produced pulps.

For the pulps produced with added xylan a higher content of xylan was detected at the surfaces, the outer surfaces and the lumen, as previously seen. The added xylan also, as expected, improved the fiber shape as well as the tensile strength properties for these pulps.

5 Recommendation

Several studies with added xylan have been performed within this project aiming to affect the sensitivity of fibres towards mechanical damage by affecting their strength properties. That the dissolution of native xylan from the fiber wall became lower, by having high xylan concentration in the cooking liquor, could not be confirmed in this study. In all these investigations the xylan added had a high Mw and the xylan has been analyzed as adsorbed on the fiber surfaces; the outer fiber walls as well as on the fiber cell lumen walls. One reason for not achieving higher penetration of the xylan may be the molecular size of the xylan, being too large to be able to penetrate into the fiber wall.

One further possibility could therefore be to investigate if xylan with shorter chains (low Mw) would be able to penetrate further into the fiber wall, affecting the sensitivity towards mechanical damage.

References

- Daniel, G., Olsson, A-M., Ander, P., Kaňuchová, A., Salmén, L., Filonova, L. Sjöström, K., Boussard, L., Berg, S., Heijnesson-Hultén, A. and Lindström, C. (2010): The influence of xylan on the sensitivity towards fiber damage, CRUW, Intern rapport nr 2.
- Daniel, G., Olsson, A-M., Ander, P., Kaňuchová, A., Salmén, L., Filonova, L. Sjöström, K., Boussard, L., Berg, S., Heijnesson-Hultén, A. and Lindström, C. (2011a): The influence of xylan on the sensitivity towards fiber damage: xylan added in the oxygen stage, Intern rapport nr 4.
- Daniel, G., Olsson, A-M., Ander, P., Kaňuchová, A., Salmén, L., Filonova, L. Sjöström, K., Boussard, L., Berg, S., Heijnesson-Hultén, A. and Lindström, C. (2011b): The influence of xylan on the sensitivity towards fiber damage analyzed on pulps cooked to high kappa numbers, Intern rapport nr 5.
- Salmén, L. and Lundqvist, F. (2011): Effects of mechanical forces for strength delivery in kraft cooking, *Appita* 64(1), 89.

Appendices

APPENDIX 1A. COOKING RESULTS

Cook ¹⁾	Shear temp.	Press mm	Compression %	OH-charge g/l	Resid.-OH impreg. g/l	Resid.-OH end g/l	OH-consumpt kg/t	Resid.-HS g/l	H-faktor	Kappa number		Pulp, g		Shives %	Total yield %	Shear temp. °C	Pulp, % dry content		
	°C	mm	%	g/l	g/l	g/l	kg/t	g/l		S ⁵⁾	N ⁶⁾	Average	S	N	%	%	°C	S	N
Ref.1	90	225	42,3	42	22,9	7	210	5,73	1300	34,6	34,8	34,7	155,0	81,4	1,4	48,8	90	32,1	25,8
Ref.2	165	230	41,0	50	28	16,3	203	7,61	1300	23,7	24,6	24,1	136,6	87,0	1,19	46,0	165	33,4	27,2
Ref.3 ¹⁾	165	240	39,7	45	17,6	10,9	205	5,36	1296	33,3	35,1	34,0	137,9	97,8	1,55	48,8	165	33,6	26,4
Ref.4 ¹⁾	165	240	38,5	45	23,9	13,5	189	6,02	1298	27,8	29,9	28,6	146,0	84,4	0,75	46,9	165	32,8	25,7
Ref.5	165	260	33,3	45	24,5	13,3	190	6,12	1300	28,6	28,9	28,7	120,3	114,2	0,88	47,8	165	29,4	26,1
Ref.6	165	260	33,3	44	20,5	13,0	188	6,72	1300	29,2	29,3	29,2	141,1	92,4	0,91	47,6	165	31,1	25,8
Ref.7	165	265	32,1	44	22,5	12,0	195	6,38	1299	29,7	31,0	30,3	125,9	108,8	0,87	47,8	165	31,4	25,3
Beech-1 ²⁾	165	265	32,1	44	21,8	8,3	216	6,36	1300	31,0	33,2	32,0	132,6	110,6	3,12	51,8	165		
Beech-2 ²⁾	165	265	32,1	45	20,5	9,3	214	6,11	1301	29,4	31,8	30,5	131,5	111,7	3,13	51,8	165	31,5	26,9
Beech-3 ²⁾	165	265	32,1	45	24,2	9,2	215	6,26	1303	29,2	30,1	29,6	132,7	105,9	3,69	51,4	165	31,4	26,3
Wheat-1 ³⁾	165	265	32,1	45	20,5	8,8	217	6,39	1298	31,3	31,7	31,5	119,7	115,3	1,73	48,7	165	30,8	25,4

Colour code



Pretrials

1. No carbonate added
2. 30 g beech xylan/liter (Poly β-D-xylopyranose[1→4], Sigma X4252)
3. 30 g wheat arabinoxylan/liter (Megazyme)

5. Top zone (shear zone)
6. Bottom (reference zone)

4. Cooking conditions:
0-120°C >5°C/min
120°C 35 min
120-165°C, 4°C/Min

- 165°C 120 min
165°C Shear treatment
Liquid/wood: 6
SH⁷⁾: 0,255 mol/l
CO₃²⁻: 0,1 mol/l

APPENDIX 1B. ANALYSIS ON THE BEECH AND WHEAT XYLANS

Carbohydrate composition, molecular weight and degree of substitution for the beech and wheat xylans used in the experiments.

		Beech	Wheat
Galactose	Rel. %	1.3	0.3
Glucose	Rel. %	1.8	0.4
Mannose	Rel. %	<0.1	0
Arabinose	Rel. %	0.1	37.5
Xylose	Rel. %	88.5	61.8
HexA	Rel. %	<1	-
GlcA	Rel. %	7.5	-
Rhamnose	Rel. %	0.8	-
Tot anhydrose sugar	%	100	100
Molecular weight	Mp	12570	55950
	Mn	9310	42670
	Mw	11095	58610
DS		0	0.6

APPENDIX 1C. COOKING LIQUOR



Analysis on cooking liquors

Precipitate day	Sample ID	Bottle weight/g	(B+sample weight)/g	Dry content	BL /ml	Precipitate, g/l	Klason lignin, %	Ara+xyl %	Xylan g/l
2011-02-28	STFI S1-ref3	11,032	14,982	0,91	500	7,187	NA	NA	NA
2011-03-08	STFI S2-ref5	10,646	14,840	0,921	500	7,725	64,84	74,7	2,03
2011-03-22	Beech2	11,023	25,127	0,75	500	21,142	37,65	91,6	12,08
2011-04-04	Wheat2	11,042	21,973	0,929	500	20,314	26,71	97,2	14,47

APPENDIX 1D. STRENGTH PROPERTIES

	Beating rev.	SR	WRV g/g	Density kg/m ³	Tensile l kNm/kg	Stretch %	Tens. energy abs. l, J/kg	Stiffness MNm/kg	Burst l kPam ² /g	Tear l mNm ² /g	Zero-span kNm/kg
Ref-N	0	13,7	1,63	541	66,1	1,47	625,12	9,3	4,0	18,4	120,1
	1000	14,1	1,61	626	84,4	2,40	1343,43	9,3	5,6	16,8	118,9
	2500	15,8	1,71	672	96,5	2,57	1628,34	10,0	7,0	14,1	119,8
	5000	18,6	1,82	702	107,1	2,87	2015,80	10,2	7,8	13,5	116,3
Ref-S	0	13,4	1,47	492	48,4	1,29	411,63	7,7	2,4	16,6	118,4
	1000	13,2	1,53	573	68,5	1,77	801,14	8,9	4,4	16,7	116,2
	2500	14,6	1,61	646	87,9	2,38	1392,81	9,6	5,8	13,1	116,2
	5000	17,3	1,71	675	97,2	2,72	1745,16	9,8	6,7	12,3	115,7
Beech-N	0	14,1	1,67	540	68,8	1,48	646,20	8,9	4,0	16,4	120,9
	1000	14,1	1,7	629	94,4	2,07	1267,52	10,2	5,8	13,8	120,9
	2500	17,2	1,81	670	106,1	2,27	1545,79	10,6	7,6	11,7	117,8
	5000	23,3	1,92	704	119,7	2,60	2013,59	11,3	8,4	10,6	117,1
Beech-S	0	13,0	1,48	497	54,0	1,41	496,55	8,1	2,9	13,8	108
	1000	13,5	1,55	630	80,6	2,05	1082,55	9,4	5,2	13,0	114,6
	2500	15,2	1,68	663	98,5	2,29	1457,57	10,3	6,6	10,7	113,7
	5000	20,1	1,78	693	105,4	2,40	1634,54	10,5	7,2	9,6	111,2
Wheat-N	1000	15,4	1,74	638	90,0	2,33	1372,25	9,7	6,3	14,6	119,3
	4000	17,8	1,9	703	107,3	2,83	1877,93	10,0	8,5	13,0	117,7
Wheat-S	1000	13,4	1,73	606	75,8	2,35	1186,49	8,6	4,8	14,3	117,8
	4000	16,1	1,83	671	96,0	3,01	2191,29	10,4	6,6	11,5	114,4

APPENDIX 1E. FIBERMASTER RESULTS

	Beating rev.	Fiber length mm	Fiber width µm	Shape factor %	Shape factor S3, %
Ref-N	0	2,63	31,6	89	93,1
	1000	2,608	30,6	86,7	90,6
	2500	2,625	30,6	86,3	90,2
	5000	2,596	30,5	85,7	89,3
Ref-S	0	2,664	29,7	86,6	90,6
	1000	2,643	29,1	86	90
	2500	2,635	29,3	85,8	89,7
	5000	2,652	29,6	85,2	89,1
Beech-N	0	2,655	31,6	89,1	93,4
	1000	2,669	31,2	87,2	91,5
	2500	2,727	31,3	85,2	89,4
	5000	2,641	31,5	85,9	89,9
Beech-S	0	2,682	30,1	87,3	91,8
	1000	2,651	29,9	86,9	91,1
	2500	2,596	30,2	86,6	90,5
	5000	2,576	30,5	86	89,8
Wheat-N	1000	2,689	30,4	86,2	90,7
	4000	2,089	30,7	91	93,7
Wheat-S	1000	2,784	29,9	85,5	89,9
	4000	2,635	29,9	85,3	89,4

APPENDIX 2. IMMUNOFLUORESCENCE MICROSCOPY AND ELISA EVALUATION OF XYLANS ON THE SURFACE OF FIBRES DERIVED FROM SPRUCE KRAFT PULPS DURING WHICH BEECH AND WHEAT XYLANS WERE ADDED AT THE COOKING STAGE

Geoffrey Daniel, Andrea Kaňuchová, Lada Filonova, SLU, Uppsala

Aims: The aim of the study was to use the *Enzyme-Linked Immunosorbent Assay* (ELISA) to indirectly quantify the presence of beech, wheat and spruce xylans on the surfaces of spruce kraft pulp fibres and to visualize its microdistribution using immunofluorescence microscopy.

Background: In previous studies (Daniel et al., 2010, 2011a,b) the ELISA method was developed to detect and indirectly quantify the absorption of birch xylan (BX) on spruce pulp fibres added during kraft cooking. The ELISA approach developed involves use of specific rat antibodies to detect xylans. Detection involves a three step process: *i*) Localization of xylan epitopes on fibre surfaces using primary rat antibodies LM11 and LM10; *ii*) Detection of the primary rat antibody using a specific enzyme-linked-FITC-conjugated secondary antibody; and *iii*) Determination of the amount of enzyme present by addition of its substrate and measurement of colour development at 405 nm using spectrophotometry over a fixed period of time. The approach allows for the determination of unknown quantities of xylan binding to pulp fibres by comparison to a standard curve where different concentrations of pure beech/wheat xylan absorbed to the surfaces of microtitre plates have been determined in a pre-examination experiment. The advantage of the ELISA approach is that it allows determination of the presence of xylan on a relatively large population of fibres (i.e. *ca* 700-1000 fibres) thereby reducing the problems of substrate (fibre) variability from the true reactivity (e.g. differences in morphological structure, chemistry) when only a few fibres or fibre areas are analyzed as is often the case with microscopy approaches. The ELISA approach therefore complements microscopy methods. In order to visualize the microdistribution of xylans on fibres, immunofluorescence microscopy was also employed using the same antibodies and methodology described previously (Daniel et al., 2010).

Material and Methods

1) Fibre materials

Spruce fibres were derived from kraft cooks in which the xylan content during the impregnation had been significantly increased to 30g/L with either beech or wheat xylan. One half of each of the pulps was also subjected to mechanical action (shear

and compressive forces) at the end of the cook in a special digester (Salmén and Lundqvist, 2011). Additional reference pulps without added xylan and with- and without mechanical action were also produced. For more details see the introductory part of this report.

2) Xylans

Beech xylan (poly β -D-xylopyranose (1-4)) was obtained from Sigma (X4252) and wheat xylan arabinoxylan from Megazyme.

3) Monoclonal antibodies

Visualization of xylan was achieved using monoclonal antibodies generated against low and unsubstituted xylans (LM10) and low and highly substituted (LM11)(1-4)- β -D-xylans. The antibodies were a generous gift from Prof. P. Knox (Leeds Univ., UK).

4) Immunofluorescence of xylans on whole spruce fibres treated with either beech/wheat xylan and reference pulps with- and without shearing

i) Whole fibres

Whole fibres were reacted with anti-xylan in eppendorf tubes as described earlier by Daniel et al., (2010).

ii) Positive and negative and substrate controls

The specificity of the anti-xylan was checked previously using a number of positive and negative substrate controls (Daniel et al., 2011a,b). Thus in the present study only the technical control where the antibody was omitted from the labeling procedure was adopted.

iii) Fluorescence microscopy

Fibre sections were placed on object glasses mounted in Fluorsave (Calbiochem) covered with coverslips and examined using a Leica DMRE fluorescence microscope fitted with a mercury lamp and I3-513808 filter-cube (Leica, excitation 450-490 nm, emission 515 nm) from Leica Microsystems, Wetzlar, Germany. Images were recorded using a Leica DC300F CCD camera and digital imaging system for professional microscopy (Leica Microsystems GmbH) at equal settings (exposure time 1s and gain 3.2).

5) Enzyme Linked Immunosorbent Assay (ELISA): This involved the development of standards using beech and wheat xylans. The reactions of the xylans on the reference pulps gave an estimation of the presence of spruce xylan.

i) Enzyme Linked Immunosorbent Assay- Standard development

The standards were developed as described previously (Daniel et al., 2011a,b) and used in the present study.

ii) Enzyme Linked Immunosorbent Assay

Presence of beech and wheat xylans on the pulps was determined in the same manner as described previously (Daniel et al., 2010). Briefly, 1.7 mg of never-dried pulp fibres taken from a filtered pulp fibre suspension suspended overnight in buffer were suspended in eppendorf tubes containing blocking agent in PBS and left for 1 hr at room temperature. Thereafter, the fibres were treated with rat-anti-xylan (LM11 or LM10) in PBS overnight at 4° C. Next day, samples were washed and treated with a secondary goat anti-rat secondary antibody IgG conjugated to alkaline phosphatase. Samples were left for 2 hr at RT in a shaker (or overnight in a cold room). After alkaline phosphatase labeling, the substrate p-nitrophenyl phosphate was added and samples incubated for 30 mins. Colour development was terminated with NaOH, the fibres centrifuged down and 100 µl transferred to ELISA plates for absorbance determination at 405 nm. Xylan concentration was then determined from the 30 min. standard equation. The assay was repeated 10 times (i.e. repeated with 10 different fibre samples from the pulp) and results shown reflect mean absorbance values. Both method and specificity controls were included. Method controls included omission of the primary and secondary antibodies in the labeling procedure; all of which were negative.

Results and discussion

1) Immunofluorescence microscopy: *Figures 3-8* show representative fluorescence images of the typical labeling of whole fibres with different two antibodies (LM11 and LM10) for low and unsubstituted and low and highly substituted xylans on the surfaces of early- and latewood fibres. It is clear from the microscopy images it is difficult to see any major trends partly because of the variable fluorescence reaction along the fibre axes and differences in amounts of xylan present. SEM analysis (*Appendix 5*) showed the presence of small and large xylan aggregates associated with the fibre surfaces which is consistent with the types of reaction seen here with areas of strong fluorescence mixed with weaker areas. The reference pulps also showed some strong fluorescence consistent with the reaction of the probes for spruce xylans remaining sorbed onto the fibre surfaces after cooking.

2) Enzyme Linked Immunosorbent Assay

i) Semi-quantitative assessment for the presence of xylans; direct analysis

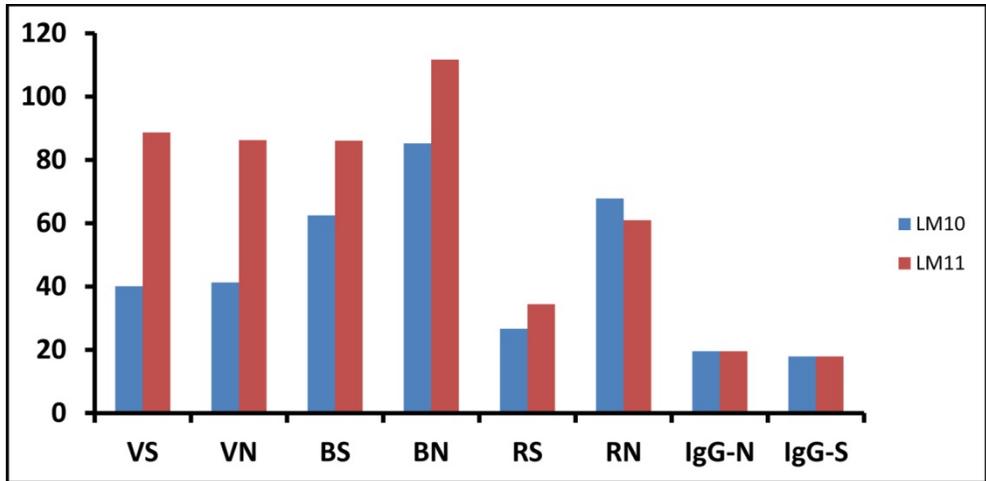


Figure 1. Uncalibrated results from the ELISA assay. Y- Axis shows absorbance at 405 nm in arbitrary units against pulp type.

Figure 1. Histogram showing direct ELISA results (i.e. not calibrated against standard beech and wheat xylans). Abbreviations: VS, Wheat sheared; VN, Wheat Normal; BN, Beech normal; BS, Beech sheared; RS, Reference sheared; RN, Reference Normal, IgG-N, normal control (no antibody), IgG-S, sheared control (i.e. no antibody).

The following trends can be observed:

- That LM11 for the presence of low- and highly substituted xylans on the surfaces of fibres gave the strongest reaction in the beech and wheat treated normal- and sheared pulps; a result consistent with the known and wider specificity of LM11. With both the beech (normal/sheared) and wheat treated pulps the reaction of LM11 was clearly stronger than for LM10;
- That for the reference pulps the difference between LM11 and LM10 was not as distinct. LM10 gave a slightly stronger reading for Ref. normal and LM11 for Ref. sheared. This result emphasizes that a range of xylans with variable substitution were also present on the surfaces of the reference spruce pulps;

- Control IgG reactions without the primary antibodies as expected showed only a weak background reaction in both normal and sheared pulps;
- That the sheared pulps show a much weaker absorbance (both beech treated and reference) than the normal treated pulps apart from wheat sheared pulps with LM10;
- That the weak reaction of the wheat normal and sheared pulps with LM10 is consistent with the weak reaction of the antibody for arabinoxylans (highly substituted xylans);
- That the strong absorbance seen with the beech-N pulp is consistent with all the other analyses (i.e. chemical analyses on xylan in pulps/xylan remaining in the cooking liquor) and microscopy methods conducted (*Appendices 3-5*);
- That the results indicate how difficult it is to distinguish between the added xylans (beech/wheat) and native spruce xylans in both the normal and sheared pulps;
- That the semi-quantitative results emphasize that considerable xylans are associated with the fibre surfaces (i.e. have apparently not penetrated into the fibres). A comparison between the reference normal with the beech- and wheat normal fibres with the LM11 probe (i.e. low/high substituted xylans) shows *ca* double and 50% more surface xylan associated with the fibres. With the LM10 probe, beech normal had *ca* 30% more xylan than that found on the reference normal pulp;
- Taken together with the results obtained with the TEM immunogold (*Appendix 4*) and immunofluorescence labeling (*Appendix 3*) studies, most of the added xylans –beech/wheat- seem to be associated with the fibre surfaces rather than penetrating into the fibre wall.

2) Quantitative assays of xylan

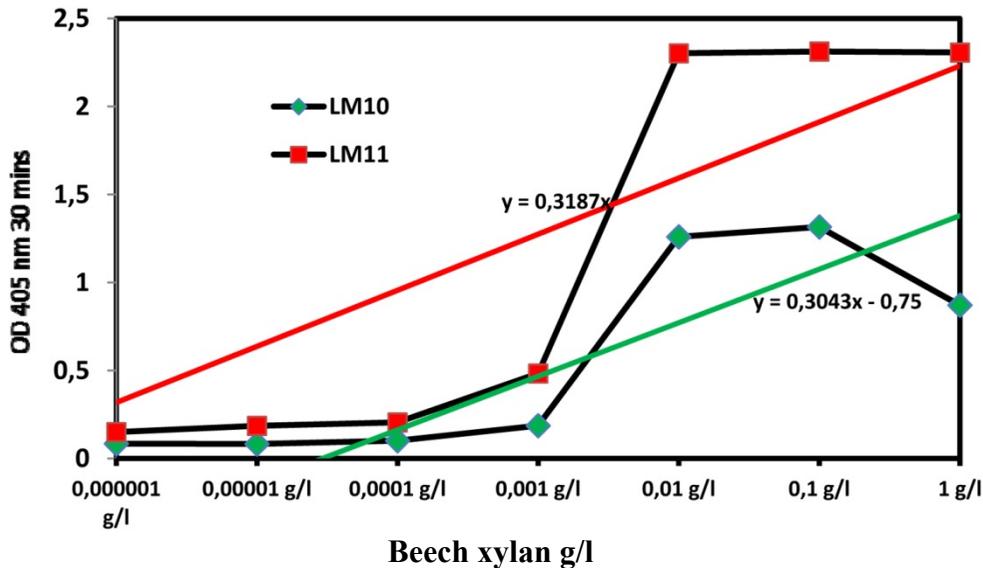
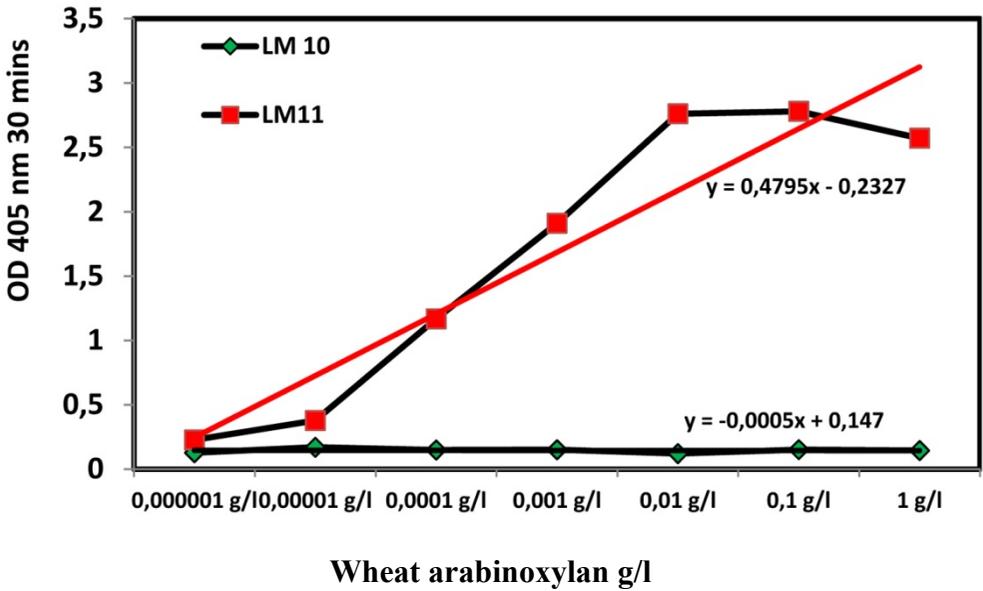


Figure 2a,b. Standard curves for purified wheat arabinoxylan and beech xylan (g/l) and their respective reaction with LM10 (low and unsubstituted xylan) and LM11 (low and highly substituted xylans) antibodies in ELISA assays.

From the standard curves (*Figures 2a, b*) for wheat and beech xylans reaction with LM10 (low and unsubstituted xylans) and LM11 (low and highly substituted xylans) the different specificities of the antibodies are apparent. LM11 reacts strongly but differently with beech and wheat xylans. In contrast, LM10 reacts strongly with the beech xylan but very poorly (*if at all*) with wheat xylans. From this standard against the pure xylan substrates we are able therefore to assume that any reactions of LM10 with fibres treated with wheat xylan are likely to be similar to that of the reference fibres with only native spruce xylan as seen in *Figure 1*. The poor reaction/negative specificity of LM10 with arabinoxylans is consistent with that reported previously (McCartney et al., 2005) and contrasts with that shown for LM11.

Quantitative estimates of low and unsubstituted xylans on the surfaces of fibres derived from the cooks with added beech or wheat xylans compared to the reference are shown in *Figure 2a,b*. The OD₄₀₅ for the wheat normal and sheared pulps was ca 0,620 and 0,644 respectively. However, from the standard curve it is not possible to attain a reading since the reaction of LM10 is poor to negative (*Figure 2a*). Therefore we can assume that the greater part of the reaction with LM10 was with the native spruce xylans absorbed/remaining on the fibre surfaces as suggested by the immunofluorescence images (*Figures 3-8*) and the results using other approaches (*Appendices 3-5*). Results from the spruce fibres treated with beech xylans and labeled with LM10 gave estimates of coverage of ca 0,1g/L (OD₄₀₅ 1,222 beech sheared; 1,309 beech normal). The reference pulp fibres with LM10 showed OD₄₀₅ estimates of xylan of 0,843 for reference sheared and 0,792 for reference normal using the standard curve in ELISA.

References

- Daniel, G., Olsson, A-M., Ander, P., Kaňuchová, A., Salmén, L., Filonova, L. Sjöström, K., Boussard, L., Berg, S., Heijnesson-Hultén, A. and Lindström, C. (2010): The influence of xylan on the sensitivity towards fiber damage, CRUW, Intern rapport nr 2.
- Daniel, G., Olsson, A-M., Ander, P., Kaňuchová, A., Salmén, L., Filonova, L. Sjöström, K., Boussard, L., Berg, S., Heijnesson-Hultén, A. and Lindström, C. (2011a): The influence of xylan on the sensitivity towards fiber damage: xylan added in the oxygen stage, CRUW, Intern rapport nr 4.
- Daniel, G., Olsson, A-M., Ander, P., Kaňuchová, A., Salmén, L., Filonova, L. Sjöström, K., Boussard, L., Berg, S., Heijnesson-Hultén, A. and Lindström, C. (2011b): The influence of xylan on the sensitivity towards fiber damage analyzed on pulps cooked to high kappa numbers, CRUW, Intern rapport nr 5.
- McCartney, L., Marcus, S.E. and Knox, J.P. 2005. Monoclonal antibodies to plant cell wall xylans and arabinoxylans. *J. Histochemistry* 53, 543-546.

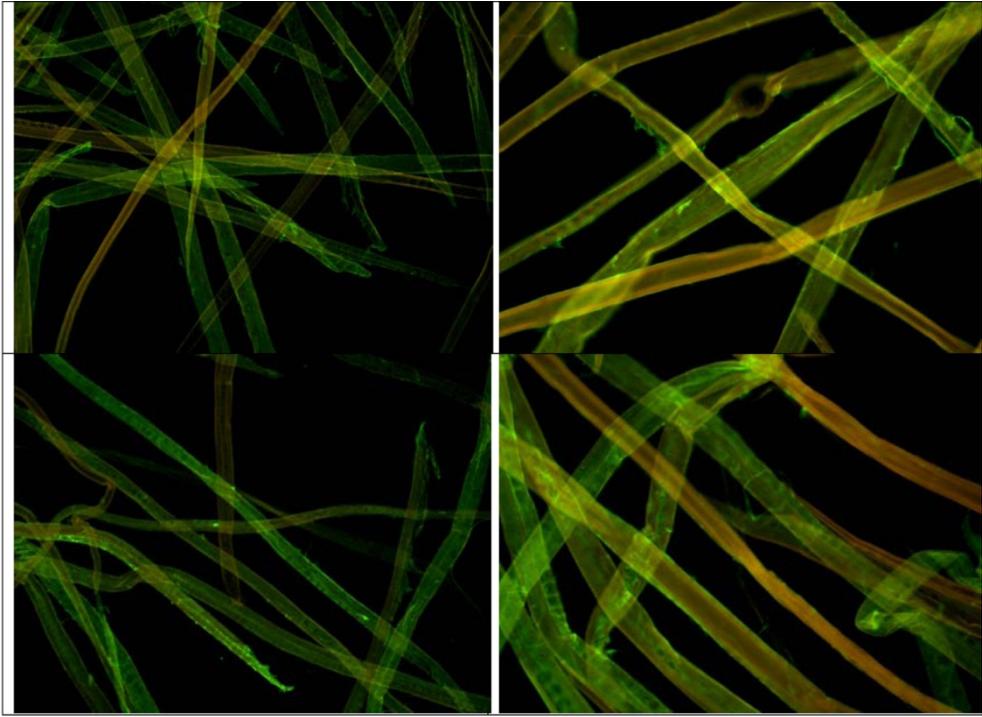


Figure 3. Immunofluorescence of whole fibres from kraft pulp with beechN xylan labeled with LM11 (upper row) and LM10 (bottom row).

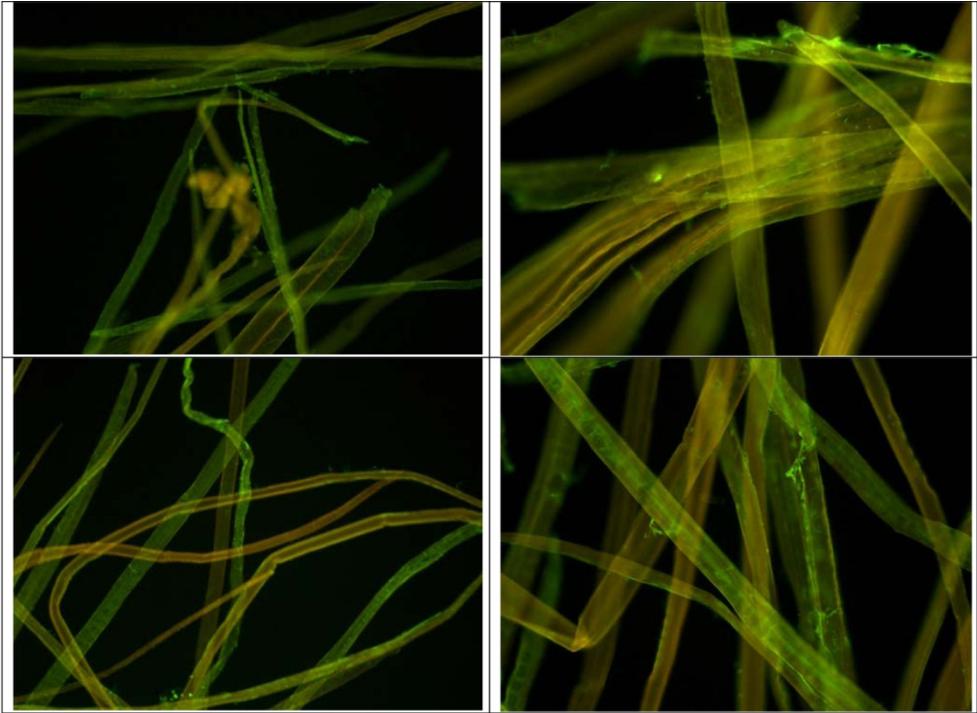


Figure 4. Immunofluorescence of whole fibres from kraft pulp with beechS xylan labeled with LM11 (upper row) and LM10 (bottom row).

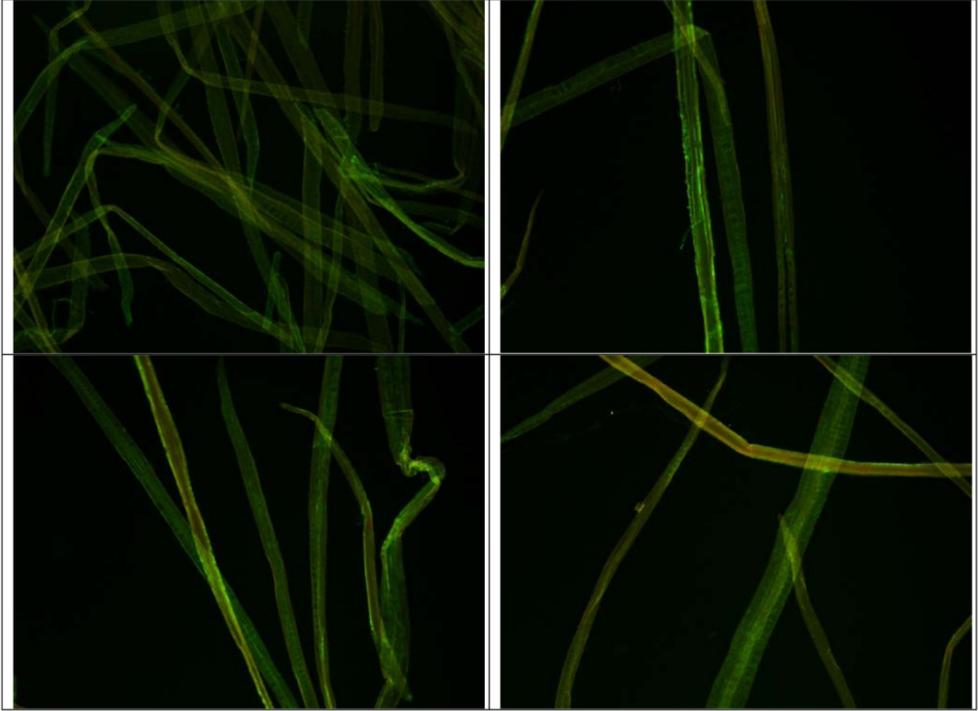


Figure 5. Immunofluorescence of whole fibres from kraft pulp with wheatN xylan labeled with LM11 (upper row) and LM10 (bottom row).

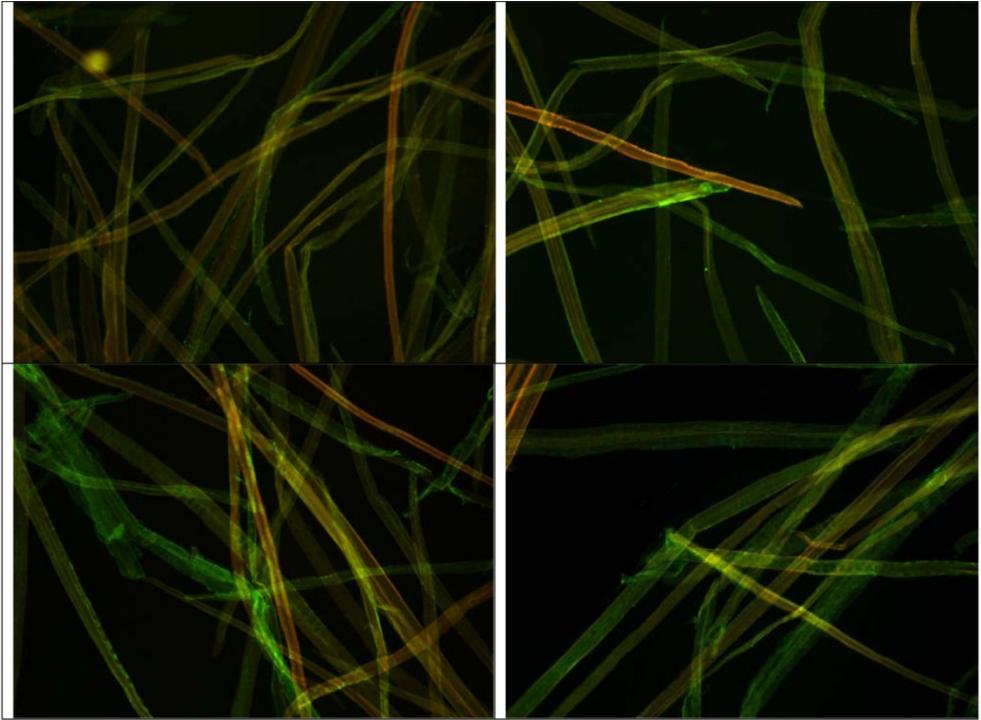


Figure 6. Immunofluorescence of whole fibres from kraft pulp with wheatS xylan labeled with LM11 (upper row) and LM10 (bottom row).

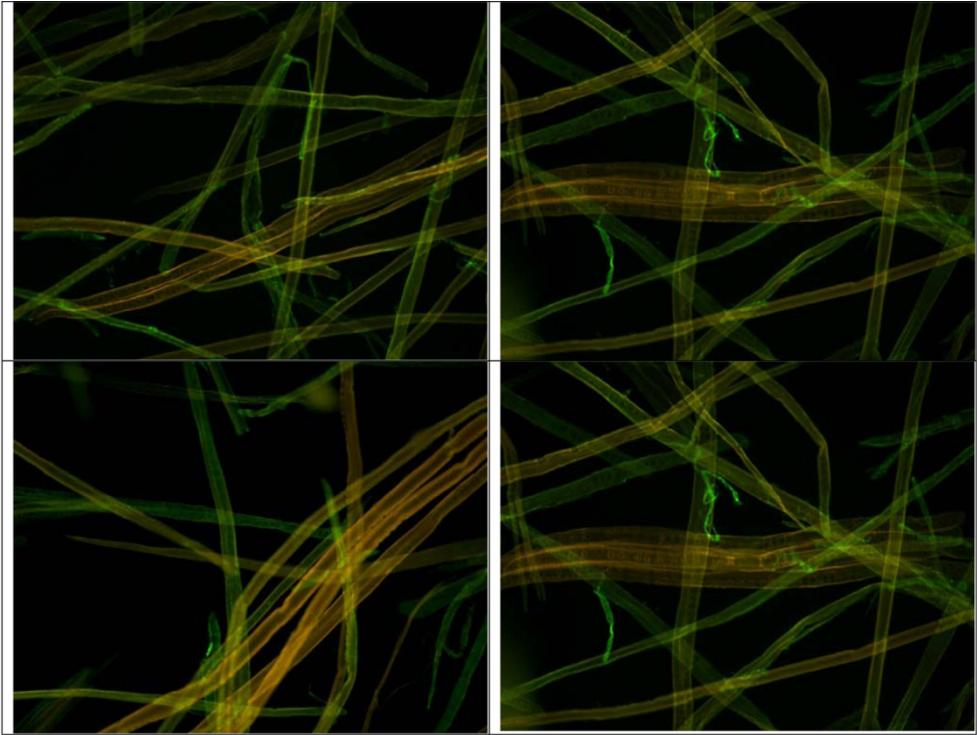


Figure 7. Immunofluorescence of whole fibres from kraft pulp with Ref. N labeled with LM11 (upper row) and LM10 (bottom row).

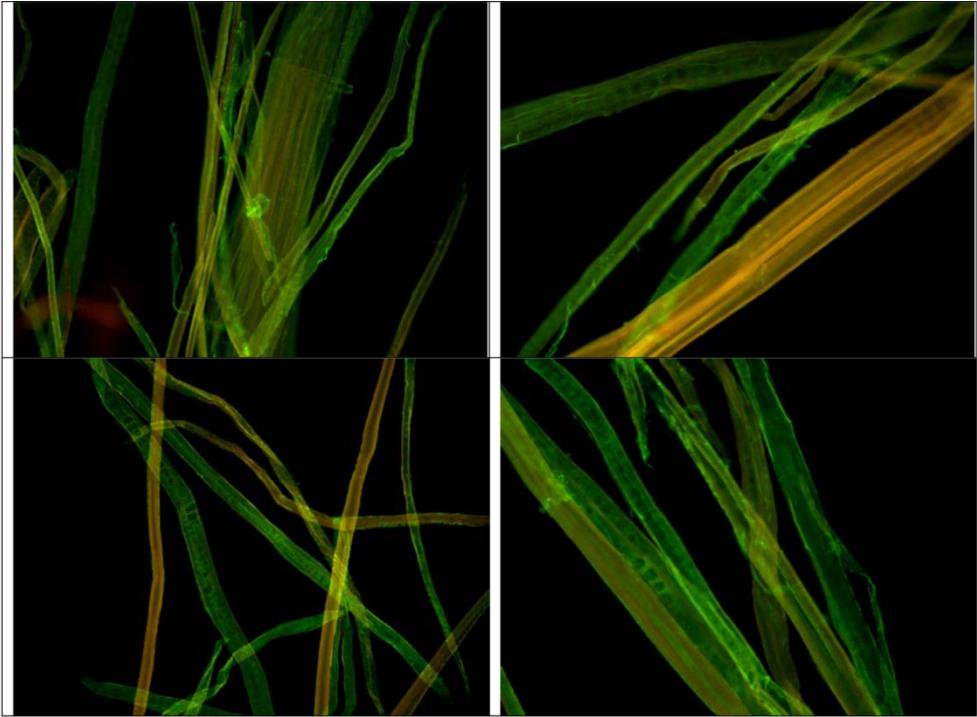


Figure 8. Immunofluorescence of whole fibres from kraft pulp with Ref. S labeled with LM11 (upper row) and LM10 (bottom row).

APPENDIX 3. SEMI-QUANTITATIVE IMMUNOFLUORESCENCE STUDIES ON THE PRESENCE OF NATIVE AND ADDED BEECH AND WHEAT XYLANS DETECTED ON CROSS-SECTIONS OF KRAFT SPRUCE PULP FIBRES USING SPECIFIC XYLAN PROBES

Geoffrey Daniel, Lada Filonova, SLU, Uppsala

Aims: The purpose of the study was to evaluate the potential of using two specific xylan probes to localize native xylan on spruce kraft pulp fibres and extraneously added beech- (poly β -D-xylopyranose (1-4) and wheat (arabinoxylan) xylans.

Background: In previous work (Daniel et al., 2010a,b; 2011), the influence of added xylans on the degree of damage introduced into spruce fibres through the mechanical action of the cook was studied using a number of different process parameters and spruce fibre types *viz:* *i)* The influence of xylan on the sensitivity towards fibre damage; *ii)* The influence of xylan on the sensitivity towards fibre damage: xylan added in the oxygen stage; and *iii)* The influence of xylan on the sensitivity towards fibre damage analyzed on pulps cooked to high kappa numbers. In the present work the effect on spruce pulp and paper properties by adding different extraneous xylans at high levels (i.e. beech and wheat xylan) during the cooking was studied. Since the extraneous beech and wheat xylans were added at the cooking stage it was envisaged that they could have effects by: *i)* their adsorption to the fibre wall as observed in earlier studies (Daniel et al., 2011); *and ii)* help hinder the diffusion of native xylans from the fibre walls into the cooking liquor. In order to evaluate this approach it was necessary to develop a method that would allow visualization of xylans in the reference pulps (i.e. native xylan) as well as the added beech and wheat xylans in treated pulps with- and without mechanical shearing. To achieve this, a method based on measuring and quantifying the immunofluorescence of labeled xylans in fibre cross-sections of reference and xylan treated spruce pulps using imageJ was evaluated. As spruce cross-sections were used some information could also be obtained on the penetration of different xylans into the pulp fibre walls could be obtained. It should be noted that with this approach a large number of fibre cross sections can be analysed and thereby better statistics obtained.

Materials and methods

1) Fibre materials

Spruce fibres were derived from kraft cooks in which the xylan content during the impregnation had been significantly increased to 30g/L with either beech- or wheat xylan. One half of each of the pulps was also subjected to mechanical action (shear

and compressive forces) at the end of the cook in a special digester (Salmén and Lundqvist, 2011). Additional reference pulps without added xylan and with- and without mechanical action were also produced. For more details see the introductory part of this report.

2) Xylans

Beech xylan (poly β -D-xylopyranose (1-4)) was obtained from Sigma (X4252) and wheat xylan arabinoxylan from Megazyme.

3) Immunolocalization of xylans on spruce fibre cross-sections

i) Monoclonal antibodies

Visualization of xylan was achieved using monoclonal antibodies generated against low and unsubstituted xylans (LM10) and low and highly substituted (LM11)(1-4)- β -D-xylans. The antibodies were a generous gift from Prof. P. Knox (Leeds Univ., UK). A pre-study using ELISA (*Appendix 2*) confirmed the reactivity of the purified beech and xylans. This study revealed poor reactivity of the LM10 probe for wheat xylan whereas LM10 and LM11 both reacted with the beech xylan, the latter v. strongly. Previous observations confirmed the strong reaction of LM10 with birch and also reactivity with spruce xylan as shown by other authors.

ii) Fibre cross-sections

Representative samples from the 6 pulps (BeechN, BeechS, WheatN, WheatS, Ref.N, Ref.S) were processed dehydrated and embedded in technovit resin as previously described (Daniel et al., 2010a, b). Semi-thin fibre cross-sections were cut using a Microm microtome HM 350, (Micron, Germany) and mounted on object glasses and labeled with the monoclonal antibodies for immunofluorescence microscopy as described previously (Daniel et al., 2010a).

iii) Positive and negative and substrate controls

The specificity of the anti-xylan was checked previously using a number of positive and negative substrate controls (Daniel et al., 2010b). Thus in the present study only the technical control where the antibody was omitted from the labeling procedure was adopted.

iv) Fluorescence microscopy

Fibre sections were placed on object glasses mounted in Fluorsave (Calbiochem) covered with coverslips and examined using a Leica DMRE fluorescence microscope fitted with a mercury lamp and I3-513808 filter-cube (Leica, excitation 450-490 nm, emission 515 nm) from Leica Microsystems, Wetzlar, Germany. Images were recorded using a Leica DC300F CCD camera and digital imaging

system for professional microscopy (Leica Microsystems GmbH) at equal settings (exposure time 1 s and gain 3.2).

4. ImageJ method

The method involves a number of steps after labeling of fibre cross-sections with antibodies Daniel et al. 2010a, b) including: *i*) Collection of representative images at 200x; with at least 30 images for each fibre treatment (*Fig. 1*); *ii*) thresholding of images; *and iii*) determination of the pixel values for the defined fibre selections. In the following work a total of ca 600 fibre cross-sections images (both late- and earlywood) were measured. In addition, studies were made on 3 separate occasions to prevent operator bias.

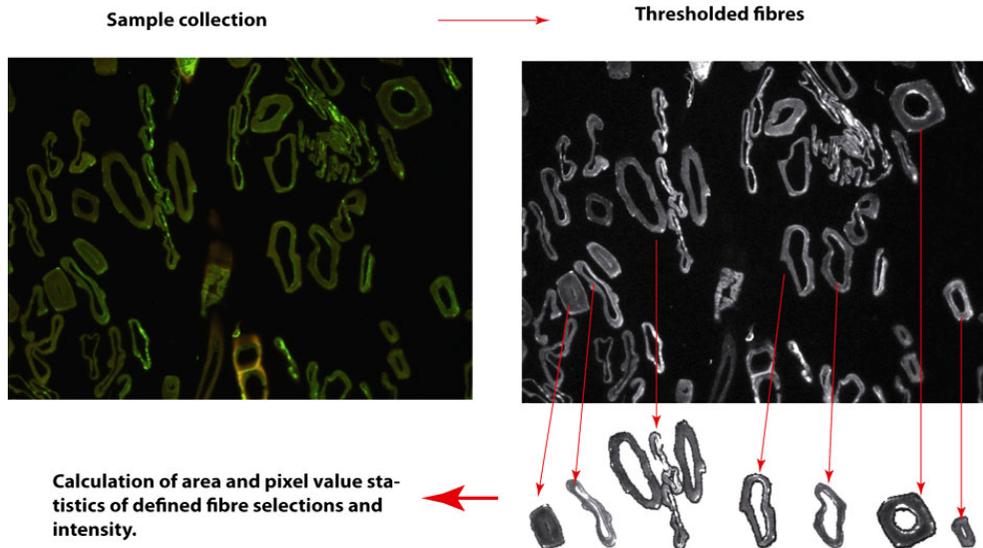


Figure 1. Outline of method for determining fluorescence intensity for presence of xylan. Note that in all semi-thin labeled section, a variety of fibre types (i.e. early- and latewood) and a number of fibres exist and these show various degrees of collapse and labeling intensity. Areas of high brightness reflect a more intense signal for the presence of xylan.

Results and discussion

Results from the chemical analyses reflected the nature of the added xylan. For example the addition of beech xylan resulted in the highest xylose, xylose anhydro sugar as well as presence of strong and weak acids. These results were consistent with the highest readings for beech xylan from the immunofluorescence and ELISA results (*see Appendix 2*). Chemical analyses further showed the addition of wheat xylan resulted in the highest arabinose, arabinose anhydro sugars as well as

total anhydro sugars. Ref. N and S pulps showed lower or similar results for all chemical analyses apart from relative higher glucose and anhydro glucose readings. In summary, the gross chemical results indicate the highest uptake of xylan for pulps treated with beech xylan followed by the wheat xylan. Very little difference was seen between the normal- and sheared pulps of the respective groups no matter whether beech, wheat xylan was added or in the reference pulps. Taking this into account, results from the immunofluorescence images were interpreted.

1) Qualitative assessment of the immunofluorescence labeling patterns

The immunofluorescence approach used here does not determine the xylans on the *expose fibre surfaces* as shown using the ELISA approach; it is not a surface technique. It can however show the presence of xylans on the cut surface, i.e. edges of the fibres. In addition, the total area available to the antibody is considerably less in comparison to the labeling of whole fibres as seen in the ELISA approach. The advantage of the present approach however is that information on the microdistribution and intensity of the presence of xylans associated with the fibres (i.e. exposed surfaces – S1, S3 layers, and S2 intracellular) can be assessed. The method is not a high resolution method like TEM immunogold labeling (*see Appendix 4*), but has the advantage in that large numbers of sections/fibres can be observed and measured.

Figures 2-5 show various general views of numerous fibre cross-sections (both early and late wood) from the different pulp types produced with- and without beech or wheat xylan added during cooking and with or without mechanical shearing and respective references. In addition, the fibre sections have been labeled with two antibodies for low and un-substituted (LM10) and low/highly substituted (LM11) xylans for xylopyranosyl residues. From an overview of many fibre cross-sections, it is very difficult to compare the different treatments especially at low magnification. Both antibodies have reacted with the fibres compared with the IgG technical control (*Figure 3; image on right*) which shows autofluorescence and that there is no or very little non-specific background labeling.

The following aspects can be discerned from the immunofluorescence labeling:

- 1) That overall, the LM11 has given a more intense immunofluorescence signal for both the beech and wheat xylan treated pulps and reference pulps (*Figures 2, 3*). This probably relates to the probes overall greater span of cross-reactivity;
- 2) That there is considerable variability in immunofluorescence for both probes but what they both have in common is that the most intense reactions are on the fibre surfaces (i.e. exposed edges) for both early- and the latewood fibres (*Figures 2-4*);

- 3) That the Ref. N and S pulp fibres also show immunofluorescence and here some fibres do show strong fluorescence of exposed edges especially thin collapsed fibers (*Figure 5*) especially with LM11;
- 4) That it is difficult to determine a major difference in the immunofluorescence between the normal and corresponding sheared fibres (*Figures 2-4*). Possibly the sheared fibres show more split and collapse fibres;
- 5) That the fluorescence with the Ref. N. and S. fibres reflects cross-reaction with the native xylan from spruce wood. Strongest intensities were on the edges (i.e. S1 layer/primary wall) and lumen wall (i.e. S3 layer) suggesting the presence of an increased level of xylan either remaining from cooking and/or a larger indigenous concentration of xylan in the outer fibre wall layers as reported previously for other softwoods;
- 6) That considering points 3 and 4 above that the strong immunofluorescence on some of the beech and wheat treated pulp fibres represents a combination of native and absorbed extracellular xylan added during cooking. The fact that the highest intensities were on the exposed edges of the fibres would indicate considerable adsorption of added xylan (i.e. beech or wheat) or hindrance to loss of native xylan during cooking or both.

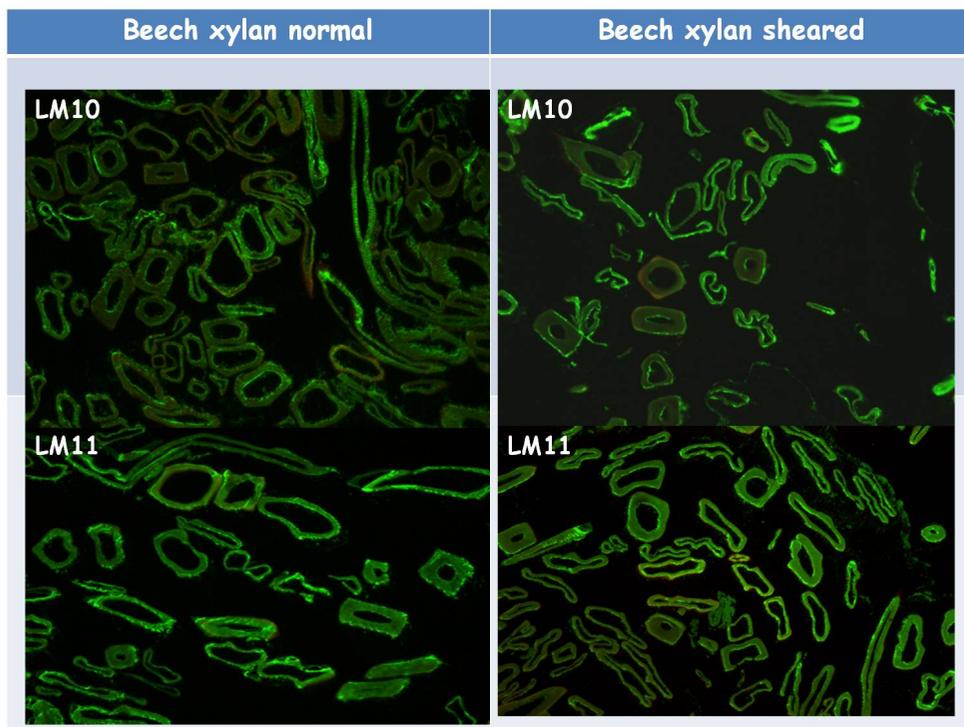


Figure 2. Representative semi-thin sections of spruce fibres previously treated with beech xylan during the impregnation stage of kraft cooking with- and without shearing and labeled with LM10 or LM11. Both early- and latewood fibres are apparent and labeled in the sections. Most intense fluorescence is derived from the edges of the fibres.

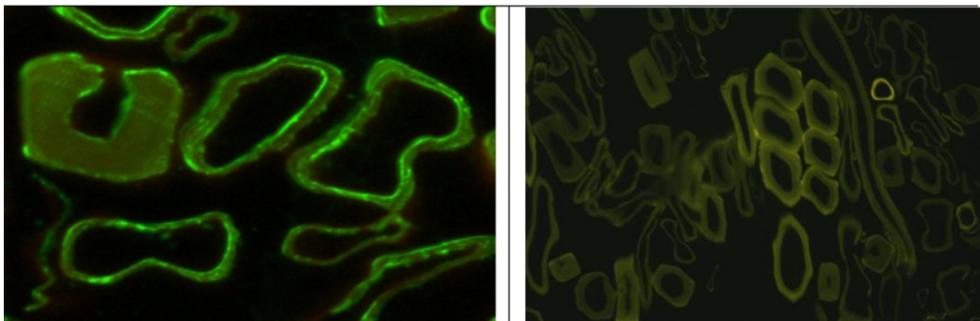


Figure 3. Representative beech-X (left) treated spruce pulp fibres after LM11 and corresponding control labeled with IgG only (i.e. no antibody probe). The beech-X early- and latewood fibres show strong immunofluorescence especially on the exposed edges (primary wall/S1, S3 (lumen wall) layers) and possibly some weaker reaction inside the fibre (S2 layer; either native xylan ?). Only autofluorescence was noted for the IgG treated fibre sections.

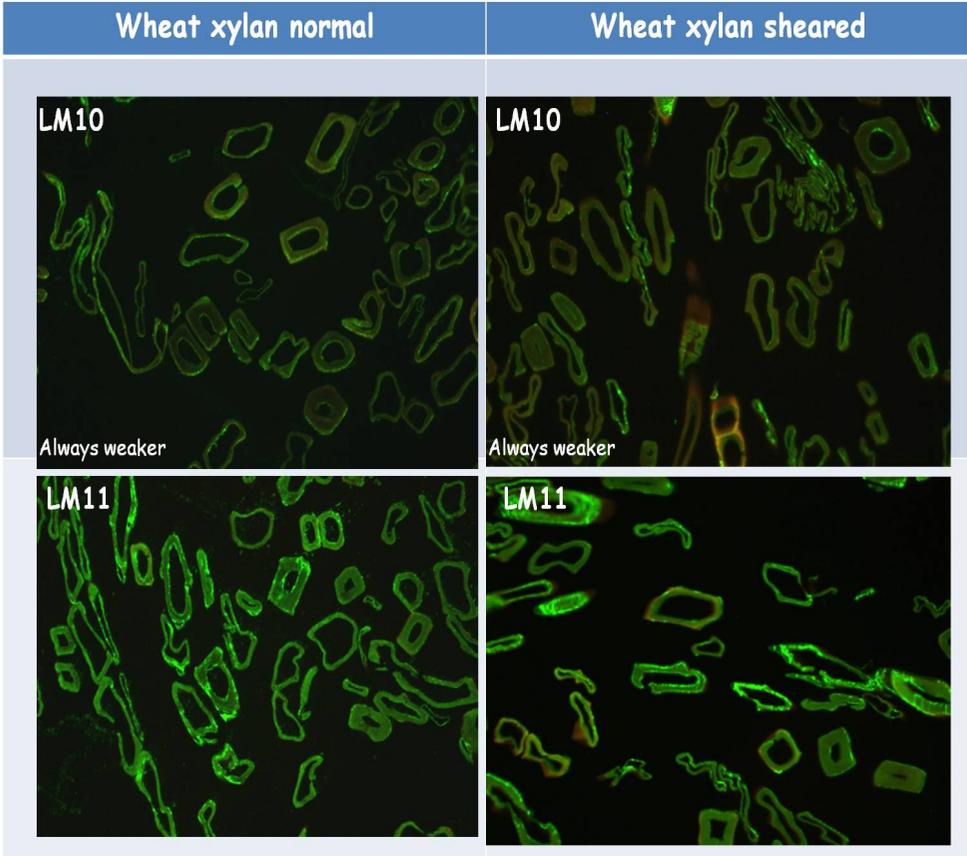


Figure 4. Representative semi-thin sections of spruce fibres previously treated with wheat xylan during the impregnation stage of kraft cooking with- and without shearing and labeled with LM10 or LM11. Both early- and latewood fibres are apparent and labeled in the sections.

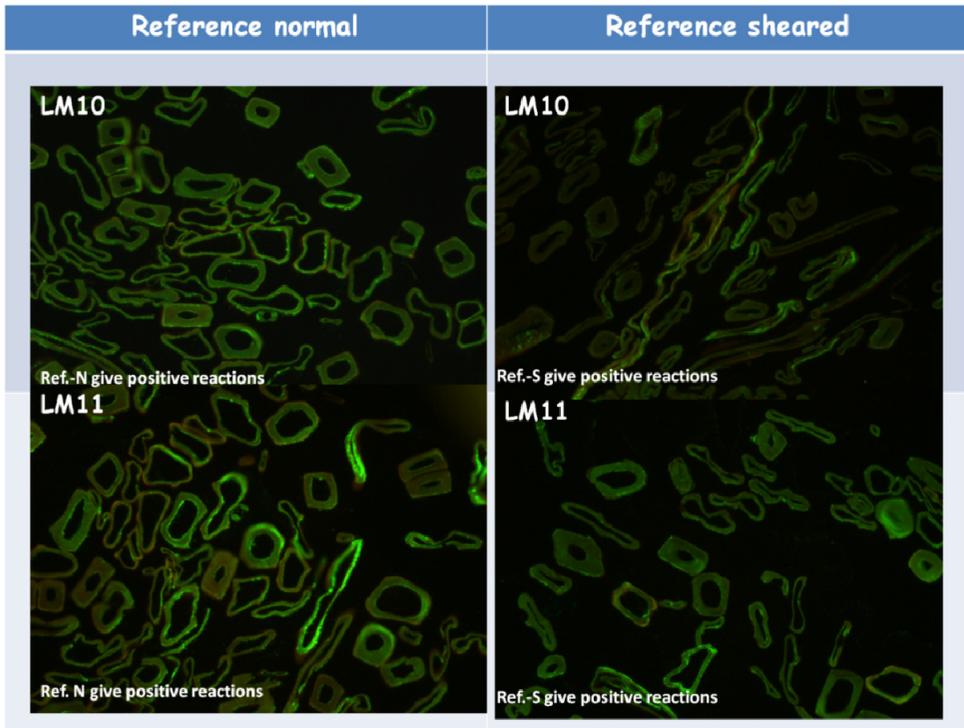


Figure 5. Semi-thin sections of spruce Ref. N and S fibres after kraft cooking with- and without shearing and labeled with LM10 or LM11. Both early- and latewood fibres are apparent and labeled in the sections. Strongest reactions are on the edges of the fibres.

2) Semi-quantitative assessment of immunofluorescence of labeled fibre cross-sections intensities from the different treatments

With the approach adopted it was not possible to distinguish between native spruce and beech and wheat xylan adsorbed to the fibres when added during cooking with the probes used. In future this could be overcome with a specific probe for detecting only spruce xylan. Nevertheless the labeled fibre cross sections showed differences between the treated- and reference pulps. In an attempt to measure semi-quantitatively differences between the pulps, imageJ software was adopted as outlined above. The approach is semi-automatic in that first high resolution images of fibre cross-sections are recorded and binarized and thereafter individual fibre cross sections are randomly selected and analysed. Some difficulties arose (e.g. representative and consistent labeling, bleaching) therefore the labeling and detection was repeated on 3 separate occasions. The results presented show analyses from *ca* 600 fibre cross-sections for each pulp category.

Table 1. Semi-quantitative analysis of fluorescence intensity on spruce earlywood and latewood fibres (immunostaining microscopy + ImageJ analysis methods). * Each result represents the mean intensity measures on 180-200 individual fibres; ** Standard deviation; *** Mean intensity values for entire group (i.e. ca 600 fibres). ^{A)} Results obtained at a different time

Presence /absence of added xylan +/-shearing	IgG control	LM10	LM11
Beech-N	19,52181^{A)} (1,02524)	36,19355* (6,350226)**	41,04946 (6,059641)
		31,75748 (7,248531)	47,33343 (5,512486)
		35,49457 (7,128081)	49,7016 (8,230849)
		34,48187*** 6,908946	46,02816 6,600992
Beech-S	17,92827 (0,84877)	52,3728 (8,917255)	45,29557 (6,521162)
		44,35525 (4,683781)	40,40509 (4,223886)
		40,76706 (9,9341)	40,6693 (4,246449)
		45,8317 7,845045	42,12332 4,997166
Wheat-N	17,94437 (2,06099)	34,64811 (3,125292)	35,0437 (4,15951)
		29,93216 (3,463093)	33,40648 (4,915789)
		40,48926 (5,634205)	42,80092 (4,80082)
		35,02318 4,074197	37,0837 4,625373
Wheat-S	17,92827 (0,84877)	40,62827 (6,83132)	47,22226 (5,610949)
		33,61331 (7,464271)	36,69882 (3,284418)
		27,0708 (4,896979)	41,96054 4,447684
		33,77079 6,397523	
Ref.-N	19,84468 (1,71543)	25,18221 (4,160338)	39,57500 (5,30261)
		28,32097 (1,334468)	37,29763 (4,309028)
		25,07672 (3,205501)	39,58324 (4,871599)
		26,1933 2,900102	38,81862 4,827746
Ref.-S.	17,92827 (0,84877)	29,12956 (3,555153)	32,32115 (6,158003)
		32,09984 (6,86583)	46,48202 (5,734218)
		30,64336 (4,739418)	40,98254 (6,54228)
		30,62425 5,053467	39,92857 6,144834

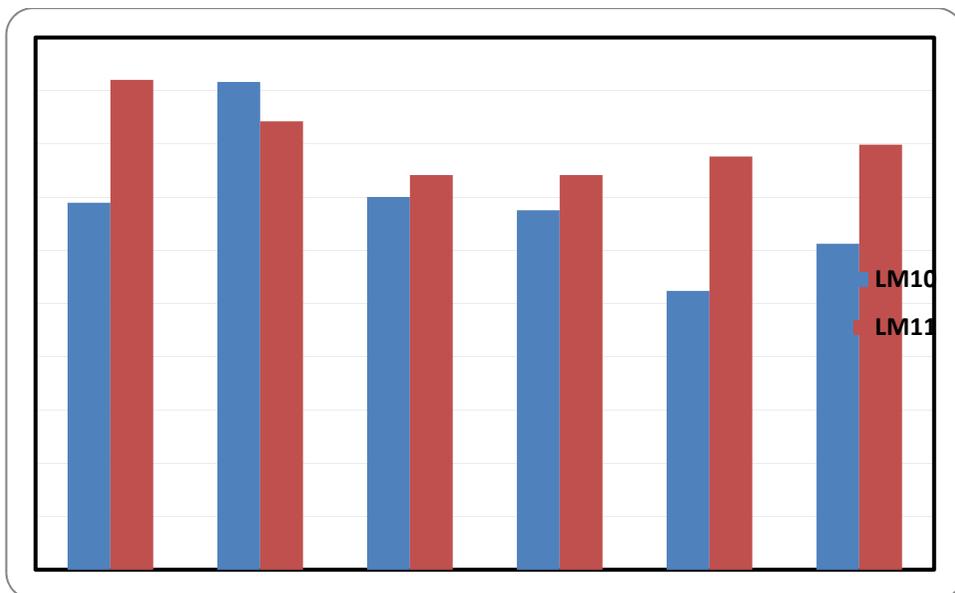


Figure 6. Values from Table 1. Abbreviations: BN, beech normal; BS, beech sheared; WN, wheat normal; WS, wheat sheared; RN, Ref. normal; RS, Ref. sheared.

Results from the semi-quantitative analyses are shown in Table 1 and Figure 6. These results show the following trends:

- 1) That the autofluorescence (i.e. only IgG) for the fibre cross sections was quite similar no matter the pulp type with a mean of *ca* 17-20 units (Table 1);
- 2) That apart from Beech-S, LM11 (low and highly substituted xylans) gave an overall more intense fluorescence with all the pulps including reference. Ref. N and S gave the lowest intensity values of all pulps with the LM10 probe (Table 1, Figure 6);
- 3) Strongest immunofluorescence was derived from the Beech-N and Beech-S pulps labeled with LM11 (Table 1, Figure 6) results that were consistent with that seen with the images (Figure 4);
- 4) That the Ref. pulps gave strong immunofluorescence intensities (Table 1, Figure 6) consistent with the appearance of images of sections;
- 5) That when compared with its respective Ref. pulp, whether normal or sheared that the beech and wheat normal xylan treatments resulted in greater immunofluorescence intensities for both LM11 and LM10 apart from Ref.-N and wheat N that were similar;
- 6) That there is considerable variability with the method as judged by the high standard deviations. However, in order to show a trend if Beech-N is compared with its Ref.-N there is a *ca* 8,4 increased in intensity value

consistent with a 32% increase in presence of xylan (*i.e. added beech-xylan*). Similarly, a comparison of Beech-S with its corresponding Ref.-S shows a 15,2 increase, alternatively 49,7% increase in presence of beech xylan. However, these results need to be proven statistically and we still do not know if the increase in xylan reflects entirely the added Beech-X during cooking or a combination of retained native spruce xylan and added Beech-X;

- 7) That from the immunofluorescence results, the greatest signals were from the edges of the fibre cross-sections. So we can assume that the majority of the added xylan –beech/wheat-was associated with the fibre surfaces. The same was apparent for the reference N and S pulp fibres;
- 8) With addition of very high levels of Beech-X and Wheat-X during the cook, it quite possible that the xylans have adsorbed to each other and built up several layers of xylan on the fibre surfaces as suggested by SEM observations of the entire fibres (*Appendix 5*);
- 9) That this approach is very different from ELIZA quantification which is a surface orientated measurement and gives an entirely different data set.

Conclusions

The above study using immunofluorescence and semi-quantification of fibre transverse-sections has confirmed that adding Beech-X and Wheat-X xylans to spruce kraft pulps during the cooking stage in high concentrations (30 g/L) results in high amounts of xylan associated with the fibres in pulps with- and without mechanical shearing treatments. However even the reference pulps without added xylans show strong fluorescence reflecting the native spruce xylans present.

Using the two xylan antibody probes (LM11, LM10) both Beech-X and Wheat-X was found associated with the exposed edges (*i.e. S1, S3 layers*) of both early- and latewood fibres in the treated pulps. With some fibre cross-sections the outer parts of the walls showed intense fluorescence. This was strongly suggested in many collapsed earlywood fibres. The quantitative results for Beech-X and Beech-S were higher than the Wheat- X and Wheat-S and Reference N and S pulps suggesting greater intensity of xylan associated with the exposed regions of the fibre walls.

Using imageJ and semi-quantification of the intensity values for *ca* 600 individual fibre cross-sections and comparison between their respective controls there was strong evidence for increase of xylan (*i.e. 32 and 49.7%*) in the Beech-X and Beech-S pulp samples. The results are consistent with gross chemical analysis/surface charge for beech N and S pulps to have the greatest amount of

xylans associated (summary report). They are also consistent with the large amounts of aggregates observed on the fibre surfaces using SEM (*Appendix 5*).

While this approach allowed for increased knowledge on the association of different xylans with the fibres, it was not possible to distinguish between the added xylan (beech/wheat) and native spruce xylan. Treatment of the fibres in the cook could have also reduced the loss of native spruce xylan and this value would be associated with the increase level of intensity. Further studies with additional specific probes to only spruce xylan would be required to distinguish between the spruce and beech/wheat xylans, although this would be difficult as the structures are similar.

References

- Daniel G., Kaňuchová A., Filonova L., Ander P. 2010a. Visualization and quantification of xylans on xylan treated kraft spruce pulp fibres using immunofluorescence microscopy, ELISA, HCL and xylanase assays. In: Proceedings of the 11 Workshop on Lignocellulosics and Pulp, Hamburg, August 16-19, 33-37.
- Daniel G., Olsson, A-M., Ander P., Kaňuchová A., Salmén L., Filonova L., Sjöström K., Boussard L., Berg S., Heijnesson-Hultén A., Lindström C. 2010b. The influence of xylan on the sensitivity towards fiber damage. CRUW Chemical Pulping; CRUW Intern Rapport nr 2, 88p.
- Daniel, G., Olsson, A-M., Ander, P., Kaňuchová, A., Salmén, L., Filonova, L., Sjöström, K., Boussard, L., Berg, S., Heijnesson-Hultén, A., Lindström, C. 2011. The influence of xylan on the sensitivity towards fiber damage: xylan added in the oxygen stage. CRUW Chemical Pulping. CRUW Intern Rapport 4, 44 pp.
- Salmén, L. and Lundqvist, F. (2011): Effects of mechanical forces for strength delivery in kraft cooking, *Appita* 64(1), 89.

APPENDIX 4. TRANSMISSION ELECTRON MICROSCOPY (TEM) ON BEECH AND WHEAT XYLAN TREATED KRAFT SPRUCE PULP FIBRES

Jong Sik Kim, Geoffrey Daniel, SLU

To visualize the presence of xylan and complement and confirm the results from ELIZA, immunofluorescence (whole fibres and sections) and SEM observations, pulp samples were processed for TEM and examined after labeling with antibodies primarily specific for highly substituted (LM11) and low and unsubstituted (LM10) xylans. Since TEM has better resolution and higher magnification possibilities than immunofluorescence microscopy, it was possible to achieve a much better overview of the spatial microdistribution and concentration (indirectly by intensity of labeling) of xylans in the kraft cooked reference spruce and where beech and wheat xylans had been added to the cook.

The aim of the present work was: *i)* Visualize the presence and microdistribution of native spruce xylans using two antibodies for substituted and unsubstituted xylans in the reference and sheared kraft spruce pulp samples –*both early- and latewood samples*; *ii)* Visualize spruce pulp fibre samples after treatment with beech xylan; both normal and sheared samples; and *iii)* Visualize the spruce pulp fibre samples after treatment with wheat xylan.

Materials and Methods

PULP FIBRE EMBEDDING AND IMMUNOGOLD LABELING

Spruce normal and sheared reference pulps and xylan (beech/wheat) treated normal and sheared pulps were processed and embedded in London resin as previously described (Daniel et al., 2004). Thereafter pulp fibres were sectioned using a Reichert Ultracut E ultramicrotome with sections collected on nickel grids. Transverse ultrathin sections (*ca* 90 nm) were incubated in blocking buffer (pH 8.2, Tris-buffered saline (TBS) containing 1% w/v BSA and 0.1% w/v NaN₃) for 30 min at room temperature. Grids were then incubated with LM10 or LM11 antibodies (PlantProbes, UK; 1:20 dilution in blocking buffer) for 2 days at 4°C. After three washes with blocking buffer for 10 min each, grids were incubated with goat anti-rat secondary antibody conjugated with 10-nm colloidal gold (BBInternational, UK) for 4 h at 35 °C for the LM10 (1:50 dilution in blocking buffer) and at room temperature for the LM11 (1:100 dilution in blocking buffer). Subsequently, grids were washed five times with blocking buffer for 10 min each, followed by washing with distilled water. After post-staining with 4% w/v uranyl acetate for 10 mins, grids were examined using a Philips CM12 transmission electron microscope (TEM, USA) operated at 80 kV. Negative TEM films were scanned using an Epson Perfection Pro 750 film scanner.

Care was always made that labeling and observations were made on cross-sections of the pulp fibres that were perpendicular to the fibre axis and not oblique as this can affect both the intensity labeling patterns. The TEM-immunogold method complements the immunofluorescence approach but provides greater magnification and detailed information at the cell wall level. In the following images, the black spots on the fibre walls indirectly show the localization and spatial microdistribution of xylans. In general, the greater the number of gold particles (av. size 10 nm) present, the greater the amount of xylan epitopes present and available for labeling. At least 20 images of different fibre cross-sections were taken in order to achieve representation.

Results

Compound TEM micrographs showing the microdistribution and intensity of labeling for substituted and unsubstituted xylans in cross-sections of early- and latewood pulp fibres are shown in *Figures 1-4*. Representative TEM images showing the microdistribution of substituted xylans in native reference spruce with- and without shearing in early- and latewood fibres are shown in *Figs 1A, B and 2A, B*. Similar micrographs after addition of beech xylan to the cook are shown in *Figs 1C, D and 2C, D*. Representative images after the addition of wheat xylan to the cook are shown in *Figs 1E, F and 2 E, F*. Representative micrographs showing the microdistribution of unsubstituted and poorly substituted xylans in cross-sections of native spruce with- and without shearing in early- and latewood are shown in *Figs 3A, B; 4A, B*. Micrographs of pulp fibres after addition of beech or wheat xylans to the cook with- and without shearing are shown in *Figs 3C, D; 3E, F; 4C, D and 4E, F*. Note all the micrographs shown in *Figs 1-4* are of approximately the same magnification (Bar line bottom right corner = 500 nm) so they can be directly compared. The magnification was chosen so as to provide a high enough magnification to see the individual gold particles/labeling pattern and largest part of the fibre wall as possible.

LM11 FOR HIGHLY SUBSTITUTED/LOW-SUBSTITUTED XYLANS

1) Comparisons of the Spruce reference normal and sheared fibres showed a similar pattern and intensity of labeling across the pulp fibre secondary cell walls no matter early- or latewood with LM11 for substituted xylans (*Figs 1A, B; 2A, B*). A similar situation was apparent for pulp samples in which either beech- or wheat xylans had been added to the cook (*Figs 1C-F, 2C-F*). The fact that a similar intensity and microdistribution of gold labeling was attained using LM11 in the native spruce cross-sections with- and without shearing indicates that the mechanical action of the latter had not caused significant xylan removal from inside the fibre secondary wall. Furthermore as all the results for the addition of beech- and wheat xylans in both early and latewood fibres in both

normal- and sheared pulp fibres appeared similar to the reference pulp (*Figs 1, 2*) it indicates either the beech/wheat xylans had not penetrated the pulp walls or that the xylans had penetrated but they cannot be distinguished from the native spruce xylans. Since the labeling patterns and intensity using LM11 were similar for the spruce reference (normal and sheared) and beech and wheat treated fibres it is not possible to distinguish between them. However, the similar patterns and intensities on the pulp fibre cross-sections would suggest against penetration.

LM10 FOR LOW & UNSUBSTITUTED XYLANS

2) Labeling with LM10 for unsubstituted xylans gave similar and contrasting results. With spruce Reference normal and with beech and wheat normal xylan treated pulps the microdistribution of xylan labeling appeared similar to that observed with LM11 (*Fig 3A-F vs Figs C-D, E-F*). In contrast, the sheared pulp samples labeled with LM10 showed different labeling intensities. Beech sheared pulps labeled with LM10 were similar to the normal non-sheared pulps (*Figs 3C, D vs 4C, D*). In contrast, the sheared spruce reference pulp samples appeared poorly labeled (*Fig. 4A, B*) as was the wheat sheared pulp samples (*Fig. 4 E, F*). This could be interpreted that during shearing considerable unsubstituted xylans are removed from the pulp fibres thus the weaker labeling compared with the reference normal spruce pulps. However, this was not the case with the beech treated pulp using LM10 (*Fig. 3B-C; Fig. 4C-D*). With the sheared wheat xylan treated pulps, the labeling pattern was more similar to the reference spruce sheared pulps (*Figs 4E, F vs 4 A, B*) suggesting that added beech and wheat xylans may perform differently when added to the cook. However, since the patterns and intensity of labeling for the spruce reference sheared (*Fig. 4E, F*) and the wheat sheared (*Fig. 4 A, B*) are similar, it would suggest that wheat xylan was unlikely to be penetrating the fibre walls during cooking.

A POSSIBLE EXPLANATION OF THE RESULTS:

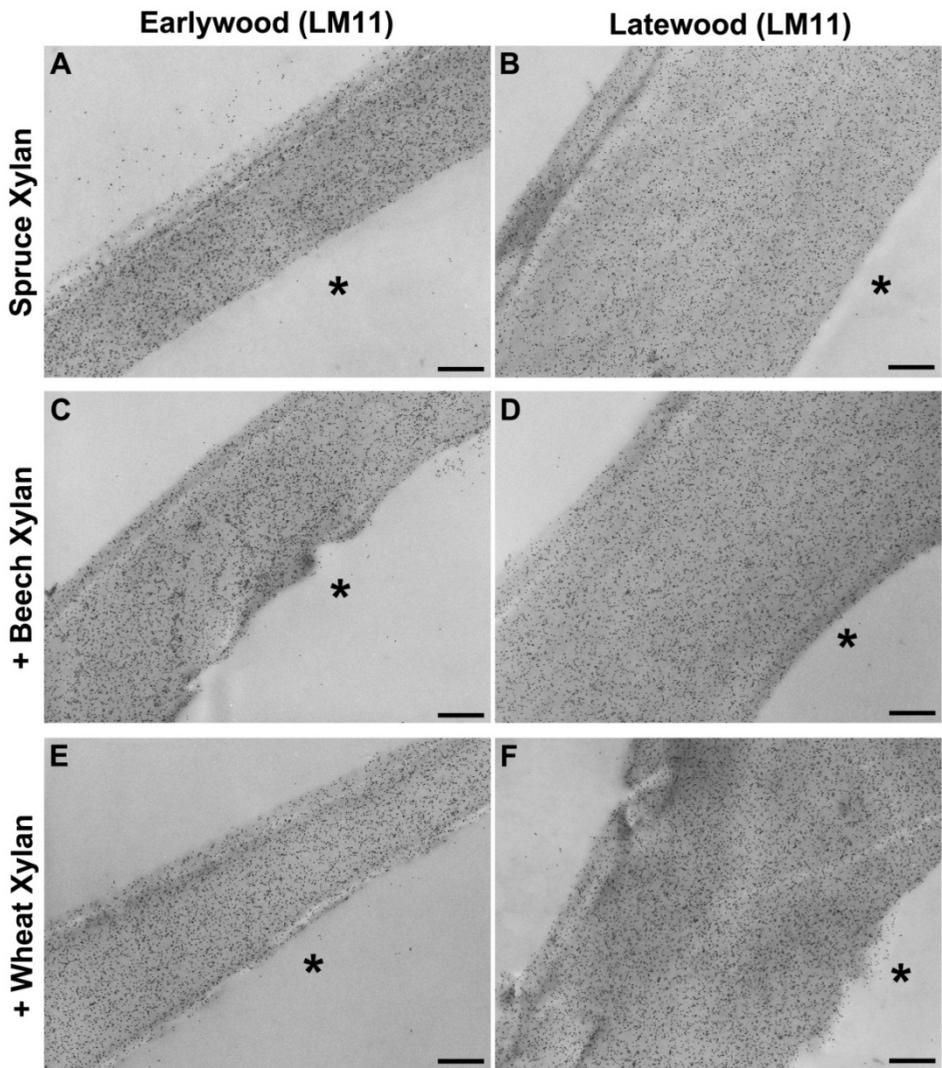
The fact that there was very little difference in the labeling with LM11 in the spruce reference and sheared fibres suggest the presence of substituted (low and high) xylans in both fibre samples and that shearing did not cause their significant removal. Since the labeling patterns and intensity with added beech and wheat xylans with- and without shearing were similar to the spruce reference pulps it is not possible to infer penetration of the fibers by the two xylans. Most probably, the results reflect the remaining native substituted xylans in the fibre walls. The poorer labeling patterns observed with LM10 on the sheared reference and sheared wheat pulps can presumably be interpreted by the loss of unsubstituted xylans during shearing while the stronger reaction with beech sheared pulps (i.e. like the normal fibre) reflecting some variation

in the cook. A complicating aspect is also the poor reaction of LM10 with wheat xylan. The only alternative explanation is that the beech xylan was penetrating the pulp fibre walls. However, if this was the case an effect of improving strength was not been realized.

References

- Daniel, G., Olsson A-M., Ander P., Kaňuchová, A., Salmén, L., Filonova, L., Sjöström, K., Boussard, L., Berg, S., Heijnesson-Hultén, A., Lindström, C. 2010. CRUW Chemical Pulping; The influence of xylan on the sensitivity towards fiber damage, CRUW Internal Report 2, 88 pp.
- Daniel, G. Volc, J., Niku-Paavola, M-L. 2004. Cryo-Fe-SEM & TEM Immunotechniques reveal new details for understanding white rot decay of lignocellulose. *Comptes Rendus, Biologues* 327/9-10, Oct. pp. 861-871

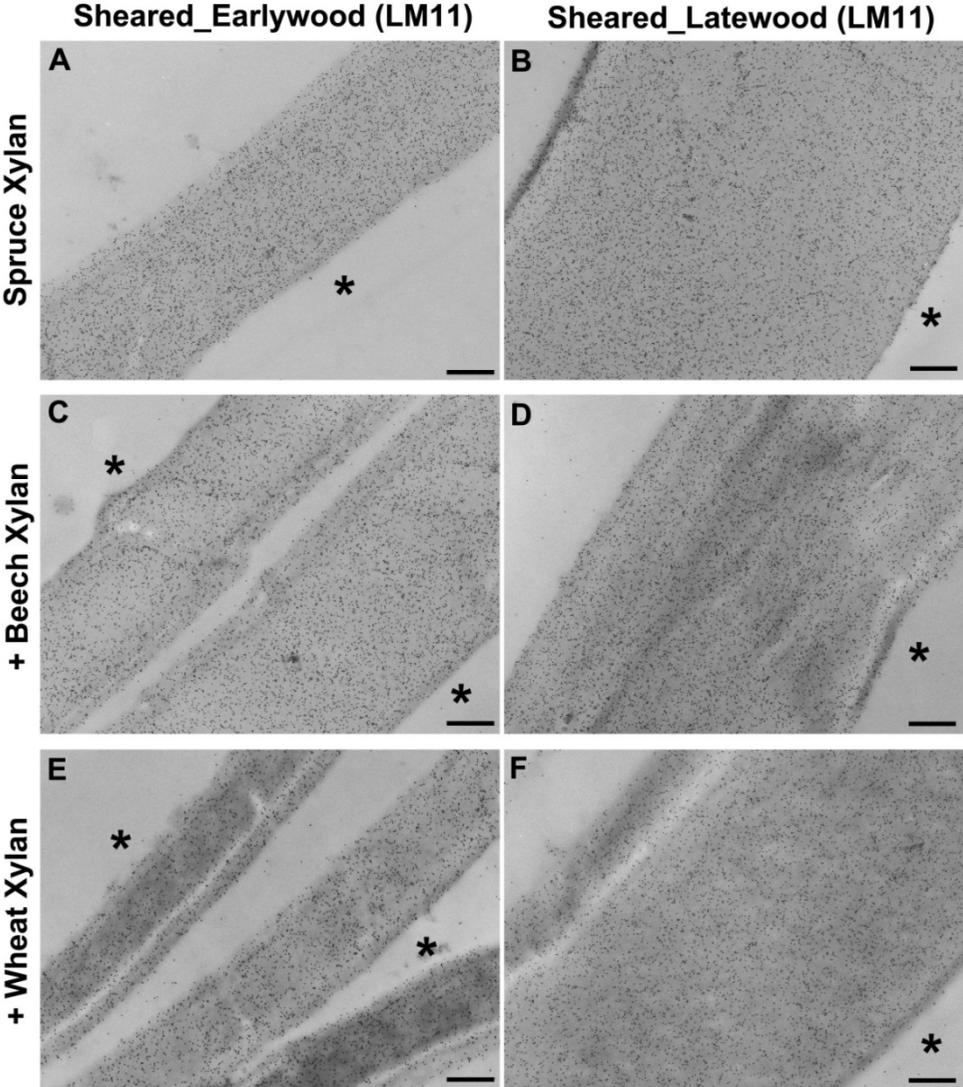
Normal Spruce_LM11



* Cell lumen side

Figure 1.

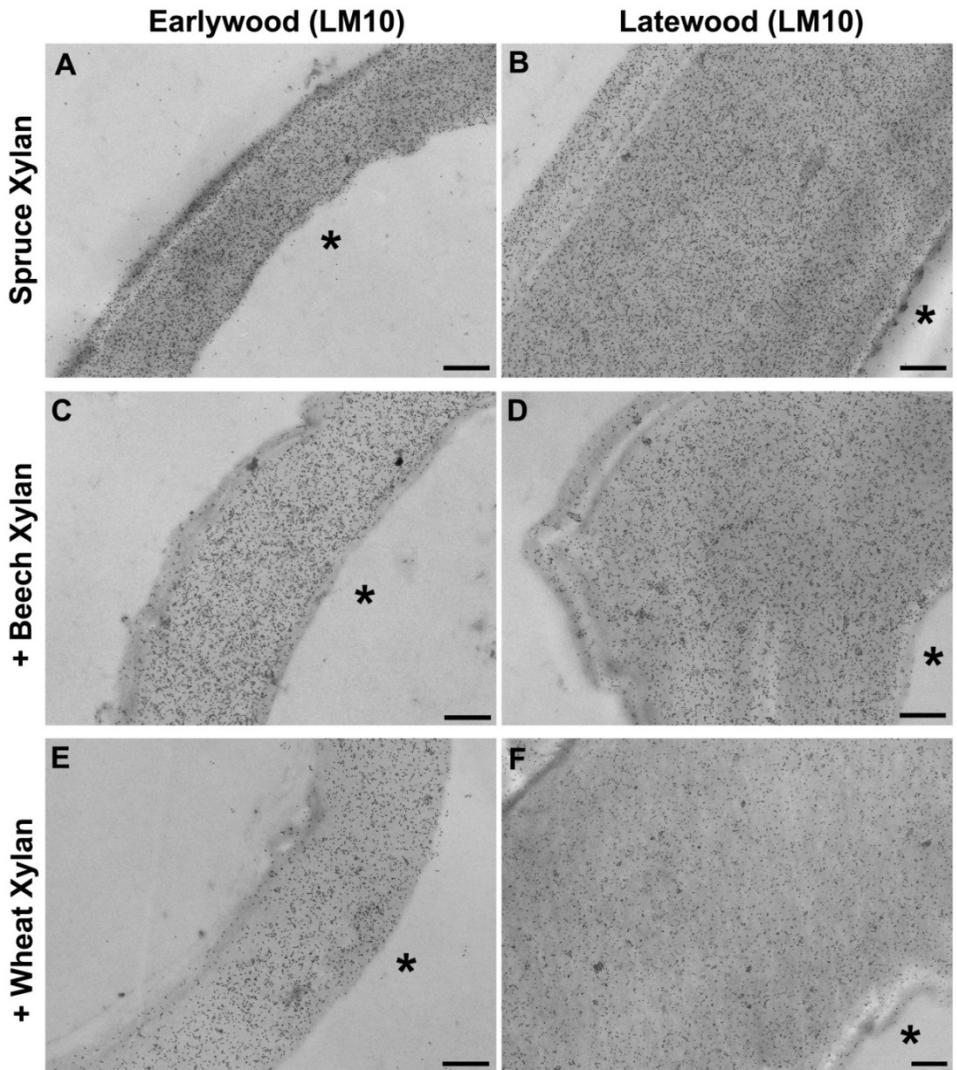
Sheared Spruce_LM11



* Cell lumen side

Figure 2.

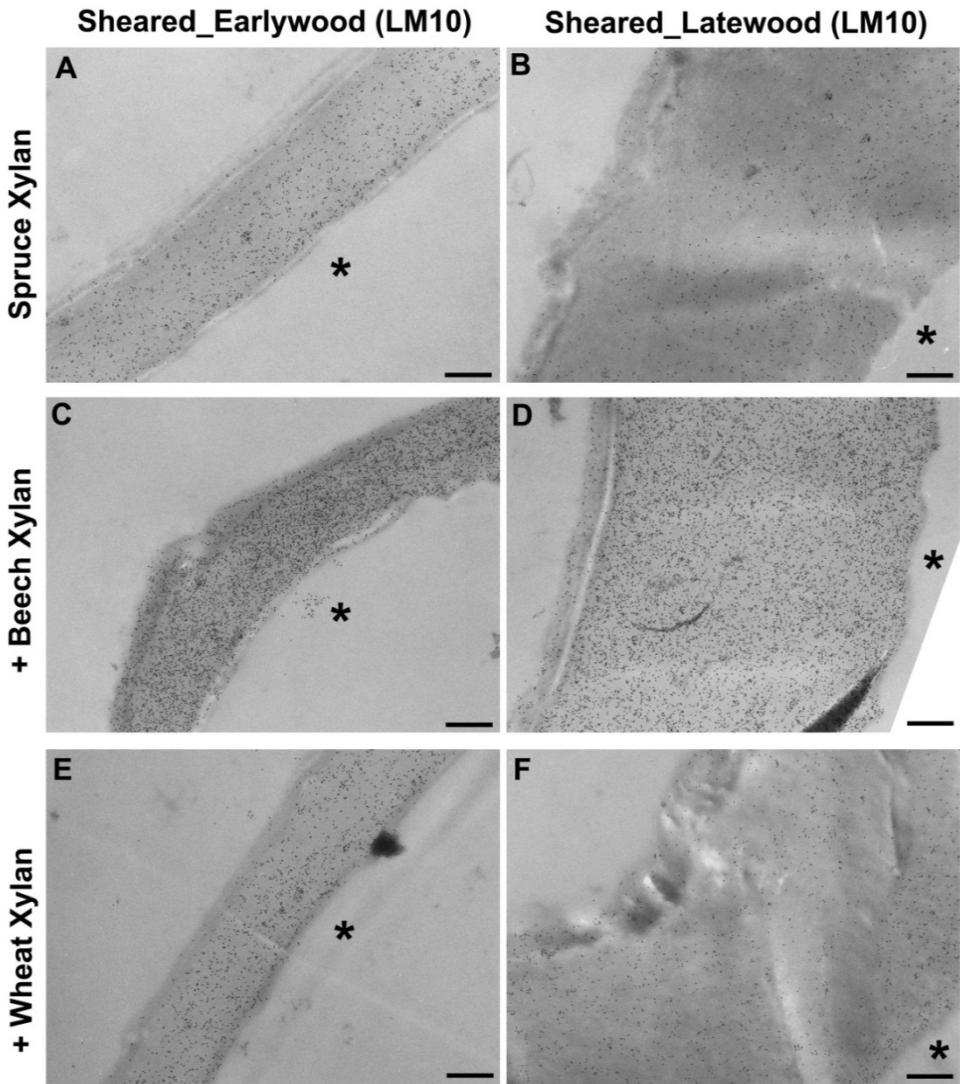
Normal Spruce_LM10



* Cell lumen side

Figure 3.

Sheared Spruce_LM10



* Cell lumen side

Figure 4.

APPENDIX 5. SEM OBSERVATIONS ON BEECH AND WHEAT XYLAN TREATED KRAFT SPRUCE PULP FIBERS

Geoffrey Daniel, SLU, Uppsala

Aims: The purpose of the study was to answer the following questions: *i)* Do exogenously added beech- and wheat xylan added at high levels during the impregnation/cooking stage of kraft pulping remain on the surface of final spruce pulp fibres and is there any evidence for a reaction with the fibre macrofibrillar structure?; *ii)* If present, does the xylan remain after mechanical treatment performed by shearing at the end of the cook?; *iii)* Do the beech and wheat xylans show any morphological differences on the fibre surfaces?

Background: During standard kraft pulping, xylans have been reported to precipitate onto the surfaces of pulp fibres from the cooking liquor (Yllner and Enström, 1956, 1957) or when applied exogenously to the cook (Daniel et al., 2010, 2011). During the present study, beech and wheat xylans were added at very high concentrations (*ca* 30g/L) at the cooking impregnation stage of spruce chips in an attempt to diminish the loss of native xylan from the fibre wall and thereby indirectly retain the inherent fibre strength. In addition, it was further expected that the added exogenous xylan could also together with the native and retained xylan help reduce the sensitivity of the fibre to mechanical damage. Therefore in the present work, one set of xylan treated pulps were also subjected to mechanical damage in the form of shearing to simulate industrial situations. The pulps were also compared with reference pulps without added xylan but with- and without mechanical shearing.

Scanning electron microscopy (SEM) was used as a means of observing the fibre surface and any changes associated with its macrofibrillar structure which could be interpreted as associated with the effect of added/retained xylans. SEM has been used repeatedly as a means of observing changes in fibre surface structure during kraft processing and fibre wall delignification (e.g. Daniel and Duchesne, 1998; Duchesne and Daniel, 2000; Daniel et al., 2004a,b; 2010; 2011). While SEM can provide a 3-dimensional overview of any changes in the morphological structure of the fibres during processing it does not allow (*i.e. without some form of marking*) for distinguishing between native- and exogenously added xylan. In the present work, this could only be done by comparison with reference samples which were cooked/sheared in the same way but in which exogenously xylan was not added.

Materials and Methods

Fibre materials: These are described in detail in the report introduction and overview given in *Table 1*.

Table 1. Cooking conditions

	Shear temp. °C	Compr, %	OH-charge g/L	H-factor	Cooking time ¹ min	Residual OH-g/L	Kappa no N ² , S ³
Ref	165	32.9	44	1300	120	12.8	29.7, 29.2
Beech	165	32.1	45	1300	120	9.2	31.0, 29.3
Wheat	165	32.1	45	1300	120	8.8	31.7, 31.3

¹at 165 °C

²N cooks without mechanical treatment, i.e. only compression

³S cooks with compression and shearing forces applied during 2 minutes, 15 minutes before the end of cook

Scanning Electron Microscopy approach for fibre analysis: For SEM the pulp samples were processed according to Daniel and Duchesne (1998) and Daniel et al. (2004) using ethanol dehydration and dried in an Agar E3000 critical point dryer (Agar Scientific Ltd, Stansted, UK) with CO₂ as the drying agent. Samples were subsequently coated with platinum gold (*ca* 6 nm) using an Agar high resolution coater and examined using Philips Environmental-SEM or Hitachi 4500 operated at variable kV. Images were digitalized using the embedded software. In order to have an overview of the effects of the different treatments, SEM observations were performed on the surface areas of at least twenty fibres including early- and latewood fibres at various magnifications.

Results and Discussion

GENERAL OVERVIEW OF PULP FIBRES; SURFACE ULTRASTRUCTURAL FEATURES OF THE DIFFERENT PULPS

Representative SEM images from the six pulps *viz* BeechX-N, BeechX-S, WheatX-N, WheatX-S, Ref.-N and Ref.-S are shown in *Figures 1-13*. In total roughly 40 images/pulp including both early- and latewood fibres were taken in order to visualize trends and make a representative appraisal.

Two assumptions are made with respect to interpretation of images that are based on previous studies (Daniel and Duchesne, 1998; Daniel et al., 2004; Duchesne and Daniel, 2000) on changes in the surface morphological ultrastructure of wood fibres during kraft pulping and delignification namely:

- i) That the true surface morphological ultrastructure of wood fibres is reflected by a complex macrofibrillar structure of cellulose microfibrils (i.e. aggregates of cellulose microfibrils) *more or less* associated with hemicelluloses (i.e. xylan, glucomannans) and lignin. With removal of lignin and consequently some of the hemicelluloses during the kraft process, the macrofibrillar cellulose structure becomes visually apparent on the outer fibre wall and is composed of either the primary wall (looks similar in morphology to the structure of pit membranes) if present and the fibre S1 layer. With processing and refining, the primary wall is often removed and even parts of the S1 layer sometimes thus revealing the S2 layer. Using SEM these aspects are readily visualized;
- ii) That if the macrofibrillar structure is not visualized, then it is likely to be covered with extraneous materials (e.g. hemicelluloses, lignin) from the cook or from that added extraneously to the cook –*in our case here beech- and wheat xylans.*

These changes in surface morphological ultrastructure are however very difficult to quantify by SEM and only trends can be realized. In addition, SEM cannot give any information of the penetration of extraneous added (or retained) xylan into the fibre wall or from the cell lumen. Therefore the following observations can be considered as trends that reflect changes in the surface ultrastructure of the outer fibre wall; i.e. the primary wall and secondary S1 layers. Similarly the cellulosic encapsulation and presence of aggregates are considered to reflect the addition of xylans and complement the localization of xylans in these fibre regions as shown from the use of specific xylan probes reported in *Appendices 2-4*.

General observations:

BeechX-N. The surface macrofibrillar structures of the fibres were typically encapsulated in a matrix-type material giving the cellulose microfibrils a reticulate appearance (*Figs 1-3*). In addition, elongated and spherical-type aggregates of the order 60-90 nm were randomly distributed over the surface ultrastructure of the fibres as single entities (*Fig. 1*) or aggregated into clumps (*Figs 2, 3*).

BeechX-S. Typically, the fibre surface ultrastructure morphology was similar to BeechX-N but possibly the cellulosic macrofibrillar structure is less encapsulated due to shearing action and lesser numbers of aggregates were present (*Figs 4, 5*). Aggregates were similar in appearance as those for BeechX-N.

WheatX-N. Fibre surface ultrastructure morphology showed a cellulose macrofibrillar structure encapsulated/covered with matrix materials and aggregates

(Figs 6, 7). Surface aggregates were of the order of 40 to 150 nm (e.g. Fig. 6c) frequently randomly distributed over the surface rather than in large clumps (Fig. 6b). Cracks in the outer wall structure suggested that the fibres were covered in xylan components (Fig. 7a,b).

WheatX-S. Fibre surface ultrastructure frequently covered with matrix materials of the primary wall that were broken during shearing or sample preparation (Figs 8a, b, 9a). The outer primary wall macrofibrillar structure appeared encapsulated in matrix materials giving a reticulate appearance (Fig. 8b, d, 9a). The outer S1 layer revealed beneath the primary wall also appeared covered in matrix materials and small aggregates (Fig. 8c). Similar sized aggregates were also present on the exposed primary wall macrofibrils (Fig. 8d). At high magnification the large aggregates are shown composed of a multicomplex of smaller aggregates (Fig. 9b).

Ref.-N and Ref.-S. Neither reference pulp fibres showed a "clean" surface cellulose macrofibrillar structure (Figs 10-13) and evidence for presence of matrix materials was quite evident. Surface aggregates were present but were less abundant (e.g. Ref. N; Fig. 10a-d) and frequently smaller than observed with the Beech- and Wheat xylan treated spruce pulps. Interestingly, exposed macrofibrillar structures from the bordered pits were highly and selectively covered with aggregates (Fig. 11b) of the order consistent with the aggregates seen from the Beech and Wheat xylan treated pulps. This emphasizes that the morphological distinction of components diffusing from the spruce fibres during kraft pulping and that added exogenously is difficult.

Conclusions

- 1) Both BeechX-N and WheatX-N treated fibres had large amounts of xylans (as aggregates) associated with their fibre surfaces –*both early and latewood fibres*. Presence of xylans was manifested in two ways: *i)* Encapsulation of the cellulosic structure obscuring the cellulose macrofibrillar structure; *and ii)* Presence of aggregates considered as precipitates of xylans (*and possibly other components; e.g. glucomannans, lignins*) present on the fibres surfaces;
- 2) Both the BeechX-S and WheatX-S treated and mechanically sheared fibres also had considerable amounts of materials associated with their fibre surfaces –*both early and latewood fibres*. As a comparison with the respective BeechX-N and WheatX-N pulps there were great difficulties in observing any particular differences. Again the cellulosic macrofibrillar structure of the fibres appeared coated and encapsulated in materials together with precipitates consisting of xylan and presumably other components on the fibre surfaces;

- 3) That there appeared to be some morphological differences in nature of the aggregates with the Beech and Wheat xylans; the latter forming more elongated aggregates and the latter more spherical structures;
- 4) That some evidence for fibrillar encapsulation and aggregates were also noted in the Ref.-N and Ref.-S pulps. However, this appeared different than that with the Beech and Wheat treated pulps and areas of “free” cellulosic structure was also noted;
- 5) That both the Beech and Wheat xylans were binding to both the primary wall and S1 secondary cell wall layer cellulosic macrofibrillar surface structure (e.g. with remaining primary wall materials on the outer fibre walls and also at pit membranes);
- 6) With addition of high levels of Beech- and Wheat xylans and mechanical shearing, the morphological surface fibre ultrastructure appears fairly similar although the total chemical analyses suggest a considerable reduction in xylans in the sheared pulps. Possibly this is seen in a reduction of the aggregates present but loss of xylan from the inside the secondary wall structure is also expected;
- 7) Both the BeechX-N, BeechX-S, WheatX-N and WheatX-S treatments gave the impression that the fibre walls were covered in a layer, *possibly ultrastructural layers* of xylan components;
- 8) Surface ultrastructure of the beech- and wheat treated fibres also often showed evidence for the primary wall remaining which may have been an indirect consequence of adding xylan at the impregnation stage.

References

- Daniel, G. and I. Duchesne (1998). Revealing the surface ultrastructure of spruce pulp fibres using field emission-SEM. In: Proceedings of the 7th Int. Conf. on Biotechnology in the Pulp and Paper Industry, pp. 81-85, Vancouver, Canada.
- Daniel, G., J. Volc, M-L. Niku-Paavola (2004). Cryo-FE-SEM & TEM Immuno-Techniques Reveal New Details for Understanding White Rot Decay of Lignocellulose. *Comptes Rendus, Biologies* 327/9-10, Oct. pp 861-871.
- Daniel, G., I. Duchesne, C. Tokoh and S. Bardage (2004). COST Action E20, “Wood Fibre Cell Walls: Methods to Study Their Formation, Structure and Properties”, Eds: U. Schmitt, P. Ander, J.R. Barnett, A.M.C. Emons, G. Jeronimidis, P. Saranpää, and S. Tschegg. Swedish University of Agricultural Sciences, Uppsala, Sweden. ISBN 91-576-6803-5, pp. 87-104. (<http://www-wurc.slu.se>).
- Daniel G., Olsson, A-M., Ander P., Kaňuchová A., Salmén L., Filonova L., Sjöström K., Boussard L., Berg S., Heijnesson-Hultén A., Lindström C. (2010). CRUW Chemical Pulping 1. The influence of xylan on the sensitivity towards fiber damage. CRUW, Intern rapport nr 2, 88p.

- Daniel G., Olsson, A-M., Ander P., Kaňuchová A., Salmén L., Filonova L., Sjöström K., Boussard L., Berg S., Heijnesson-Hultén A., Lindström C. (2011). CRUW Chemical Pulping 1. The influence of xylan on the sensitivity towards fiber damage analyzed on pulps cooked to high kappa numbers. CRUW, Intern rapport nr 5, 58p.
- Duchesne, I. and G. Daniel, 2000. Changes in surface ultrastructure of Norway spruce fibres during kraft pulping – visualization by field emission-SEM. NPPJ., 1, (15), 54-61.
- Yllner, S. and B. Enström, 1956. Studies on the adsorption of xylan on cellulose fibres during sulphate cook. Part 1. Svensk Papperstidn 59, (6) 229-232.
- Yllner, S. and B. Enström, 1957. Studies on the adsorption of xylan on cellulose fibres during sulphate cook. Part 2. Svensk Papperstidn 60, (15) 449-454.

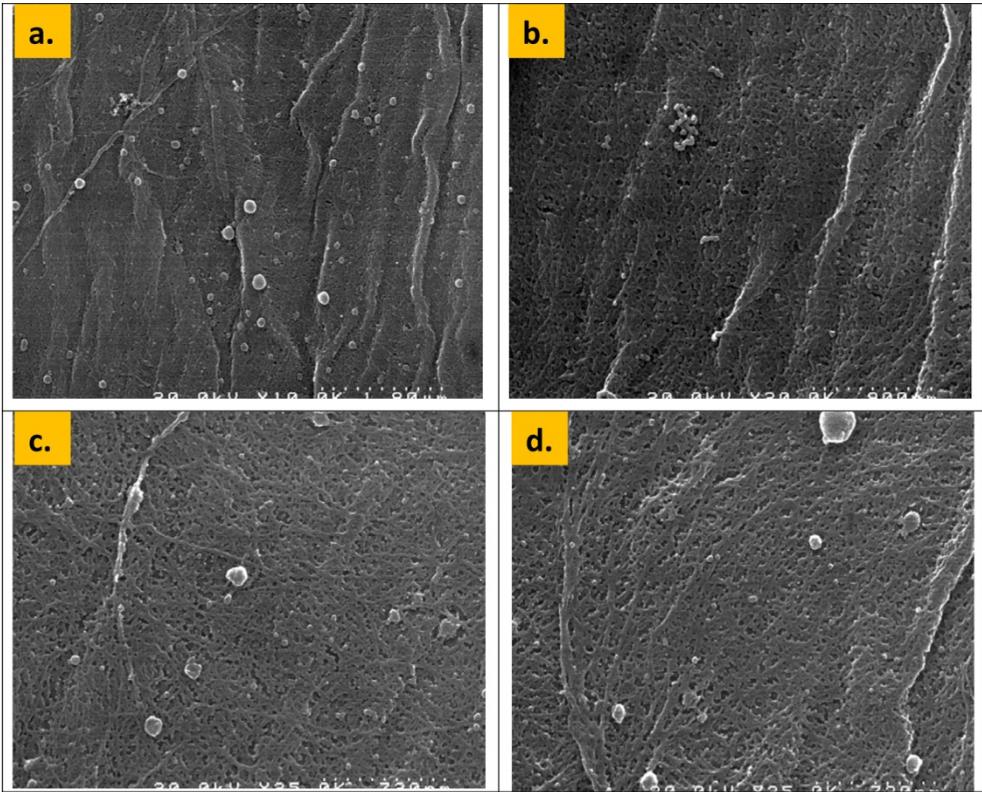


Figure 1. BeechX-N treated spruce pulp fibres. Surface ultrastructure of the fibres appears highly packed with matrix materials and the cellulosic microfibrillar structure coated. Images show coating of the primary wall. Aggregates are associated with the fibre surface.

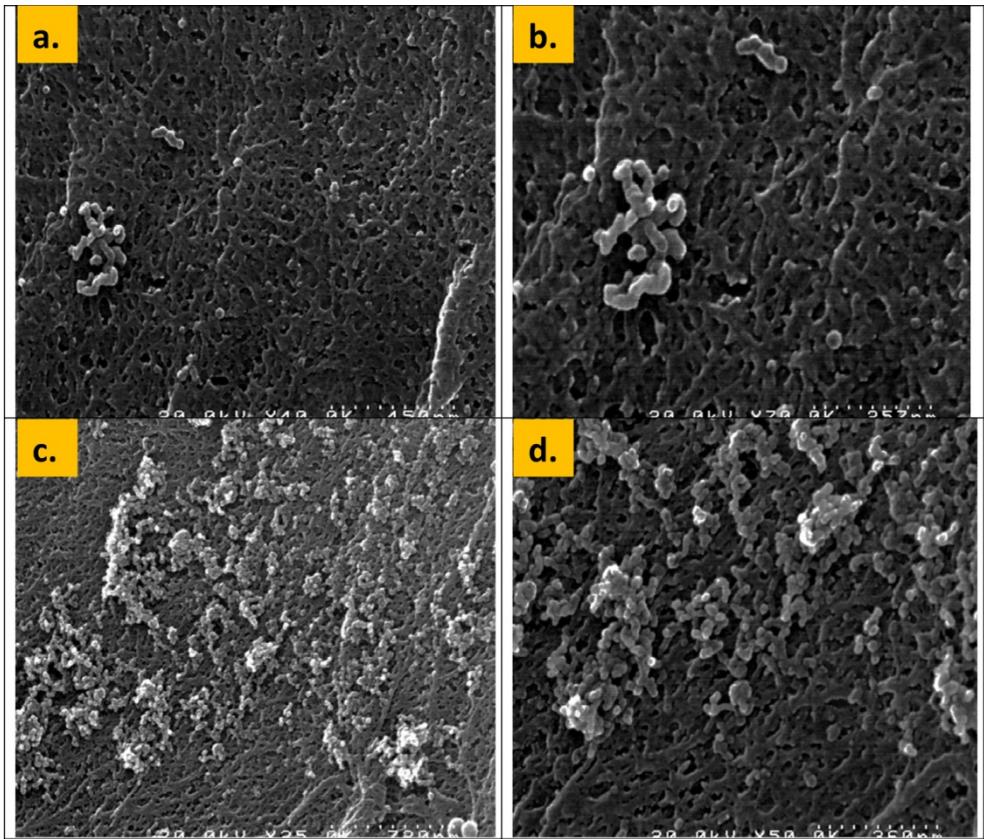


Figure 2. Beech-X-N treated spruce pulp fibres. Cellulosic microfibrillar ultrastructure appears coated. Many of the fibres had large amounts of aggregates associated with the surface structure.

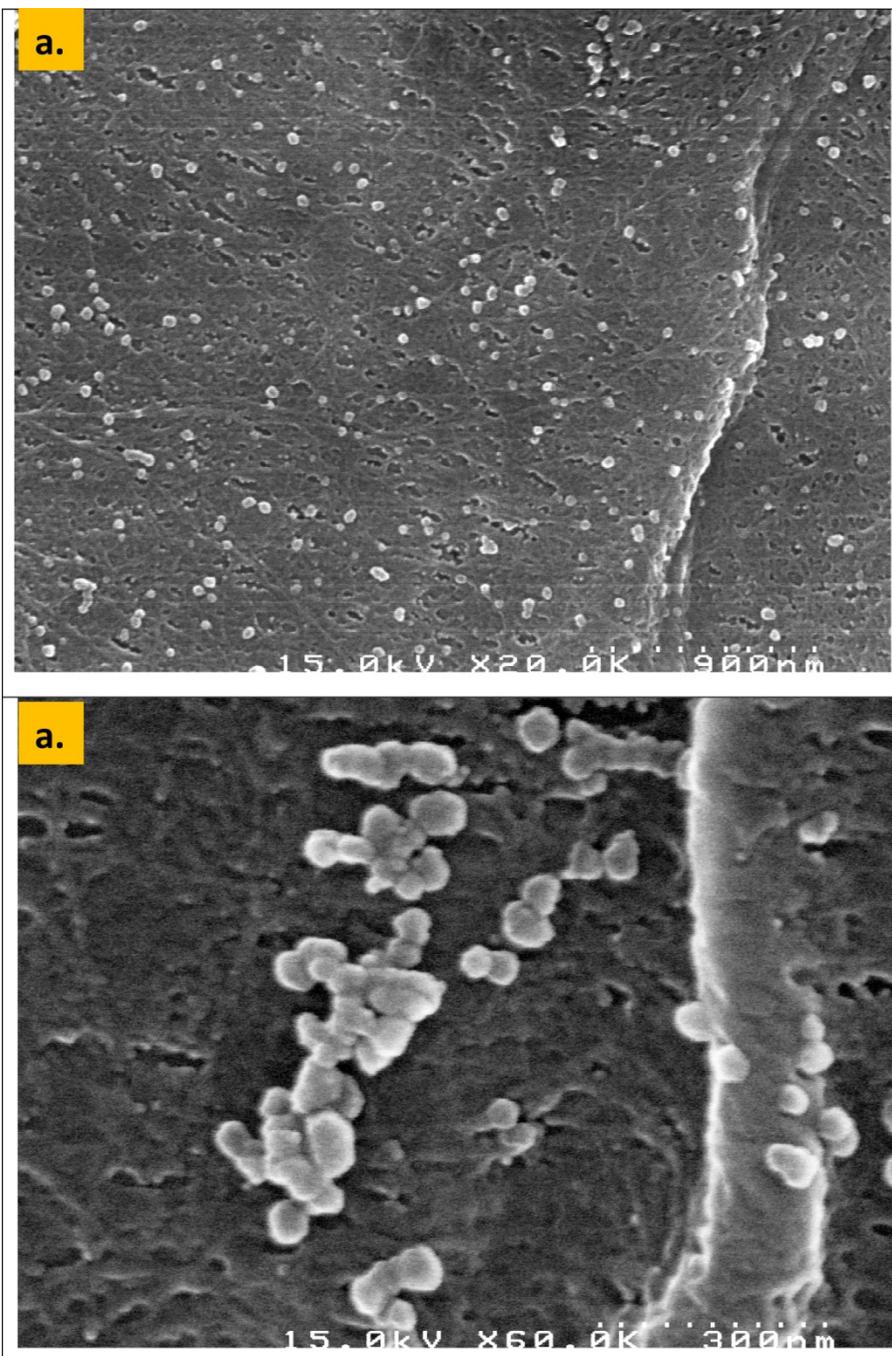


Figure 3. Beech-X treated spruce pulp fibres.

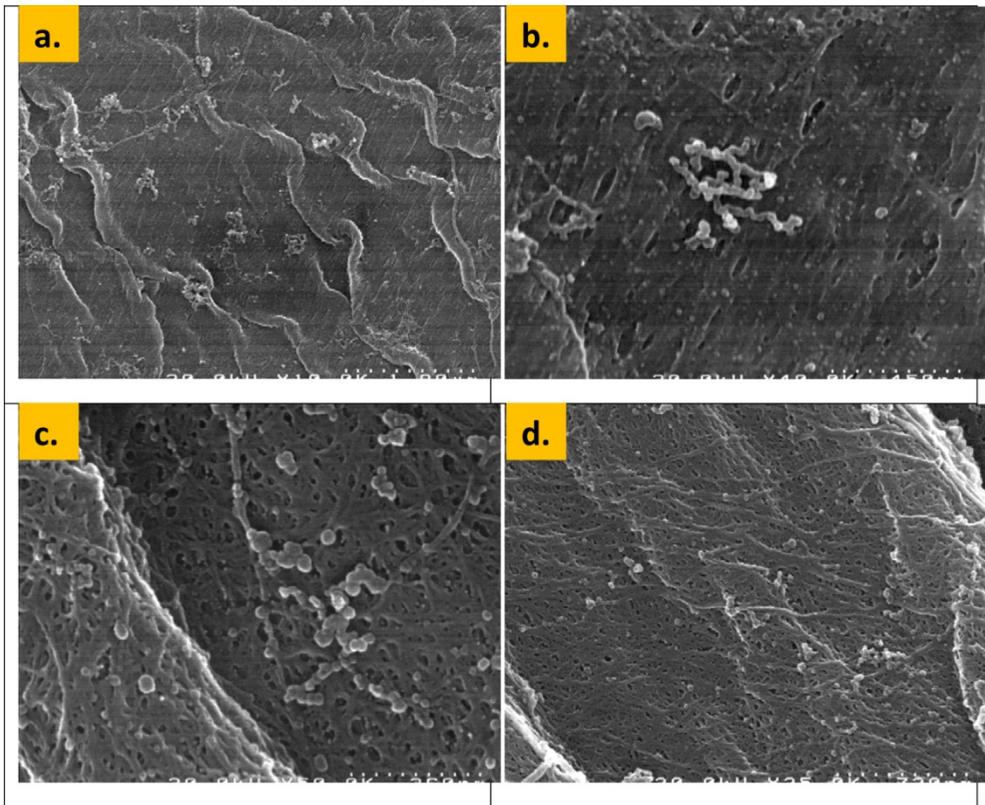


Figure 4. BeechX-S fibres.

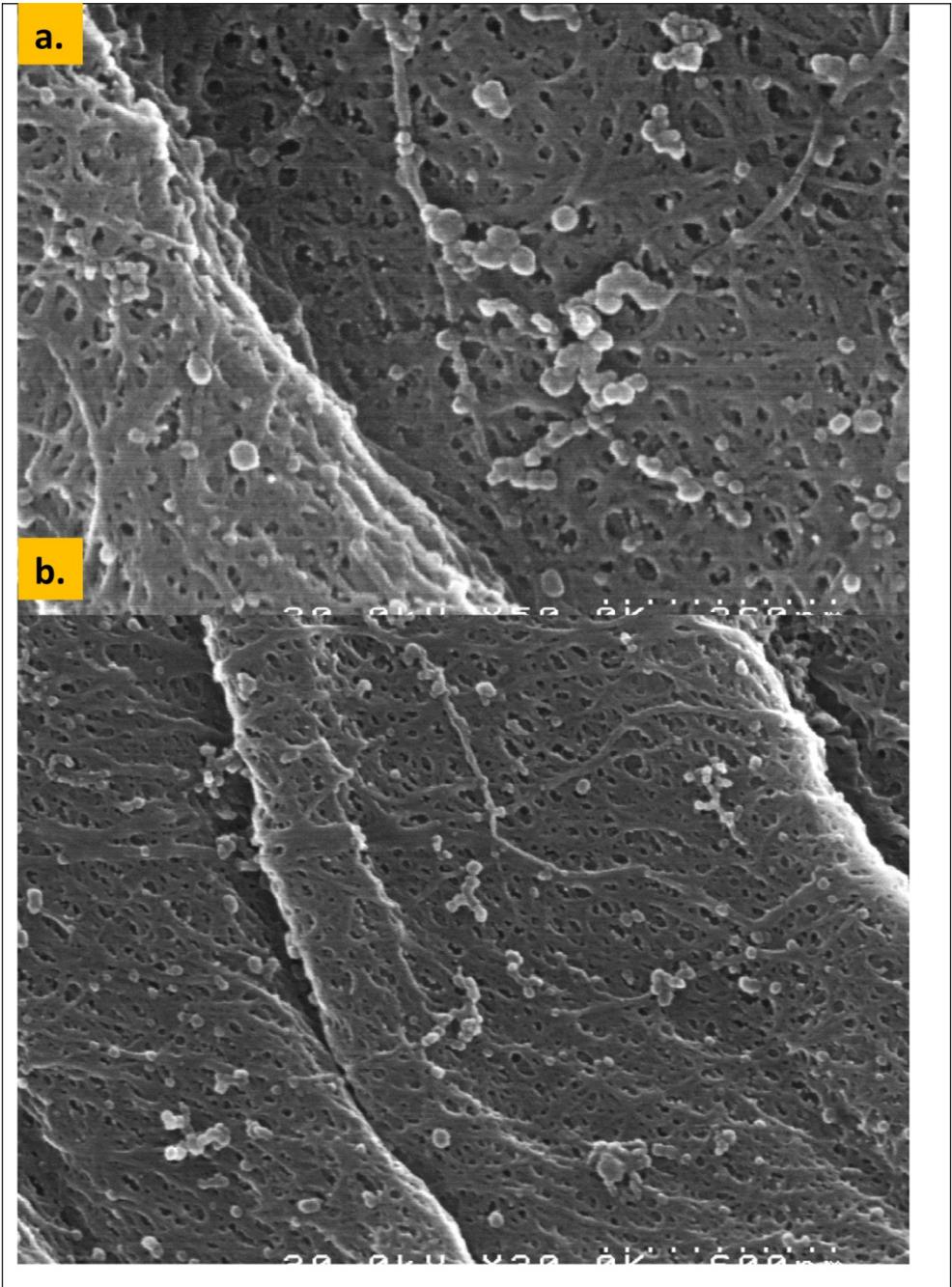


Figure 5. BeechX-S fibre surfaces.

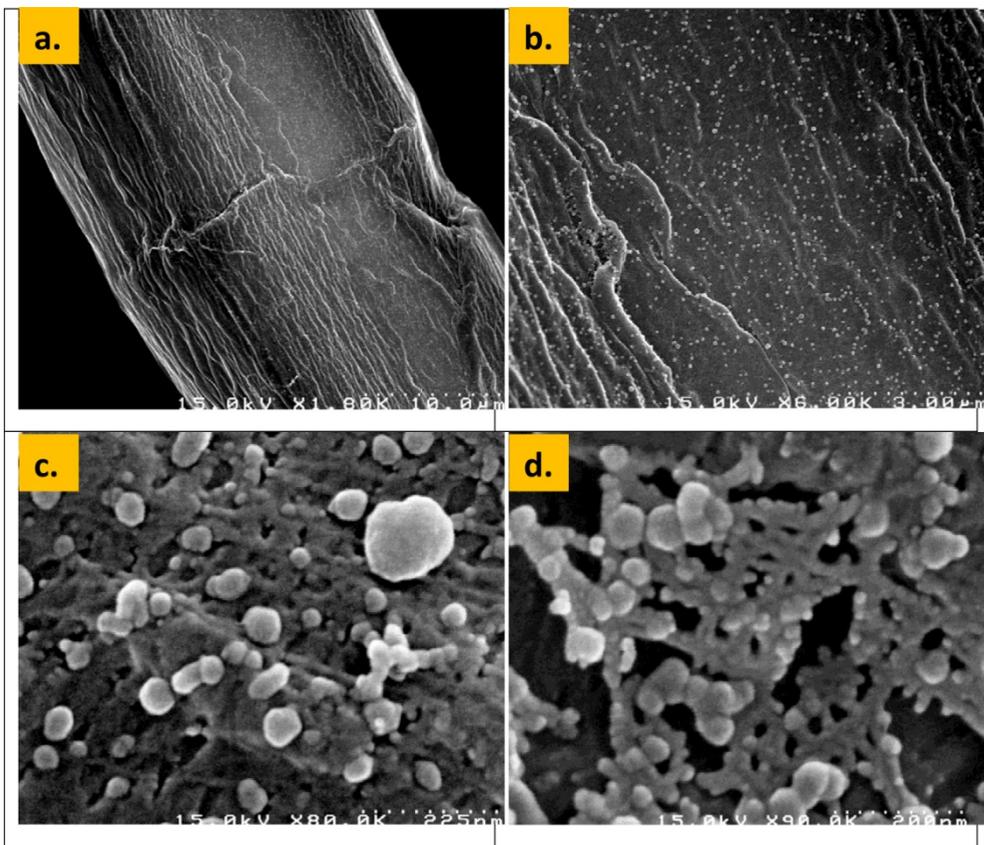


Figure 6. WheatX-N fibre surfaces.

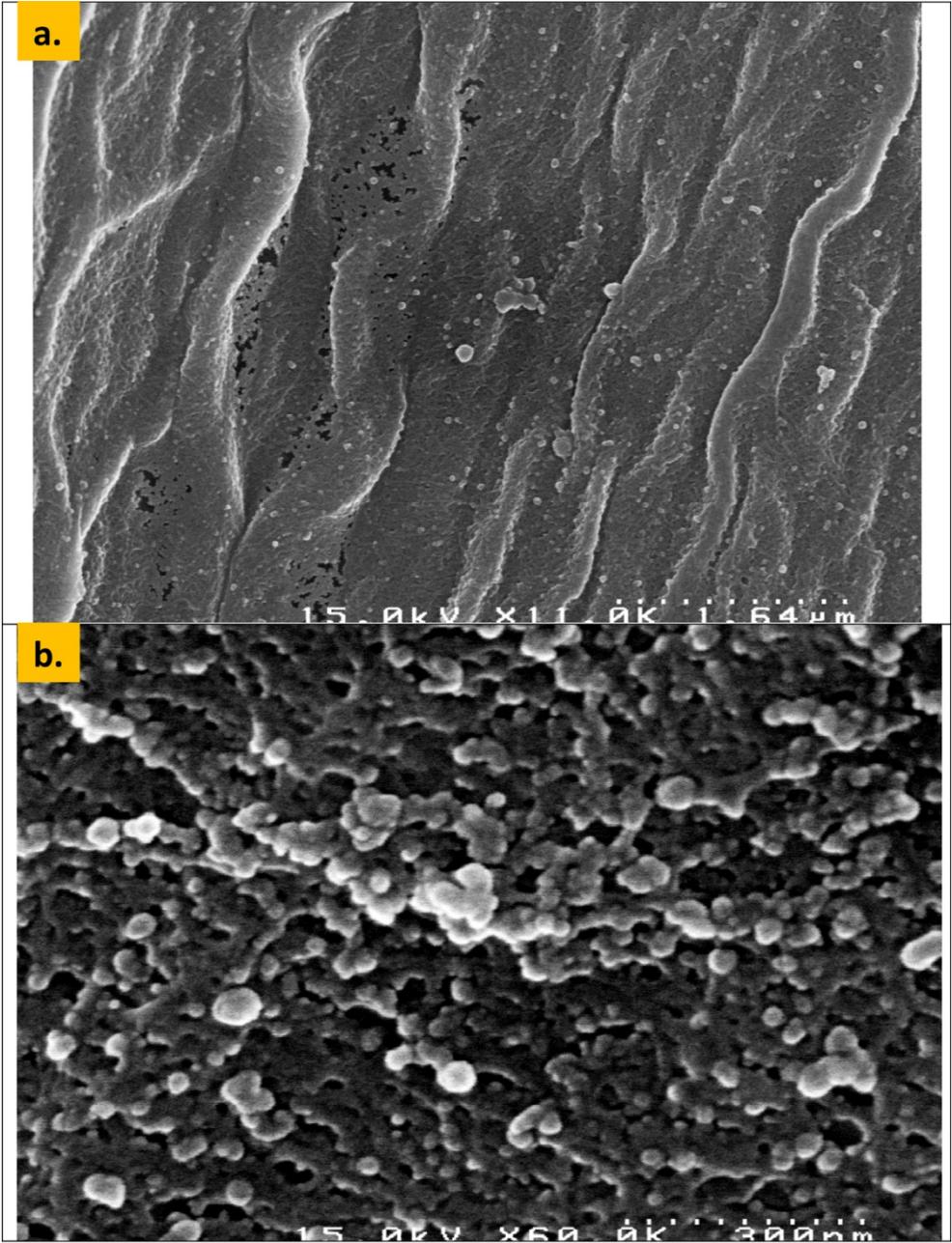


Figure 7. WheatX-N fibre surfaces.

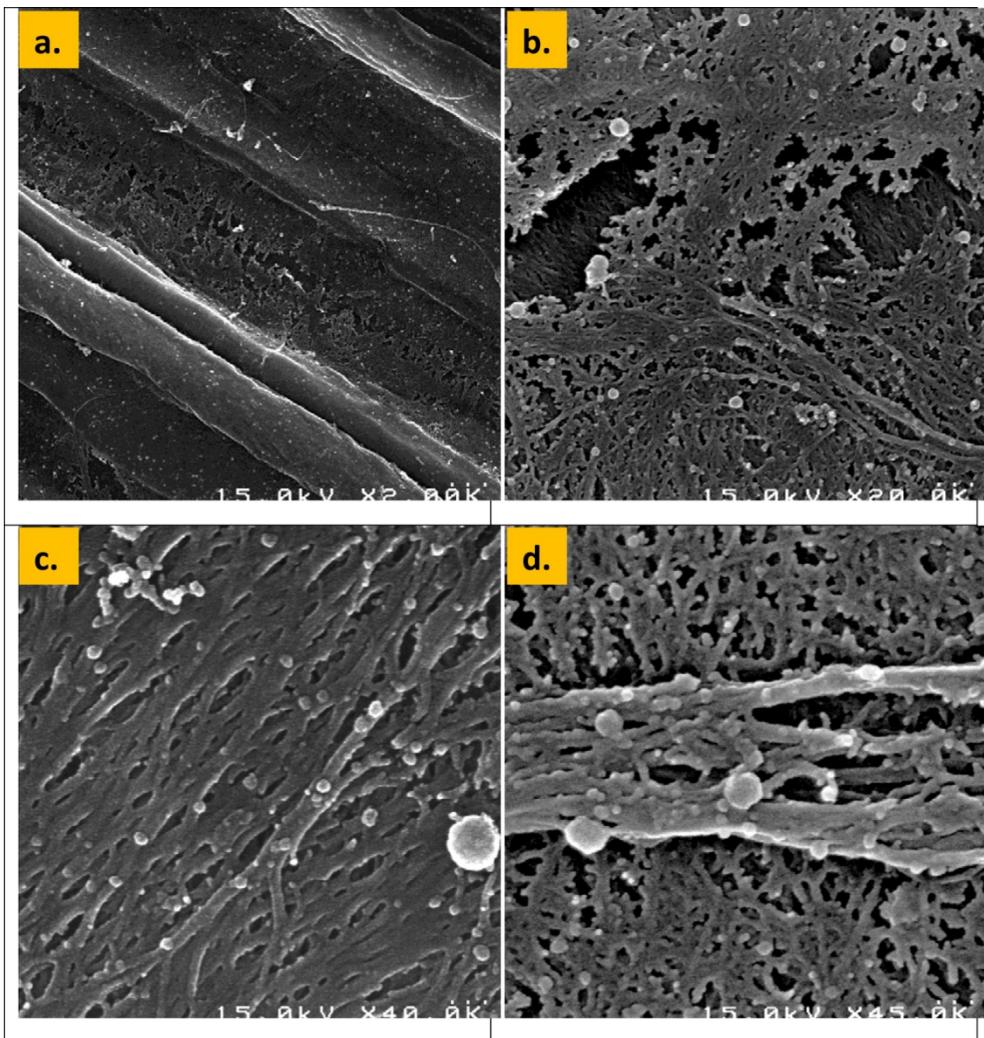


Figure 8. WheatX-S fibre surfaces.

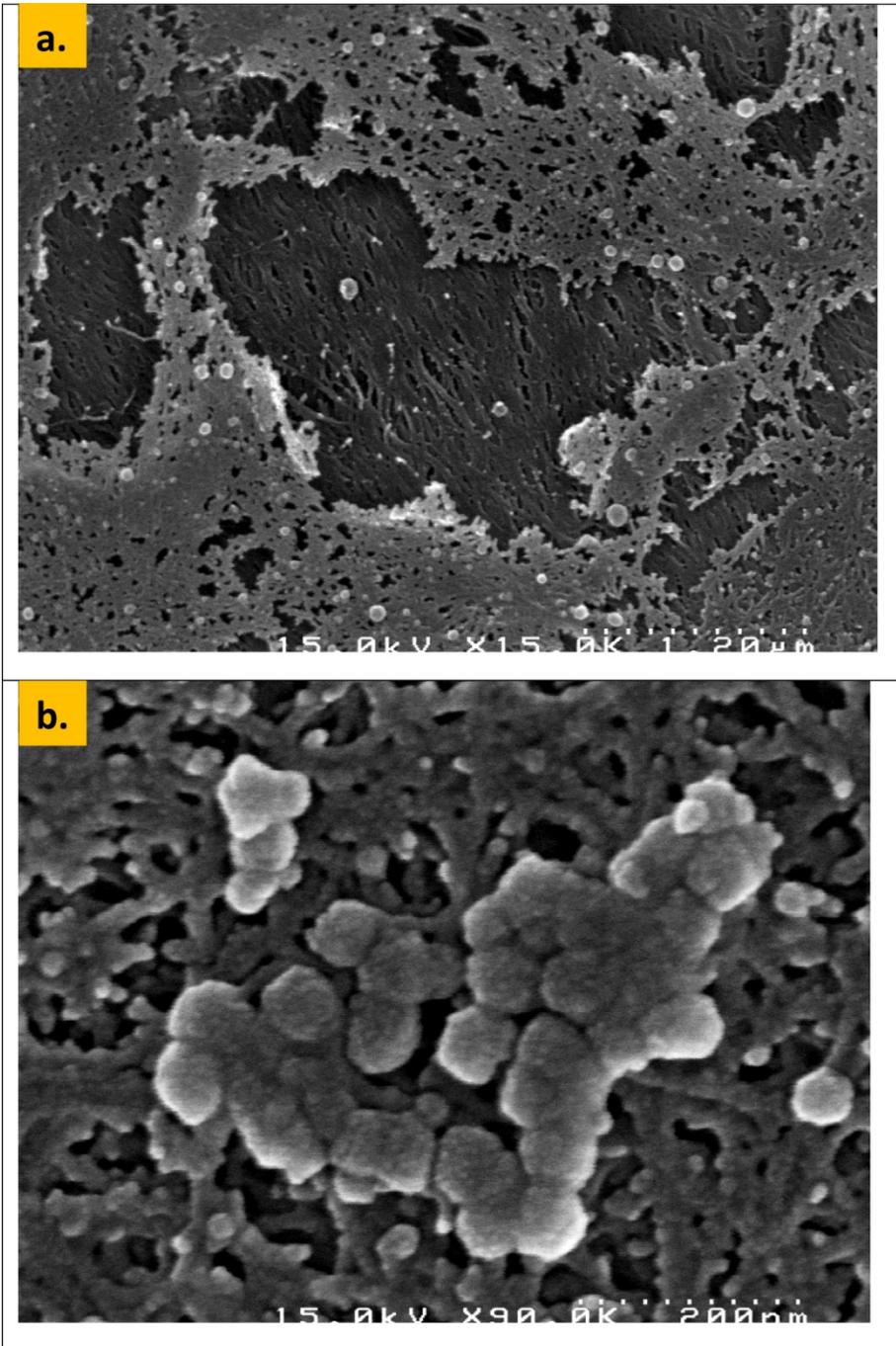


Figure 9. WheatX-S fibre surfaces.

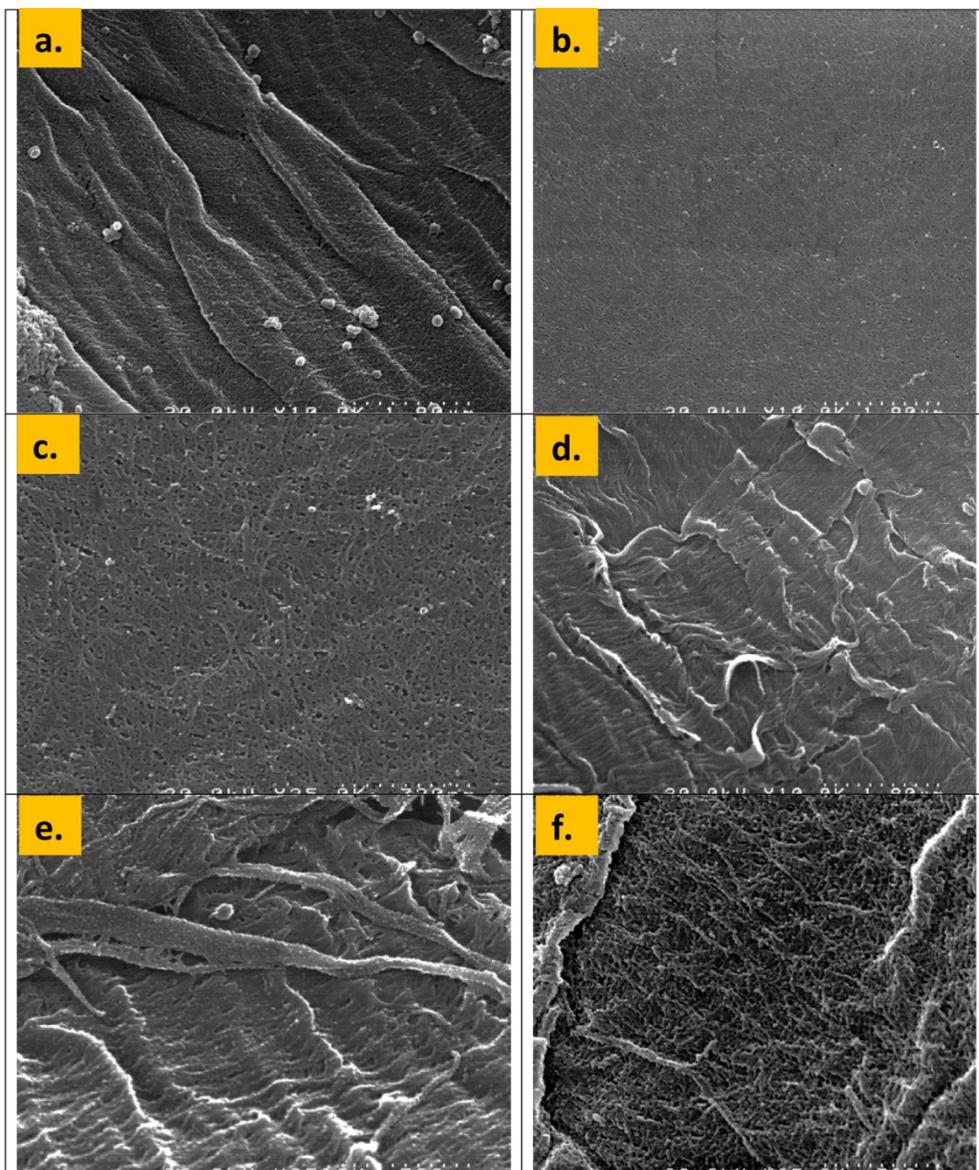


Figure 10. Ref.-N fibre surfaces.

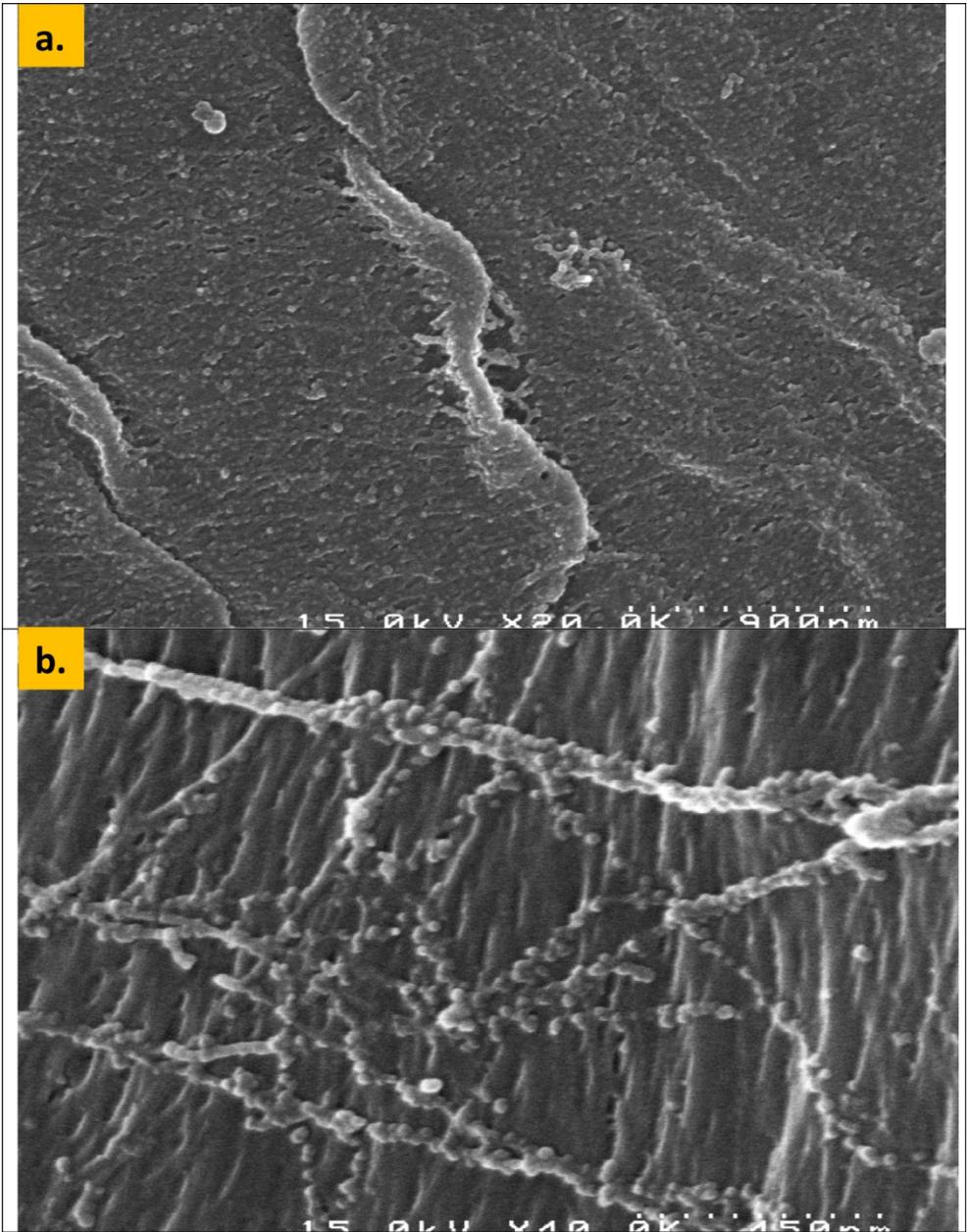


Figure 11. Ref.-N fibre surfaces.

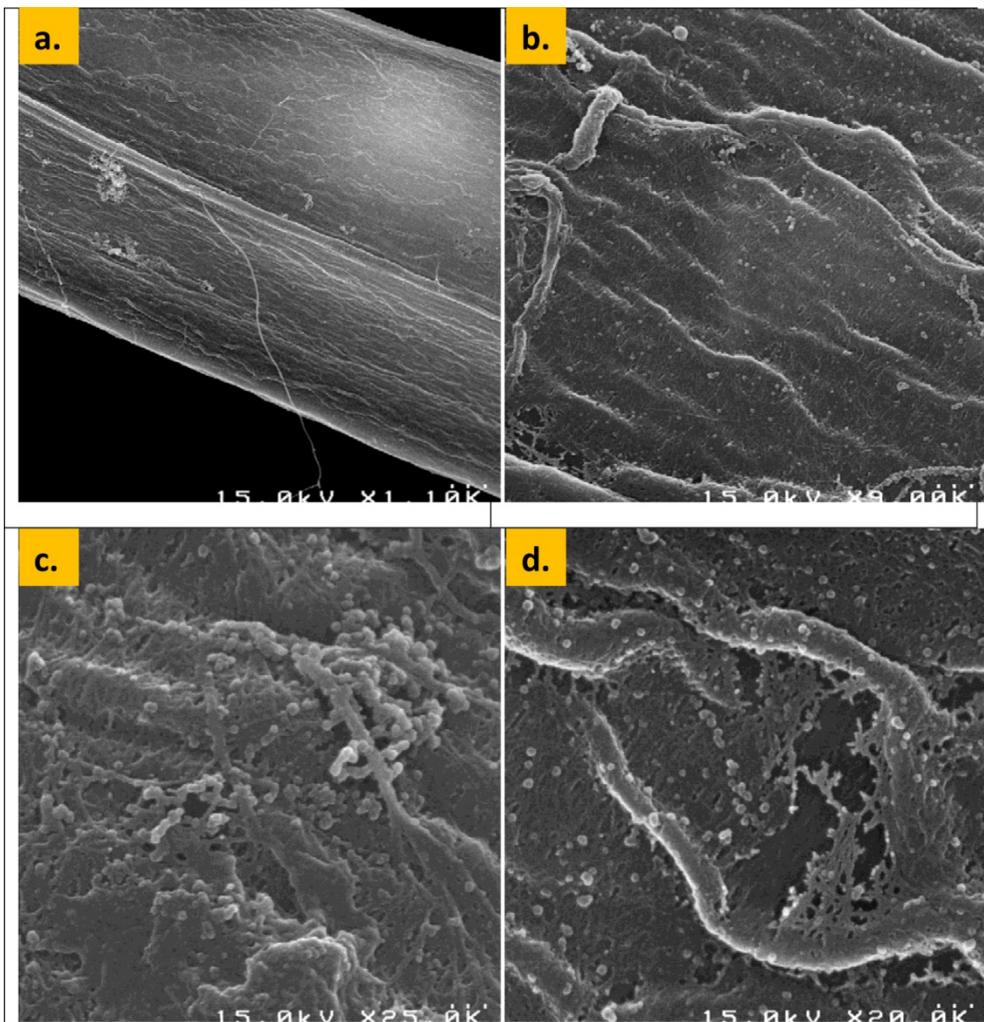


Figure 12. Ref.-S fibre surfaces.

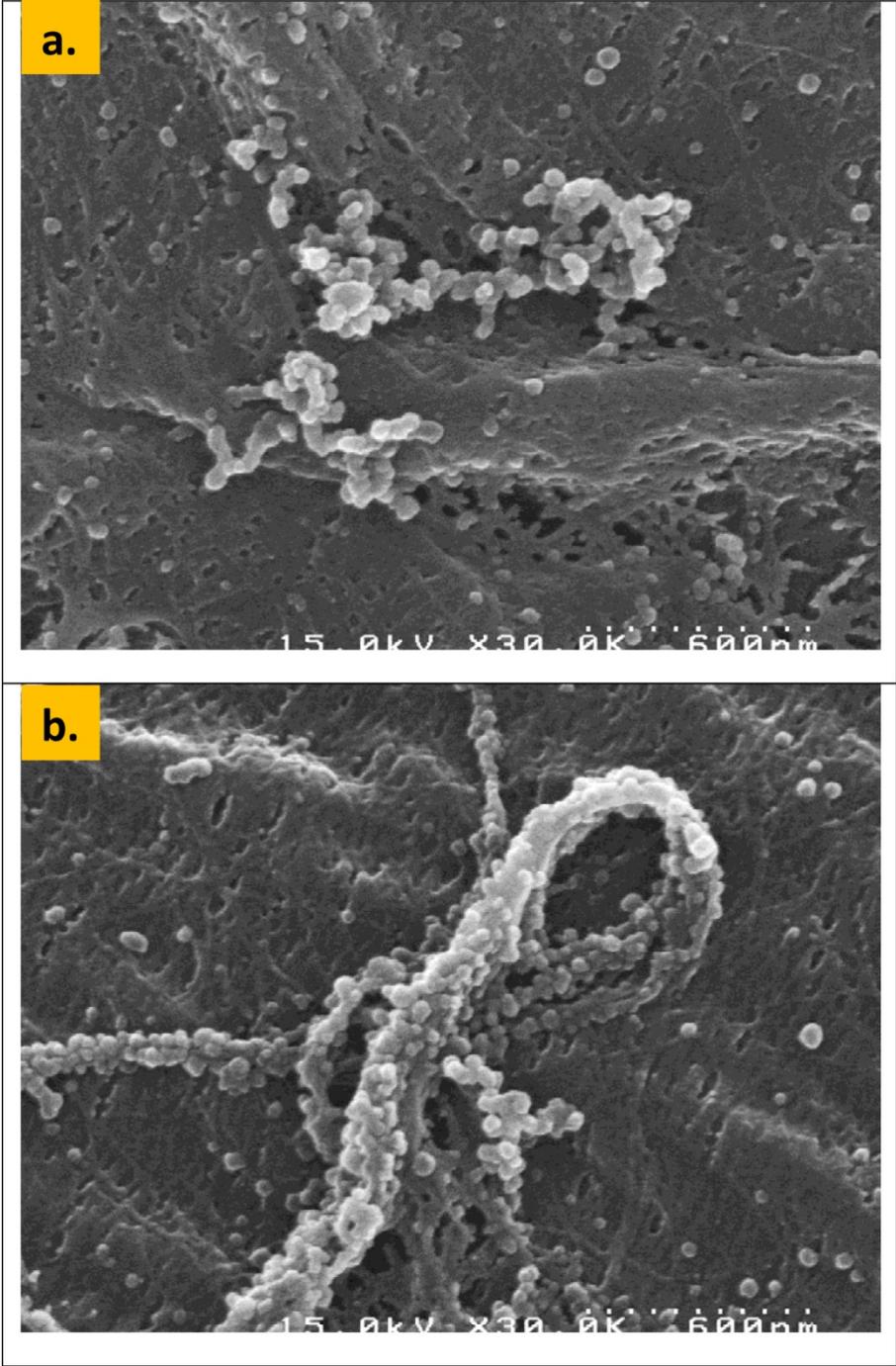


Figure 13. Ref.-S fibre surfaces.

APPENDIX 6. USE OF SIMONS STAINING FOR CHARACTERIZING XYLAN TREATED CHEMICAL PULPS

Dinesh Fernando, Geoffrey Daniel, SLU, Uppsala

Aim: To evaluate if Simons staining (Fernando and Daniel, 2010) could provide morphological evidence for differences in fibre development and porosity of the beech- and wheat xylan treated spruce kraft pulp fibres, with- and without shearing in comparison to reference pulps with- and without shearing.

Background

Use of Simons staining (SS) for fibre analysis was initiated already in the 1950's (Simons, 1950) as a method for the microscopic evaluation of beaten- and unbeaten pulp fibres. Most of the early applications have been directed to chemical pulp refining, but over the years the number of applications have been extended to include biomechanical, recycled and enzymatically modified fibres. The SS method allows for evaluation on how well papermaking fibres are treated or modified in terms of fibre wall damage and/or cell wall fibrillation and delamination (i.e. opening of the fibre wall structure) during processing (Esteghlalian et al., 2001; Akhtar et al., 1995, Blanchette et al., 1992; Fernando and Daniel 2010). It has also been used as a relatively simple and easy method to estimate the porosity of lignocellulose substrates/pore volume distribution of the fibre wall (Yu and Atalla, 1998; Joutsimo and Robertson, 2005) in comparison to more time-consuming quantitative techniques such as nitrogen adsorption and solute exclusion. Recently a quantitative SS method was developed and applied for assessing the development of mechanical pulp fibres and this method is applied here (Fernando and Daniel, 2010).

SS is a double staining method composed of a mixture of two dyes direct blue 1 (DB) and direct orange 15 (DO). DB has a well-defined structure and low molecular weight (*ca* 992.82 kD) while direct orange 15 (DO) is a polymeric mixture containing a high molecular weight (HMW) fraction (> 25,000 kD)(Yu et al., 1995).

In the present study, we used the Fernando and Daniel (2010) SS approach to assess whether an effect on fibre porosity was achieved during treatment with a high a concentration of beech/wheat xylans added during kraft cooking of spruce chips.

Principle of the method

During pulping, wood fibre cell walls are subjected to morphological/structural modifications and opening (i.e. internal delamination/fibrillation (D/IF)) of the wall structure leading to the generation of voids and changes in fibre pore size and shape. When pulp fibres are stained with SS they normally develop various colours -blue to yellow/orange- (see Fernando and Daniel, 2010) depending on process conditions and treatment. During Simons staining, the low molecular weight DB dye initially enters the fibre cell walls through the pores and stains the whole fibre blue, representing fibres with no (i.e. untreated fibres) or minimal structural modification. Intensely refined fibres will however develop extensive internal delamination and fibrillation in the wall causing the native pores to increase in size to produce large spaces in the wall allowing access for the larger-molecular-weight DO dye to penetrate. Severely treated fibres will adsorb the high molecular orange dye preferentially in the accessible regions of the fibre wall that will displace the blue dye because of its greater affinity for cellulose. As a consequence, the stained fibres will appear green to yellow/orange. Some fibres also stain green reflecting a combination of staining with both the low- and high molecular weight dyes indicating fibres with moderate structural changes. This results from the orange dye gaining access to the interior of the fibre wall so that fibres adsorb both dyes presumably in more or less equal amounts.

It is also important to examine individual fibres over their entire length during characterization as localized D/IF normally exists along segments of fibres. In the present work this was typically shown at sites of fibre dislocation (*Figs 1, 2*). The amount and/or severity of localized D/IF will strongly influence fibre properties (e.g. fiber flexibility) and thereby ultimately pulp properties.

Materials and methods

SIMONS STAINING

SS stain consists of a 1% w/v aq. solution of Chicago Sky Blue (DB) and 1% w/v aq. Pontamine Fast Orange (DO) mixed in a 1:1 ratio. Never-dried beechX-N, beechX-S, wheatX-N, wheatX-S, ref. N, ref. S fibres (*ca* 1g) were placed onto glass slides with a few drops of SS added and heated at 60°C until dry and then rinsed with distilled water to remove excess stain. Stained fibres were examined using a Leica DMLB light microscope and images recorded digitally with a Leica DC300 camera.

During processing of any pulp type, different fibre populations (*sub-fibre populations S-FP*) exist representing different degrees of fibre wall development (i.e. D/IF). By examining fibres along their length after SS, it is possible to classify

the staining into five categories: 1) light blue, 2) dark blue, 3) green, 4) yellow/orange colouration along less than half the fibre length (denoted as “< ½ fibre”), and 5) yellow/orange staining of more than half the fibre length (denoted as “> ½ fibre”). Light microscopy was used to identify the different S-FPs in the six pulp types by examining a total of 200 stained single fibres (i.e. long (> 0.6 mm) and median long (~ 0.2-0.6 mm) fibres but not short fibres or fibre fragments in each pulp sample using a few random fields of view at 100 x (Fernando and Daniel, 2010). Each fibre examined was categorized in one of the five S-FPs and data from each sample in relation to the identified S-FPs recorded. For simplicity the five sub-fibre populations were reduced to three S-FPs combining populations 1 and 2 and 4 and 5. Normally the data for such studies is analyzed using SAS software (SAS/STAT, Version 9.1.2 for Windows (XP-Pro platform), SAS Institute Inc., Cary, NC, USA) (Fernando and Daniel, 2010) using the GENMOD procedure of SAS software. GENMOD is a general statistical modeling tool for fitting generalized linear models to data, including OLR to assess differences in degree of D/IF for different pulps. In the present study however, the results shown are based on raw data analyses and statistical analyses have not been performed.

Results and discussion

Representative SS staining of the beechX-N pulp fibres are shown in *Figure 1*. All pulps showed a fairly similar staining with greatest indication for presence of highly developed fibres (i.e. yellow orange fibres) and green fibres (i.e. moderately developed fibres). Blue coloured fibres (i.e. non-developed fibres) were generally poorly represented in the pulps indicating that the majority of the fibres were well processed (i.e. opened up) with moderate to high porosity (*Fig. 1*) which is consistent for kraft pulp fibres. This was the same for both early- and latewood fibres with the fibres fairly uniformly stained along their length. A typical feature observed was at sites of dislocations/kinks, fibres showed a different staining reaction. For example in green fibres, the sites of dislocations were primarily yellow (*Figs. 1, 2*), while in yellow/orange staining fibres the sites of dislocations/kinks were poorly stained (*Figs 1, 2*). Overall, this indicates that fibres were quite homogeneously processed but at sites of dislocations/kinks along fibres that the fibre wall structure was more opened and structurally different than the rest of the fibre wall. In the blue fibres noted, sites of dislocations showed a darker blue staining indicating an increase in DB adsorption and thereby porosity (*Fig. 2*).

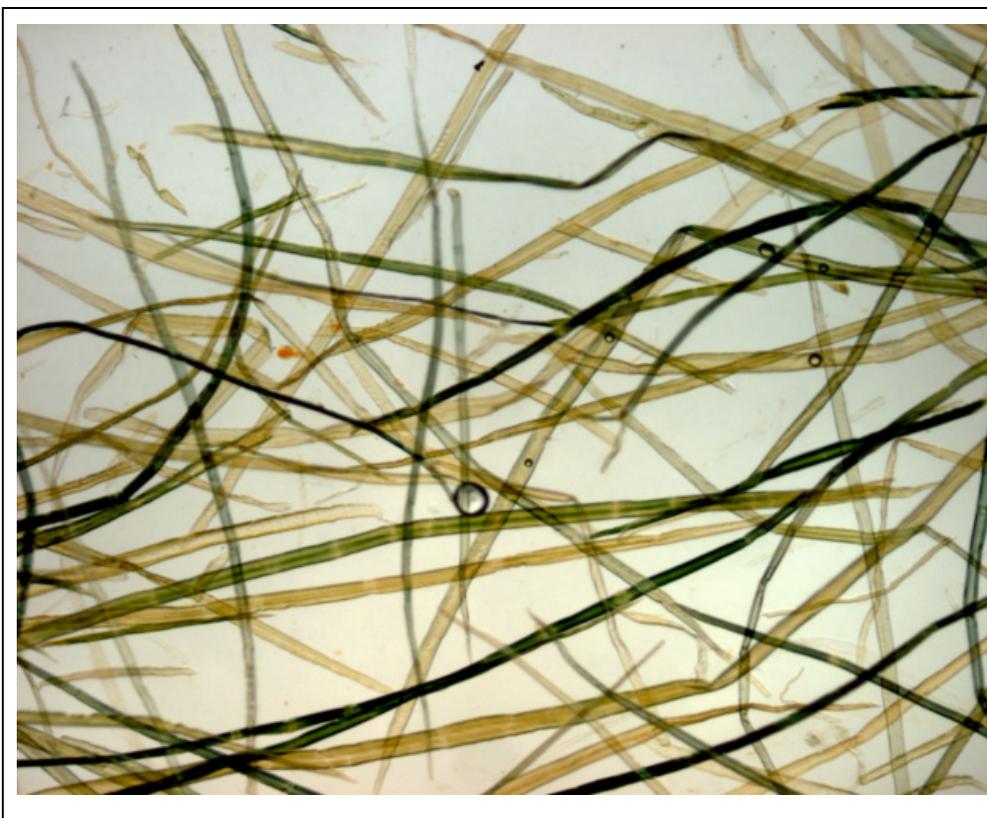


Figure 1. Typical SS staining of the xylan treated (here beechX-N) fibres showing predominantly a fairly uniform yellow/orange or green staining consistent with highly and moderately developed fibres. Some of the yellow and green staining fibres show characteristic differences in staining at sites of fibre dislocations/kinks.

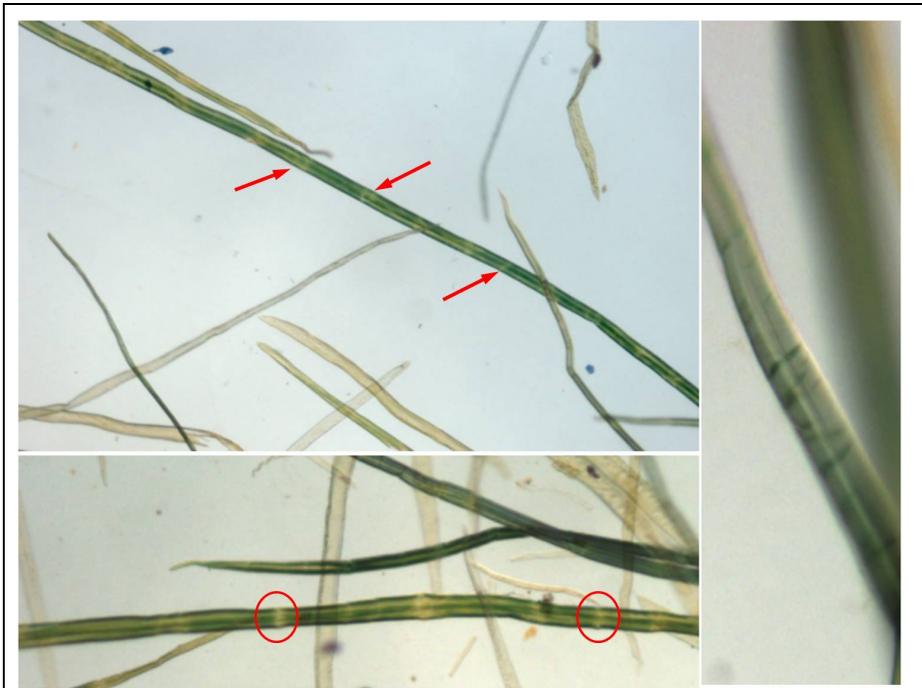


Figure 2. High magnification images showing representative fibres with characteristic differential staining of fibre dislocations (red arrows and rings).

Quantitative analysis of SS staining from the beechX- and wheatX treated fibres with- and without shearing and with comparison with the ref.-N and ref.-S pulps is shown in *Figure 3a*. Only marginal differences were observed with SS indicating that the fibres from all pulps showed fairly similar internal fibre (delamination/fibrillation (D/IF) development. The sheared pulps (i.e. beechX-S, wheatX-S, ref.-S) as a group however showed slightly improved D/IF compared to the normal pulps (i.e. beechX-N, wheatX-N, ref.-N) with slightly more developed fibres and improved porosity (i.e. less non-developed fibres shown as blue). Dislocations/kinks present in the fibres showed localized differential staining indicating the presence of a more opened fibre structure (*Figure 2, arrows and rings*).

For comparison, the typical development of fibres from populations of thermomechanical pulp fibres in which different pressures were used between the refiner plates (for more details see Fernando et al., 2010, 2011) is shown in *Figure 3b*. The fibre populations show a completely different type of fibre development consistent with high yield pulping with larger populations of non-developed and moderately developed and reduced numbers of highly developed fibres. Here the porosity of the fibres is also reduced.

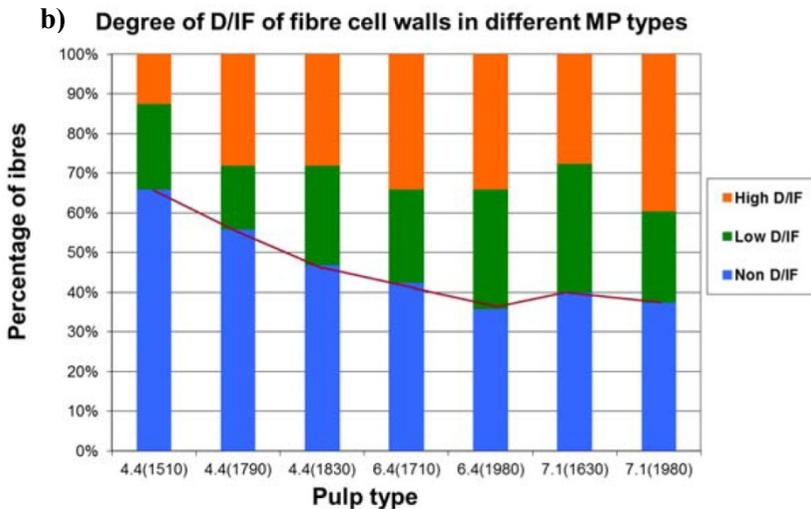
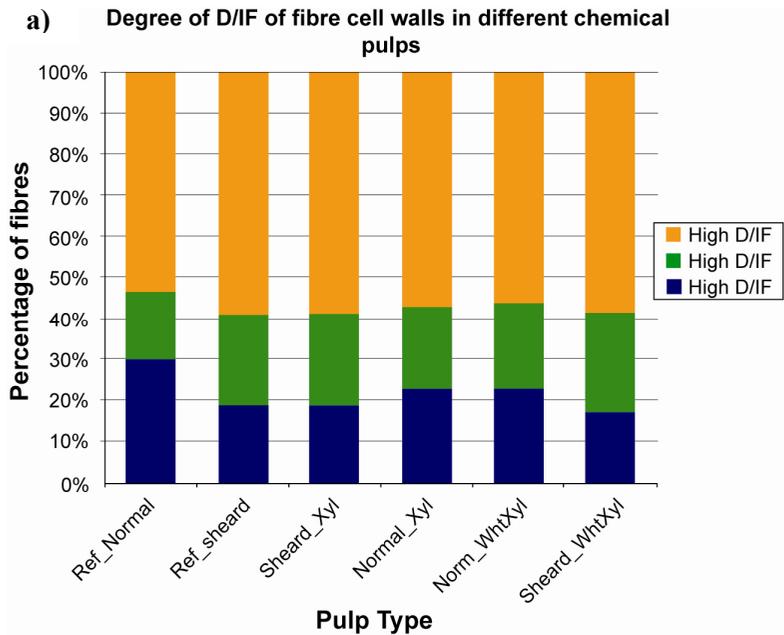


Figure 3. a) Quantitative analysis of Simons staining from the beech- and wheat xylan fibres (200 fibres/pulp) with- and without shearing and with comparison with the ref. N and ref.-S pulps. B) Typical development of fibres from populations of thermomechanical pulp fibres in which different pressures were used between the refiner plates. Nomenclature: High D/IF relates to highly developed internal delamination (orange); Low D/IF relates to low/moderately developed internal fibre delamination; Non D/IF relates to the fibre population without or with only very poorly developed internal delamination/fibrillation and thereby porosity.

Conclusions

1. The pulp fibre populations from beechX-N, beechX-S, wheatX-N, wheatX-S, ref.-N, ref.-S all showed advanced fibre development (i.e. strong internal fibre D/IF) and porosity as shown by the predominantly yellow/orange staining of the fibres;
2. The sheared pulps showed slightly more fibre development than the corresponding non-sheared pulps both as entire group and when compared with their non-sheared pulp;
3. Little difference was observed between the beechX-S, wheatX-S and ref.-S sheared pulps in fibre development;
4. With the normal xylan treated pulps, the ref.-N showed slightly less fibre development and porosity than beechX-N and wheatX-N with *ca* 30 % vs *ca* 22-23 % non-developed fibres;
5. All pulp types showed fibres with characteristic dislocations/kinks that showed different staining consistent with a more open structure. As this was consistent for all pulp types it is most likely related to the inconsistencies in fibre structure. *The fact that xylan treatments did not change the staining characteristics is further consistent that the xylan probably did not affect the porosity of these structures (or the fibres) significantly or help to “heal” (i.e. close) the internal open macrofibrillar structure known at these sites.* This is further consistent with an overall coating of the fibres with xylan as suggested from SEM observations (*Appendix 4*);
6. Results indicate that the beech- and wheat xylan treatments with- and without shearing have had little effect on changing the porosity of the fibres as judged by SS.

References

- Akhtar, M., Blanchette, R.A., Burnes, T.A. (1995). Using Simons stain to predict energy savings during biomechanical pulping. *Wood Fiber Sci.* 27(3), 258-264
- Blanchette, R.A., Akhtar, M., Attridge, M.C. (1992) Using Simons stain to evaluate fiber characteristics of biomechanical pulps. *Tappi J.* 75:121-124.
- Esteghlalian, A.R., Bilodeau, M., Mansfield, S.D. and Saddler, J.N. (2001) Do enzymatic digestibility and Simons stain reflect the differences in the available surface area of lignocellulosic substrates. *Biotechnology Progress* 17:1049-1054.
- Fernando, D., Daniel, G. (2010) Characterization of spruce thermomechanical pulps (TMP) at the fibre cell wall level: a method for quantitative assessment of pulp fibre development using Simons stain. *Tappi J.* 9:47-55.
- Fernando, D., Muhić, D., Engstrand, P., Daniel, G. (2011) Fundamental understanding of pulp property development during different TMP refining conditions using the

application of new Simons stain method and fibre surface ultrastructure.
Holzforschung 65:777-786.

Simons, F.L. (1950). A stain for use in the microscopy of beaten fibers. *Tappi J.* 33:312 – 314.

Joutsimo, O. and Robertsen, L. (2005). The effect of mechanical treatment on softwood kraft pulp fibers. Fiber surface layer. *Pap. Puu* 87 (1): 41.

Yu, X. and Atalla, R.H. (1998). A staining technique for evaluating the pore structure variations of microcrystalline cellulose powders. *Powder Technol.* 98 (2): 135.

Yu, X., Minor, J.L., Atalla, R.H. (1995). Mechanism of action of Simons staining *Tappi J.* 78(6): 175.

APPENDIX 7. XYLAN ABSORPTION EVALUATED BY POLARISED FTIR MICROSCOPY

Anne-Mari Olsson, Lennart Salmén, Innventia

Aim: The purpose of this work was to investigate if the amount of xylan deposited during kraft processing could be differentiated from xylan originally present in the fibre wall by examining the orientation distribution vis-à-vis the cellulose microfibrillar orientation. The hypothesis was that the xylan deposited during cooking would be more randomly orientated.

Background

It is well known that xylan present in the native fibre wall has an orientation similar to that of the cellulose microfibrils (Stevanic, Salmén 2009, Simonović et al. 2011). Xylan added in the kraft cooking is deposited on the surfaces of the fibres. However, so far little is known with regard to the distribution of this added xylan as well as to the form of the added xylan depositions. Imaging FTIR-microscopy offers here a possibility for investigating the organization of this xylan by comparing possible changes in the orientation distribution of the xylan absorption peaks for fibres with no xylan deposition and those for which xylan has been deposited. Previous studies using imaging FTIR-microscopy xylan have shown that the orientation distributions of xylan may be followed using specific absorption bands (Stevanic, Salmén 2009, Simonović et al. 2011).

Experimental

MATERIALS

Single fibres from kraft pulps performed with beech xylan added in the impregnation stage have been compared with fibres from a similar reference kraft cooks, both type of cooks performed with or without shearing during cooking at 165°C. The beech- xylan concentration was high, about 30 g/L. Originally also studies of fibres from cooks performed with the addition of wheat-xylan were planned but due to the low amount of absorbed wheat-xylan onto the fibres no such measurements were performed. The fibres chosen from the different pulps were mounted on a sample stage, as parallel as possible to the orientation of the 0° polarization direction of the imaging FTIR-microscope.

METHOD

The xylan distribution were analyzed by FTIR microscopy measurements using a Spectrum Spotlight 400 FTIR Imaging System (Perkin Elmer Inc, Shelton, CT,

USA), in transmission using a MCT detector with a spectral resolution of 4 cm^{-1} . The incident IR radiation was polarized by a gold wire grid polarizer, with -90° to $+90^\circ$ polarization in relation to the fibre orientation, at intervals of 15° . A well oriented part of each fibre was chosen and an area of 10 times $100\mu\text{m}$ in the middle of the fibre encompassing the double cell wall was selected for the measurement, see *Figure 1*. Three fibres from each pulp were measured.

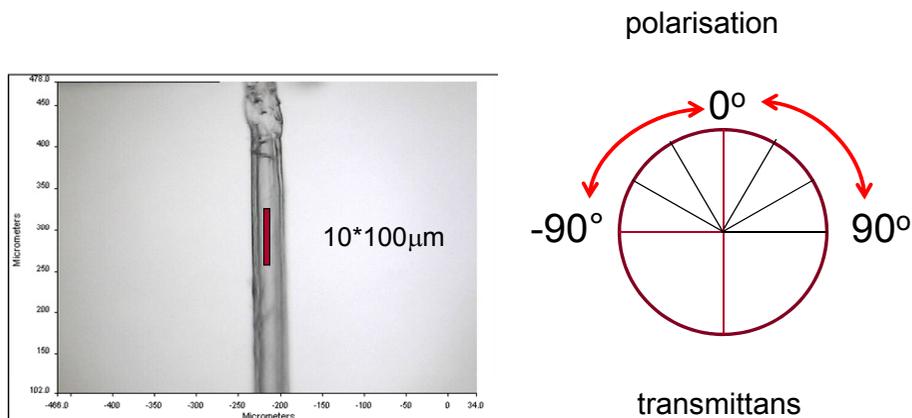


Figure 1. Light microscopy image of pulp fibre with the area of measurement indicated as well as the degrees of polarization measured in relation to the fibre axis.

An average spectrum of the added beech-xylan was also taken in ATR –mode (Attenuated Total Reflection) of FTIR.

SPECTRAL EVALUATION

The spectra were recorded between the wavenumbers 4000 cm^{-1} to 700 cm^{-1} , and baseline corrected to 0 intensity at 2000 , 1536 , 1192 , and 780 cm^{-1} .

Orientation dependency curves were constructed as the relative absorbance for the peaks of interest according to the following:

$$RA = \left(\frac{I_P - I_{\min}}{I_{\max} - I_{\min}} \right)$$

where RA is the relative absorbance, I_P is the intensity of the absorbed I_R radiation at a given angle of the polarization (P), I_{\max} is the maximal intensity observed for a given vibration and I_{\min} is the minimal intensity observed the same vibration. These

relative absorbance values were presented in relation to the angle of the incident IR polarization from 0 to 90° (Stevanic and Salmén 2009).

Results

The beech-xylan used in these studies had a M_w of 11400 with a polydispersity of 1.2. The content of glucuronic acid was 7.2 weight %. The FTIR spectra clearly reflected the content of charged groups at 1734 cm^{-1} as well as a clear signal of the CH_2 wagging of the xylose units at 1450 cm^{-1} .

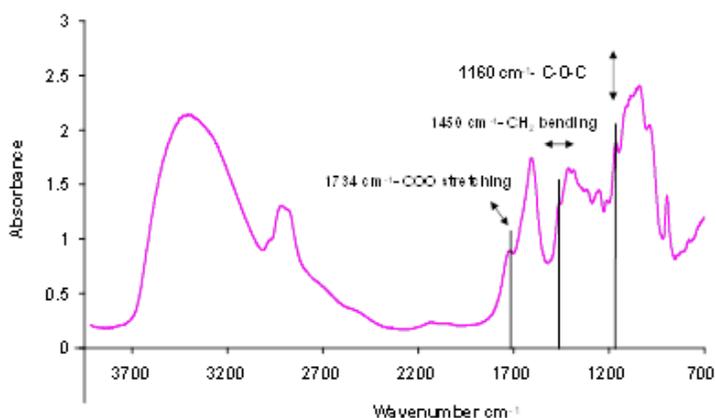


Figure 2. FTIR spectra of beech xylan indicating specific vibrations with its polarization angle to the polymer chain indicated by arrows.

FTIR-spectra from fibre wall areas were taken from selected straight sections of the fibres. As an example of the raw data spectra are illustrated in *Figure 3* showing clear differences in the 1734 cm^{-1} region. For the fingerprint region between 1500 and 700 cm^{-1} also clear difference with respect to the angular dependence is seen in *Figure 4*.

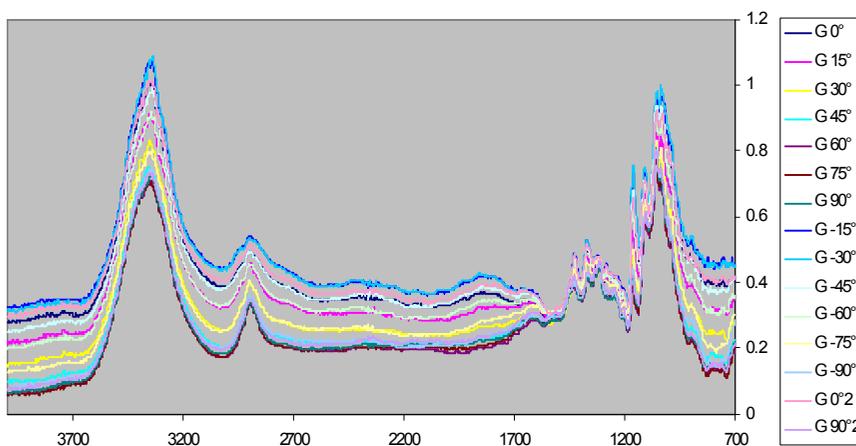


Figure 3. FTIR spectra in transmission from a beech-xylan treated fibre at different polarization angles. Unprocessed FTIR data.

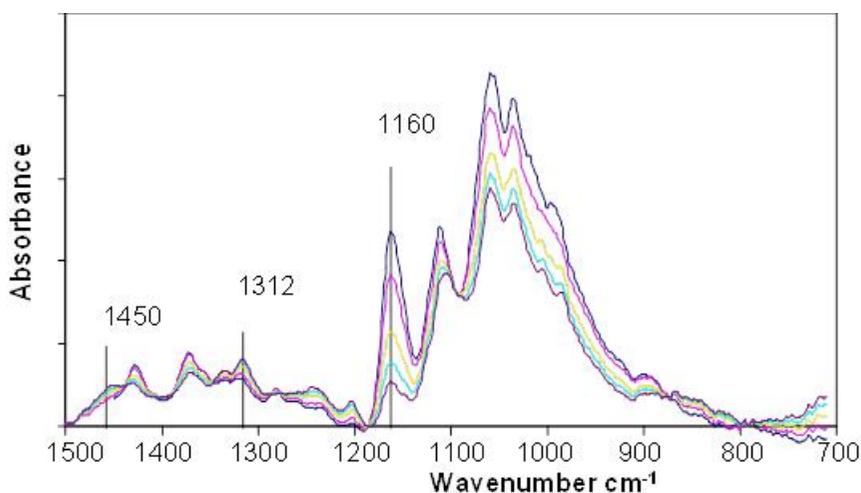


Figure 4. FTIR spectra in transmission from the finger-print region of the spectra from a beech-xylan treated fibre. The spectra have been base-line corrected. The specific absorbance peaks of the C-O-C bond at 1160 cm^{-1} (mainly related to the cellulose), the CH_2 -wagging of cellulose at 1312 cm^{-1} and the CH_2 -bending of xylan at 1450 cm^{-1} are indicated.

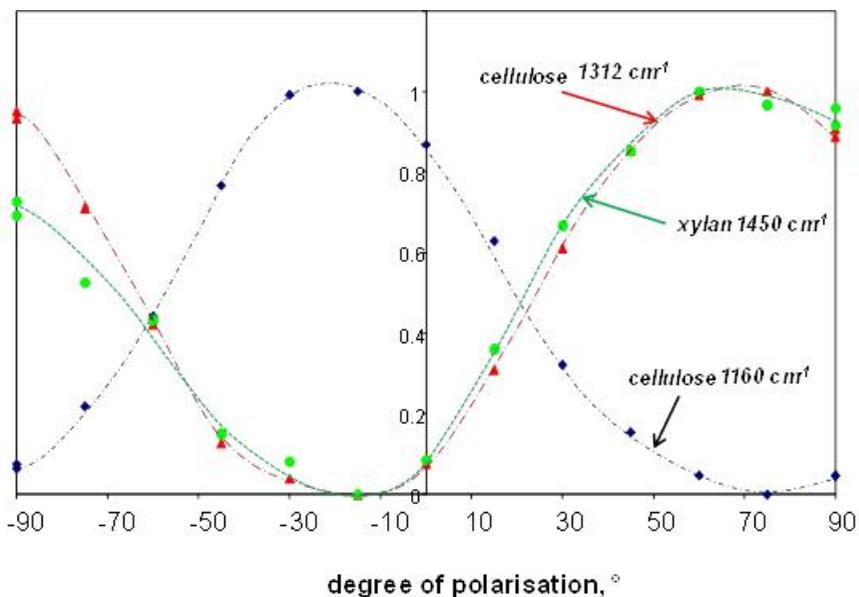


Figure 5. Orientation distribution of specific absorbance peaks for cellulose and xylan from a reference kraft fibre. The peak values for different degrees of polarizations have been normalized to the interval of 1 to 0.

By analyzing the peak height variation as a function of the degree of polarization the orientation of specific groups of the polymers may be obtained. This fact is exemplified in Figure 5 where the orientation of the main backbone C-O-C bond at 1160 cm^{-1} , primarily from cellulose, is shown together with the orientation of the orthogonal cellulose CH_2 wagging at 1312 cm^{-1} as well as the orthogonal CH_2 bending of xylan at 1450 cm^{-1} . In this set-up only the orientation from one of the fibre walls are displayed. It is here clear that the orientation of the native xylan in the fibre wall is very similar to that of the cellulose microfibrils, i.e. xylan is oriented in parallel with the cellulose microfibrils.

When more closely looking at the absorbance variation with regard to xylan content we have focused on the spectra in the wavenumber-region between 1550 and 1200 cm^{-1} , see Figure 6. Both the orthogonal CH_2 bending of xylan at 1450 cm^{-1} and the C-O stretch at 1244 cm^{-1} were used and compared to the C-H bending vibration of carbohydrates at 1370 cm^{-1} . Both the xylan vibrations have an orientation 90° to the backbone while the C-H bending has a vibration at 0° to the polymer backbone.

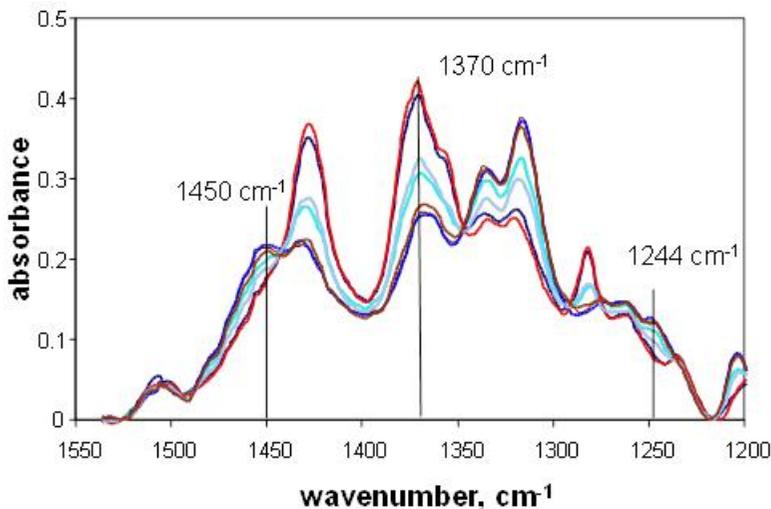


Figure 6. FTIR spectra in transmission from the wavenumber region 1550 to 1200 cm^{-1} of spectra from a beech-xylan treated fibre. The spectra have been base-line corrected. The specific absorbance peaks of the CH_2 -bending of xylan at 1450 cm^{-1} , the C-O stretch at 1244 cm^{-1} and the C-H bending vibration of carbohydrates at 1370 cm^{-1} are indicated.

In Figure 7 and 8 the absorbance of the two xylan peaks compared to the C-H bending vibration of carbohydrates are examined as a function of polarization degree. By comparing the absorbance to the more general C-H vibration of carbohydrates effect of the thickness of the sample is corrected for and thus the absorbance level can be compared between the samples. Although the number of fibres tested is small there is an indication for the 1450 absorbance peak that the fibres with beech xylan added shows higher absorbance indicating a higher content of xylan present in the fibres. For the 1244 peak the scatter between the fibres is very large and really no conclusion can be drawn. In general the absorbance level is much lower than for the 1450 peak meaning that the general noise in the spectra may be a disturbing factor.

In Figures 9 and 10 the absorbance of the two xylan peaks has instead been normalized with the average absorbance itself. In this way the degree of orientation can be evaluated between the samples without the disturbance from different amounts of xylan present in the sample. For the C-O vibration, 1244 cm^{-1} , in Figure 10 it seems clear that a higher degree of orientation is indicated for the samples with beech xylan sorbed in the cook. For the CH_2 vibration at 1450 cm^{-1} the situation is not so clear, Figure 9. The sample with beech xylan sorbed and not sheared showed the lowest degree of orientation. Unfortunately this result is only based on one fibre due to bad spectra for some polarizations in the other samples

studied. For fibres that have been sheared the behavior is very similar for the one with and without xylan added. This could indicate that the xylan sorbed onto the fibres is likely to be sorbed with an orientation that is similar to that of the cellulose microfibrils of the secondary wall of the fibre structure.

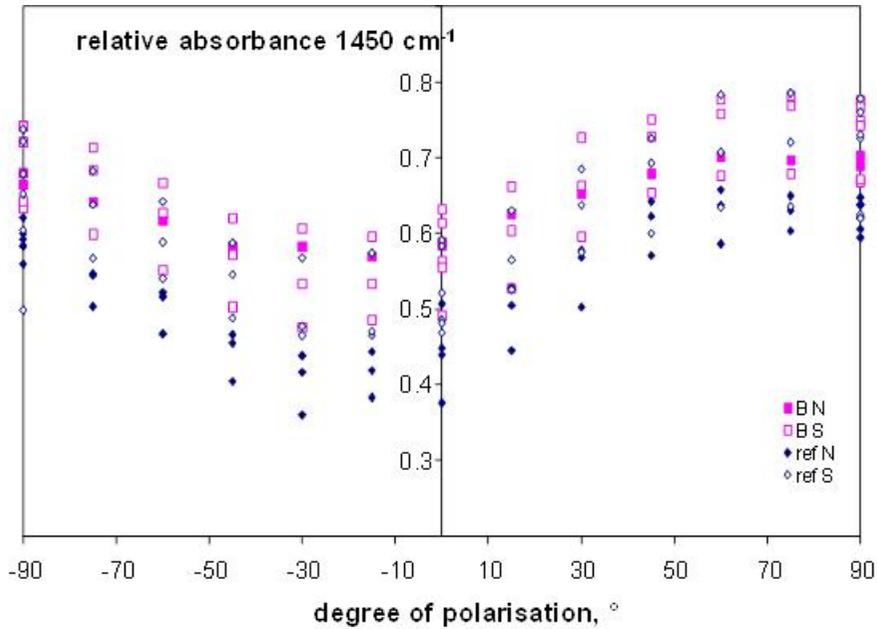


Figure 7. Orientation distribution of the xylan CH₂-bending absorbance peak at 1450 cm⁻¹ for different fibres. The peak values for different degrees of polarizations have been normalized with the average absorbance of the C-H bending vibration of carbohydrates at 1370 cm⁻¹, to compensate for different fibre thickness

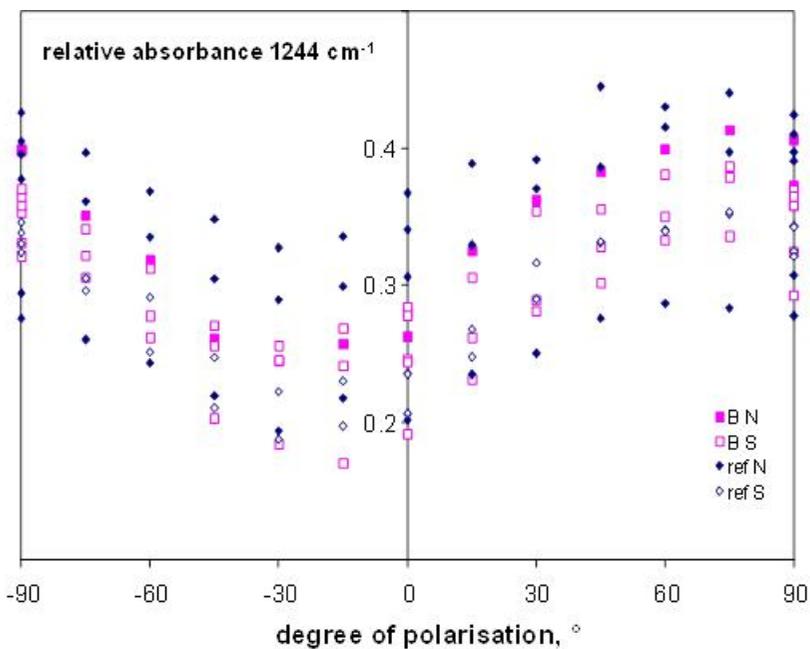


Figure 8. Orientation distribution of the xylan C-O stretch absorbance peak at 1244 cm⁻¹ for different fibres. The peak values for different degrees of polarizations have been normalized with the average absorbance of the C-H bending vibration of carbohydrates at 1370 cm⁻¹, to compensate for different fibre thickness.

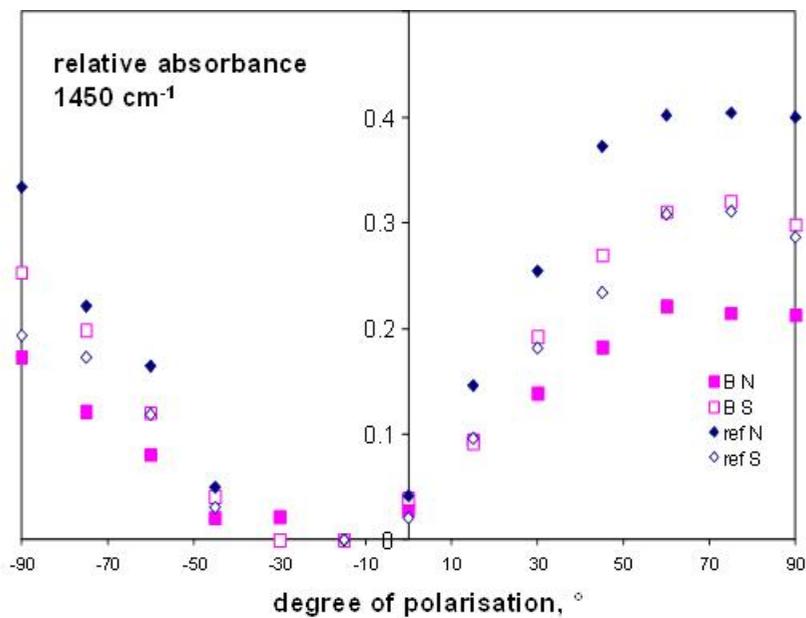


Figure 9. Orientation distribution of the xylan CH₂-bending absorbance peak at 1450 cm⁻¹ for different fibres normalized with regard to the average xylan peak height of the same CH₂ peak, indicating the orientation regardless of xylan content.

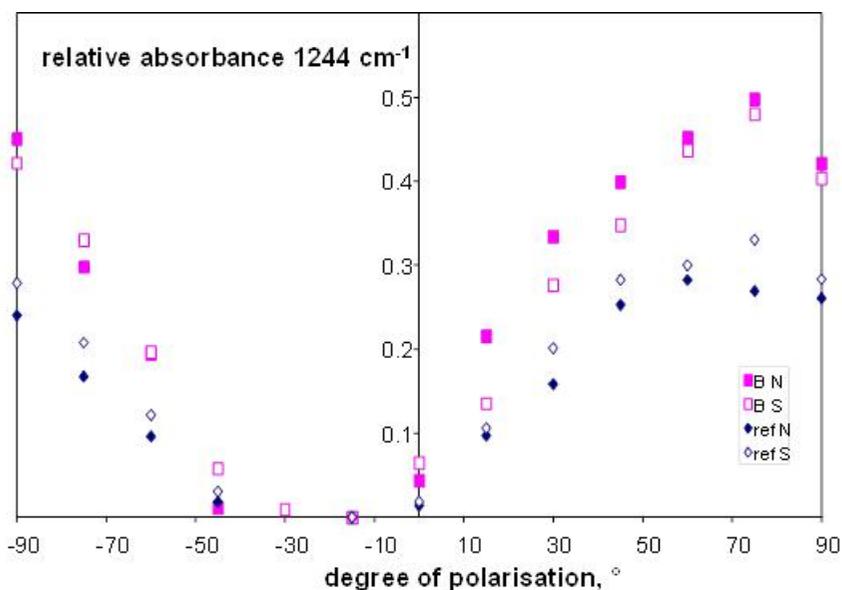


Figure 10. Orientation distribution of the xylan C-O stretch absorbance peak at 1244 cm^{-1} for different fibres normalized with regard to the average xylan peak height of the same C-O peak, indicating the orientation regardless of xylan content.

Conclusions

In the measurements performed here, no significant difference can be distinguished between orientation of xylan on fibres with sorbed xylan and fibres where no xylan has been added. This indicates that the majority of the xylan sorbed onto the fibres will most probably be sorbed onto cellulose microfibrils with an orientation similar to the orientation of the microfibrils in the secondary cell wall.

References

- Stevanic S., J. and Salmén, L. 2009. Orientation of the wood polymers in spruce wood fibres. *Holzforschung* 63, (5) 497-503.
- Simonović J., Stevanic J., Djikanović D., Salmén L., Radotić K. 2011. Anisotropy of cell wall polymers in branches of hardwood and softwood: a polarized FTIR study. *Cellulose* 18, 1433–1440.

APPENDIX 8. INFLUENCE OF WHEAT AND BEECH XYLANS ON SHEARING AND HCL SENSITIVITY OF SPRUCE KRAFT PULPS

Paul Ander, SLU, Uppsala

Introduction

Wheat and Beech xylans were added to spruce kraft pulps with or without shearing during cooking at 165 °C. The idea was to evaluate whether high concentrations (30 g/l) of these xylans could decrease the mechanical effects of fibre shearing. Evaluation was conducted measuring length weighted fibre lengths and fibre cleavage before and after HCl treatment (Ander et al. 2008; Heinemann and Ander 2011).

Results

The results are shown in Table 1 and in *Figures 1* and *2*.

Table 1. HCl-sensitivity of spruce kraft pulps with or without addition of wheat or beech xylan (both 30 g/l), or mechanical treatment (shearing). Cooking at 165°C 120 min to the same lignin kappa 30. Shearing at 165 °C

Kraft pulps (Figs 1 & 2)	Kraft Pulps	LWFL (mm) L0 H2O	LWFL (mm) L HCL	Cleavage per fibre
Ref N	Ref Normal	2.113/2.204 2.159	1.098/1.115 1.107	0.951
Wh N	Wheat N	2.232/2.294 2.263	1.233/1.169 1.201	0.884 (7%)
B N	Beech N	2.328/2.232 2.280	1.480/1.311 1.400	0.633 (33%)
Ref S	Ref Shear	2.355/2.182 2.269	0.706/0.765 0.736	2.084
Wh S	Wheat Sh	2.341/2.279 2.310	0.84/0.88/0.855/0.861 0.859	1.689 (19%)
B S	Beech Sh	2.309/2.301 2.305	1.030/1.048 1.039	1.218 (42%)

Fibre lengths before HCl were similar with no effect of shearing but with a trend of fibres with added xylans being slightly longer. After HCl the fibres were much shorter, with sheared fibres shortest (as before for many pulps). With both xylan types longer fibres were obtained with both water and HCl. The degrading effect of HCl against sheared pulps as compared with normal pulps could however not be alleviated by added xylans.

HCl cleavage: For normal fibres, addition of Beech xylan gave less cleavage **0.633**, while Reference or Wheat xylan gave 0.9-0.95 in cleavage per fibre. After Shearing, cleavage for Reference fibres increased from 0.95 to 2.084. Adding 30

g/l of Wheat or Beech xylan resulted in only **1.69 and 1.22 cleavages per fibre**. These trends are clearly shown in Figure 2. The smaller cleavage with added Beech xylan is probably related to the higher concentration of xylose on and/or in Beech treated fibres as compared with Wheat xylan treated fibres (Table 3, page 9). Better encapsulation of cellulose macrofibrillar structure by Beech xylan may also contribute.

Conclusions

Addition of 30 g/l of Wheat or Beech xylan to spruce kraft pulp reduced the degrading effect of HCl looking separately on normal fibres and sheared fibres. Strongest effect was with Beech xylan and cleavage per fibre decreased from 2.084 for Reference shear to 1.22 for Beech xylan shear. This is a **42%** reduction in acid sensitivity of sheared pulps. Wheat addition gave a 19% reduction in cleavage per fibre for sheared pulps.

Also without shearing, that is for normal pulps, a **33%** decrease in acid sensitivity was obtained by Beech xylan added at high concentration. Improvement with added Wheat xylan was smaller only 7%.

Comparing sheared and non-sheared pulps, the degrading effect of shearing was however still present regardless addition of Wheat or Birch xylyans.

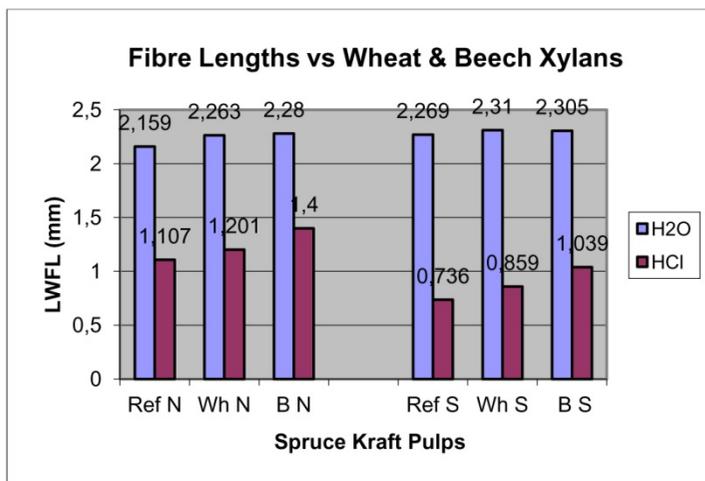


Figure 1. Fibre lengths.

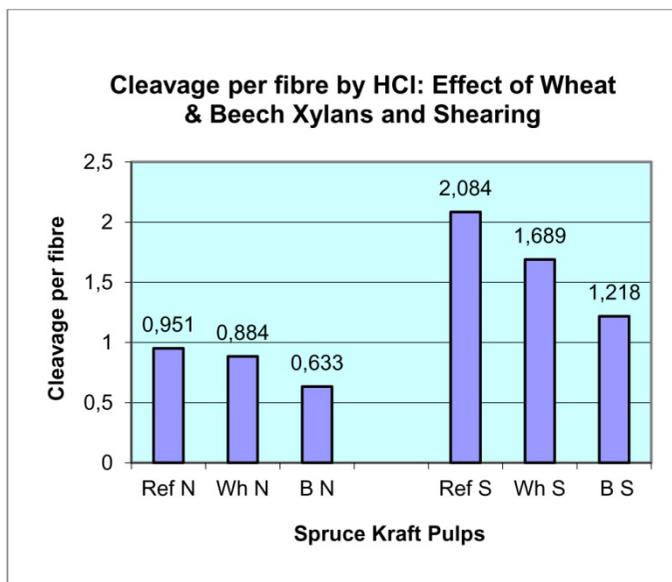


Figure 2. Cleavage per fibre. Wheat (Wh) and Beech (B) xylans added to give 30 g/l. Quota Ref S/Ref N; WH S/WH N & B S/B N = 2.19; 1.91 & 1.92 respectively. These quota should be near 1.

References

- Ander, P., Hildén, L. and Daniel, G. (2008). Cleavage of softwood kraft pulp fibres by HCl and Cellulases. *Bioresources* 3(2), 477-490.
- Heinemann, S. and Ander, P. (2011). Standard pulp and paper tests. In *Fine Structure of Papermaking Fibres, COST Action E54 Characterisation of the fine structure and properties of papermaking fibres using new technologies*. Eds: Ander P., Bauer W., Heinemann S., Kallio P., Passas R. and Treimanis A. Swedish University of Agricultural Sciences. ISBN 978-91-576-9007-4, p. 211-232.

Collaborative Research on the Ultrastructure of Wood Fibres (CRUW)

CRUW represents a collaborative research program between the Swedish Forest Industries Akzo Nobel, Holmen, Smurfit Kappa Packaging, SCA, Stora Enso, Södra, SLU, Innventia, KTH and Mid Sweden University. The program is directed towards energy efficient processes for mechanical pulping and retention of the full fibre potential in chemical pulping. It is believed that research ideas based on insight into fibre ultrastructure can provide openings for breakthroughs in the applied area. The program forms part of the VINNOVA and Industry *"Branschforskningsprogram för skogs- och träindustrin"*.