Chemical extraction from beech wood, douglas fir and pine using acid, base extractions to obtain the purest cellulose to improve radiocarbon dating

Bachelor research project at the University of Groningen

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1. Abstract

Three kinds of woods, beech wood, douglas fir and pine were selected to investigate which protocol yields the purest cellulose to increase the effectiveness of radiocarbon dating. The basic protocol consists of an acid, base, acid extraction and a chlorite oxidation step to remove the lignin and hemicellulose from the wood. To eventually obtain pure cellulose, but so far insufficient cellulose purity was obtained for this protocol. Therefore, other protocols had been conceived starting from the basic protocol by adding two different organic washes to remove the resin of wood or by adding an alkali extraction to remove more hemicellulose and lignin by breaking more linkages. After every protocol, complete acid hydrolysis was performed to hydrolyse the carbohydrates into monosaccharides, which were analysed by high performance anion-exchange chromatography with pulsed amperometric detection. The purest cellulose for beech wood was yielded after applying the basic protocol with the additional alkali extraction. For beech wood, this was an amount of 96.1% (w/w) of cellulose and a carbohydrate recovery of 96.1% (w/w). More linkages of hemicellulose and lignin were broken, thus more hemicellulose and lignin were removed and purer cellulose was obtained. For douglas fir and pine, the purest cellulose was yielded after the basic protocol with an additional organic wash A (methanol, acetone and chloroform). The resins were removed due to this organic wash and an amount of 87.0% (w/w) of cellulose for douglas fir was obtained and for pine 69.9% (w/w) of cellulose. Their carbohydrate recovery was 106.6% (w/w) for douglas fir and 89.6% (w/w) for pine. In general, a result of a high weight percentage of the extracted cellulose and a high overall carbohydrate recovery yields the purest cellulose. Then, the α-cellulose extraction protocol is enhanced and radiocarbon dating is improved.

2. Table of contents

3. Introduction

3.1 General introduction

Radiocarbon dating is used to estimate the age of organic material. The unstable isotope carbon-14 (^{14}C) is the basis of this method. In the atmosphere, ^{14}C was formed due to the emission of cosmic radiation by the sun reacting to the nitrogen (N) in the atmosphere, which creates nuclear reactions to produce ^{14}C [1]. Organic material absorbs ^{14}C, because ^{14}C tends to form carbon dioxide (CO_2) with oxygen in the atmosphere, which is absorbed by organic material [1]. Since then, the amount of ^{14}C was the same as in the atmosphere. If an organic material has died the absorption of ^{14}C stops and ^{14}C was decayed back into nitrogen. Due to comparing the amount of ^{14}C in the atmosphere and in the dead organic material, the age of the organic material was estimated [1].

In this bachelor research project, wood is used to estimate its age. Wood consists of three main components, namely lignin, hemicellulose and cellulose. These three components contain ^{14}C, but only cellulose is used as the carbon source for this method. A tree grows every year and every year an annual ring grows. When this happens lignin, hemicellulose, extractives (resins) tend to migrate throughout the structure of annual rings. Constituents and the ^{14}C of these components will then be found in other annual rings than where they came from, except for cellulose. Therefore, the purest cellulose is used as a carbon source and needs to be extracted from wood to optimize this method [2]. In previous research, an α-cellulose extraction protocol was already applied to other wood samples to obtain the purest cellulose, but so far insufficient cellulose purity was obtained [3]. Therefore, in this project, the aim is to optimize the α-cellulose extraction protocol for three kinds of woods by adding an organic wash or an alkali extraction to obtain the purest cellulose to enhance radiocarbon dating.

3.2 Wood composition of hardwood and softwood

The polysaccharides cellulose, hemicellulose and lignin are the three main components of wood. Wood can be divided into two different types of wood, namely softwood and hardwood. They are different in their wood composition. For example, the amount of cellulose in hardwood is higher than in softwood and the amount of hemicellulose, lignin and extractives in softwood is higher than in hardwood (Table 1).

Table 1: The composition of cellulose, hemicellulose, lignin and extractives of softwood and hardwood compared to each other [4, 5, 6].

	Softwood	**Hardwood**
Cellulose	33-42%	38-51%
Hemicellulose	22-40%	17-38%
Lignin	27-32%	21-31%
Extractives	2-3.5%	2.5%

In this project, three different kinds of woods are used namely beech wood, douglas fir and pine. Beech wood is a hardwood and douglas fir and pine are softwoods.

The main component is cellulose and is a polysaccharide consisting of glucose (Glc) monosaccharides linked through β-1,4-glycosidic bonds in the linear backbone without branches (Table 1). Cellulose has a crystalline structure, because of the hydrogen bonding between the monosaccharides [4]. The degree of polymerization also influences strength. The longer the Glc chains, the more hydrogen bonding, the more difficult it is to hydrolyse cellulose [7]. The hydroxyl groups of the Glc molecules are cross-linked to each other to form microfibrils. This leads to a strong and compact structure [8]. This is the carbon source used to perform radiocarbon dating because it will not contain constituents and ^{14}C of other components.

Hemicellulose in contrast has a non-crystalline structure, because of the shorter chains and the presence of branches (Table 1). Hence, hemicellulose is more susceptible to hydrolysis by diluted acids and bases [9]. Depending on which type of wood, the monosaccharides content of hemicellulose and their way of branding varies. In general, hemicellulose can consist of several monosaccharides, namely out of xylose (Xyl), arabinose (Ara), glucuronic acid (GlcA), mannose (Man) and galactose (Gal) and rhamnose (Rhm). In hardwood, the type of hemicellulose is called glucuronoxylan which consist mainly of a Xyl backbone with 4-*O*-methylglucuronic acid branches linked through α-1,2-glycosidic bonds (Table 2), [10]. In softwood, the type of hemicellulose is called galactoglucomannan which contains Man, Glc and Gal (Table 2). The backbone consists of Glc and Man monosaccharides which are randomly linked via β-1,4-glycosidic bonds. The Gal monosaccharides are linked via α-1,6-glycosidic bonds attached to Man monosaccharides [10].

Table 2: The differences in the types of hemicellulose for hardwood and softwood [11].

	Hardwood	**Softwood**	
Wood	Beech wood	Douglas fir	Pine
Hemicellulose	Glucuronoxylan	Galactoglucomannan	
Monosaccharides	Xylose	Mannose	
	4-*O*-methylglucuronic acid	Glucose	
		Galactose	

The last component is lignin, located in the cell wall and binds hemicellulose and cellulose to each other (Table 1). It can be compared to natural glue between cellulose and hemicellulose to hold the complex matrix together and to increase the strength of the wood structure. Lignin consists of three monolignols namely coniferyl alcohol, *p*-coumaryl alcohol, and sinapyl alcohol cross-linked by ester, ether and carbon-carbon linkages [11]. Their composition depends on the type of lignin. In softwood, the lignin composition contains coniferyl alcohols and in hardwood, the composition consists of coniferyl and sinapyl alcohols [10]. Compared to hemicellulose and cellulose, lignin is hydrophobic and therefore soluble in some organic solvents and in alkali solutions [7]. Besides this, it also has a non-crystalline structure due to its complex and branched construction [12].

Extractives are a minor part of the composition of wood (Table 1). They are found in the heartwood [13]. Extractives consist of fats, fatty acids, fatty alcohols, phenols, terpenes, steroids, rosin, waxes, many other minor organic compounds and resin acids. Resins need to be removed in this project by applying an organic wash to improve the α-cellulose extraction protocol. They can be extracted by different solvents depending on their solubility [10]. In

general, softwoods have a higher extractive content than hardwoods (Table 1). Therefore, softwood contains more resin than hardwood.

3.3 α-cellulose extraction protocol

In this project, the aim is to develop a protocol to obtain the purest cellulose by applying different protocols to three different kinds of wood. The commonly used protocol is the α-cellulose extraction protocol. It is used in the Centre for Isotope Research (CIO) in Groningen to perform radiocarbon dating, but so far insufficient cellulose purity was obtained [3]. In this project, it is called the basic protocol. The steps are as follows:

3.3.1 Milling of wood

The physical pre-treatment is necessary to do the extraction. During this pre-treatment the wood was cut into smaller pieces and then milled to enhances the effectiveness of the cellulose extraction [3].

3.3.2 Additional organic washes

Organic washes existing of organic solvents are an optional step and are normally only applied to resinous wood, which is softwood, containing more resin than hardwood (Table 1). In this project, it is also applied to hardwood to see if it has some effect on it as well. Organic solvents are applied to wood samples to remove the resin and additives like glues and preservatives from the wood [3]. In this project, two different kinds of washes were applied before the basis protocol, namely wash A and wash B. In wash A, three different kinds of organic solvents (acetone, methanol and chloroform) were used and applied in three steps to the wood [13]. This will remove more resin and additives like glues and preservatives instead of applying one wash with one organic solvent. In wash B, a solution of 90% acetone and 10% water was used. An aqueous mixture with acetone is one of the most suitable organic solvents for washes. The combination of water and organic solvents increase the removal of resin and additives like glues and preservatives because some compounds are better soluble in water and others are more soluble in acetone [15].

3.3.3 Acid, Base, Acid (ABA) extraction

The acid, base, acid (ABA) extraction is applied to the wood to extract the hemicellulose and lignin of the wood. The acid used for this extraction is hydrochloric acid (HCl) and the used base is sodium hydroxide (NaOH), [3]. The last acid extraction is to prevent NaOH from absorbing atmospheric CO_2 [2].

3.3.4 Additional alkali extraction

To yield purer cellulose, an additional alkali extraction of a stronger base could help to remove more hemicellulose and lignin. The first added base removes already a part of the lignin and hemicellulose, but if a stronger base like potassium hydroxide (KOH) is added, more ester and glycosidic linkages between hemicellulose, lignin and cellulose will be broken [16]. Therefore, more hemicellulose and lignin can be removed and more cellulose will be yielded.

3.3.5 Chlorite oxidation step

After the ABA extraction, the chlorite oxidation step or the bleach step is applied. This step removes the lignin of the wood. Lignin is responsible for the colour of the wood. $NaClO_2$ with a few drops of HCl is used to perform this step [3].

3.3.6 Complete acid hydrolysis

During complete acid hydrolysis, carbohydrates are hydrolysed into monosaccharides by breaking their chemical bonds using diluted sulfuric acid (H_2SO_4) and water. Then, these monosaccharides are analysed via high performance anion-exchange chromatography with pulsed amperometric detection HPAEC-PAD.

3.3.6 High Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD)

After complete acid hydrolysis, high performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) is used to analyse and quantify the amount of each monosaccharide for every wood sample. Therefore, it is important to know what the composition of hardwood and softwood is and how they react [17].

3.4 Research objectives

The purpose of this research is to find the most effective protocol based on the basic protocol to obtain the purest cellulose from beech wood, douglas fir and pine to improve radiocarbon dating. This was done by adding an organic wash or alkali extraction to the basic protocol consisting of the ABA extraction and the chlorite oxidation step. The additional alkali extraction is expected to improve the α-cellulose extraction protocol the most for beech wood, douglas fir and pine because it breaks more linkages between lignin, hemicellulose and cellulose. Thus more lignin and hemicellulose should be reduced and more cellulose will be yielded. Complete acid hydrolysis and HPAEC-PAD were chosen to separate and analyse the compositional differences between the different woods and their different protocols. This means that attention was given to cellulose and hemicellulose because they contain monosaccharides separated by complete acid hydrolysis which are detectable via HPAEC-PAD. Lignin is not detectable, because it does not contain monosaccharides, but monolignols.

4. Materials and Methods

4.1 Materials

Wood:
Milled beech wood, douglas fir and pine from the stock of the University of Groningen bioproduct engineering group was used and provided by Ph.D student Lisanne Hameleers.

Chemicals:
The chemicals used were purchased from Sigma Aldrich. Dilutions of HCl, NaOH, KOH and H_2SO_4 needed for these protocols were prepared beforehand.

4.2 Methods

4.2.1 Classic α-cellulose extraction protocol

30 mg milled wood was weighed and put in glass vials and the HCl (5.47% w/vol (1.5 M)) was added for 20 min on the dry block heater at 80°C, (n=2). HCl was carefully decanted off and the samples were rinsed three times with demiwater. Then the NaOH (17.5%, w/vol) was added and the samples were ultrasonicated under a nitrogen gas (N_2) atmosphere for 60 min at room temperature. NaOH was carefully decanted off and the samples were rinsed five times with demiwater. HCl (5.47% w/vol (1.5 M)) was added again for 20 min on the dry block heater at 80 °C. After 20 min, HCl was carefully decanted off and the samples were rinsed three times with demiwater. Lastly, the $NaClO_2$ (1.5% w/vol in HCl (0.06M)) was added for 16 hours on the dry block heater at 80°C. After, 16 hours the $NaClO_2$ was carefully decanted off and freshly prepared $NaClO_2$ (1.5% w/vol in HCl (0.06M)) was added for four hours on the dry block heater at 80°C. $NaClO_2$ was decanted off and the samples were rinsed three times with demiwater. During the last rinse, a small layer of demiwater was left in the glass vial. The samples were closed with parafilm and a small hole was perforated in it. Then the samples were put in the freezer for six hours. Lastly, the samples were taken out of the freezer and put in the freeze-drier for 16 hours.

4.2.2 Additional organic washes to improve α-cellulose extraction protocol

4.2.2.1 Wash A (acetone, methanol and chloroform)

Firstly, 500 mg milled wood was weighed and put in small glass vials, (n=2). Pure acetone was added, until half of the glass vial was filled, to the wood samples and left on the dry-block heater for 45 min at 45 °C. Acetone was decanted off and methanol was added to the wood samples and left on the dry-block heater for 45 min at 45 °C. Methanol was also decanted off. In the last step of the wash, chloroform was added to the wood samples and left for 45 min at room temperature and decanted off (or use a pipette to be more accurate) after 45 min and the wood was dried for four days in the fume hood. Once fully dry, cellulose was extracted following the basic protocol.

4.2.2.2 Wash B (90% acetone and 10% water)

Firstly, 500 mg milled wood was weighed and put in small glass vials, (n=2). A solution of 90% acetone and 10% demiwater was made. This solution was added to the samples until they

were half filled and they were left for six hours on the dry block heater at 45 °C. They were left to dry for four days in the fume hood. Afterwards, the basic protocol was followed to extract cellulose.

4.2.3 Additional alkali extraction to improve α-cellulose extraction protocol

After weighing 40 mg milled wood, the ABA extraction was performed and the additional alkali extraction was introduced, (n=2). The second stronger base, namely KOH (22.4% w/vol, (4 M)), was added to the wood samples. These samples were ultrasonicated under a nitrogen gas (N_2) atmosphere for 60 min at room temperature. After one hour, KOH was carefully decanted off and the samples were rinsed five times with demiwater. For the third time, HCl (5.47% w/vol (1.5 M)) was added for 20 min on the dry block heater at 80°C and afterwards carefully decanted off. The samples were rinsed three times with demiwater. Once this was done, the basic protocol was continued again starting from the chlorite oxidation step.

4.2.4 Complete acid hydrolysis for sugar composition

After the extraction, the complete acid hydrolysis was performed, (n=2 or 4). For the freeze-dried wood samples, 5 mg was weighted and for the untreated wood samples, 10 mg was weighed into glass vials. 450 µL of H_2SO_4 (72% w/w, 12M) was added while the glass vials were standing on ice. Then the samples were put in a water bath for one hour at 30 °C. After three hours, 4.55 ml milli-Q water (MQ) was added to the samples and there were put for 3 hours in a shaking water bath at 95°C. Afterwards, the samples were cooled down to room temperature. The samples were prepared for analysis. Firstly, take 1 mL of the acid hydrolysis of the samples (AH) and transfer it into a 1.5 mL Eppendorf Tube. The Eppendorf tubes were centrifuged for 2 min at a speed of 14680/rpm. The samples with particles in them were filtered by using a 13mm syringe filter. Then the samples were diluted by a dilution factor of 40, thus 975 µL MQ and 25 µL of the particle free supernatant of the samples were transferred into HPAEC-PAD vials.

4.2.5 HPAEC-PAD to quantify the monosaccharide composition of extracted cellulose fractions

The HPAEC analysis was performed on a Dionex Ultimate 6000 system, (n=2 or 4). (Thermo Scientific, Sunnyvale, CA, USA) equipped with a CarboPac PA-1 column (2 mm x 250 mm ID) in combination with a CarboPac PA-1 guard column (2 mm x 50 mm ID) and PAD detection. The system was controlled and analysed by the Chromeleon 7.3.1 software (Thermo Scientific, Sunnyvale, CA, USA). First, the sequence of the samples was determined and the eluents were checked. Before running these wood samples, two sets of standards STD1 (Ara, Glc, Xyl, GlcA) and STD2 (Rha, Gal, Man) in different concentrations (1, 5, 10, 20, 30 µL/mL*)*. With a constant flow rate of 0.25 mL min-1 the elution of monosaccharides was performed and a multi-step-gradient using the following eluents: A: 0.1M NaOH, B: 1M NaOAc in 0.1M NaOH, C: 0.2 M NaOH, and D: milli-Q water. In the first 20 min, most monosaccharides elute with 16% A and 84% D. In the second part of the gradient, a linear increase over 20 min to 40% B elutes uronic acids. The column was flushed for 10 min with 100% C to regenerate it. In the end, the column is equilibrated for 10 min with 16%A and 84%D. Peak areas were determined for the standards with known concentrations by integrating them using Chromeleon 7.3.1 software (Thermo Scientific, Sunnyvale, CA, USA). Next, the standard curves for each monosaccharide were plotted to obtain standard curve equations (equation 1). The intercept at the y-axis was set to zero to allow quantification of very low monosaccharides concentrations.

$$A = slope * concentration + intercept$$

Equation 1: Standard curve equation for STD1 and STD2 and these concentrations 1, 5, 10, 20, 30 µL/mL. The intercept was set to zero.

The weight percentage (% (w/w)) of each monosaccharide of the total weight was calculated by this formula:

$$\%\left(\frac{w}{w}\right) = \left(\frac{A - intercept}{slope}\right) * df * starting\ V * \left(\frac{weight\ correction f}{starting\ m}\right) * 100\%$$

Equation 2: This is the formula to calculate the weight percentage of the extracted monosaccharides from cellulose and hemicellulose for beech wood, douglas fir and pine per protocol. The intercept was set to zero. A is the peak area and df is the dilution factor used in during the complete acid hydrolysis. Starting V is the starting volume at the beginning of the complete acid hydrolysis the same holds for the starting m which is the starting volume at the beginning of the acid hydrolysis. Lastly, the weight correction factor is the factor that takes the removal of water during acid hydrolysis into account.

5. Results and discussion

5.1 The purity of extracted cellulose determined by the Glc weight percentage after complete acid hydrolysis

After performing complete acid hydrolysis and HPAEC-PAD, the purity of the extracted cellulose in every wood sample for every protocol was determined by calculating the weight percentage of cellulose (Glc) and hemicellulose (Xyl, Ara, GlcA, Man, Gal). The higher the weight percentage of cellulose in a sample, the purer the cellulose in this wood sample is. If the weight percentage of cellulose in a sample is very low and it contains a higher weight percentage of monosaccharides of hemicellulose, the less pure the cellulose in this sample is.

Untreated wood was analysed to obtain the initial monosaccharide composition. This helps to understand which monosaccharides need to be removed to yield the purest cellulose. In untreated beech wood, Glc, Xyl, Ara, GlcA and Gal were detected (Table 3). Glc, Xyl and GlcA were expected and Ara and Gal were not expected. (Table 2) In untreated douglas fir, Glc, Xyl, Ara, GlcA, Man and Gal were found (Table 3), but Glc, Ara, GlcA were not expected (Table 2). Pine contains Glc, Man and unexpected Xyl, GlcA and Gal (Table 3) (Table 2). These unexpected monosaccharides were not anticipated, because in general, the majority of hemicellulose in softwood consists of galactoglucomannan and hemicellulose in hardwood consist of glucuronoxylan. (Table 2) This is correct, but softwood can contain other types of hemicelluloses like 5-15% (w/w) of methylglucuronoxylan, 15-30% (w/w) of arabinomethylglucuronoxylan, 1-5% (w/w) of glucomannan and 1-15% (w/w) of arabinogalactans and hardwood can also contain other types of hemicelluloses like 0.1-1% (w/w) of arabinomethylglucuronoxylan, 1-5% (w/w) of glucomannan, 0.1-1% (w/w) of galactoglucomannan and 0.1-1% (w/w) of arabinogalactans. [18] Therefore, these unexpected monosaccharides are present in beech wood, douglas fir and pine in small amounts called traces (Table 3). Comparing the obtained amounts of cellulose and hemicellulose of the untreated woods after HPAEC-PAD with the literature, they are almost equal to each other (Table 2). They are not exactly the same, because these amounts are average data for hardwoods and softwoods. Thus no specific data for beech wood, douglas fir and pine which can differ from other softwoods and hardwoods.

Table 3: Weight percentages of cellulose and hemicellulose of beech wood, douglas fir and pine per protocol and their overall carbohydrate recovery percentage. Peak areas ≤ 10 nC*min = traces(TR), n.d. = not detected, n=2 or 4

Sample	**Cellulose [% (w/w)]**	**Hemicellulose [% (w/w)]**						**Carbohydrates [% (w/w)]**
	Glucose	Xylose	Arabinose	Glucuronic acid	Mannose	Galactose	Total amount hemicellulose	**Total carbohydrate recovery**
Beech wood								
Untreated	**47.7 ± 10.2**	11.8 ± 2.5	TR	TR	n.d.	TR	11.8	**59.5**
Basic (no wash)	**103.2 ± 2.9**	8.9 ± 0.6	TR	TR	n.d.	TR	8.9	**112.1**
Wash A	**87.5 ± 11.0**	TR	n.d.	TR	n.d.	TR	n.d.	**87.5**
Wash B	**45.0 ± 2.0**	TR	TR	n.d.	n.d.	TR	n.d.	**45.0**
Extra base step	**96.1 ± 8.3**	TR	n.d.	n.d.	n.d.	n.d.	n.d.	**96.1**

Douglas fir								
Untreated	**40.6 ± 6.8**	TR	TR	TR	19.9 ± 12.2	TR	19.9	**60.5**
Basic (no wash)	**58.2 ± 5.0**	n.d.	n.d.	n.d.	13.2 ± 0.9	TR	13.2	**71.4**
Wash A	**87.0 ± 2.3**	n.d.	TR	n.d.	19.6 ± 6.5	TR	19.6	**106.6**
Wash B	**27.8 ± 4.5**	TR	n.d.	n.d.	TR	n.d.	n.d.	**27.8**
Extra base step	**68.4 ± 7.2**	n.d.	n.d.	n.d.	9.7 ± 3.6	n.d.	9.7	**78.1**
Pine								
Untreated	**30.4 ± 8.7**	TR	n.d.	TR	17.2 ± 13.5	TR	17.2	**47.6**
Basic (no wash)	**68.0 ± 0.0**	n.d.	n.d.	TR	20.1 ± 0.0	TR	20.1	**88.1**
Wash A	**69.6 ± 1.4**	n.d.	n.d.	n.d.	20.0 ± 0.3	TR	20.0	**89.6**
Wash B	**27.2 ± 1.1**	TR	n.d.	n.d.	TR	n.d.	n.d.	**27.2**
Extra base step	**61.3 ± 8.7**	n.d.	n.d.	n.d.	11.7 ± 1.8	n.d.	11.7	**73.0**

Knowing the initial monosaccharide composition of every wood, it is possible to see what kind of effect every protocol has on the monosaccharides of the wood samples. In this case, the results of the basic protocol are compared to the results of untreated wood, because the other protocols are based on this basic protocol. Therefore, it is interesting to see what the basic protocol has already removed to obtain pure cellulose and what the other protocols did to enhance that basic protocol after applying it to untreated wood to obtain purer cellulose to optimize radiocarbon dating. After performing the basic protocol, beech wood contains 103.2% (w/w) of Glc and 8.9% (w/w) of Xyl (Table 3). The Glc amount was increased by 55.5% (w/w) and the Xyl amount was decreased by 2.9% (w/w). In general, samples that have a higher amount than 100% (w/w), which is not possible, may result from several causes. For example, the integration of the peak has not been done precisely, or the peak was higher than the linear range. Then the peak is flattened and the detection becomes oversaturated. There is no obvious reason for this and it is also not reported in the literature. In douglas fir, 58.2% (w/w) Glc and 13.2% (w/w) Man was obtained (Table 3). The Glc amount was increased by 17.6% (w/w) compared to the untreated douglas fir and there was a decrease of 6.7% (w/w) Man. Looking at the composition in pine, after the basic protocol, 68% (w/w) of Glc and 20.1% (w/w) Man was detected (Table 3) and there was an increase of 37.6% (w/w) of Glc, but the amount of Man had a small increase of 2.9% (w/w). Due to the basic protocol, lignin and a part of hemicellulose are removed for beech wood and douglas fir, because there is an increase of Glc and for beech wood a decrease of Xyl and for douglas fir a decrease of Man. As expected, after the basic protocol insufficient cellulose purity was obtained. This was the α-cellulose extraction protocol that was used by CIO to perform radiocarbon dating and what needed to be improved. [3] For pine, there was also an increase of Glc but, the amount of Man was increased as well with 2.9% (w/w). The amount of Glc with a standard deviation of the Man for the untreated pine reflects that the amount of Man can be 17.2 ± 13.5% (w/w) (Table 3). Therefore, 20.1% (w/w) can be an amount of Man for the untreated pine and thus stayed the same after applying the basic protocol which had an amount of 20.1% (w/w) Man (Table 3). In the literature, there is no other reason found to explain this.

The following protocols are compared to the basic protocol to see what kind of improvement they made to obtain the purest cellulose. To improve the cellulose extraction an additional wash A (methanol, acetone and chloroform) was applied before the extraction to remove the resin of

the wood and thus obtain purer cellulose. After applying the wash on beech wood, only an amount of 87.5% (w/w) Glc was obtained (Table 3). The amount of Glc was decreased with an amount of 15.7% (w/w), and Xyl was reduced by a very small amount because 8.9% (w/w) was reduced to traces. It is not possible to conclude something about the amount of Glc for the protocol with additional wash A relative to the basic protocol, because the amount of Glc in the basic protocol was extremely high due to an error. In general, hardwood does not contain hardly any resin, thus it was not expected that purer cellulose was obtained. The amount of Xyl was about the same for both protocols. Therefore, it could be assumed that the amount of Glc for the basic protocol is 87.5% (w/w) instead of 103.2% (w/w), but this is only an assumption. For douglas fir, the amount of Glc is increased with an amount of 28.8% (w/w) to 87% (w/w) compared to the basic protocol (Table 3). The obtained amount of Man was 19.6% (w/w) thus there was a little increase of 6.4% (w/w) (Table 3). Due to the high increase of Glc and a smaller increase of man, not only resin is removed to obtain purer cellulose (Table 1). The increasing Glc and decreasing Man are due to the solubility of Man in the wash. Man is, in fact, soluble in acetone [19] and methanol [20]. Hence, a part of Man was possibly dissolved due to the additional organic wash A. Therefore, the increase of Glc and the decrease of Man. The wash did improve the α-cellulose extraction protocol by removing the resin, and a part of Man. Purer cellulose was yielded. After applying this wash A to pine, about the same amounts were obtained as for the basic protocol (Table 3). The resin of pine is soluble in acetone [21], methanol [22, 23] and chloroform [22, 24]. Thus it is not, that the resin is not removed. Previous research showed that the basic protocol with $NaClO_2$ and NaOH was sufficient to remove resins and the additional wash A was not needed to remove the resin [25]. Therefore, the amounts of Glc and Man were the same after the basic protocol and after the basic protocol with additional wash A. For douglas fir and pine, the highest amount of cellulose was obtained for this protocol and therefore the purest cellulose as well. The protocol with the additional organic wash A enhanced the α-cellulose extraction protocol the best for douglas fir and pine. Thus the radiocarbon dating method was improved as well

A protocol with another additional organic wash B (90% acetone and 10% water) was applied to remove resin as well. The difference between organic wash B and organic wash A is that in this organic wash B there is also water used because some compounds are better soluble in water and others are more soluble in acetone (organic solvents). After this protocol was applied, beech wood contained 45% (w/w) Glc (Table 3). Compared to the basic protocol, the amount of Glc was decreased by 58.2% (w/w) (Table 3). In douglas fir, the amount of Glc was 27.8% (w/w), thus increased by 12.8% (w/w) compared to the basic protocol (Table 3). In pine, the amount of Glc decreased by 40.8% (w/w) to 27.2% (w/w) (Table 3). For all three wood samples, only Glc was obtained, so pure cellulose was obtained. The amount of Glc has been reduced to such an extent, that something must have gone wrong. Possibly, the samples were not dried enough after applying the wash and there was still some water or organic solvent in them. It is possible that compounds were lost due to the additional wash, that the samples were not long enough in the freezer or the freeze-drier, or that the samples had been exposed to the air for too long allowing other compounds to join it. The complete acid hydrolysis was performed well, thus that can not be it.

The last protocol contains an additional alkali extraction of a stronger base (KOH) to improve the α-cellulose extraction protocol. After applying this protocol to beech wood, the highest and purest amount of cellulose was yielded, namely, 96.1% (w/w) Glc and all the traces were removed, except for the Xyl trace (Table 3). The stronger base had broken more glycosidic and ester linkages due to its strength, thus all the hemicellulose monosaccharides were removed (except for the trace of Xyl) and the purest cellulose was obtained. For douglas fir, the amount

of Glc was 68.4% (w/w) with an increase of 10.2% (w/w) and the amount of Man was 9.7% (w/w) with a decrease of 3.5% (w/w) compared to the basic protocol (Table 3). The composition in pine after this protocol was applied, contains 61.3% (w/w) Glc, but a decrease of 6.7% (w/w) Glc and 11.7% (w/w) Man with a decrease of 8.4% (w/w) Man (Table 3). For douglas fir and pine, this protocol has removed some Man. Thus the additional alkali extraction has removed more hemicellulose, which was expected because KOH is a stronger base and breaks thus more linkages. Therefore, an increase in Glc was obtained. Besides this, all the traces were removed, thus the Gal side chains from galactoglucomannan of the softwoods as well. It is easier to remove the side chains because they are less rigid than the backbone. It is harder to hydrolyse the backbone which consists of Glc and Man, due to the branches that interfere with the surface area of the backbone. Therefore, the branches were removed first. [9]. The amount of Glc for douglas fir was increased and Man was decreased, but for pine, the amount of Glc was decreased and the amount of Man was decreased. The weight percentage of Glc with standard deviation for pine for the protocol with additional alkali extraction is 61.3 ± 8.7% (w/w) (Table 3). Therefore, the weight percentage of Glc for this protocol can be 68.0% (w/w) like in the basic protocol (Table 3). Thus then there would not be a decrease in Glc. In the literature, there is no other reason found to explain this. There is also another factor that should be taken into account, namely the overall carbohydrate recovery after complete acid hydrolysis before determining which protocol enhanced the radiocarbon dating method the best.

5.2 Total carbohydrate recovery of extracted cellulose after complete acid hydrolysis

Around 100% (w/w) were the expected weight percentages for total recovery carbohydrates. This is not the case for every sample. It is important to look at this number because if the recovery carbohydrate is 100% (w/w), it means that everything in the sample is detectable via HPAEC-PAD thus it means that there is only cellulose and hemicellulose in this sample.
If the carbohydrate recovery percentage is very small, it means that there are other compounds than cellulose or hemicellulose in the sample which are not detectable via HPAEC-PAD. For example, lignin can not be detected via HPAEC-PAD, because it contains different alcohols. Alcohols are detected via nuclear magnetic resonance spectroscopy (NMR spectroscopy) [26]. There are also other compounds that are not detectable via HPAEC-PAD, like water.

The total amount of carbohydrates for the untreated wood was 59.5% (w/w) for beech wood, 60.5% (w/w) for douglas fir and 47.6% (w/w) for pine (Table 3). This amounts consists of cellulose and hemicellulose. For lignin, it is difficult to say that it has been removed because it is not detectable via HPAED-PAD, but untreated wood contains lignin thus the other 40-50% (w/w) to obtain a carbohydrate recovery of 100% (w/w) is lignin and the extractives (Table 1).

After performing the basic protocol, the recovery of beech wood was extremely high, namely 112.1% (w/w) (Table 3). This is due to the Glc amount which was too high, due to an error. For douglas fir and pine, the basic protocol yielded a recovery of 71.4% (w/w) and 88.1% (w/w) (Table 3), but still, some undetected compounds were obtained. Probably, there is still some water or lignin in the samples. This was also the α-cellulose extraction protocol that had to be improved, thus as expected, insufficient cellulose purity was obtained. If the overall carbohydrate recovery is high, the extraction was done nicely, because all the other compounds are removed except for cellulose and hemicellulose. If the carbohydrate recovery is low, then the extraction was not perfectly performed, because other compounds are in there which should have been removed before applying the complete acid hydrolysis.

After applying the protocol with additional organic wash A, the highest recovery was obtained in general for this protocol. Thus the protocol was performed nicely but not perfectly, because the carbohydrate recovery is still not 100% (w/w), but comes close. For beech wood, the carbohydrate recovery was 87.5%, but this is not the highest amount obtained for beech wood. (w/w) (Table 3). The weight percentage of Glc in douglas fir is 87% (w/w) and in pine 69.6 % (w/w) (Table 3). The carbohydrate recovery for douglas fir is 106.6% (w/w) and for pine, it is 89.6% (w/w) (Table 3). This was compared to the other protocol which resulted in a high Glc weight percentage as well, which was the protocol with the additional alkali extraction. The amount of Glc for the protocol with additional wash A in douglas fir was 68.4% (w/w) and in pine 61.3% (w/w) (Table 3). Their recovery percentage was 78.1% (w/w) and 73.0% (w/w) (Table 3). Comparing the amounts of Glc and their carbohydrate recovery for the protocol with the additional alkali extraction to the protocol with the additional wash A, there could be concluded that for douglas fir and pine the purest cellulose was obtained for the protocol with the additional wash A and that this protocol was better performed due to a higher carbohydrate recovery. The recovery amount for pine for the basic protocol and the protocol with the additional organic wash A is the same. This wash was not necessary, because NaOH for performing the base extraction step and $NaClO_2$ for performing the chlorite oxidation step, was already enough to remove the resin [25].

Thus, the purest cellulose for douglas fir and pine was obtained and the α-cellulose extraction protocol was enhanced to improve radiocarbon dating. After applying the protocol with the additional alkali extraction for beech wood the carbohydrate recovery was 96.1% (w/w) and its Glc weight percentage is 96.1% (w/w) as well (Table 3). This is not the highest carbohydrate recovery, but all the detectable compounds are 100% (w/w) Glc because the Glc weight percentage is equal to its total carbohydrate recovery. This is the protocol that has improved the α-cellulose extraction protocol the most for beech wood. Therefore, the purest cellulose is obtained and the radiocarbon dating method is highly improved for beech wood.

The carbohydrate recovery for the protocol with the additional wash B was not performed well. The recovery of beech wood was 45.0% (w/w), for douglas fir 27.8% (w/w) and pine 27.2% (w/w) (Table 3). For these three wood samples, the carbohydrate recovery is the same as the amount of Glc in these samples. Thus, everything that was detectable in the samples is 100% (w/w) Glc, but the carbohydrate recovery is so low that the protocol was not performed well. Therefore, they do not contain pure cellulose, due to other undetectable compounds in there. It is obvious that during this protocol something went wrong. Possibly, the samples were not dried enough after applying the wash, compounds were lost due to the additional wash, the samples were not long enough in the freezer or the freeze-drier, or the samples had been exposed to the air for too long allowing other compounds to join it.

6. Conclusion

This bachelor research project had focused on the optimization of the α-cellulose extraction protocol to yield the purest cellulose to eventually increase the effectiveness of radiocarbon dating. Different protocols were performed on three different kinds of woods, namely, beech wood, douglas fir and pine. It was demonstrated that the protocol with the additional alkali extraction was the most efficient and allowed the purest cellulose in beech wood. 96.1% (w/w) cellulose was obtained and a carbohydrate recovery of 96.1% (w/w). Due to the additional alkali extraction to the basic protocol, more bonds between cellulose and lignin and hemicellulose were broken. Therefore, more lignin and hemicellulose were removed and purer cellulose was yielded. For douglas fir and pine, the protocol with the additional organic wash A yielded the purest cellulose. In douglas fir 87.0% (w/w) cellulose with a carbohydrate recovery of 106.6% (w/w) was obtained and in pine 69.9% (w/w) cellulose and a carbohydrate recovery of 89.6% (w/w) was obtained. The protocol with additional wash A has improved the α-cellulose extraction protocol by removing the resin of douglas fir. The resin of pine was already removed by NaOH and $NaClO_2$ during the base extraction and chlorite oxidation step. The overall carbohydrates recoveries allowed an overview of compounds that were detectable during HPAEC-PAD. The carbohydrates recoveries showed that the protocols were well performed because they were almost 100% (w/w). For further research, it can be interesting to combine the protocol with additional organic wash A and the protocol with additional alkali extraction for douglas fir to obtain purer cellulose. To conclude, the purest cellulose was obtained and radiocarbon dating was improved by applying the protocol with additional alkali extraction to beech wood and by applying the protocol with additional wash A to douglas fir and pine.

7. References

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