

The Study of Glucose and Xylose Content by Acid Hydrolysis of Ash Wood (*Fraxinus excelsior* L.) after Thermal Modification in Nitrogen by HPLC Method

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This study aimed to determine glucose and xylose content by acid hydrolysis of wood samples, both unmodified and thermally modified (modification time was 2, 6, 10 hours), using high performance liquid chromatography. Optimization of the hydrolysis process on the native ash wood samples showed that 3 h was the best time in the hydrolysis process. After that time, 58.8% of glucose and 20.8% of xylose were obtained. In turn, chromatographic analysis showed incomplete hydrolysis of ash wood samples, which were modified in a nitrogen atmosphere, especially at shorter times (2 and 6 h) of modification. With longer modification times (10 h), the hydrolysis of ash wood samples was completed. The above mentioned problem was caused mainly by the increase of cellulose crystallinity degree. The decrease of this parameter was observed only after 10 h of thermal modification, which would facilitate the process of acidic hydrolysis. Additionally, it was observed that the thermal modification of ash wood at 190 °C in a nitrogen atmosphere for 10 h caused a drastic decrease in the xylose content (from 20.8% to 8.0%) and only a slight decrease in the glucose content (from 58.8% to 54.9%).

Keywords: Acidic hydrolysis; Thermal modification; Ash wood; HPLC; Glucose and xylose content

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INTRODUCTION

Thermal treatment is an alternative to chemical treatment in wood preservation and has been used to some extent for improving timber quality. One way to improve its properties is thermal modification under nitrogen. This type of modification has been successfully introduced and is being implemented on a fairly large scale in France (Vernois 2001). Among the benefits associated with this technique is an improvement in dimensional stability (Kamdem *et al.* 2000; Rapp and Sailer 2001; Awoyemi and Jones 2011). Additionally, the strength properties of wood are changed, the bending strength decreases, but the modulus of elasticity remains at a similar level (Viitaniemi and Jämsä 1996; Kubojima *et al.* 2000). Thermal modification changes the colour of wood. This applies to the entire cross-section of wood and the colour is confusingly similar to some species of tropical wood. The darkening of the wood becomes more intensive with increasing time and temperature of thermal modification (Sundqvist 2002; Bekhta and Niemz 2003). It is believed that the main cause of the wood color change under the

influence of thermal modification is the formation of colored degradation products from extractives and hemicelluloses (Sundqvist and Morén 2002; Sundqvist 2004). Additionally, the formation of oxidation products (*e.g.* quinones) is also considered as the reason for color change (Mitsui *et al.* 2001; Bekhta and Niemz 2003). Research studies on thermal modification are still ongoing; there are various conditions of modification and methods to analyze modified wood (Zaman *et al.* 2000; Alén *et al.* 2002; Sivonen *et al.* 2002; Nuopponen *et al.* 2003, 2004; Wikberg and Maunu 2004). Thermal wood modification is typically conducted within a temperature range from 160 °C to 260 °C under conditions of limited supply of oxygen. Currently, several thermal modification technologies in various conditions of gas atmosphere (vacuum, steam, nitrogen, combustion gases) and also by using an oil were developed (Hill 2006; Esteves and Pereira 2009).

During thermal modification, large changes occur in the chemical structure of the wood compounds. In particular, there is a degradation of amorphous carbohydrates, mainly hemicelluloses (Rowell *et al.* 2002; Udaka and Furuno 2003; Boonstra and Tjeerdsma 2006; Gawron *et al.* 2011). Depending on the conditions and the environment of thermal modification, the amount of carbohydrates decreases. The content of polysaccharides in the thermally modified wood can be determined by hydrolysis and high performance liquid chromatography (HPLC) methods. The hydrolysis process is a reaction that allows the conversion of polysaccharides to monosaccharides. Concentrated and dilute acids or specific enzymes are used for obtaining simple sugars from the polysaccharides (Wyman *et al.* 2005; Carvalheiro *et al.* 2008). Mineral acids such as sulfuric, hydrochloric, phosphoric acids, and organic trifluoroacetic acid are the most commonly used. The advantages of the use of acids are their low price and minimal consumption. In turn, the enzymatic hydrolysis process uses highly specialized enzymes. In the case of cellulose three enzymes are used, which act synergistically: endocellulases (hydrolyzing β glycosidic bond within the cellulose chain), exocellulases (splitting off the cellobiose molecule from the end of the chain), and β -glucosidase (decomposing cellobiose molecule to glucose). On the other hand, the enzymes for the hydrolysis of hemicelluloses are much more complex and include endo-1,4- β -D-xylanases, exo-1,4- β -D-xylosidases, endo-1,4- β -D-mannanases, β -mannosidases, acetyl xylan esterases, α -glucuronidases, α -L-arabinofuranosidases, and α -galactosidases (Jorgensen *et al.* 2003).

A number of different analytical methods can be used to separate and analyze sugars, such as thin layer chromatography, gas, liquid chromatography, or capillary electrophoresis. Among the instrumental methods, HPLC is a technique often used in the analysis of sugars (Aglevor *et al.* 2004, 2007; De Muynck *et al.* 2006; Rybak-Chmielewska 2007; Rahman *et al.* 2008; Salman *et al.* 2011). This method has many advantages; it has high accuracy, the sample requirement is very low (about tens to hundreds of mg), and there is no need for sample derivatization. In the available literature, there is information regarding the use of the HPLC method for analysis of sugars in wood subjected to thermal modification (Inari *et al.* 2007; Karlsson *et al.* 2012; Cademartori *et al.* 2013). But unfortunately, there is no information about the use of this method to examine the content of sugars in ash wood. The ash wood is an important species, because it is often used for thermal modification. Therefore, in this case, from a scientific and practical point of view, the use of the HPLC method is interesting and worthy of attention.

In this work, an attempt was made using HPLC to examine the chemical composition of the main saccharides (xylose and glucose) obtained from ash wood (*Fraxinus excelsior* L.) unmodified and thermally modified under nitrogen.

EXPERIMENTAL

Materials

The thermal modification was carried out on the ash wood (*Fraxinus excelsior* L.). The age of the tree was about 80 years. Uncoloured wood taken from the heartwood was used. The average density of the tested ash wood was 731.6 kg/m³ and the average standard deviation 40.4 kg/m³.

Methods

Thermal modification in nitrogen atmosphere

Thermal modification in nitrogen atmosphere was carried out in three-necked round bottom flask (250 cm³) placed in an oil bath. In the modification process, ash wood ground to the form of sawdust (fraction 0.43 to 1.02 mm) was used. Duration of the respective modification process was 2, 6, and 10 h at 190 °C. The modification was preceded by drying the samples to an absolutely dry state. Then, the temperature was raised to 130 °C at the rate of 1 °C/min, and nitrogen with a purity of 99% was supplied to the flask from a cylinder. Nitrogen flow was maintained at a constant rate throughout the thermal modification process, including the cooling of samples. Temperature was maintained at 130 °C for 30 min. In the next stage, the temperature in the flask was raised to 190 °C at the rate of 1 °C/min. When the temperature in the flask reached 190 °C, the appropriate thermal modification occurred, which lasted for 2, 6, or 10 h. After the specified time, the samples were cooled for 30 min to ambient temperature and then placed in a desiccator. The samples, which were taken after the modification process, were subsequently subjected to hydrolysis, in order to determine the content of the saccharides (xylose and glucose) in the tested ash wood by HPLC.

Hydrolysis of wood samples

Acid hydrolysis (Kačik and Solár 1999; Antczak *et al.* 2012) was performed for ash wood samples unmodified and thermally modified under nitrogen at 190 °C for 2, 6, and 10 h. Each wood sample was then subjected to a second series of hydrolysis. In the hydrolysis process, finely ground wood in the form of sawdust (fraction below 0.43 mm) was used. At the beginning, wood dust was extracted with a mixture of chloroform (Chempur, Poland) and 96% ethanol (Chempur, Poland) in a weight ratio of 93:7. It was then dried to constant weight in a vacuum drier at 60 °C and under pressure 0.4 kPa. After drying, the extracted wood samples (approximately 100 mg) were placed in flasks with a capacity of 100 cm³, which were poured with 1 cm³ of 72% sulfuric acid (Chempur, Poland), and tightly stoppered. Then, samples were heated at 30 °C for a period of 1 h, cooled to room temperature, and 28 cm³ of distilled water was added to each sample.

After connecting to a reflux condenser, the flasks were heated at 120 °C in an oil bath. In order to determine the optimal hydrolysis time for the control sample (unmodified thermally), various heating times (from 1 to 4 h) were examined. Each sample, after cooling to room temperature, was filtered through a G3 Schott filter; the

filtrate was quantitatively transferred to 50 cm³ volumetric flasks. From each flask, 25 cm³ of the hydrolysate was collected. Then, using sodium carbonate (Chempur, Poland), the pH of the hydrolysate was adjusted to about 5. After neutralization, in order to concentrate the hydrolysate, each sample was evaporated to dryness in a vacuum evaporator and then transferred using distilled water to a volumetric flask with a capacity of 10 cm³. The prepared solutions of hydrolysates were analyzed by HPLC for separation of sugars (xylose and glucose).

HPLC analysis of standards and wood hydrolysates

A liquid chromatograph (SHIMADZU Company) with refractometric detector RID-10A was used. Column Phenomenex Luna 5 μ NH₂ 100Å was used to analyze the distribution of simple sugars standards (xylose and glucose) (Sigma-Aldrich, Germany) and hydrolysates. Analysis conditions were as follows: mixture of acetone (Chempur, Poland) and redistilled water in a volumetric ratio of 90:10 as the eluent, oven temperature: 50 °C, eluent flow: 2.5 cm³/min, and sample volume: 0.2 cm³. Each sample was 2 or 3 times injected, and the values were averaged.

Results were elaborated by using LC Solution v.1.21 SP1 software. For the quantitative determination of monosaccharides in hydrolysates, previously developed calibration curves were used (Antczak *et al.* 2012). Concentrations of calibration solutions were as follows: for xylose (10.02, 6.68, 5.01, 3.34, 2.00 mg/cm³) and for glucose (10.08, 6.72, 5.04, 3.36, 2.02 mg/cm³). Equations of xylose and glucose calibration curves are presented below:

$$y=276567x; R^2=0.9984 \text{ (xylose)} \quad (1)$$

$$y=458503x; R^2=0.9983 \text{ (glucose)} \quad (2)$$

Analysis of cellulose crystallinity degree by WAXS

The study of crystallinity degree was carried out on cellulose isolated from unmodified and thermally modified ash wood (*Fraxinus excelsior* L.) by the Kürschner-Hoffer method (Saeman *et al.* 1954; Krutul 2002). The analysis was performed by wide angle x-ray scattering (WAXS). For each sample two analysis of the crystallinity degree were done.

Tests were performed on a TUR M-62 apparatus (Carl Zeiss AG company), equipped with a goniometer HZG-4. The source of radiation was anode Cu - K α ($\lambda = 1.5418 \text{ \AA}$), with a voltage of 30 kV and a current of 25 mA. The nickel filter was used during the analysis.

The resulting rentgenograms are a function of the intensity of the angle 2θ . The spectra registration was carried out in the angle range $2\theta = 5 - 30^\circ$ with the counting rate of $0.04^\circ / 3$ seconds.

Separation of peaks was made using the method proposed by Hindeleh and Johnson (1971), as modified by Rabiej (1991). After separation of the peaks, the degree of crystallinity was calculated on the basis of area ratio derived from the crystalline regions to the sum of the areas derived from crystalline and amorphous regions (Segal *et al.* 1959; Isogai and Usuda 1990).

RESULTS AND DISCUSSION

HPLC was used to determine the percentage content of saccharides (xylose and glucose) obtained from ash wood (*Fraxinus excelsior* L.) before and after thermal modification in a nitrogen atmosphere at 190 °C. To elaborate the sugars profile, hydrolysis method with 72% sulfuric acid was used. In order to obtain reliable results, the optimization of hydrolysis time was done. Optimization was performed on unmodified wood.

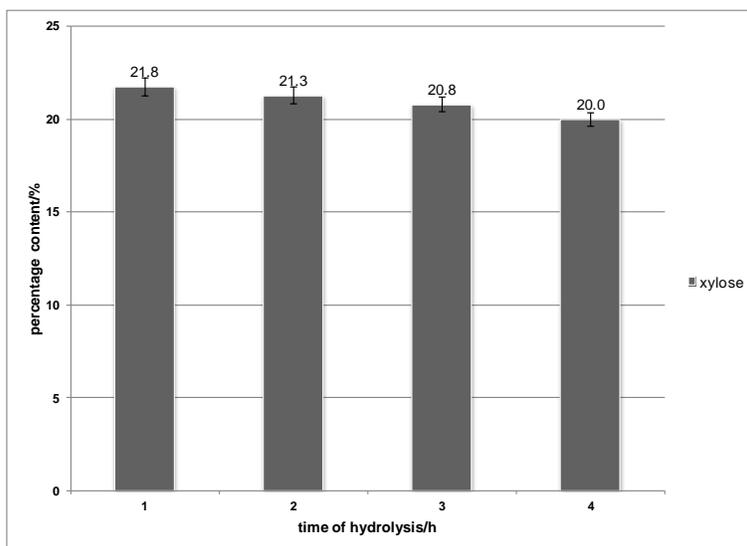


Fig. 1. Average percentage content of xylose from unmodified ash wood (*Fraxinus excelsior* L.) in relation to time of hydrolysis with using 72% H₂SO₄

The results for the average percentage content of sugars after various hydrolysis times are presented in Figs. 1 and 2. The results were presented by plotting the average percentage content of xylose and also glucose versus time of hydrolysis. Additionally, Fig. 3 shows the HPLC chromatograms of unmodified ash wood samples subjected to different times of hydrolysis in 72% sulfuric acid.

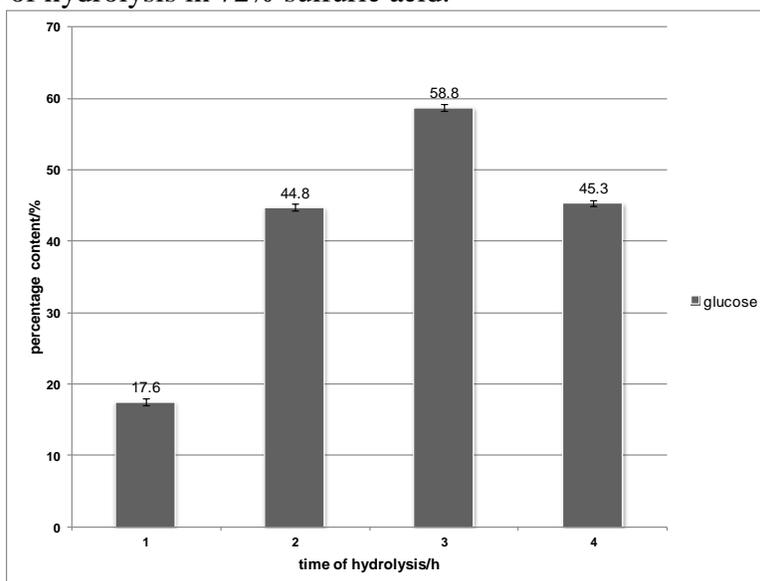


Fig. 2. The average percentage content of glucose from unmodified ash wood (*Fraxinus excelsior* L.) in relation to time of hydrolysis with using 72% H₂SO₄

The results (Figs. 1 and 3) indicate that after 1 h of hydrolysis, the greatest quantity of xylose (21.8%) was produced. Prolongation of hydrolysis led to the reduction of the sugar content, which is associated with the degrading influence of concentrated sulfuric acid.

Based on the data presented in Figs. 2 and 3, it can be concluded that in the case of glucose, the highest content (58.8%) was formed after 3 h of hydrolysis. Extension of the hydrolysis time, similar as before, resulted in a decrease of the glucose content to 45.3%.

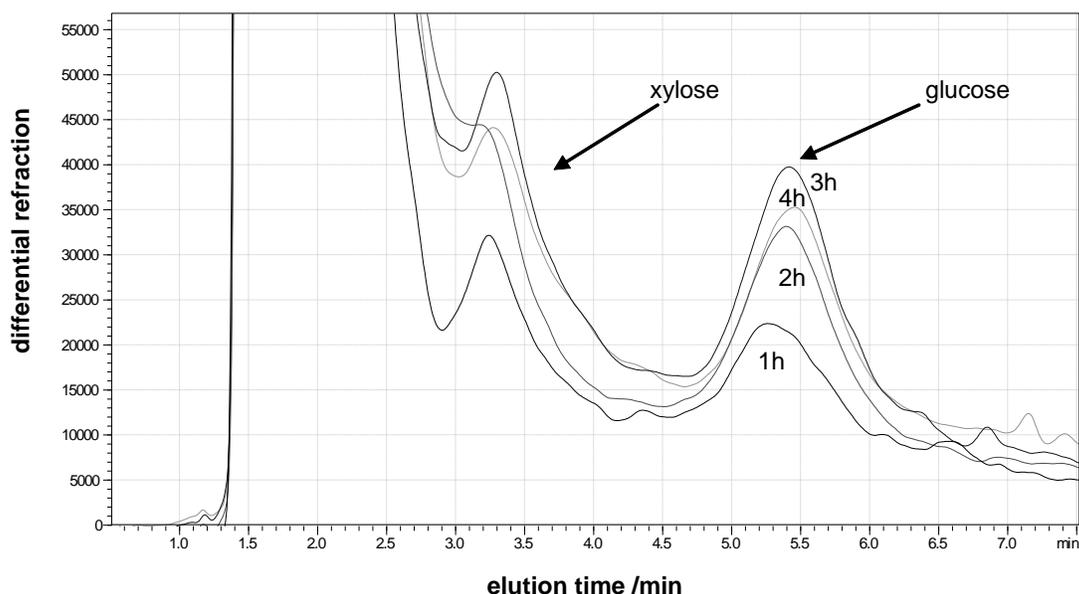


Fig. 3. Chromatograms from HPLC analysis of unmodified ash wood (*Fraxinus excelsior* L.) after hydrolysis with 72% H₂SO₄. Designations: 1 h, 2 h, 3 h, and 4 h – the various times of hydrolysis.

Based on the results from the optimization, it can be concluded that 3 h is the best time in the hydrolysis process. After that time, the highest amount of glucose was obtained, with a slight decrease of xylose at the same time. Therefore, further studies on the determination of the sugars profile in thermally modified ash wood in an optimized hydrolysis time (3 h) were performed. The results of the average percentage content of determined sugars are presented in Table 1.

Table 1. Results of the Average Percentage Content of Monosaccharides Obtained from Unmodified and Thermally Modified Ash Wood (*Fraxinus excelsior* L.) in Nitrogen Atmosphere

Modification Time	Xylose /%	Glucose /%	M* /%
Control	20.8 ± 0.4	58.8 ± 0.4	79.6
2h	-	5.0 ± 0.3	5.0
6h	7.1 ± 0.3	45.9 ± 0.5	53.0
10h	8.0 ± 0.3	54.9 ± 0.5	62.9

M* – the sum of monosaccharides content.

The errors are expressed as standard deviations on two or three replicates.

Based on the results in Table 1 and Fig. 4, it can be concluded that the glucose content increased with time of thermal modification of wood, which was carried out in a nitrogen atmosphere. Similar results were reported by Esteves *et al.* (2008), who also observed an increase of glucose content in hydrolysates due to more selective hemicelluloses degradation. Also in this work it was found that the hemicelluloses were much more susceptible to thermal degradation than cellulose. Evidence of this was a decrease of xylose content from 20.8% (for a control sample) to 8.0% (for the sample subjected to 10-hour thermal modification). In the case of the glucose content decrease was not so high, and it ranged from 58.8% (for a control sample) to 54.9% (for the sample subjected to 10-hour thermal modification).

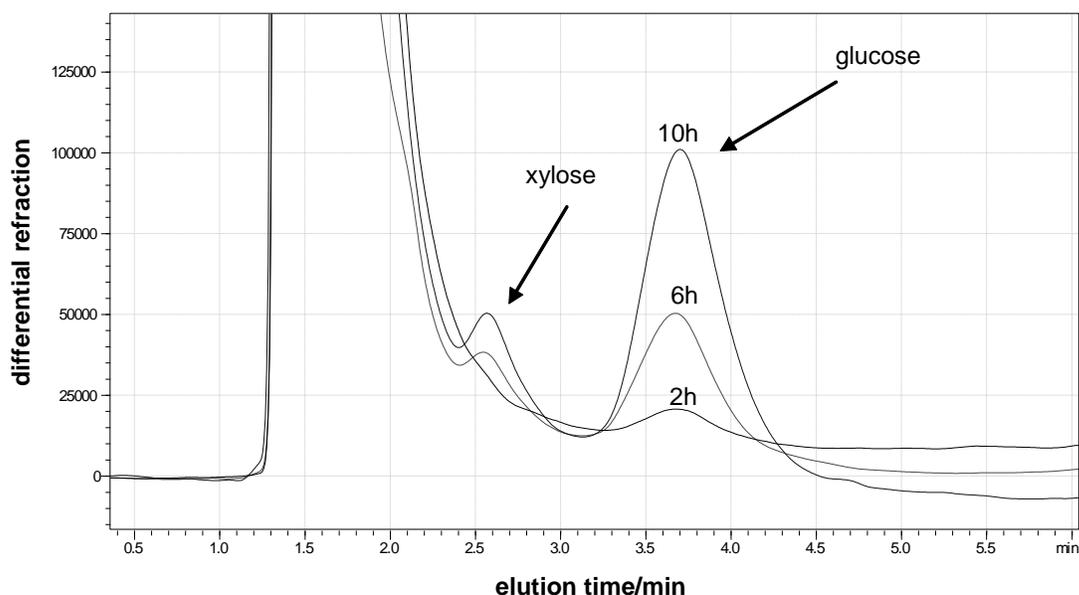


Fig. 4. Chromatograms from HPLC analysis of thermally modified ash wood (*Fraxinus excelsior* L.) after hydrolysis with 72% H₂SO₄. Designations: 2 h, 6 h, and 10 h – the various times of thermal modification in nitrogen atmosphere.

A noteworthy result is the apparent lack of xylose and low content of glucose obtained from the wood sample thermally modified for 2 h under nitrogen (Table 1). In this case, the apparent absence of xylose may be due to greater susceptibility of xylan than cellulose to hydrolysis in sulfuric acid, which may further result in a greater exposure of formed xylose to the degradation in acid. Thus, it is likely that a certain amount of xylose (about a few percent) that that was formed was subsequently degraded in sulfuric acid at 120 °C. The remaining small amounts of xylose in the applied analytical system could not be separated and determined.

Additionally, based on the results in Table 1 and Fig. 4 it can be concluded that thermally modified wood had a different susceptibility to hydrolysis in concentrated sulfuric acid. The least susceptible to the saccharification process was wood after a 2-h thermal modification, while the most susceptible was wood following a 10-h modification. So, the most understated results of saccharides content were obtained for wood after a 2-h thermal modification. According to information from the literature, it is known that the application of high temperatures (for example: accelerated aging) may

cause a phenomenon that is referred to as "hornification" (Kato and Cameron 1999; Borrega and Kärenlampi 2010; Suchy *et al.* 2010; Zauer *et al.* 2014). This phenomenon has also been observed in studies related to accelerated aging of cellulose (Antczak 2010). Aged cellulose at elevated temperature was less susceptible to swelling in water, followed by the less soluble in a solution of 8% LiCl/DMAc. The above problem is probably due to the existence of additional hydrogen bonds inter- and intra-molecular. The hornification phenomenon results in lower flexibility, lower water retention, and increases of the fiber embrittlement. During the appearance of hornification, there is often an increase of cellulose crystallinity. Presented characteristics of the aged fiber are associated with thermally modified wood. Thus, hornification may explain why the wood, after a 2-hour thermal modification, was less susceptible to the action of concentrated sulfuric acid than the unmodified wood. Similar problems in the process of enzymatic hydrolysis were observed by other researchers (Luo and Zhu 2011; Sun 2013). Additionally, the cause may be autocondensation of lignin under the influence of high temperature, resulting in the increase of the number of active centers in the aromatic ring by elimination of methoxyl groups (Tjeerdsma *et al.* 1998; Wikberg and Maunu 2004; Zhu *et al.* 2014). Created centers can form durable connections with carbohydrates contained in wood. The resulting lignin carbohydrate systems (lignin complex carbohydrates - LCC) may make it difficult for hydrolysis in concentrated acid, especially for a shorter time of modification. Confirmation of the LCC formation are problems with the lignin content determination in heat-treated wood which were observed by other authors (Tjeerdsma and Militz 2005; Boonstra and Tjeerdsma 2006; Esteves *et al.* 2008). It was suggested that the reason for the apparent increase in the lignin content are its polycondensation reactions with other components of cell wall. In addition to the autocondensation process, the formation of a methylene bridge linking aromatic rings of lignin may occur. These changes cause stiffening of the lignin structure by the increase of crosslinking (Funaoka *et al.* 1990; Tjeerdsma and Militz 2005; Esteves and Pereira 2009). Such transformations can also cause difficulties in the hydrolysis process.

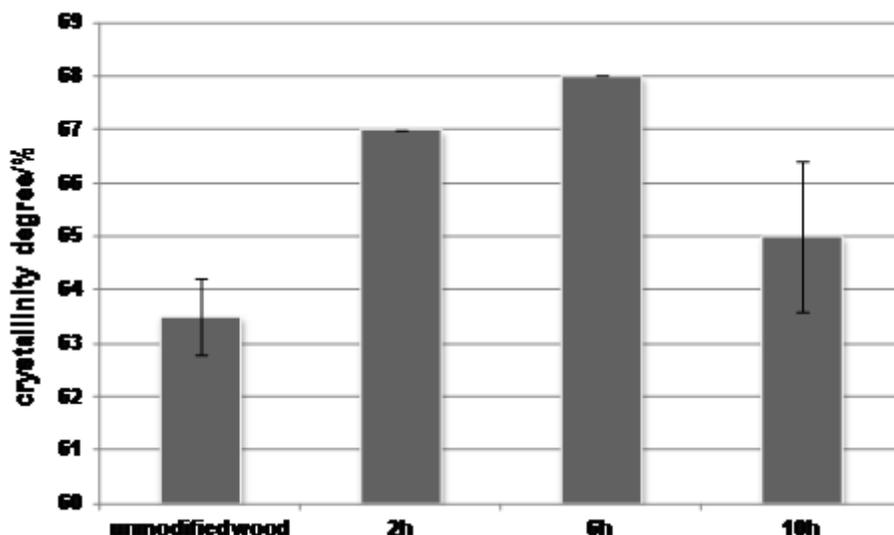


Fig. 5. The average crystallinity degree of cellulose from ash wood (*Fraxinus excelsior* L.) thermally modified in nitrogen atmosphere

In addition to the previously mentioned hypotheses, understated results may be caused by the ability of xylan and cellulose to undergo crystallization (Bhuiyan *et al.* 2000). Reorganization and crystallization may occur in xylan and cellulose quasicrystalline regions due to high temperatures, which also may cause an increase of resistance to hydrolysis in concentrated sulfuric acid. Many studies have reported on the idea that wood thermal modification leads to an increase in the crystallinity of cellulose (Kubojima *et al.* 1998; Akgül *et al.* 2007; Yildiz and Gümüşkaya 2007). These findings were confirmed by the analysis of crystallinity degree of cellulose isolated from unmodified (native) and thermally modified wood. The analysis was performed by WAXS, and the results are shown in Fig. 5. It can be seen that the wood thermal modification under nitrogen at 190 °C resulted in an increase of cellulose crystallinity degree. The decrease of this parameter was observed only after 10 h of high temperature.

Prolonging the thermal modification to 6 and 10 h is associated with increased depolymerisation of polysaccharides; in the first place hemicelluloses (Militz 2002; Fengel and Wegener 2003; Hill 2006), and consequently leads to an increased susceptibility of thermally modified wood to the effects of sulfuric acid. Moreover Bhuiyan *et al.* (2000) demonstrated that decrease of crystallinity is a result of cellulose degradation. So, because of the degradation, more free space is present between the polysaccharides macromolecular chains (Fig. 5 – reduced crystallinity after 10 h), which will facilitate the process of acidic hydrolysis. The obtained results of glucose and xylose content from thermally modified wood for 10 h in nitrogen (54.9% and 8.0%) can be considered reliable. Similar results were obtained by other researchers. Brito and colleagues (2008) found that the content of glucose and xylose obtained from eucalyptus wood subjected to thermal modification at 180 °C is respectively 51.94% and 8.51%. According to our results and the literature, the application of heat causes a dramatic decrease in hemicelluloses content. Yildiz and his collaborators (2006) examined that the thermal treatment of spruce wood for 10 h at 200 °C in air results in a very high decrease in hemicelluloses content from the value of 21.43% (for a control sample) to 1.99%. For comparison, in the same samples cellulose content decreased from 54.12% (control sample) to 50.39%. Candelier and colleagues (2013) reported that under the influence of thermal modification at 230 °C in nitrogen atmosphere the content of xylose obtained from beech wood underwent a high decrease from 30.42% (control sample) to 18.47%. In turn, regarding the content of glucose only a slight decrease from 49.75% (control sample) to 48.23% was observed. Generally, it is known that xylan is the least heat-resistant substance of the polysaccharides, and pentosans (xylan and arabinan) are very sensitive to degradation and dehydration reactions (Kotilainen 2000). The results obtained in this study confirm these observations.

Summing up, the obtained results indicate that thermal modification of ash wood (*Fraxinus excelsior* L.) at 190 °C in a nitrogen atmosphere for 10 h causes a drastic decrease in the xylose content. However, in the case of glucose, the content reduction is not so large.

CONCLUSIONS

1. Optimization of the hydrolysis process on unmodified ash wood samples showed that 3 h is the best time. After that time, the highest amount of glucose was obtained, with a slight decrease of xylose at the same time.

2. Chromatographic analysis showed incomplete hydrolysis of thermally modified ash wood with shorter modification time (2 h and 6 h). The above mentioned problem is caused mainly by the increase of cellulose crystallinity degree. In this case, additional studies are required to develop an appropriate method of wood hydrolysis using sulfuric acid.
3. Chromatographic analysis showed complete hydrolysis of thermally modified ash wood with a longer modification time (10 h). The results of carbohydrates content are consistent with the literature findings.
4. From the results obtained, it was observed that under the conditions used, thermal modification of ash wood causes a dramatic decrease in xylose content and a small reduction in glucose content.

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