The identification & upgrading of water-soluble products in a pyrolysis waste stream

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Biobased Chemistry and Technology

BSc Thesis Biotechnology

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Summary

Chemical substances, for many industrial applications, are derived mainly from fossil fuels. Fossil fuel exploitation is known to have detrimental effects on the current, hospitable environment due to their immensely long carbon cycles [1]. Biomass utilization for fuels and chemicals shortens this carbon cycle to less than one year. However, biomass by itself does not have the right properties for energy generation or chemical production. Pyrolysis, the decomposition of biomass at elevated temperature without oxidation, can output more usable material[2]. The applications of this material are still limited and its composition is not fully encompassed yet.

The aqueous fraction of a pyrolysis process (pyrolytic sugars) was investigated in this research by means of HPLC, TGA, LC-MS, and more. First, the composition was determined, then processing methods and applications were explored.

The dry weight of pyrolytic sugars is 36%, of which 43% consisted of carbohydrate and 16 % of organic acid. More abundant compounds include: 45 g/L glycolaldehyde, 21 g/L phenolic compounds, 5 g/L 5-HMF and 11 g/L ethylene glycol.

Hydrolysis occurred readily when the pyrolytic sugars was supplied with 0.1 M sulfuric acid and placed at 120°, with 80% levoglucosan conversion within 15 minutes. Conversion also occurred without acid addition and even when diluting 2 times, reaching 80% levoglucosan within 15 and 4 hours respectively.

Detoxification was observed to be most effectively facilitated by solvent extraction with either MIBK or ethyl acetate (both had similar effects), the polar phase gave a significantly reduced absorption at 210 nm on the HPLC after treatment. However, fermentation was not possible on this polar phase of both extractions, possibly due to extractant dissolution in the polar phase. Detoxification with activated carbon, overliming and deacidification all gave improved fermentation results with Saccharomyces Cerevisiae.

Table of Contents

1. Background

The dependence of mankind upon unsustainable energy systems has grown steadily ever since the onset of the industrial revolution in the 18th century. The elimination of this dependence is of importance for future welfare and environmental health[3], as both the energy crisis of the 1970s and the anticipated global warming have shown. Although plenty of alternatives exist[4], the real challenge is making the alternative economically viable which can ensure commercialization[5]. Part of this comes from utilizing every single outgoing stream of a production process, because more material exploitation or less waste equals more economic viability[5].

1.1 Pyrolysis

Biomass in itself is an energy rich product of photosynthesis, which, in comparison to fossil fuels, has a very short carbon cycle if used right away. This last property makes it carbon neutral. However, in contrast to fossil fuels, biomass is unfit for combustion engines and current fuel infrastructures due to its energy density and general properties. Pyrolysis could offer an outcome to this problem.

Pyrolysis is the process of organic material decomposition at elevated temperatures (generally around 400-600 °C), without the occurrence of oxidation. Pyrolysis has been carried out for thousands of years for the production of charcoal, however, it has gained much interest for alternative applications such as biofuel production from biomass in recent decennia[6]. Bio-oil produced by pyrolysis is compatible with existing refinery technology, and this can be further upgraded (hydrogenation) and separated (heavy and light crude) for co-feeding or even direct application in combustion engines. Initially, before fractionation, a pyrolysis product is highly complex in its composition and many unwanted by-products are present, such as biochar and an aqueous fraction containing various organic acids and simple carbohydrates. Finding applications for these by-products could play an important role in achieving economic feasibility.

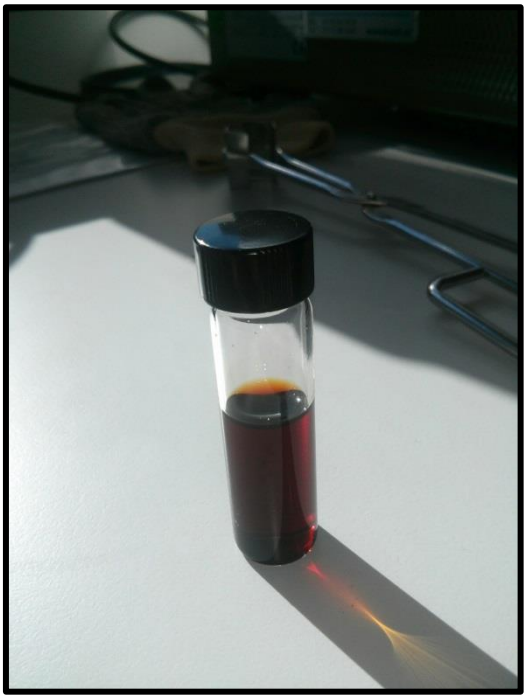

Figure 1) Sample of the aqueous fraction received from BTG

BTG, or: Biomass Technology Group, is a company situated in the Netherlands specialised in the conversion of biomass into useful fuels and energy. Pyrolysis is an important field for BTG. In this particular case, a fast pyrolysis process performed at BTG on soft wood produces an aqueous fraction, next to gas, biochar and bio-oil. This aqueous fraction is the main topic of this research, its composition and potential applications are explored.

Dry wood has the following three main constituents: cellulose, lignin and hemicellulose, in a 4:3:3 ratio [7]. When this gets subjected to fast pyrolysis, these polymers are separated into smaller polymers, oligomers, monomers, organic molecules, and finally water/gasses (H_2, CH_4,

CO, etc), in that order. Naturally, when the process of pyrolysis is stopped at an arbitrary point, all of these will be formed in varying amounts, depending on the residence time [6]. Different pyrolysis processes with different parameters and properties will yield different products, despite similar inputs. This makes the aqueous fraction from BTG unique from the many pyrolysis products discussed in literature. Moreover, this is only one fraction of a complete pyrolysis product, as it is separated from e.g. bio-oil, biochar and syngas.

1.1.1 Hypothesized composition of aqueous fraction

Much research has already been performed in order to find the composition of pyrolysis products. Although this research does not say anything conclusive about the sample in this particular project, some similarities with our sample are more than likely. Generally, the most abundant oxygenated chemicals in pyrolysis oils are the following (in decreasing order of abundance): Levoglucosan, glycolaldehyde, acetic acid and acetaldehyde. Though there are many hundreds more, these 4 compounds alone usually comprise more than half of all oxygenated chemicals in pyrolysis oils.

Purification or upgrading is necessary to add value to our pyrolysis product. Below, applications are described to achieve this via various methods.

1.2 Application: Fermentation of pyrolysis products

Fermentation of pyrolysis products is an interesting option and has been attempted a great deal of times, as seen in literature[8-14]. Here, often Saccharomyces is used for the production of ethanol. Although promising, these attempts have thus far not lead to full scale implementation. Problems such as toxicity of the to-be-fermented pyrolysis product prove hard to overcome, because this causes inhibition of growth and metabolism of micro-organisms. Many compounds contribute to the toxicity of pyrolysis products, among them are phenolic compounds and furans, and acidity also contributes to inhibition of microbes{Chi, 2013 #173}. Nonetheless, pyrolysis products contain multiple possible c-sources, such as levoglucosan and organic acids, that could be upgraded into more valuable products by micro-organisms.

1.2.1 PHA

Next to ethanol, a relatively cheap chemical, other chemicals may be produced from the pyrolysis product using microbes, PHA is one such chemical. Polyhydroxyalkanoates(PHAs) are polymers produced by a wide variety of microbes under specific conditions. PHAs are an energy storage polymer for bacteria, but when extracted and purified they can have a vast range of applications [15], among them are fine chemicals, bioplastics and medical implants. Many bacteria have PHA hydrolases, which is why PHA is biodegradable.

Bacteria are not all capable of synthesizing PHAs, and most that are do this best under specific circumstances. Thus, some selection pressure and specific circumstances must be present in order to stimulate optimal PHA synthesis. This is generally done by implementing a cycle in the fermentation known as the 'Feast and Famine Phase', where at one point there is excess food, and the next there is hardly any. This kills of most non-PHA producing bacteria and stimulates PHA production in ones that do.

On top of toxicity, there is another problem for micro-organisms: the levoglucosan and oligomeric carbohydrates, one of the most promising candidates for the energy/ c-source for the micro-organism, cannot be utilized by most microbes. In the case of levoglucosan (a monomer), this is because of an extra bond in the molecule that is formed when glucose is dehydrated, and the oligomers simply consist of glucose molecules linked by 1,4-glycosidic bonds, making them unavailable to the micro-organism.

Thus, a large fraction of the pyrolysis product is likely to be unusable by microbes unless it is hydrolysed first. However, in order to achieve this, addition of a strong acid and heating is presumed to be essential for optimal yield [16]. Whether this is also the case for the BTG sample of this project will be researched, and the added value of hydrolysis by strong acid will be reviewed, since acid and subsequent base neutralization entails another economic burden on the process. Considering that the mixture has PH of 2.18, it is not unlikely that at least some hydrolysis will already occur without additives.

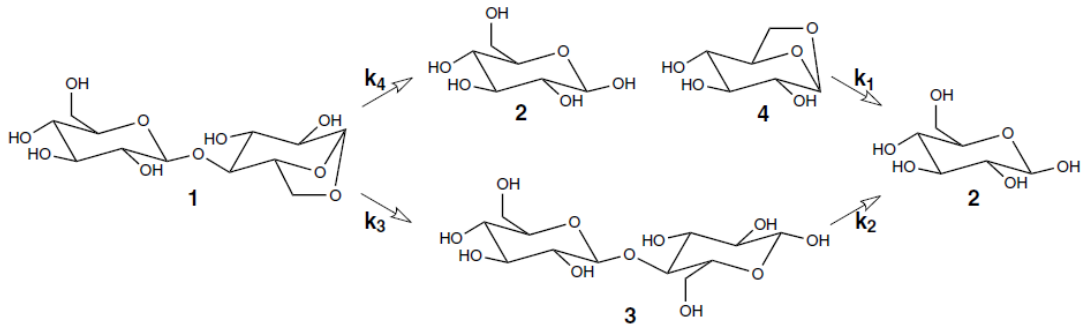

Figure 2. Two pathways from cellobiosan to glucose, one (2 and 4) via levoglucosan and glucose and one (3) via cellobiose. Source: [17]

The activation energy of breaking a glycosidic bond in a cellobiose molecule (see figure 2) is around 103 KJ/mol, whereas the hydrolysis of levoglucosan to glucose is about 114 Kj/mol [17]. Remarkably, the breaking of a glycosidic bond in a cellobiosan molecule has a lower activation energy of 98

When for example cellulose is broken down into monomers, it does not necessarily produce glucose monomers (as one might think, considering cellulose is a polymer of glucose subunits). Instead, upon cleavage of the cellulose, usually an anhydroglucose radical is formed that reacts further into a levoglucosan molecule[18]. This explains why the bulk of carbohydrate found in pyrolysis products is made up of levoglucosan[18].

1.2.2 Detoxification & deacidification

Detoxification and deacidification is required before inoculation to shorten lag phase, increase growth rate and prevent microbial death [19]. In this research, solvent extraction with both MIBK and ethyl acetate and adsorption using activated carbon is tested and evaluated. Deacidification is attempted using both overliming and sodium hydroxide addition.

1.2.3 Activated carbon

'Activated carbons are known to be excellent and versatile adsorbents and therefore used to remove a broad spectrum of dissolved organic and inorganic species from both gas phase and liquid phase' cited from {Menéndez, 1996 #235}. This property of activated carbons is related to their physical and chemical properties, namely their vast surface area and the plenitude of various functional groups that are capable of binding and reacting with many substances[20]. Naturally, selectivity of activated carbon plays an important role in this case, for instance the loss of carbohydrates must be minimized.

1.2.4 Solvent extraction

Liquid-liquid extraction will be deployed to get rid of as many inhibitory solutes as possible. This method works by introducing a driving force which pushes the solute into the organic phase, and the point at which this is in equilibrium, is given by the distribution ratio (Kd)[21]. In an ideal scenario, the inhibitory solutes all have large Kd's, whereas the useful, non-inhibitory components all have small Kd's. Furthermore, the solvent should not dissolve in water, because dissolved solvent is excluded from reuse and perhaps causes toxicity in the to be fermented raffinate.

Unfortunately, both ethyl acetate and MIBK do dissolve slightly in water, 83 g/L and 19 g/L respectively (from International Programme on Chemical Safety).

1.2.5 This research

In this research, fermentations are carried out, initially just with saccharomyces. Perhaps the aqueous fraction that we are working with does not have the problems described in literature to such a large extent, although it is likely that various detoxification measures must be performed before fermentation is even possible. Similarly, maybe glucose concentrations are already high enough for a fermentation without hydrolysis. However, if this is not the case, multiple approaches will be carried out to increase fermentable substrate (glucose).

1.3 Application: Extraction

Isolation of individual compounds from pyrolysis products has been attempted in literature. In those research attempts, high purity levels were not easily reached, unless a cost intensive multi-step process was utilized. Extraction is challenging because of the complex nature of a pyrolysis product, thereby increasing the number of extraction steps needed before reaching an acceptable purity level [22]. Naturally, only compounds that are abundantly present in pyrolysis oils are selected for extraction, such as glycolaldehyde and acetic acid to make it worthwhile [23].

1.3.1 Glycolaldehyde

Glycolaldehyde is the simplest carbohydrate of all, and has many uses. Trough hydrogenation or fermentation, glycolaldehyde can be turned into biobased ethylene glycol. Pyrolysis oils are known to contain glycolaldehyde, up to 17.5 wt%. In 2012, a PHD was published that proposed a process for glycolaldehyde extraction, using solvent extraction with 1-octanol as the main step.

1.3.2 Acetic acid

Acetic acid is an organic acid that has a high industrial demand, in part due to production of derivatives like vinyl acetate monomer. Pyrolysis oils are reported to contain acetic acid concentrations between 2-10 wt%. A one-step solvent extraction of acetic acid from pyrolysis oil was proposed by F. H. Mahfud et al. in 2008, gaining the best results using TOA diluted with toluene.

1.3.3 This research

Although these particular extractions will not be the main focus of this research, it is interesting to see what happens to glycolaldehyde and acetic acid throughout the process of upgrading, whatever that process may be. Perhaps, if glycolaldehyde or acetic acid concentrations in certain waste-streams prove to be sufficiently high, this may be refined to high purity substances.

1.4 Application: Catalytic upgrading

Catalytic upgrading involves the addition of a catalyst to a mixture, thereby initiating or stimulating certain chemical reactions to take place, yielding a compositionally different mixture after treatment. Catalytic upgrading of pyrolysis oils provides a seemingly quick and easy road to improved substance properties, however, problems such as coke formation and steep material/energy inputs are hard to overcome.

1.4.1 Reduction

Reduction of the pyrolysis oils completely alters the entire composition. In 2015, an article was published wherein a sugar fraction from pyrolysis was upgraded in supercritical methanol and ethanol, using copper doped porous metal oxide (Cu-PMO). This catalytic treatment was carried out at 275 °C and 50 bar H_2 . The author's propose that the resulting product might be used for co-feeding in methanol-to-gasoline processes [24] .

Table 1. Composition before and after catalytic treatment. Source:[24]

Sugar fractions	Area percentage (%)	Upgraded sugar fraction	Area percentage (%)
Acids	5	Alcohols	51
Ketones and aldehydes	41	Esters	16
Sugars	44	Diols	22
Phenolics	8	Ethers	8
Furanics	2	Furanics	3

1.4.2 Oxidation

Little is known about oxidising pyrolysis products. However, in particular one component is often selected for catalytic oxidation: 5-hydroxymethylfurfural. Upon oxidation, this component is converted to 2,5-Furan-dicarboxylic acid, see figure 3. This is done using for example a platina on activated carbon catalyst, in combination with strong basic conditions (0.01 M NaOH) and oxygen bubbling[25].

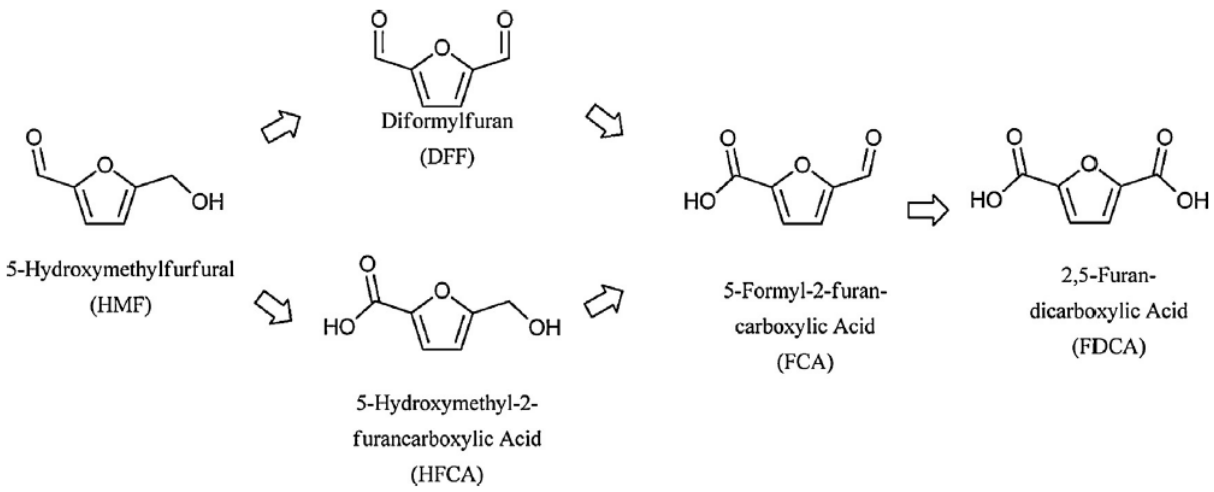

Figure 3. Conversion of 5-HMF into 2,5-FDCA. Source:[26]

FDCA is less toxic and could even be used as a substrate for fermentation in some cases. In literature, however, a simple solution of pure 5-HMF is used instead of a complex pyrolysis product, which could mean a lot of side reactions and perhaps polymerization/ coke formation in this case.

1.4.3 This Research

In this research, oxidation of the pyrolysis product is attempted. The composition of the output material is determined and various conditions are used for the conversion.

2. Aim

The aim of this thesis is to characterize the composition of an aqueous fraction produced in the fast pyrolysis of soft wood. Moreover, it is attempted to find meaningful and perhaps commercially interesting techniques for upgrading this particular by-product. Fermentation and catalytic upgrading are explored most of all.

3. Results & Discussion

3.1 Composition analysis

To guide the process of choosing appropriate applications and define required procedures, it is imperative to first characterize the composition of the mixture. Several techniques are applied to gain compositional knowledge about this mixture, some general, some more specific/detailed.

3.1.1 Brix and dry weight

The first and most basic measurements were brix, pH and dry weight. Dry weight and brix measurements give insight into how concentrated the mixture is, because most usable/valuable organic components generally found in pyrolysis products are a lot less volatile than water[22] and these same compounds usually show up in brix measurements[27].

pH of pyrolytic sugars is 2.2.

Table 2. Volatile fraction, derived from weighing before and after oven/freeze drying. It should be noted that the volatile fraction is likely to contain more than just water.

Description	Freeze dried	Oven dried
Sample undried (g)	1.19	3.39
Sample dried (g)	0.44	1.25
Volatile fraction	62.7%	63.2%

Table 3. The brix value and deduced carbohydrate content in dry fraction, by combining with found value of volatile fraction.

Description	Refractometer
Average out of 3	36.0
Derived Carbohydrate content in dry fraction	97.4%

As can be seen in table 2, both oven and freeze drying give similar numbers that indicates a dry number of 63.15% and 62.66% respectively. It is important to keep in mind that the substance that has evaporated does not necessarily consist purely of water but may represent many other volatile compounds as well. The value of 36.0 in table 3 suggests that we have about 36 grams of carbohydrate per 100 ml solution, considering 1 degree brix is equal to 1 gram of sucrose per 100 ml. However, it is unlikely that the dry fraction is 97.38% carbohydrate, because the brix measurement does not discriminate between carbohydrate and most organic compounds[27].

3.1.2 Phenol-Sulfuric acid assay

The amount of carbohydrates in this mixture will in large part determine its value for a wide range of applications. Thus, a phenol-sulfuric acid assay is performed for quantification. This method works by first hydrolysing all possible carbohydrate forms such as polysaccharides, oligosaccharides and disaccharides, including their respective anhydro forms to mono-saccharides with sulfuric acid. Then, pentoses are dehydrated to furfural and hexoses to hydroxymethylfurfural. These compounds react with phenol to produce a complex that gives a yellow colour[28]. The extinction was measured at a wavelength of 485 nm.

Table 4. The absorptions, dilutions and final concentrations of the initial sample. The first two samples seem to deviate beyond reasonable extent, but are still included in the average calculation.

Sample	Dilution	Absorption @ 485nm	mg/ml
1	2000x	1.43	164.6
2	2000x	1.46	168.2
3	2000x	1.56	178.7
4	2000x	1.53	175.4
5	4000X	0.79	180.5
6	4000x	0.79	180.5
7	8000x	0.39	178.4
Average	-	-	175.2

The average value found is 175.2 mg/ml (or 175.2 g/L), as seen in table 4. It is likely that this number is an overestimation of reality, because pyrolysis products often contain furfural and hydroxymethylfurfural by themselves already[19], these arise in pyrolysis due to glucose dehydration at elevated temperatures. Therefore, it is likely that the values presented are an overestimation of actual carbohydrate content.

3.1.3 Folin-Ciocalteu assay

The phenol content is highly relevant for fermentation, considering that phenols are found to be the most contributing to the inhibitory properties of pyrolysis products[19]. The phenol content is quantified using the Folin-Ciocalteu assay, this reagent consists of a mixture of phosphomolybdate and phosphotungstate, which react with all phenolic compounds. The resulting molecules show absorption at 550 nm. Gallic acid is used for calibration.

Table 5. The interpolated concentrations of total phenols in the initial sample taking into account dilution and absorbance.

Pyrolytic Sugar Sample	Dilution	Absorption @ 550 nm	Total in 1 L (g)
1	40x	1840	21.3
2	40x	1819	21.1
Average	40x	1830	21.2

Table 5 shows an average of 21.2 g/L total phenols. This is amount of phenols is likely to interfere with fermentation [11]. Folin-Ciocalteu assay gives an impression of total reducing capacity. Each particular phenolic compound has a specific reducing capacity, and gallic acid is a representative average of all phenolic compounds[29]. Therefore, it is a quite rough estimation. On top of this, Folin-Ciocalteu reagents react with more reducing substances next to phenol, further skewing the values found[29].

3.1.4 Glucose and Levoglucosan quantification by HPLC

The first target for quantification were both glucose and levoglucosan, which were both hypothesized to be in this mixture. Using an HPLC device, an external calibration was done and retention times for glucose and levoglucosan on a Aminex HPX 87H herecolumn at 35°C were found to be about 11 and 15 minutes respectively.

Table 6. The quantities of glucose and levoglucosan of unedited pyrolytic sugar, test is performed three times.

Pyrolytic Sugar Sample	glucose (g/L)	levoglucosan (g/L)
1	2.1	48.4
2	2.3	49.0
3	1.8	48.2

A similar approach was used for quantifying other compounds that were expected to be present[22], again using an external calibration method.

Table 7. The found quantities of 9 different compounds. Performed in duplo.

Pyrolytic Sugar Sample	Glucose (g/L)	Levoglucosan (g/L)	Acetic acid (g/L)	Formic acid (g/L)	Glycolaldehyde (g/L)
1	1.7	49.0	44.7	17.7	44.8
2	1.8	48.3	44.3	17.9	46.9
Pyrolytic Sugar Sample	**5-HMF (g/L)**	**Fructose (g/L)**	**Methanol (g/L)**	**Ethylene glycol (g/L)**	
1	5.2	2.6	0.4	11.0	
2	5.2	2.6	0.5	10.6	

On the UV spectrum, the largest peak remained unknown (figure 4), even after injecting 20 of the most commonly found pyrolysis oil products, no compound was found to have a similar retention time as this peak (37.925).

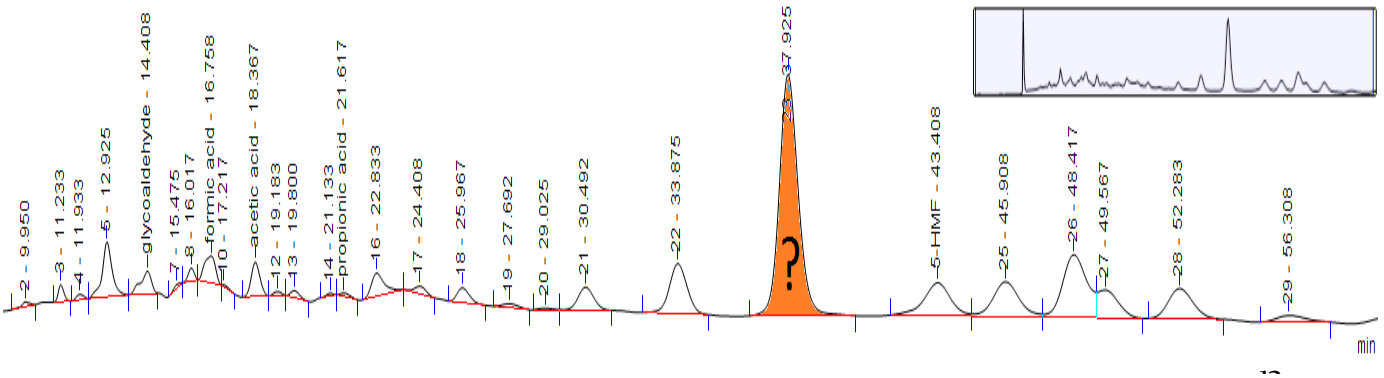

Figure 4. UV absorption @210 nm over the course of 60 minutes. HPLC was operated at 50°C to retrieve this chromatogram.

3.1.5 Titration

A titration with the strong base NaOH was performed in order to get an impression of the amount of acid that is present in the pyrolytic sugars. The sample was diluted 5 times with Milli-Q (MQ).

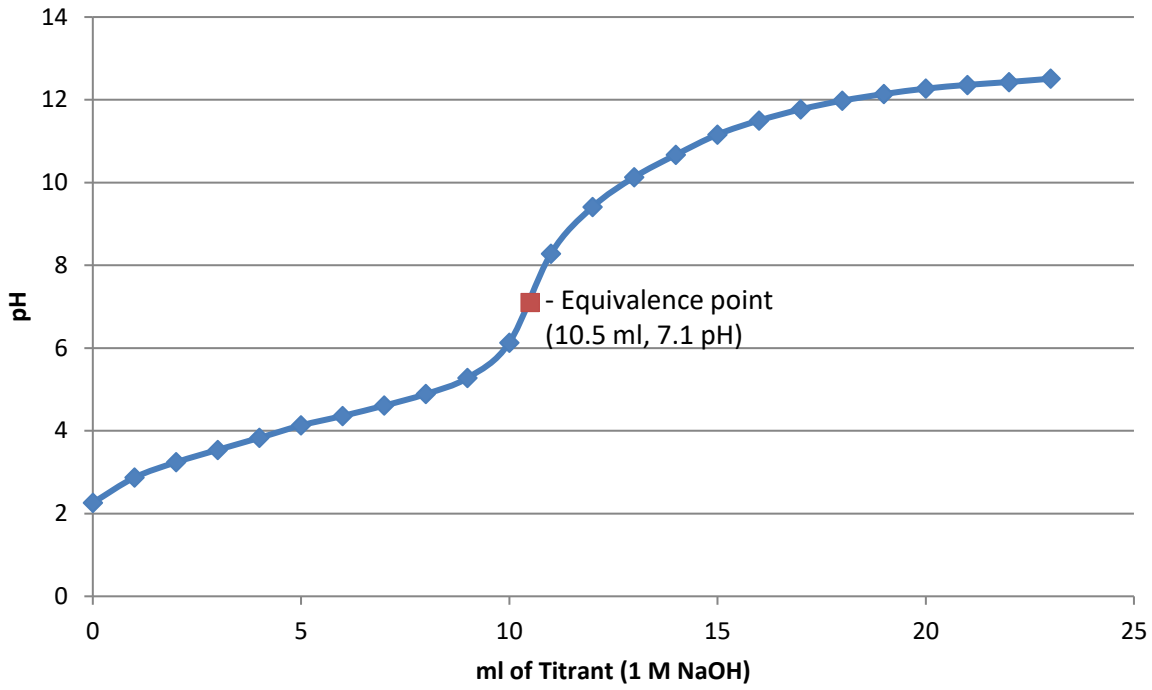

Figure 5. Titration curve, pH versus titrant added

The equivalence point of this titration was found to occur after having added 10.5 ml of titrant and reaching a pH of 7.1, see figure 5. Considering strong base is used, and pyrolytic sugars are hypothesized to only contain weak organic acids, an indication of mole acid can be given. According to the titration curve, about 0.0105 moles of strong base is required to neutralize all acid, in other words, 0.0105 moles of weak acid is present in 10 ml of pyrolytic sugars. If this were all consisting of acetic acid, this would mean 63 g/L of organic acid. This is in agreement with results found with the HPLC, formic acid and acetic acid comprise the largest portion of pyrolytic acids, and they add up to 62 g/L according to HPLC results.

3.1.6 Fourier Transform Infra-Red

Two samples, that are prepared using ATR technique, are analysed using the Fourier Transform Infra-Red (FTIR) method. One sample unedited pyrolytic sugars, is directly pipetted onto the device. The second sample is a dried pellet, yielded from a centrifuge operation onto a 10x diluted pyrolytic sugar sample. The spectrum was recorded from 500 cm^{-1} to 4000 cm^{-1} and differences in absorption were observed between the two samples.

FTIR is in principal designed for the identification of pure substances, and complex mixtures such as this yield such a vast array of peaks that drawing of any meaningful conclusions is unlikely, nonetheless, differences between samples are significant enough to include them in this report.

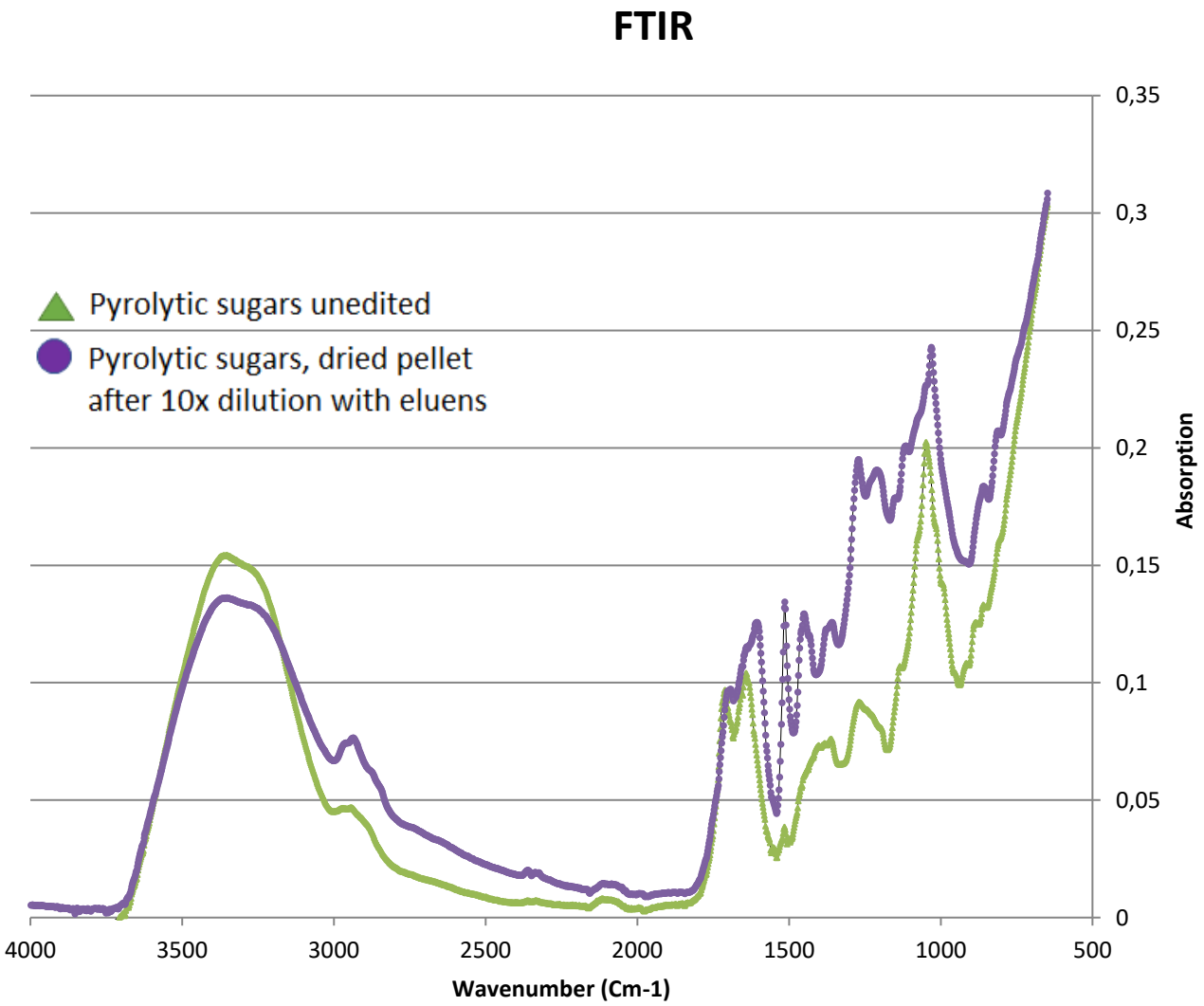

Figure 6. Infrared absorption at various wavelengths of unedited pyrolytic sugars and the dried pellet.

The spectrum seen in figure 6 is rather complex and will only give a very rough quantification of functional groups present in pyrolytic sugars. Generally, it can be stated that the absorption in the area between 3000 and 3500^{cm-1} are a result of –OH functional groups. Since water is likely to be a part of at least the pyrolytic sugars fraction, this is probably responsible for that peak. What is remarkable however, is that seemingly the unedited pyrolytic sugars contains just as much –OH groups as does the pellet sample, which is dried. This leads to the belief that perhaps different compounds are present in the pellet to larger extent, that also contain –OH groups. Phenolics are such compounds, which are often found in pyrolysis products.

In the area between 1000 and 1500 cm^{-1} another large variation between the two samples can be observed. Although these peaks are too rough to say anything impactful about them, it could be that more compounds are present with C-O bonds, such as carboxylic acids and alcohols in the pellet sample.

3.1.7 TGA-MS

The thermogravimetric analysis gives insight into the degradation properties of a sample under increasing temperature. By monitoring the weight of the sample and by connecting a mass spectrometer to it, data is gathered. In this instance, the device is set up to increase temperature 20 ° C per minute.

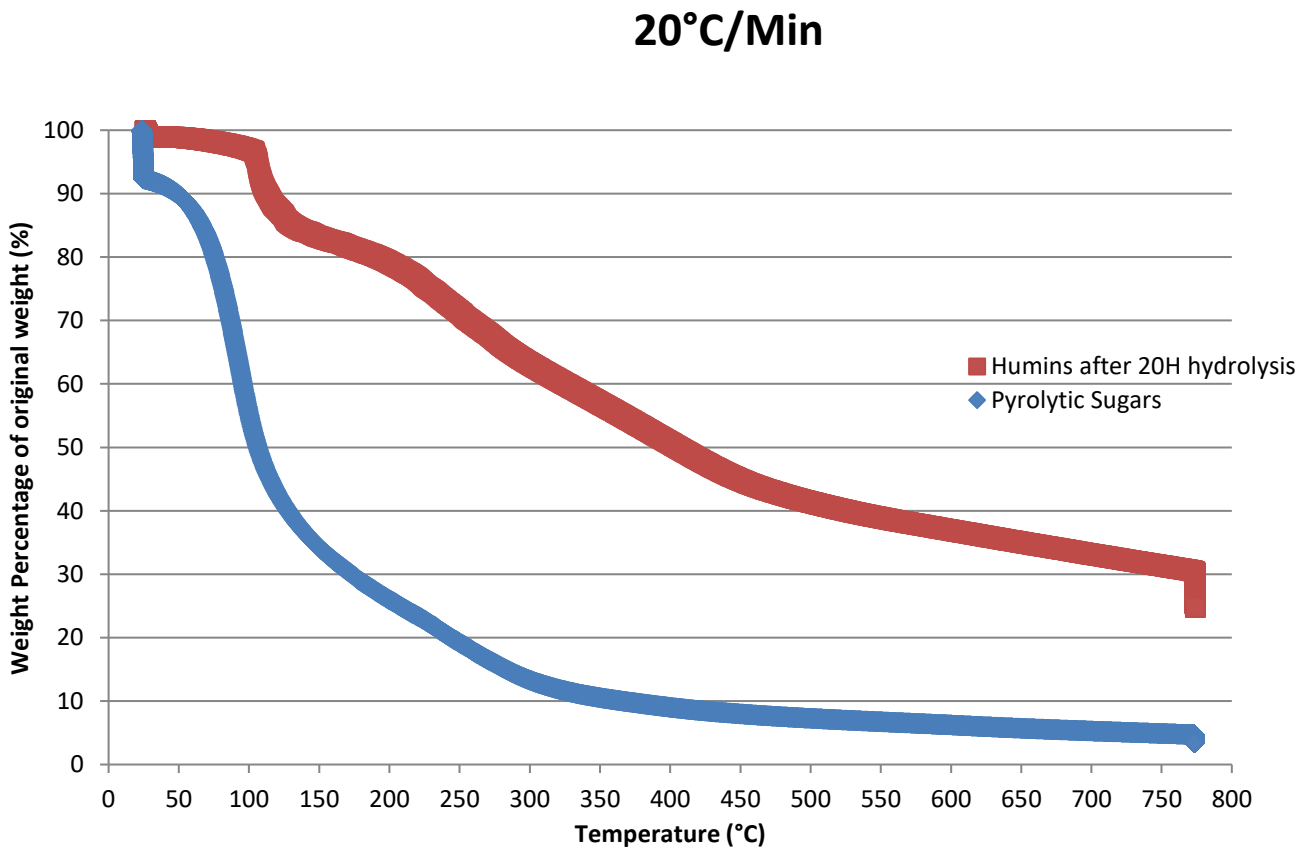

Figure 7. Weight percentage plotted against temperature. The initial weight was 25,7 mg.

The initial highly steep part the graph of figure 7 is not more volatile then what comes after, it is merely that the temperature is kept constant by the device for a couple of minutes. In these few minutes, components such as methanol, ethanol or acetaldehyde but also perhaps water are evaporating.

It can be observed that the part from 24-100 degrees decreases much quicker than what comes after, this is probably because all the volatile components/water has evaporated when it reaches 40% of its weight – this notion is in compliance with previously found results, where the non-volatile fraction was observed to be around 40% also.

The part between 110 and 300 °C is hypothesized to be mainly glycolaldehyde, organic acids and furfurals, considering their boiling points (glycolaldehyde = 131 °C, acetic acid= 118 °C and hydroxymethylfurfural(5-HMF)=115°C). Although simultaneously the 6 carbon sugars are probably degrading into smaller molecules (such as 5-HMF) and therefore also adding to the stream of evaporation. Phenolic compounds are also evaporating at this point, but might also act as reactants.

At 300-400°C most of the carbohydrate monomers are starting/have reacted to react via various degradation pathways. These pathways eventually lead to more glycolaldehyde, HMF, levulinic acid, lactic acid, etc. These compounds may polymerize to form complexes (humins) but may also evaporate due to their relatively low boiling points. These complexes in turn degrade again once the temperature is high enough.

The final weight that is achieved is about 4% of the original, however, given more time this could have probably reached less than 1% where the only thing left are inorganic substances (minerals, metals, etc.). In other words, the remains would be ashes. This was confirmed by heating a sample of pyrolytic sugars to 900°C and keeping it there for 10 minutes, the remains were less than 1% of the original weight.

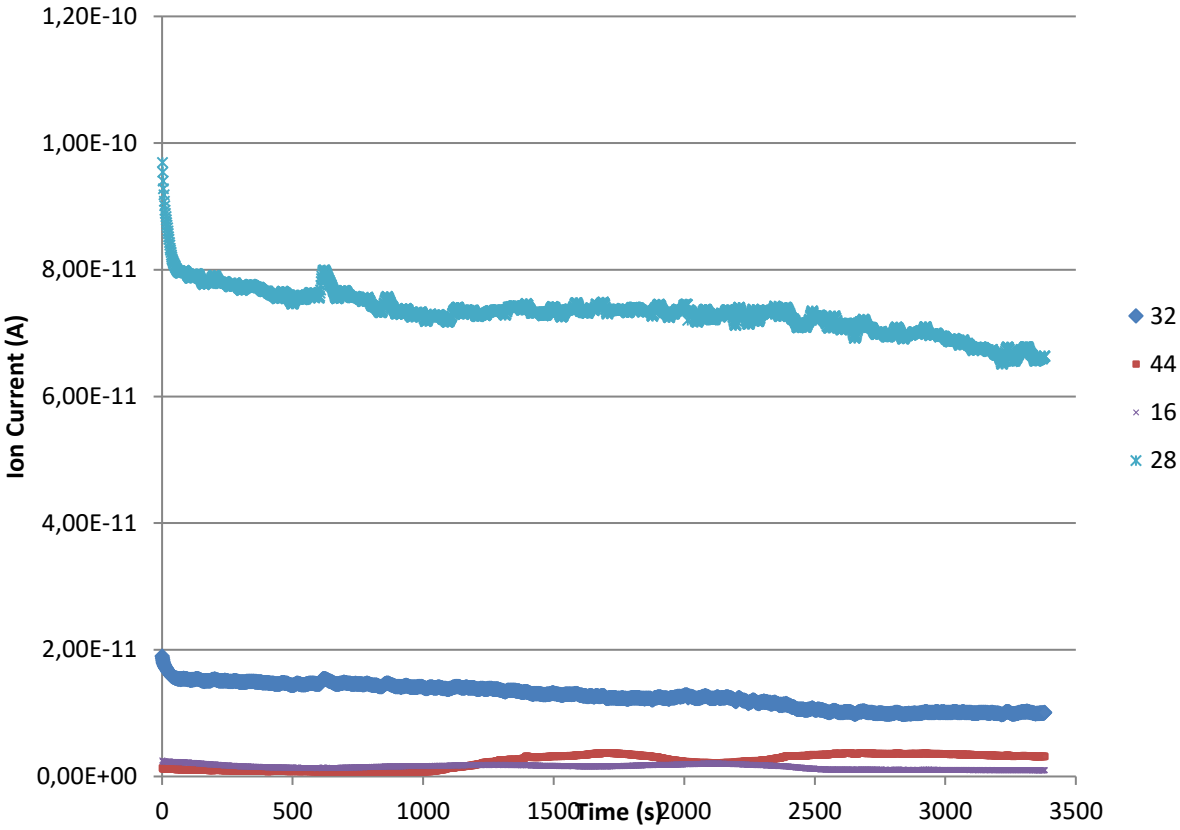

Figure 8. Ion current over time from various masses, as measured by the MS device.

The vapour stream that the TGA produced was also guided through an MS device, with the help of 50 ml/min argon gas as a carrier. This argon gas, which is also detected by the mass spectrometer, is left out of the graph in figure 8. The four masses are most likely: O_2 (32), CO_2 (44), atomic oxygen/CH_4 (16) and CO (28). The fact that all these masses are detected at rather constant levels throughout the process of thermal breakdown is surprising, considering different compounds contribute differently to the stream of vapour. Perhaps this could be explained by how compounds are broken down: a polymer like cellulose does not suddenly turn into vapour, it first breaks down into monomers which in turn break down into smaller carbohydrates, which in turn break down into organic acids, etc. The eventual compounds that evaporate are always the same. Nonetheless, e.g. organic acids could not be determined using the MS, due to too much background noise.The plenitude of oxygen in the hypothesized vapour stream suggests an aerobic environment and thus a leakage in the TGA-MS set up.

3.1.8 LC-MS

An LC-MS in combination with a UV/VIS spectrometer was used to identify unknown compounds. The UV/VIS chromatogram gives the absorption at 210 nm over the course of 60 minutes, when comparing this chromatogram to earlier found chromatograms from the HPLC, it is found to be very similar. For example, the largest peak occurs at around 35 minutes (see figure 9), which is similar to the retention time of the unknown peak in the HPLC UV/VIS chromatogram, which was 33.9 minutes. This is why it was assumed when inspecting the LC-MS data, when looking for specific compounds, that the retention times are more or less the same. This is no surprise because here, the eluent is 5 mM formic acid instead of sulfuric acid in MQ whilst the column is the same.

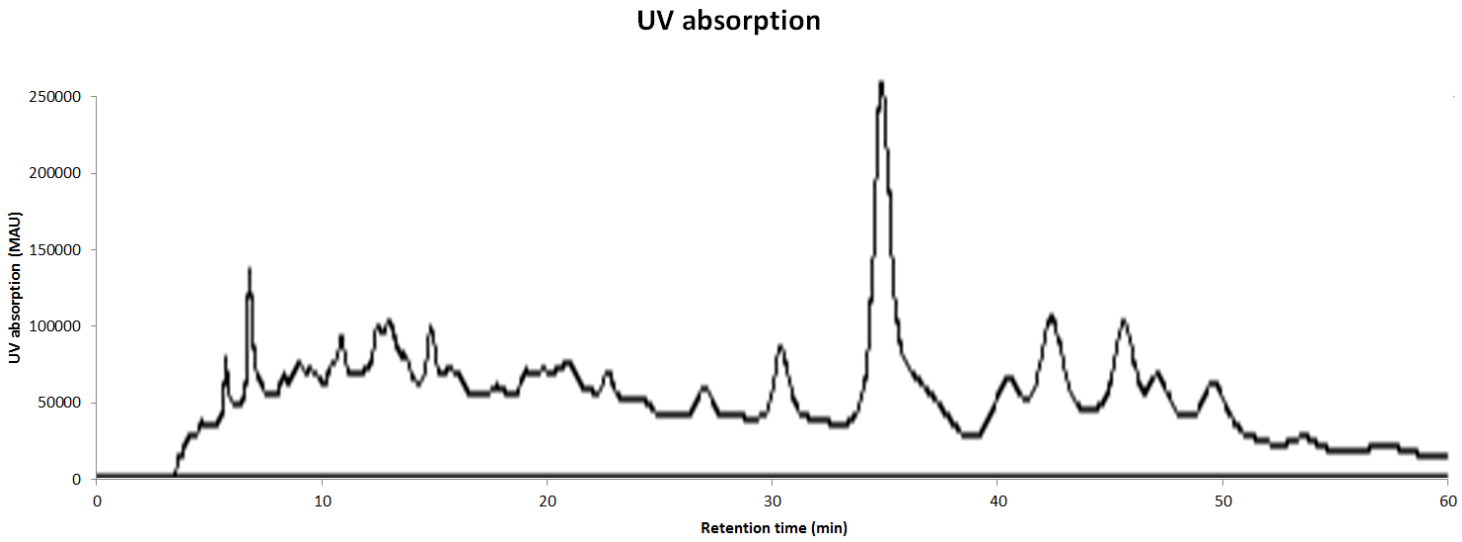

Figure 9. UV/VIS chromatogram, recorded @ 210 nm.

Another chromatogram was produced by the LC-MS analysis. Here, the relative abundance of compounds, with a mass between 50-2000 Da is plotted against the retention time, the resulting graph can be seen in figure 10. It is clear that the highest number of compounds leave the column before 20 minutes. This makes sense because acetic acid, levoglucosan, glycolaldehyde together comprise by far the largest portion of the non-volatile fraction, and these leave the column all before 20 minutes, according to the HPLC analysis.

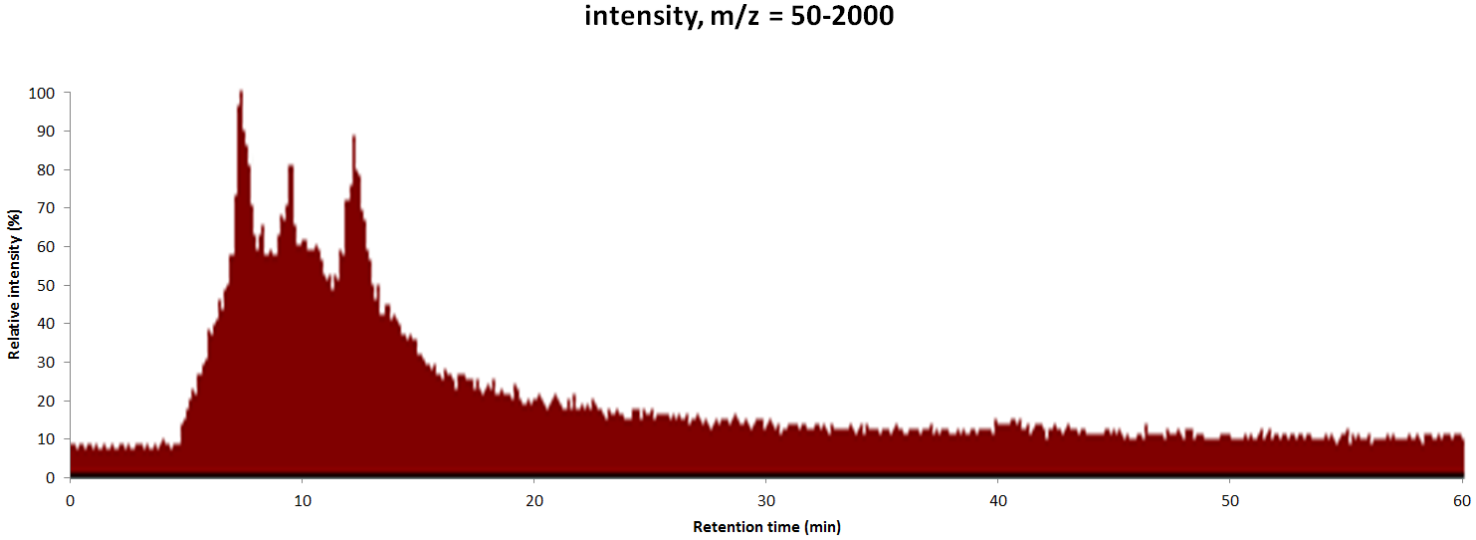

Figure 10. Relative abundance of compounds with masses between 50-2000 Da

Two compounds were identified with high accuracy: levoglucosan and catechol. Their retention times indeed matched those found in the HPLC analysis. Below, an explanation is given for how levoglucosan was identified and how the spectra were interpreted.

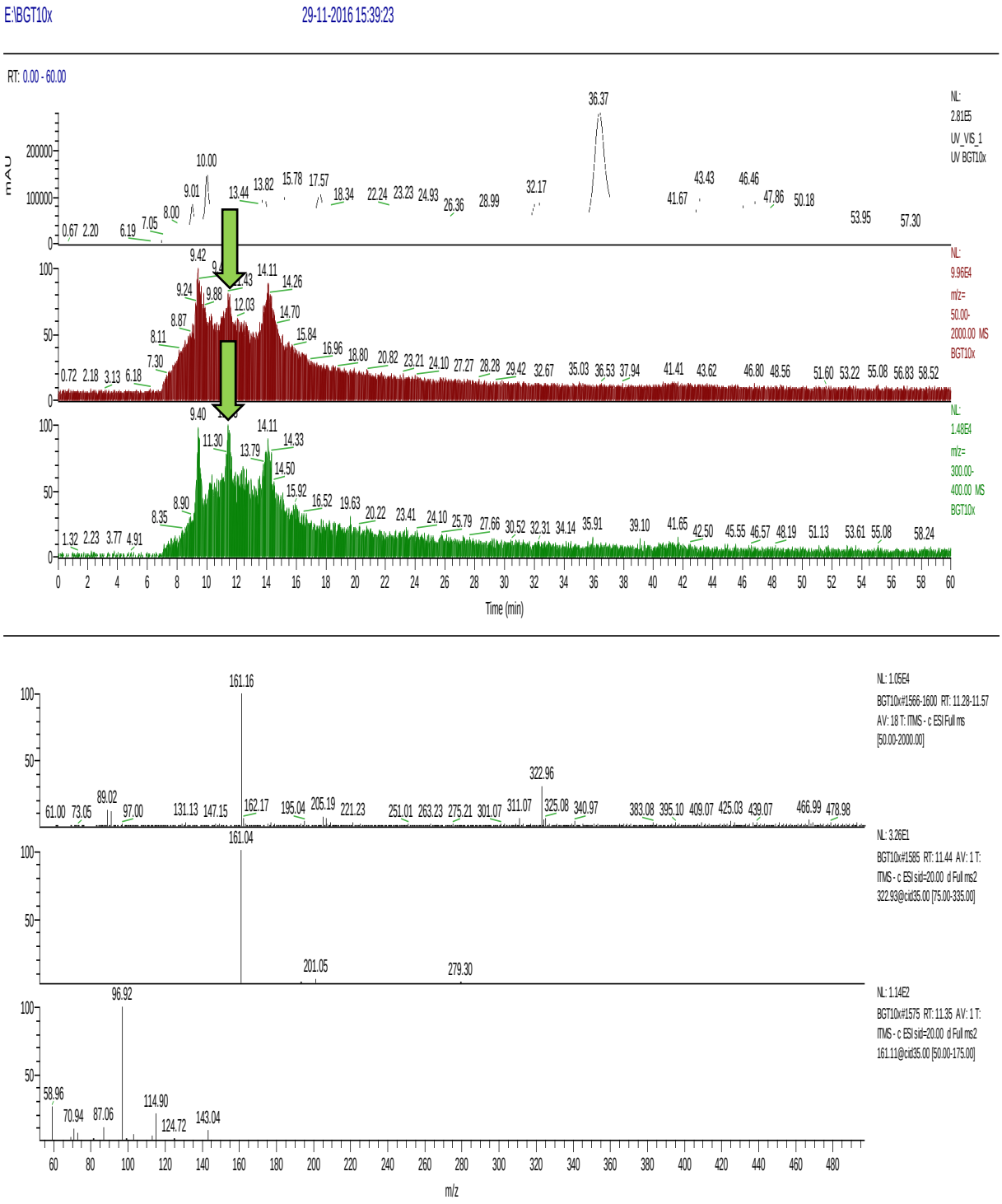

Figure 11. The three chromatograms are: UV/VIS absorption @ 210 nm (white), Relative abundance for masses between 50-200 DA (red), relative abundance for masses between 300-400 DA (green). The upper mass spectrum is the relative abundance of masses between 11.28 and 11.57 minutes, the middle one is the same but at exactly 11.44 minutes, the bottommost mass chromatogram is the MS2 spectrum.

A clear peak can be distinguished at around 11 minutes in the relative abundance spectra (figure 11), both in the 50-200 Da chromatogram and the 200-400. This is surprising considering levoglucosan has a mass of 162 Da and therefore is not expected in the 200-400 spectrum. However, this peak can be the result of levoglucosan forming a dimer after it becomes charged in the mass spectrometer.

The first and second mass spectrum (figure 11) show an expected highest peak: 161 Da, this is the mass of levoglucosan minus one hydrogen (due to the way this spectrometer operates).

The fragmentation that can be observed in the MS2 graph (figure 11), is not completely as expected. The highest peak indicates a mass of 97 Da, this can possibly mean a fragment with a mass of 64 has separated from the complete molecule. Nowhere in literature does this get proposed as a fragmentation mechanism of levoglucosan, so it remains a mystery as to what these molecules are made up of. The small 143 Da peak is likely a result of levoglucosan dehydration (161-18=143) and the 87 Da peak is first a separation of C_3H_1O or C_2O_2 and then a dehydration (161-56-18=87).

3.1.9 Ageing

The pyrolytic sugars were stored throughout the project at 4°C, however, whether this alone was sufficient to prevent any change in composition, or so called ageing, was not known. Hence, when a fresh batch came in from BTG, the old and new pyrolytic sugars were immediately analysed on the HPLC. Below the chromatograms of both the RI detector and UV/VIS spectrophotometer can be found.

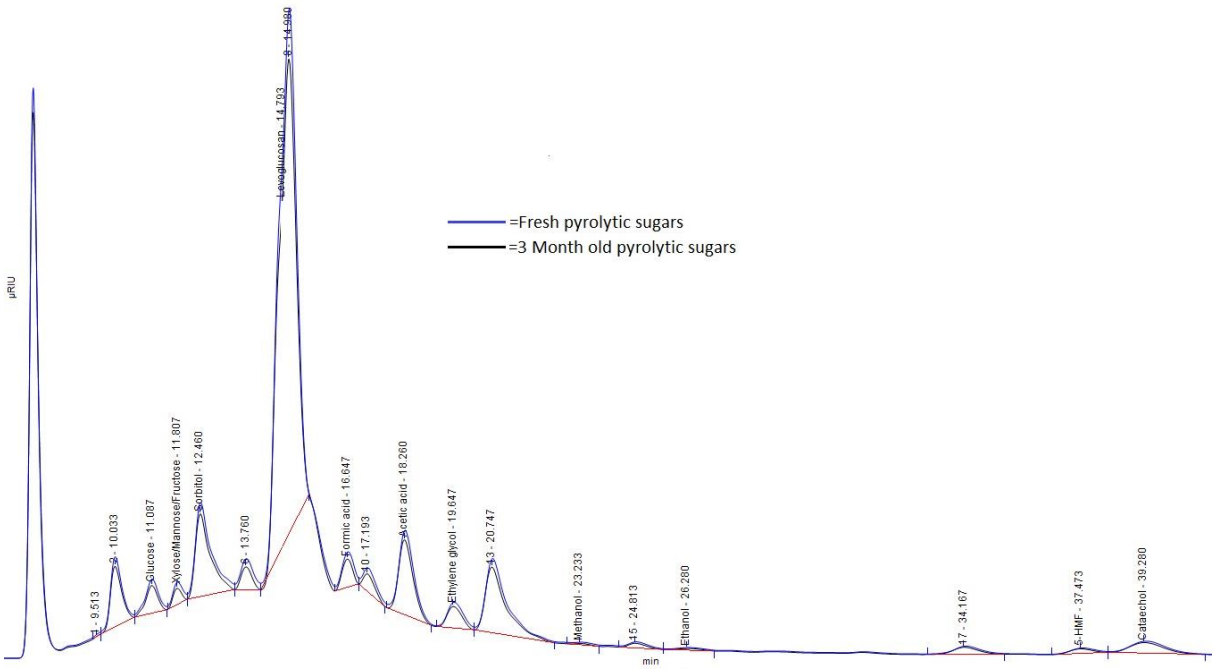

Figure 12. The RI chromatogram from the HPLC. A larger version can be found in appendix A.

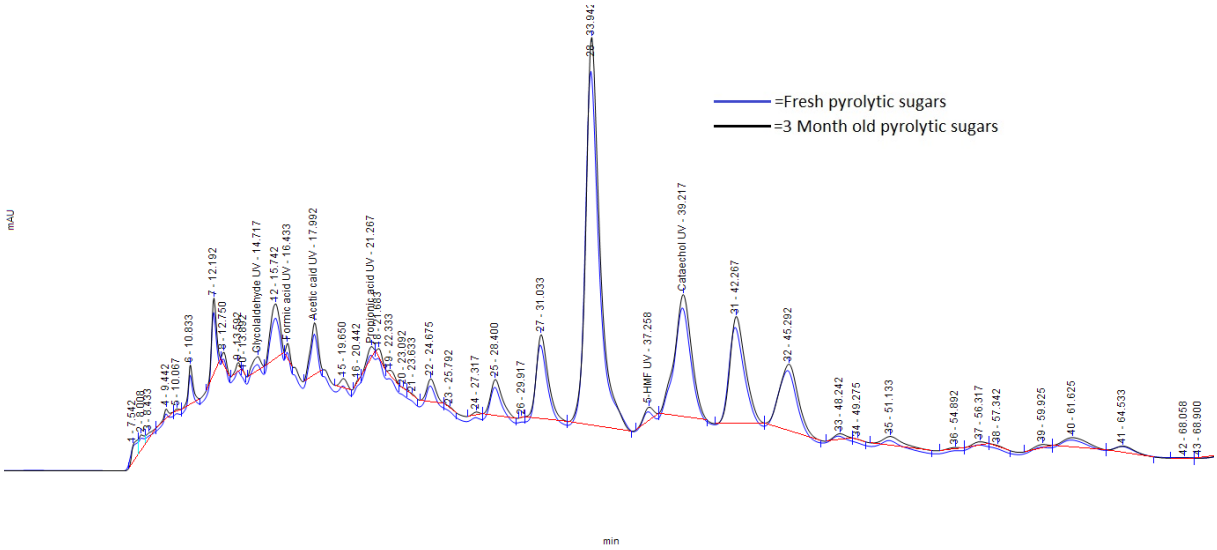

Figure 13. The UV/VIS chromatogram from the HPLC. A larger version can be found in appendix B.

From this analysis, it can be said that 3-month-old and new pyrolytic sugars are highly similar in composition. The small differences in peak areas can probably be attributed to pipetting errors rather than difference in composition, because they occur in all peaks to about the same extent. It must be noted however that components that are not visible on both RI and UV/VIS are excluded from the measurement.

3.2 Hydrolysis

3.2.1 Auto acid hydrolysis

Firstly, hydrolysis was performed without any addition of acid at 120°C, to see if hydrolysis of levoglucosan/oligomers takes place merely by presence of organic acids. Via HPLC analysis the change in composition was monitored and the increase in glucose concentration quantified. This change over time in percent converted levoglucosan (where an initial concentration of 42 g/L levoglucosan is assumed) is plotted.

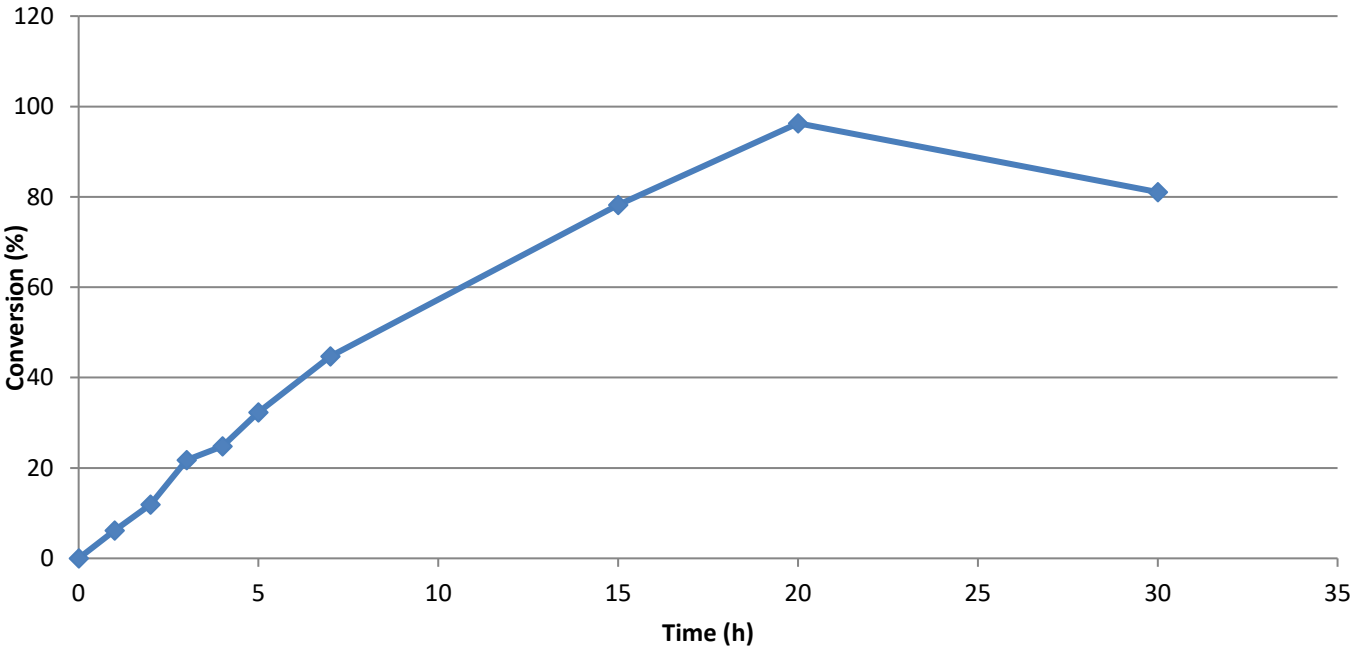

Figure 14. Percentage levoglucosan converted into glucose after exposure to 120°C heat treatment (acquired from HPLC via external calibration) plotted against time in hours. No acid was added in this instance. Results are average of duplo measurements.

In figure 14, a near linear increase in glucose concentration can be observed from 0 to 20 hours. Somewhere between 20 and 30 hours, an optimum of glucose is reached and the glucose concentration starts falling again. This decrease in glucose can be the result of various reaction pathways, some of which can be found in figure 14. When glucose concentration declines, this means the rate of formation of glucose is smaller than its conversion. Therefore, it is likely that before the optimum is reached the conversion of glucose into something else occurs at a slower rate than does the formation of glucose.

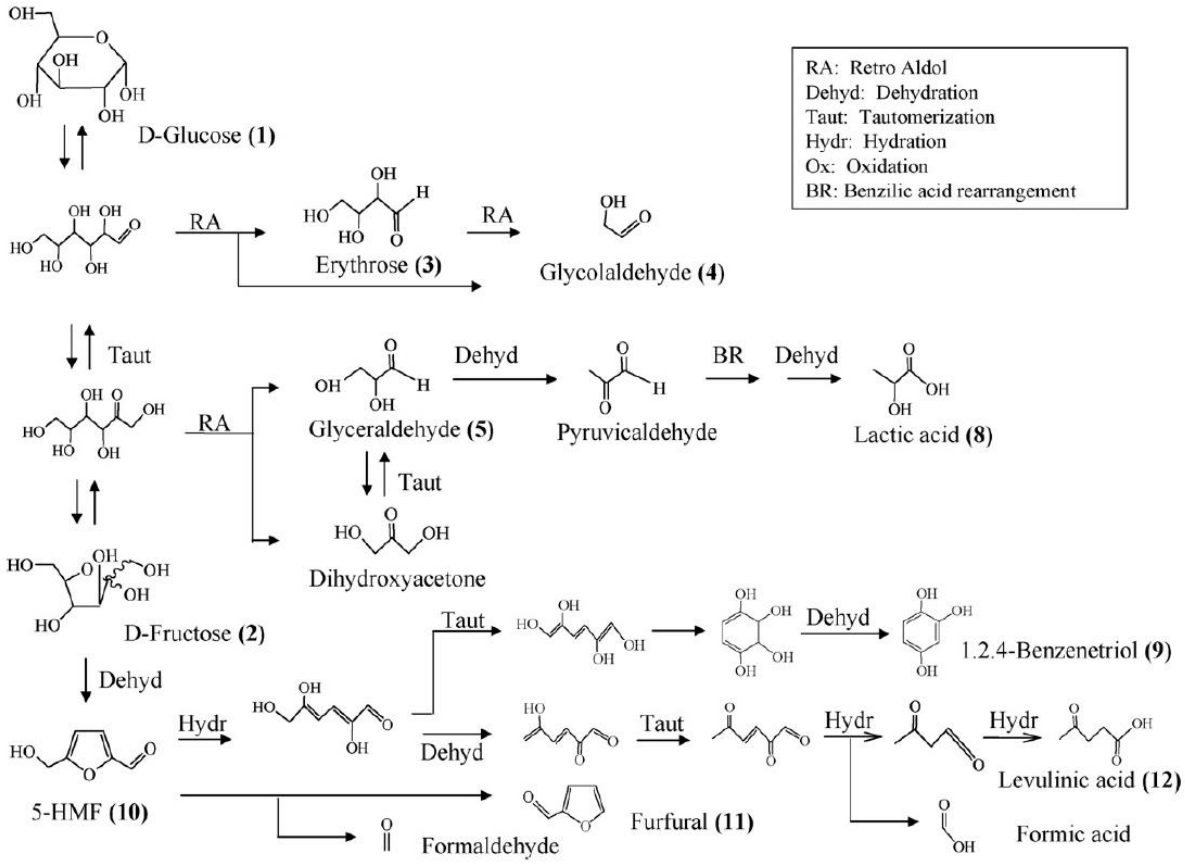

Figure 14. Breakdown of glucose can result in various compounds. Source: [30]

Remarkably, no significant increase in 5-HMF, furfural, formic acid or levulinic acid was observed upon further inspection of the HPLC chromatograms. This leads to the belief that these compounds are perhaps participating in polymerization reactions rather than remaining in solution as monomers, thereby contributing to solids that were found in the reactors after the oven treatment. This effectively excludes them from HPLC analysis due to a necessary filtration step before injection onto the column.

The increase in concentration cannot all just be attributed to the hydrolysis of levoglucosan or oligomers. Solid formation was observed in all samples past 2 hours, this meant a reduced liquid volume and therefore perhaps an unwarranted increase of glucose in the results, which could have only been the result of less water/liquid rather than hydrolysis. In the 20-hour sample, the remaining liquid was measured to be about 60% of the original liquid, the other 40% being solid.

3.2.2 Hydrolysis with 0.1M H_2SO_4

Strong acid catalyses hydrolysis of levoglucosan and oligomers. Thus, sulfuric acid was added to the samples before heat treatment to reach a concentration of 0.1 M. The resulting mixture is heated to 120 °C for various lengths of time. Again, percent of converted levoglucosan is plotted against time, see figure 15.

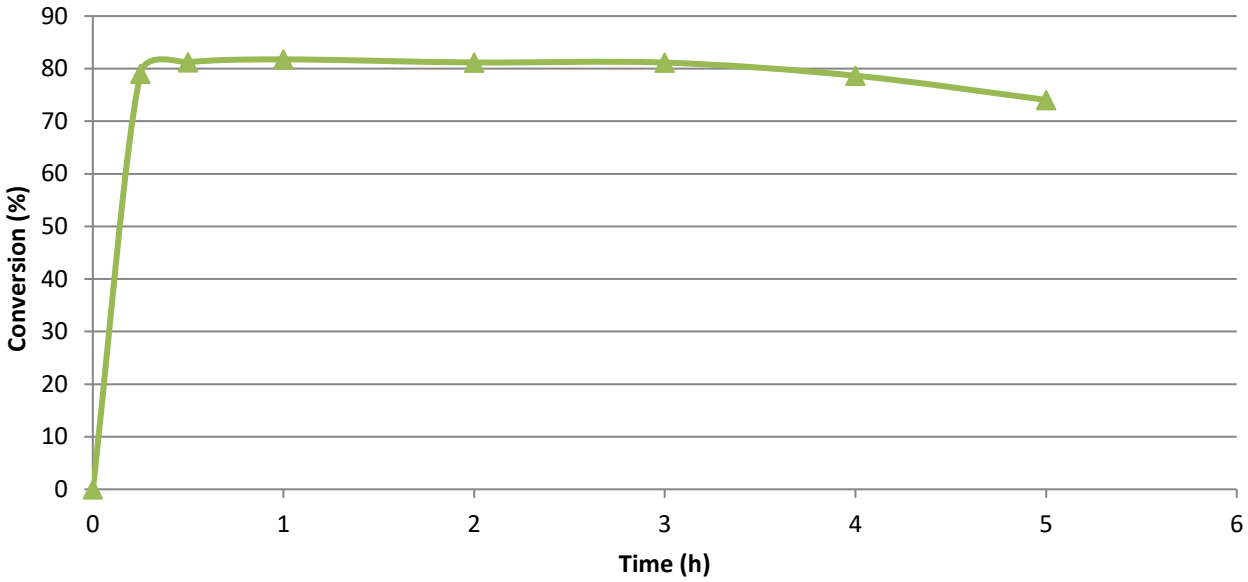

Figure 16. Percentage levoglucosan converted into glucose (acquired from HPLC via external calibration) plotted against time in hours. H_2SO_4 was added to reach a concentration of 0.1 M before heat treatment.

In figure 16 it can be seen that already within the first 30 min of hydrolysis, the highest yield is achieved. This confirms the function of strong acid as a catalyst. Remarkably, there is a plateau which is sustained between 0.5-3 hours. This can mean two things: Either the glucose concentration is kept constant because simply no formation nor breakdown is occurring within this timeframe, or the formation rate and breakdown rate are more or less equal for 2+ hours. Both options are unlikely. After 3 hours, the glucose concentration is declining – again, breakdown reactions are dominating here.

3.2.3 Hydrolysis after 1:1 dilution with 5mM H_2SO_4

A strong precipitation occurs when pyrolytic sugars are diluted 1x with 5mM H_2SO_4. This gave the insight that maybe these precipitated compounds would normally contribute to the polymerization and other unwanted side-reactions. On top of this, dilution can alter the equilibriums of various involved reactions, possibly in favour of glucose concentrations.

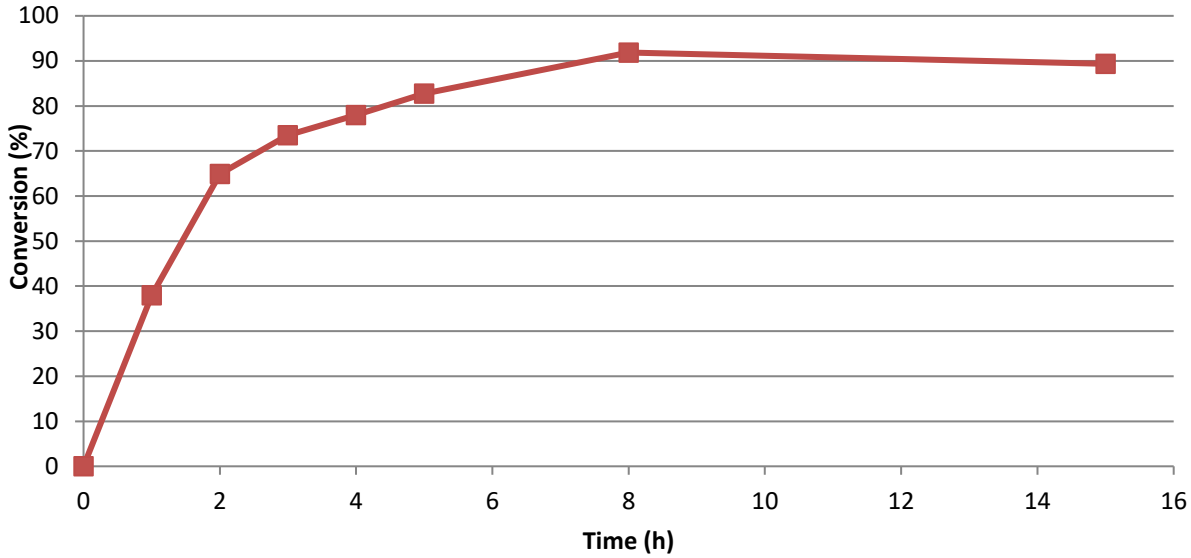

Figure 17. Percentage levoglucosan converted into glucose (acquired from HPLC via external calibration) plotted against time in hours.

In figure 17 it is observed that within 5 hours, a conversion percentage is reached that the undiluted pyrolytic sugars only reach after 15 hours. This is interesting, considering both the catalyst (assumed to be weak organic acids) and the substrate are present in half the concentration. This occurrence can be explained by the fact that not just the substrate and catalyst are diluted, so are the phenols, alcohols, furans, etc. which could all be stimulating or participating in unwanted reaction pathways. Furthermore, the precipitation caused by dilution is filtered out using a 0.2 µm filter, these compounds would normally remain in solution and perhaps interfere with hydrolysis or stimulate breakdown of glucose. The downside to this method is that the final glucose concentration is only half of what has been achieved with undiluted pyrolytic sugars, this could be an inconvenience for fermentation. Furthermore, it must be kept in mind that there is in fact more catalyst present in these samples considering 5 mM sulfuric acid was used for dilution, not merely water. However, the final concentration of sulfuric acid in the samples is 0.0025 M, which is 40-fold less than in the acid hydrolysis experiment.

3.2.4 Reactivity of separate components

Glycolaldehyde was observed to decrease when heating the sample at 120 degrees centigrade for more than 5 hours. This gave the idea that glycolaldehyde participates in certain reactions with other components which in turn may lead to new complexes or solid formation. To gain more knowledge about this phenomenon, the following liquid was prepared: 5 g/L 5-HMF, 50 g/L acetic acid, 5 g/L methanol, and 50 g/L glycolaldehyde. This was prepared two times: one vial was placed in the refrigerator, the other two were placed in a 120°C oven for 5 hours. Then, both the cooled and heated samples were injected onto an HPLC column.

When the sample came out of the oven, a change in transparency/colour was observed, see figure 18.

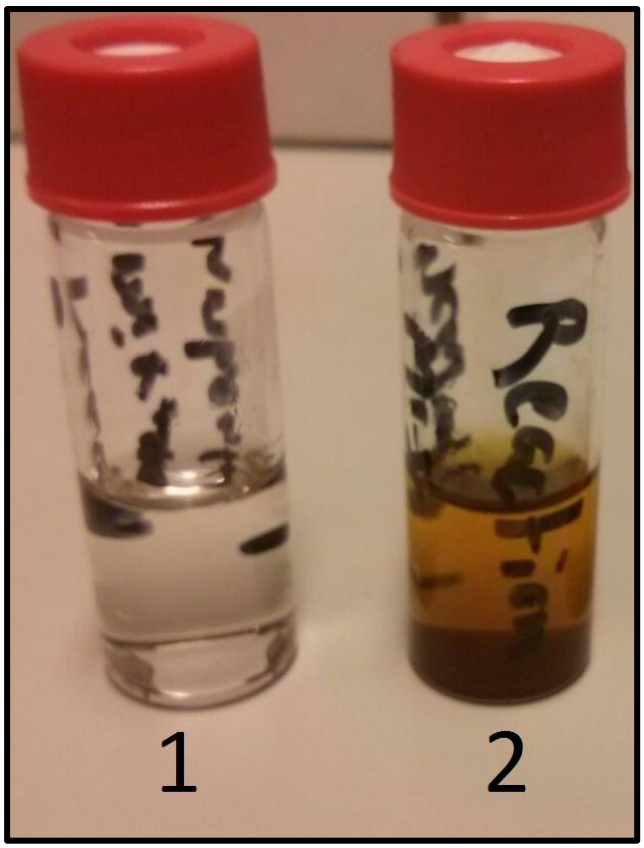

Figure 18. Unheated (1) and 5 hour long heated @ 120°C (2) reaction mixtures.

The HPLC chromatograms (which produced the numbers showns in table 8) show that acetic acid remains nearly identical in concentration before and after heating. This might indicate that acetic acid acts as a catalyst for reactions that take place during heating, or that it simply plays no role in any of these reactions. Glycolaldehyde concentration decreases significantly during heating, and it is not clear what the products of this reaction or degradation are, although a new series of peaks have formed in the heated sample, this is visible both on the RI chromatogram and UV chromatogram. 5-HMF also decreases, although to a lesser extent. Methanol peaks were observed but were too small for a reliable integration.

Table 8. Differences in concentration of three compounds between unheated and heated reaction mixtures.

	Glycolaldehyde (g/L)	Acetic acid (g/L)	5-HMF (g/L)
reaction mix unheated	47.04	48.46	5.24
reaction mix heated 5h 120C	23.22	48.27	3.35

3.2.5 Dilution behaviour

Pyrolytic sugars had the peculiar property of showing precipitation when it was diluted with water. This precipitation was even more noticeable when the pyrolytic sugars were diluted with HPLC eluent, which was 5mM sulfuric acid. To verify that this was indeed a true precipitation, and not suspended solids that only becomes visible due to dilution (because of the opaque appearance of the initial solution), the pyrolytic sugars was first filtered using a 0.2 µm filter and subsequently diluted. The results can be seen in figure 19. The unfiltered and filtered pyrolytic sugar are identical in precipitation behaviour, although this is only confirmed by inspection with the naked eye. In this same figure, the difference between using MQ and eluent for dilution can also be seen: the solid particles that are formed in the case of MQ dilution are apparently not heavy enough to form a sediment like the solid particles do in case of eluent dilution.

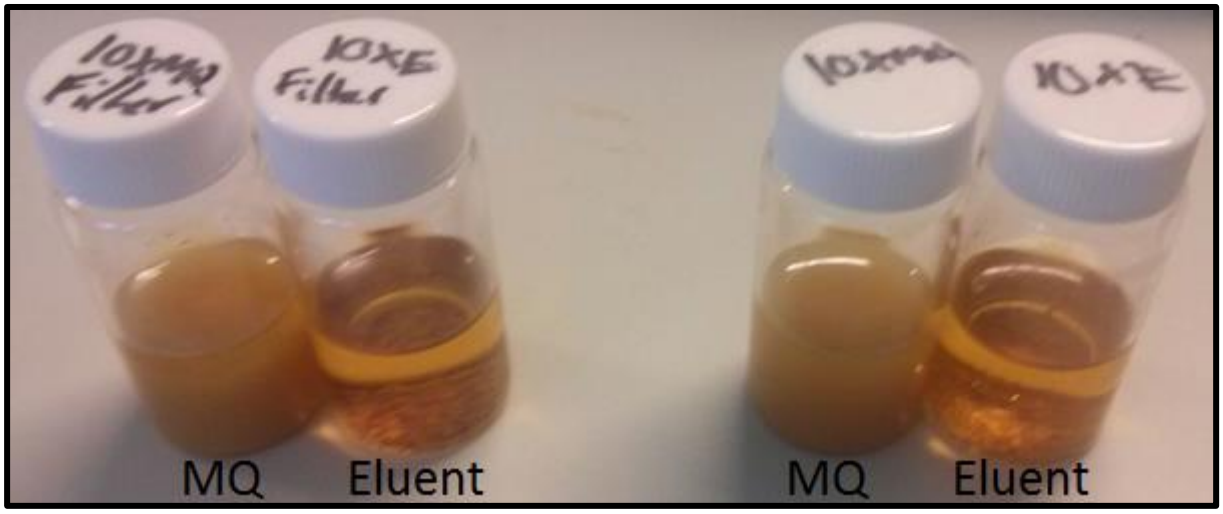

Figure 19. Pyrolytic sugar filtered using a 0.2 µm filter (left) and pyrolytic sugar unfiltered (right), then diluted 10x with MQ or Eluent.

The pyrolytic sugars that are diluted with eluent are much easier to filter than the MQ one, perhaps due to the same reason. When filtration is attempted with the MQ dilution, the filter gets clogged very quickly in contrast to the eluent dilution, which does not have this. Once filtration is completed however, the filtrates from both MQ and eluent dilution have similar clarity, see figure 20.

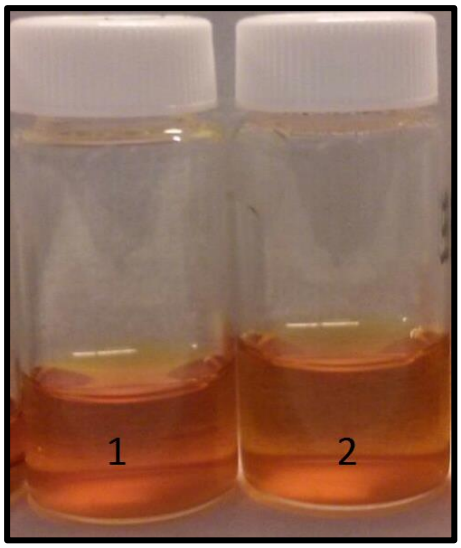

Figure 20. Filtered MQ dilution (1) and filtered eluent dilution (2), both with 0.2 µm filters.

3.3 Detoxification

3.3.1 Activated Charcoal

The composition of the pyrolytic sugar fraction is analysed before and after a treatment of 10 wt/wt% and 30 wt/wt% activated carbon using the HPLC. A UV chromatogram, recorded at 210nm is retrieved. Absorption at this wavelength is representative for inhibitory properties, see section on solvent extraction.

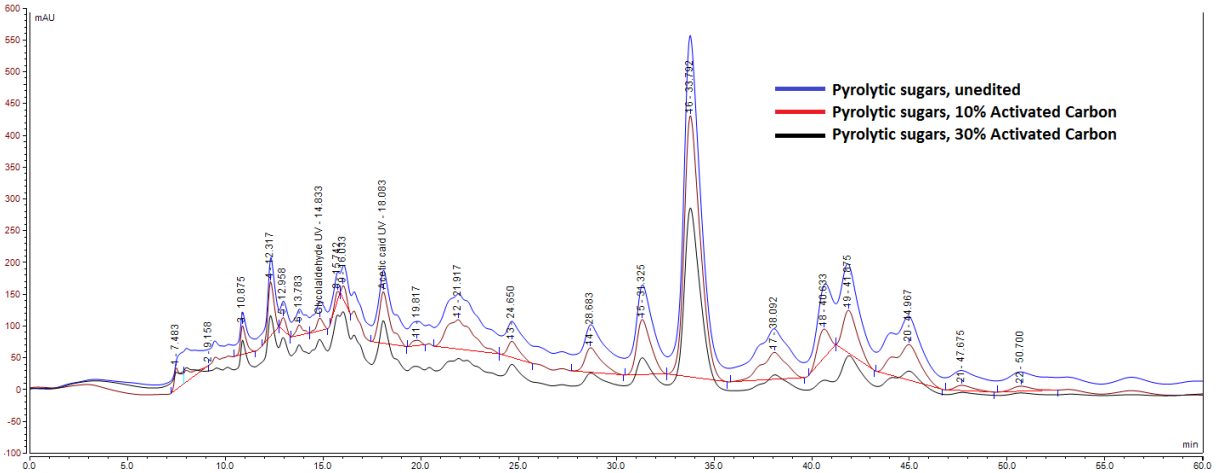

Figure 21. A UV/VIS chromatogram of pyrolytic sugars and pyrolytic sugars treated with 10% and 30% (by weight) activated carbon. Although some peaks shrink in size due to the treatment, none disappear completely. A larger version can be found in appendix E.

It can be observed in figure 21, that the absorption at 210 nm is decreased when pyrolytic sugars is treated with 10% and 30% activated carbon. 30% activated carbon is more effective at removing inhibitors than 10%.

3.3.2 Solvent extraction

Liquid-liquid extraction was carried out on undiluted pyrolytic sugars, in a ratio of 2:1 (solvent: pyrolytic sugars). The two phases were analysed, the polar phase with HPLC and the organic phase with GC-MS. Methyl isobutyl ketone (MIBK) and ethyl acetate (EA) were used as extractants.

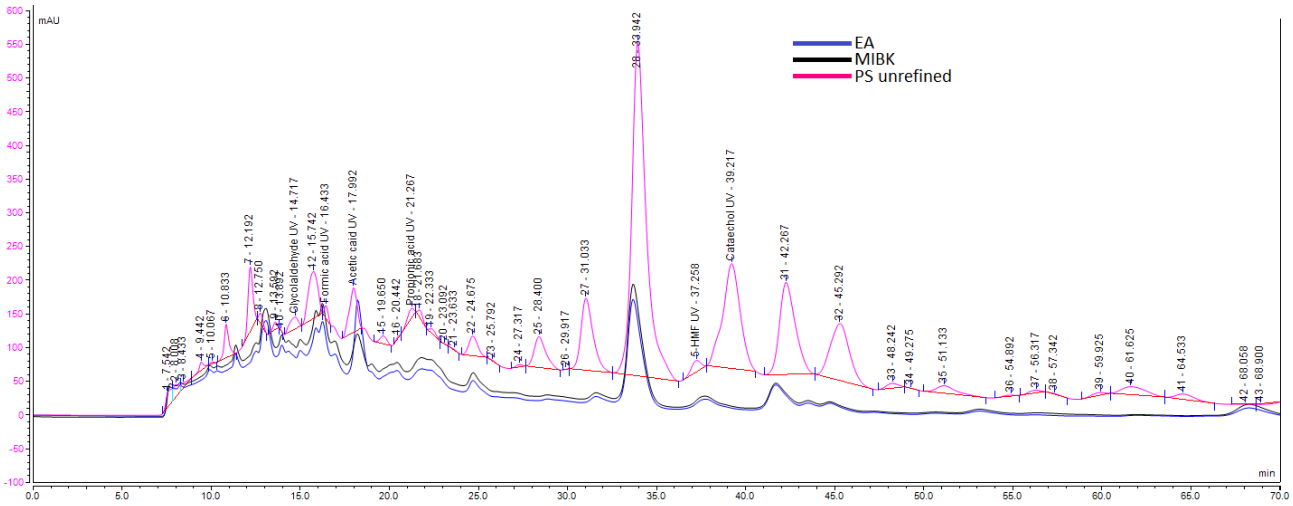

Figure 22. 3 UV/VIS chromatograms overlaid: EA treated PS, MIBK treated PS, and untreated PS. Recorded at 210nm. A larger version can be found in appendix C.

The UV/VIS detector was operated at 210 nm because absorption at this wavelength is representative for overall toxicity of pyrolysis products [19]. Phenols, furfurals, furans and more inhibitory compounds all show up at this wavelength.Hence, ideally, the UV/VIS chromatogram should show no absorption at all after solvent extraction, whereas the RI chromatogram should have an unchanged levoglucosan peak.

In figure 22 it can be observed that, the MIBK and ethyl acetate both show a significant decrease in UV absorption, especially from 20 minutes onward. Therefore, inhibitory solutes such as phenols and furfurals have migrated from the polar to the organic phase. The chromatograms are slightly misaligned, i.e. the peaks from the polar phases come slightly earlier than the PS chromatogram, this is due to somewhat different conditions such as pressure and the hydrophobicity of the column.

Table 9. Areas of various compounds, including 'unknown peak' as defined in the HPLC section.

	Levoglucosan (µRIU)	Unknown Peak retention time 33.942 (mAU)	Catechol (mAU)	5-HMF (mAU)
PS Unrefined	26.5	465.8	155.8	10.4
EA Polar Phase	24.0	145.9	0	21.9
MIBK Polar Phase	25.0	163.1	0	24.4

Table 9 shows a slight change in levoglucosan concentration in both MIBK and EA treated PS, a 6% and 10% decrease respectively. Both the unknown peak and catechol show significant reductions in area, the catechol even diminishes to zero during the solvent extraction.

HMF shows an increase instead of a decrease which is highly implausible. Upon further inspection of the chromatogram, it was found that this inconsistency was possibly the result of how the peaks were integrated and not per se by what quantity was actually in the sample. This is clarified in figure 23. The 5-HMF peak is flanked by two other peaks, this causes part of the 5-HMF peak to disappear into the belly of these two peaks. This problem has been encountered many times in this project and this is not surprising considering the complexity of pyrolytic sugars.

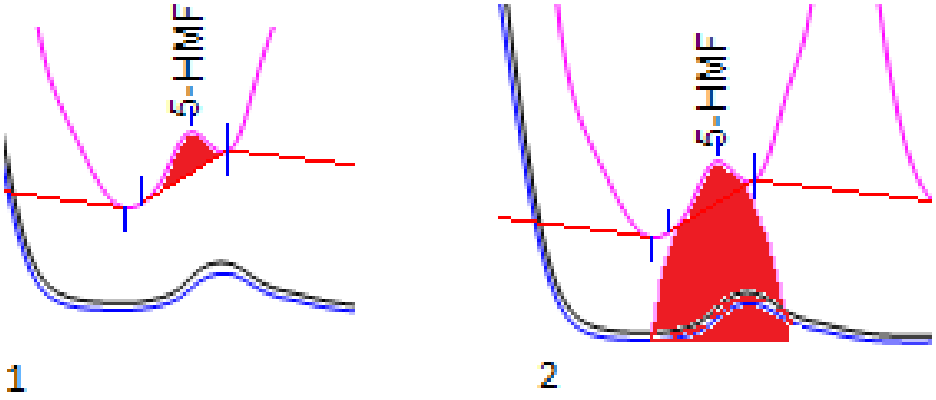

Figure 23. An example of how the 5-HMF is integrated (1) and what area 5-HMF could in reality comprise (2).

3.4 Fermentation

Yeast fermentations were carried out to test for inhibitory properties of the BTG mixture and to test for potential in a co-feeding setup for ethanol production. Effects of preparatory steps can be observed in the graph below.

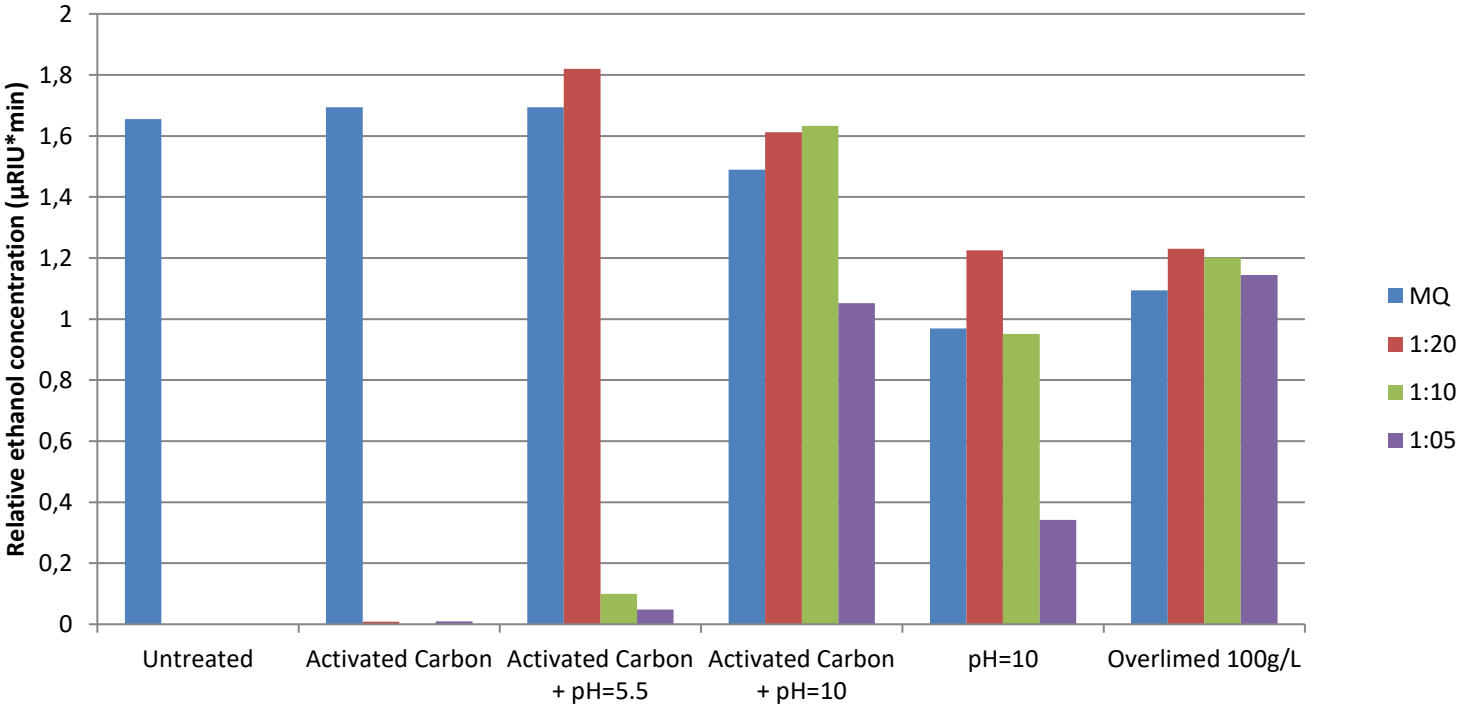

Figure 245. Ethanol production after 40 hours of fermentation on varying media. All fermentations start with 40 ml of medium and a 0.5 g inoculation with baker's yeast. About an hour after inoculation, either 2, 4 or 8 ml of (detoxified) pyrolytic sugar is added to the medium, then, a 'before' sample is taken for the HPLC. 40 hours later the second sample for the HPLC is taken.

In figure 24, the 6 blue bars represent the blank of each experiment. In these fermentations, only 4 ml MQ was used to supplement the 40 ml of medium and no pyrolytic sugars was added. Therefore, these bars give an impression of what ethanol concentrations could be achieved if no inhibition were to exist at all. Remarkably, a large variation between these blanks can be observed in each separate experiment which indicates an error. This could be attributed to a slightly different medium composition each time or variation in yeast vitality. Pipetting errors might be responsible as well. The red, green and purple bars represent the different volumes of pyrolytic sugars that was added to the 40 ml medium: 2,4 and 8 ml respectively. Thus, it makes sense that ethanol concentrations decrease accordingly: namely, they decrease once pyrolytic sugars addition increases, because pyrolytic sugars contain microbe inhibitors.

Activated carbon treatment seems to bring about very little improvement in fermentability by itself, seemingly only upon combination with deacidification it becomes effective. However, only deacidified (pH=10) pyrolytic sugars shows significant improvement in ethanol production, therefore it could be said that the contribution of activated carbon to detoxification is rather meagre, though not negligible. This is in accordance with the HPLC

results performed on before and after activated charcoal treated pyrolytic sugars, see figure 20.Overlimed pyrolytic sugars gives the cleanest substrate, considering a 1:5 addition of this substrate still gives significant ethanol production. The fermentations with added pyrolytic sugars show more ethanol content than the one with only MQ. This could be a result of more fermentable substrate from the pyrolytic sugars such as glucose, acids and more. Furthermore, the pyrolytic sugars by itself may already contain a small amount of ethanol, which also contributes to the detected amount.

3.5 Catalysis

Catalytic upgrading of the pyrolytic sugars was attempted using a platina catalyst. The catalysis was performed under basic conditions with 2 bar oxygen pressure. The 5-HMF conversion, as described in the background, was hypothesized to occur in the pyrolytic sugar sample as well. However, 5-HMF is not the only thing present in the pyrolytic sugar and therefore on top of 5-HMF, it was deemed interesting to see how the other compounds were affected as well.

The catalysis was performed at various pH levels, though only the pH 9 catalysis will be displayed here, because this was the only case that gave consistent data in the two separate attempts. It must be noted however that generally these types of catalytic oxidations are suggested in literature to take place best at 0.1 M NaOH, and therefore a pH of 13, not 9[31].

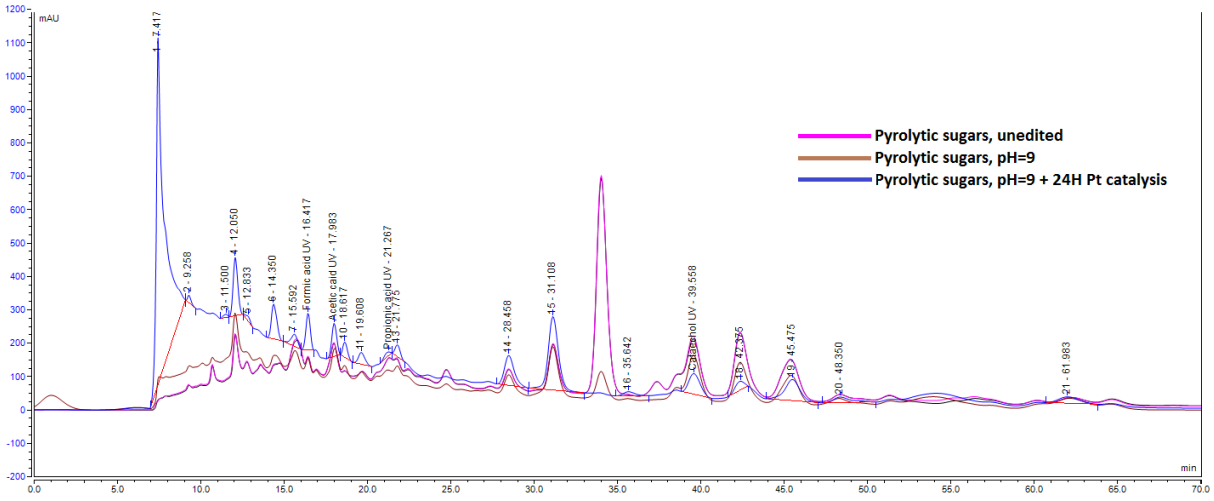

Figure 25. HPLC chromatogram recorded @ 210 nm. A larger version can be found in the appendix F.

Significant change in composition can be observed in the HPLC chromatogram from figure 25: both as a result of the base addition and catalysis. This immediately illustrates the problem that was identified in the initial experimental set up: one does not know whether the base or the catalyst is responsible for the changes observed. This is why the pH=9 sample has actually also been subjected to 2 bar o_2 pressure for 24 hours. A separate sample was then also deacidified to pH=9 and in this instance the catalyst was added.

The HPLC method is clearly lacking in substance annotations which makes it hard to draw well-grounded conclusions from this chromatogram alone. Nonetheless, it seems that the organic acids are increasing as a result of the Pt catalyst and the phenolic compounds (occurring around the catechol peak) are reduced in quantity. This is in line with the

approach of this experiment: 5-HMF was hypothesized to be converted to FDCA, an acid. A standard for FDCA was not made however, so this cannot be confirmed with certainty. Generally, it can be stated that acid elute earlier from the column, the massive increase in surface are between 5-15 min for the Pt catalysed pyrolytic sugars therefore might point towards an oxidation into acids.

Remarkably, the unknown peak from 34 minutes already decreases significantly only with addition of base, this might give another hint as to what this compound is.

4. Conclusion

4.1 Composition

Pyrolytic sugars is dark brown liquid with a dry weight of 36 % , a pH of 2.2 and a density of 1100 g/L. The carbohydrate content was ascertained using a phenol-sulfuric acid assay, and found to be 175 g/L, which means about 43 % of the dry weight consists of sugars. The phenol content, the most contributing factor to inhibitory properties of pyrolytic sugars, was determined to be 21 g/L by a Folin-Ciocalteu assay. Of the carbohydrates, levoglucosan, glucose and fructose were determined (with HPLC) to be 43 g/L, 2 g/L and 2.5 g/L respectively. This means 63% of the carbohydrate remains undefined. The total titratable acid content is 1.05 Mol/L. 71% of this is acetic acid, 37% formic acid. More interesting and abundant compounds include: 45 g/L glycolaldehyde, 5 g/L 5-HMF and 11 g/L ethylene glycol, these quantifications were made using HPLC analysis.

4.2 Dilution behaviour

Pyrolytic sugars are often diluted in order to make fermentation more feasible. Dilution with water or 5 mM sulfuric acid of pyrolytic sugars gives a solid precipitation that can be filtered out. These solids are not filtered out when filtering the undiluted pyrolytic sugars, and thus are truly formed upon dilution. Water dilution is different from 5 mM sulfuric acid dilution in that water does not cause a precipitation that collects on the bottom of the flask, but instead, these solids reside as suspended solids.

4.3 Application

Hydrolysis at 120°C was observed to occur fastest with added acid (0.1 M H_2SO_4), this gave 80% levoglucosan to glucose conversion within 30 minutes, tough much of this glucose could have come from other carbohydrate than levoglucosan, as was the case in all these hydrolysis experiments. Hydrolysis at 120°C also occurred without adding any acid, the maximum yield was higher in this case, but it took longer to get there: 96% conversion in 20 hours. Diluting the pyrolytic sugars with 5 mM sulfuric acid 1:1 and then filtering it increases the rate of conversion, within 5 hours this sample had reached 82 % conversion, compared to the undiluted pyrolytic sugars, which had only reached 32% conversion at 5 hours. Thus, the optimal solution to the hydrolysis problem completely depends on what the priorities are: to save time (add acid to 0.1 M), to save material costs (hydrolyse without any additives) or a little bit of both (dilute with 5 mM sulfuric acid).

Detoxification using 10% w/w activated charcoal has little effect both in terms of fermentability with saccharomyces and in terms of HPLC UV absorption @ 210nm. MIBK and ethyl acetate

give a significant decrease in inhibitory compounds, but were not possible to ferment with yeast due to extractant dissolution in the polar phase.

Deacidification to pH 10 with NaOH is more effective than 10% w/w activated charcoal treatment, and combining them provides even greater fermentation results: adding detoxified pyrolytic sugars at a ratio of 1:10 to the fermentation medium gives a slightly higher ethanol yield than adding the same amount of water. Overliming at 10% w/w, is the best detoxification method from this research, yielding similar fermentation results as normal medium even when the detoxified pyrolytic sugar is added at a ratio of 1:5.

5. Recommendations

5.1 Application

Pyrolytic sugars is an interesting substrate for the production of especially PHA's, because of the carbohydrate and organic acids that can both be utilized in such fermentations. Though ethanol production by co-feeding with pyrolysis products is promising as well. In both cases, some form of detoxification must be implemented such as deacidification or over liming, and even then, it will unlikely be fermentable in undiluted state. The hydrolysis that has to be performed before such a fermentation does not necessarily require strong acid addition, as often implied by literature. Hydrolysis by solely heating or diluting and then heating is a perfectly effective option, although this does require more time.

5.2 Future Research

Future research to this pyrolysis product should revolve around characterizing more of its compounds because only then will it become apparent what application is most suitable. For example: the large unidentified peak on the UV spectrum from the HPLC analysis might be a powerful inhibitor of microbes, however, since it is unknown, this cannot be targeted specifically for removal via detoxification.

Selection of the appropriate microbe is another interesting field for future research, considering some microbes are naturally more apt at dealing with the hostile conditions provided by pyrolytic sugars. Genetic modification could play an important role in tolerance as well as metabolizing properties, as the modified e-coli bacterium that is capable of breaking down levoglucosan [32] has shown.

5.3 Recommendations for practitioners

Pyrolytic sugars is a tough nut to crack in terms of analysis. The vast range of compounds in pyrolysis products, of which to this day only generally 10-20% has been identified, make this a tedious task. Often, problems were encountered during HPLC operation such as clogging of the column and overlapping of peaks. Varying temperature, eluens and column type could provide better separation and proper elution.

In fermentation, there are many variables which might have meaningful impact on the results, this should not be underestimated. Inoculum size, time of fermentation, medium composition, time of addition of pyrolytic sugars, etc. all introduce new variables into the equation.

As described in the section 'dilution behaviour', pyrolytic sugars show the unpredictable property of precipitating under specific conditions. One must realize that this means a

significant portion is excluded from analysis once it precipitates, and that, when using pyrolytic sugars in a fermentation, perhaps valuable or on the contrary, inhibitory compounds are lost because of this.

6. Materials and Methods

6.1 Brix and oven/freeze drying

The brix measurement was performed using the Atago pal-3 refractometer, it was repeated three times and an average was calculated.

For oven drying, the sample was pipetted on small absorbent rocks inside an aluminium cup. This cup was then placed inside a 60-degree oven, and the cup was weighed every 24 hours until no further weight decrease was observed.

The sample was placed inside a plastic container for freeze drying. A christ alpha 2-4 LD plus was used. The same method as for oven drying was applied.

6.2 Phenol-sulfuric acid assay

A calibration curve was made using 5 concentration of glucose (10, 20, 30, 40 and 80 ug/ml) and one blank which contained all components except glucose, the calibration was carried out in duplo. The sample was measured a total of 7 times, at three different concentrations.

For extra information retrieval and determination of the peak wavelength, the first calibration set and 3 of the samples were measured at all wavelengths between 450 and 550 nm. The blank was used to zero at all wavelengths from 450 to 550.

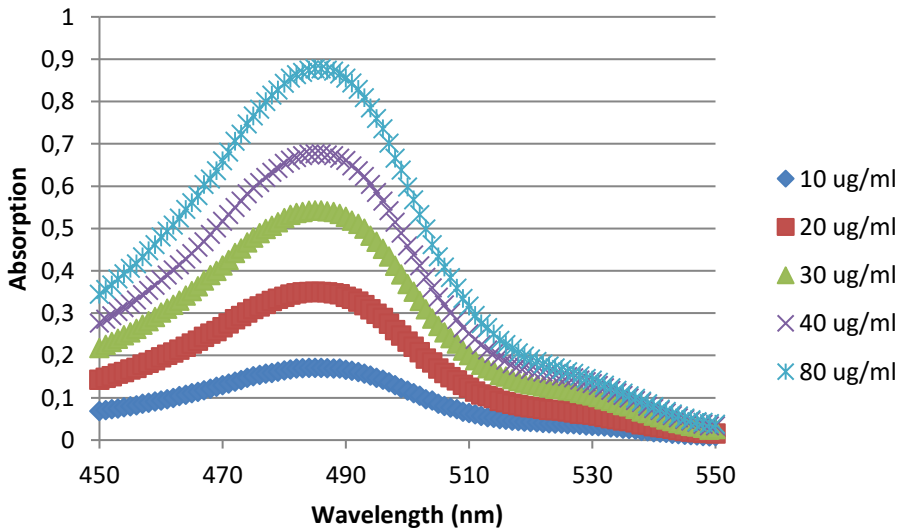

Figure 26. The profile of the glucose concentration 10-80 ug/ml after phenol and acid treatment.

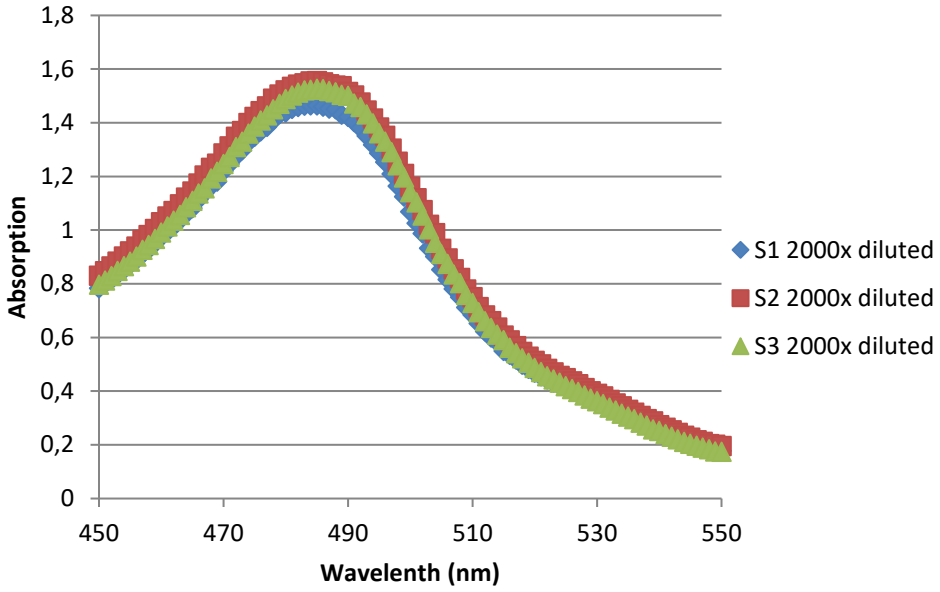

Figure 27. The profile of 2000x diluted sample after phenol and acid treatment. 485 nm was determined to be the peak of most samples (both in the calibration set and 3 samples).

485 nm was determined to be the best representative wavelength of carbohydrate content for the samples, and so it was used in the calibration and subsequent concentration determination.

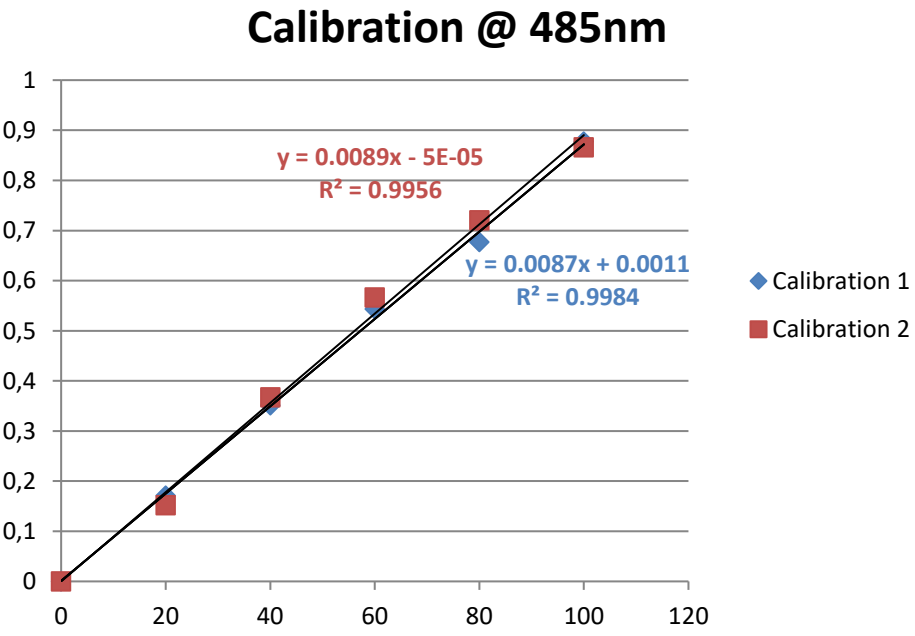

Figure 28. Calibration curve of two separate sets. A small deviation of the two can be seen, implying a slight error.

6.3 Folin-Ciocalteu

A calibration curve was made using 5 different concentrations of gallic acid (100, 200, 300, 400 and 500 ug/ml), a sixth sample contained everything except the gallic acid, this was the blank. The absorbance was measured at 550nm with a Beckman Coulter DU 720 UV-VIS spectrophotometer, the sample was measured in duplo.

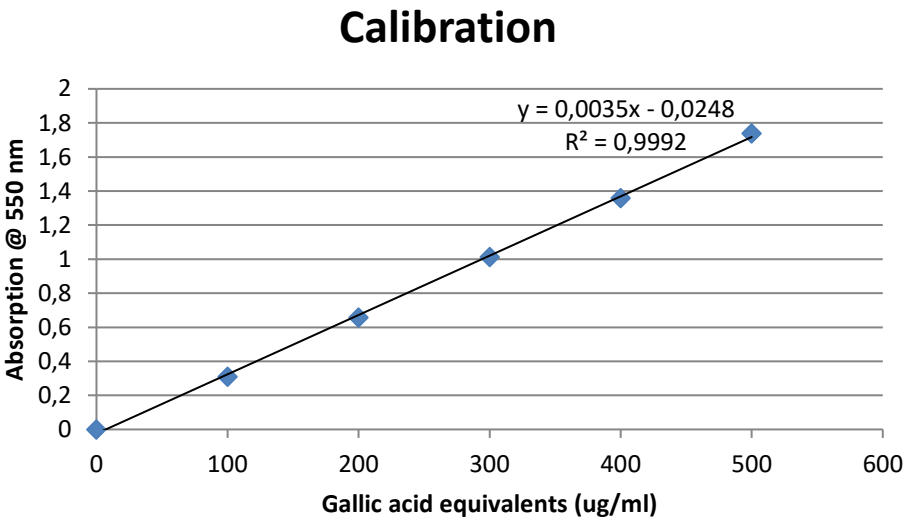

Figure 29. The calibration curve resulting from the absorbance @ 550nm -gallic acid concentration relationship. The formula depicted in the figure describes this curve and allows us to extrapolate the concentrations of the sample via absorbance, expressed in gallic acid equivalents.

6.4 High performance liquid chromatography

6.4.1 Properties

All HPLC runs were carried out using a Thermo Scientific Ultimate 3000, equipped with a Aminex HPX 87H. The device was operated at a flowrate of 0.5 ml/ml, the eluent was 5mM H_2SO_4. Oven temperature was 35°C in most earlier runs, but oven temperature was increased to 50°C in later runs to induce quicker elution from the column. For example, sugars were detected using the refractometer Shadex RI-101, and e.g. acids were detected using an Ultimate 3000 RS variable wavelength detector.

6.4.2 Calibration

The HPLC quantification was done using an external calibration of both glucose and levoglucosan, to get an impression of not just the increase of glucose over the course of hydrolysis but also the decrease of levoglucosan. The following concentrations were injected: Glucose 1g/l, levoglucosan 1g/l, mix 0.5 g/l, mix 0.25 g/l, mix 0.1 g/l, mix 0.05 g/l. Mix is glucose and levoglucosan in equal amounts.

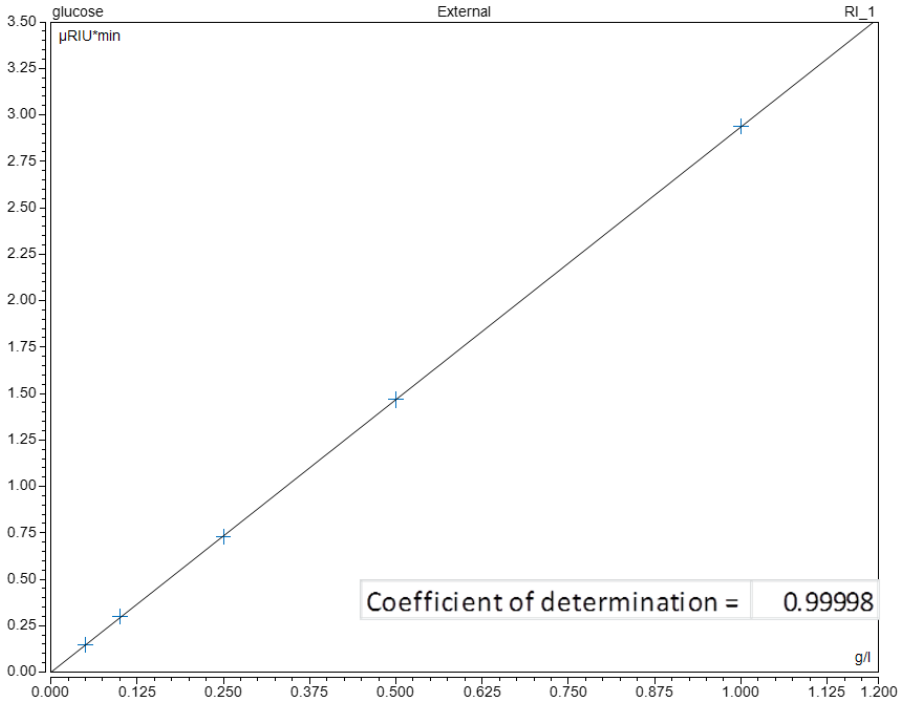

Figure 30. The calibration curve of glucose, with RI detection.

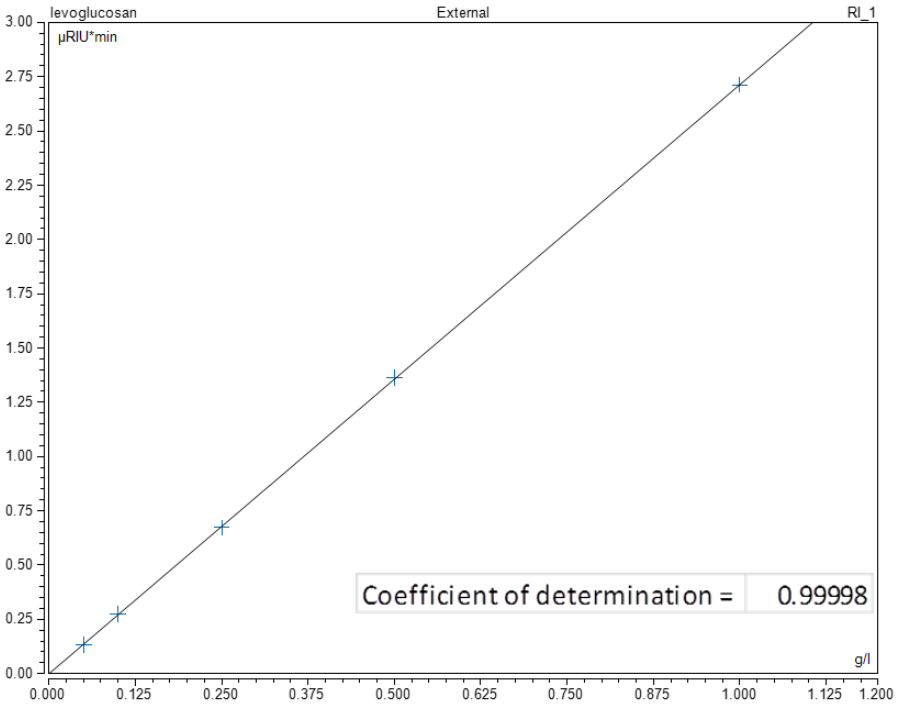

Figure 31. The calibration curve of levoglucosan, with RI detection.

From this information, the untreated sample can be analysed for its glucose and levoglucosan contents. It was diluted 1000X, injected on the column, and analysed by RI detection.

A similar method was applied for calibration of all other substances, this will therefore not be elaborated upon, considering it is more of the same.

6.5 Pt catalysis

20 ml of pyrolytic sugars is deacidified to various pH levels using 10M NaOH. The precipitate that is formed is filtered out using vacuum filtration, after which 20ml of the filtrate is pipetted into a plastic cup to which a magnetic stirrer is added as well. 50 mg of platinum on activated carbon is added. A Sample for HPLC analysis is taken at this point. The plastic cup is inserted into a metal reactor which is sealed by tightening the screws. An oxygen lead is attached to a valve on the metal reactor, which provides a total of 2 bar oxygen pressure. The reactor is flushed shortly with the oxygen and then is sealed, so that pure oxygen is inside the 'atmosphere' of the reactor. Pressure is maintained in the course of the catalysis, so that any depleted oxygen is replenished. After 24 hours, the reactor is opened and another sample is taken for HPLC analysis.

6.6 TGA-MS

For TGA analysis a <here> was used in combination with an <here> mass spectrometer. White plastic cups were used to hold the sample, often with a weight of around 200-300 mg. Samples with a weight of around 20-30 mg were transferred into the cup. Argon gas was used as carrier gas, the flow rate of the device was set at 50 ml/min.

6.7 FT-IR

For fourier transform infrared spectroscopy analysis, a Varian 1000 FT-IR scimiter series device was used. Samples (liquid and solid) were simply scooped or pipetted onto the glass and measured with the Resolutions Pro software. 64 scans were done per sample, to give an average absorption over a range of 400-5000 cm^{-1}.

6.8 Activated Carbon Treatment

Activated carbon, which has been regenerated in an oven for 2 hours at 120°C, is added to the BTG mixture to reach a concentration of 10%. For example: for 10 ml pyrolytic sugars, 1 gram of activated carbon is added. This mixture is stirred with a magnetic stirrer for one hour at 1200 RPM. After this, the solids are filtered out using vacuum filtration: a büchner funnel and flask are used for this. The filtrate can be used for fermentation, analytical purposes or further detoxification.

6.9 Deacidification

The mixture's pH is monitored real time using a pH meter, after which gradually the base NaOH is added. Precipitate is filtered out using a paper filter, Büchner funnel/flask and a vacuum pump.

6.10 Overliming

A falcon tube is filled with pyrolytic sugars and a specified amount of solid $Ca(OH)_2$ is added to it. Then, it is shaken and heated for 4 hours at 400 RPM and 60°C respectively. The tube is centrifuged and the supernatant is pipetted of and filtered using a 0.45 µm cellulose filter.

6.11 Fermentation

Each fermentation was carried out in a 100 ml erlenmeyer with an airlock. Medium was prepared using 50 g/L YPD agar medium and 30 g/L glucose, this was shaken in a sealed scott bottle to mix it up and to aerate the medium. 40 ml medium was pipetted into each Erlenmeyer and was subsequently inoculated with 0.5 g baker's yeast. The mixture is then left to ferment for 1 hour, only then is the pyrolytic sugar added, this is done to allow the yeast to 'awaken' from their lag phase before the inhibitors are introduced. Right after addition of pyrolytic sugar a sample is taken for HPLC analysis, and 24 hours later again a sample is taken. The difference in make-up between these two samples will solely include changes that were caused by fermentation.

6.12 Titration

Pyrolytic sugar was diluted 10 times with distilled water. Then, whilst with a magnetic stirrer and measuring the pH with an electrode, 1 M NaOH was added in aliquots of 1 ml. After each addition, the pH was written down.

6.13 Hydrolysis

Hydrolysis was performed initially in glass vials with red caps, that supposedly were designed for their superior pressure-bearing qualities. All samples that were heated at 120°C for longer than 4 hours failed due to leakage, this left the researcher only with a dry heap of black syrup that couldn't be analyzed – the entire volatile fraction had evaporated and left the bottle. Thus, when curiosity was drawn toward vanilla hydrolysis (no strong acid addition, that is), some other container had to be obtained. Steel reactors, that could tightly be sealed by means of a wrench, were selected as perfect candidates for hydrolysis samples beyond 4 hours. These worked like a charm and didn't let out a single stream of vapor, their downside being the difficult cleaning process: these steel reactors couldn't be thrown out after just one use like the glass reactors could, and the pyrolytic sugar residue comes off barely after 10 hours of hydrolysis.

Samples were taken from only the liquid part of the hydrolysates, simply because that is the only way one can analyze on a HPLC.

6.14 LC-MS

An LCQ Fleet™ Ion Trap Mass Spectrometer was operated in negative mode to acquire mass spectra from 10x diluted pyrolytic sugars, cateachol, levoglucosan and formic acid. Formic acid was injected to see it in relation to the background, because the eluens also consisted of a 1% formic acid solution. The LC column was an Aminex HPX 87H. The results were analysed with dedicated software.

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8. Appendices

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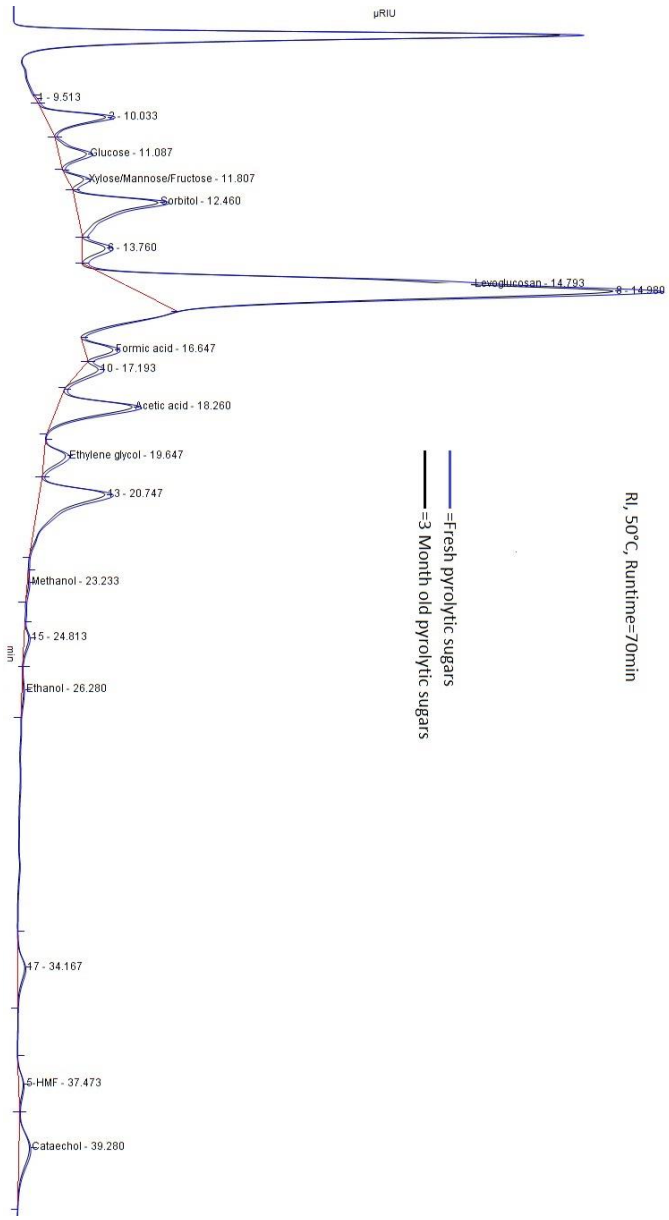

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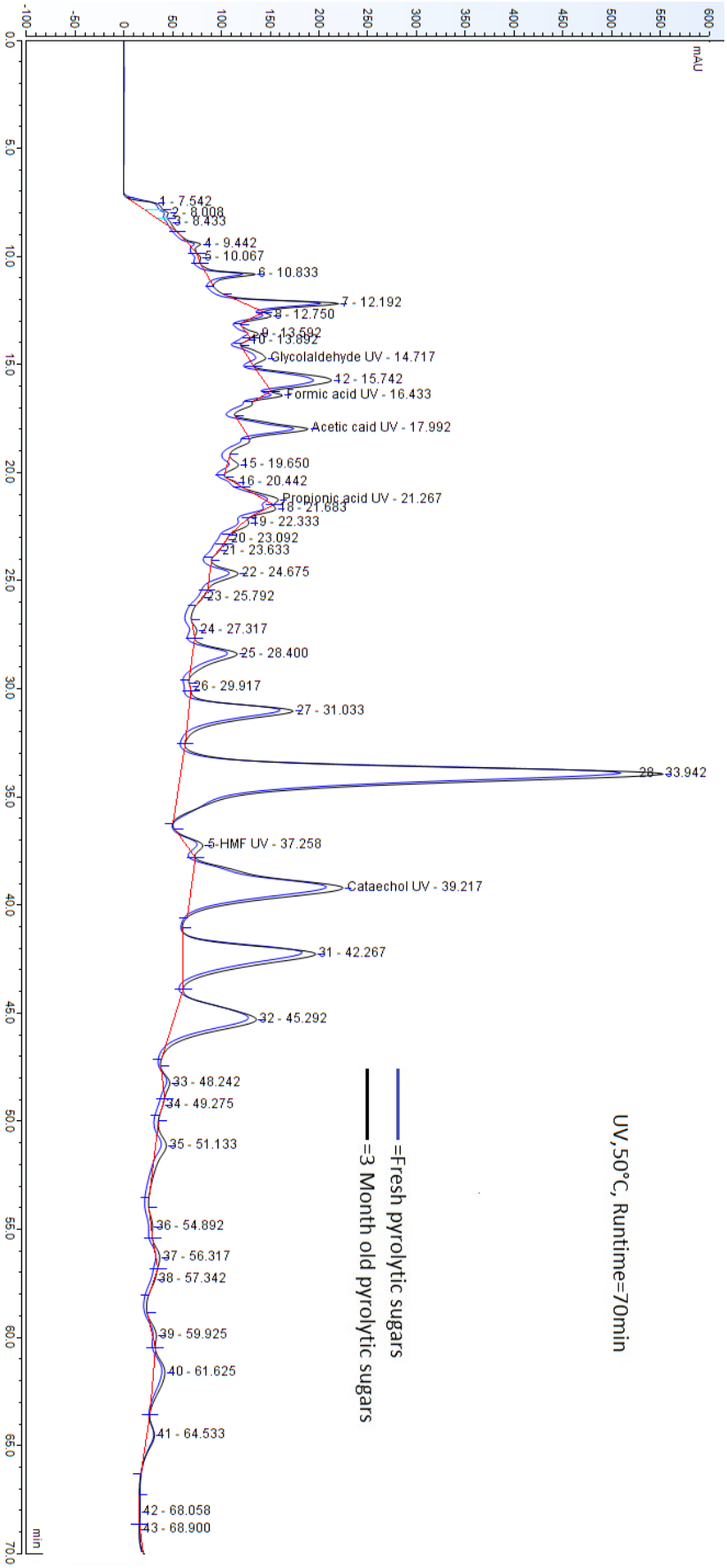
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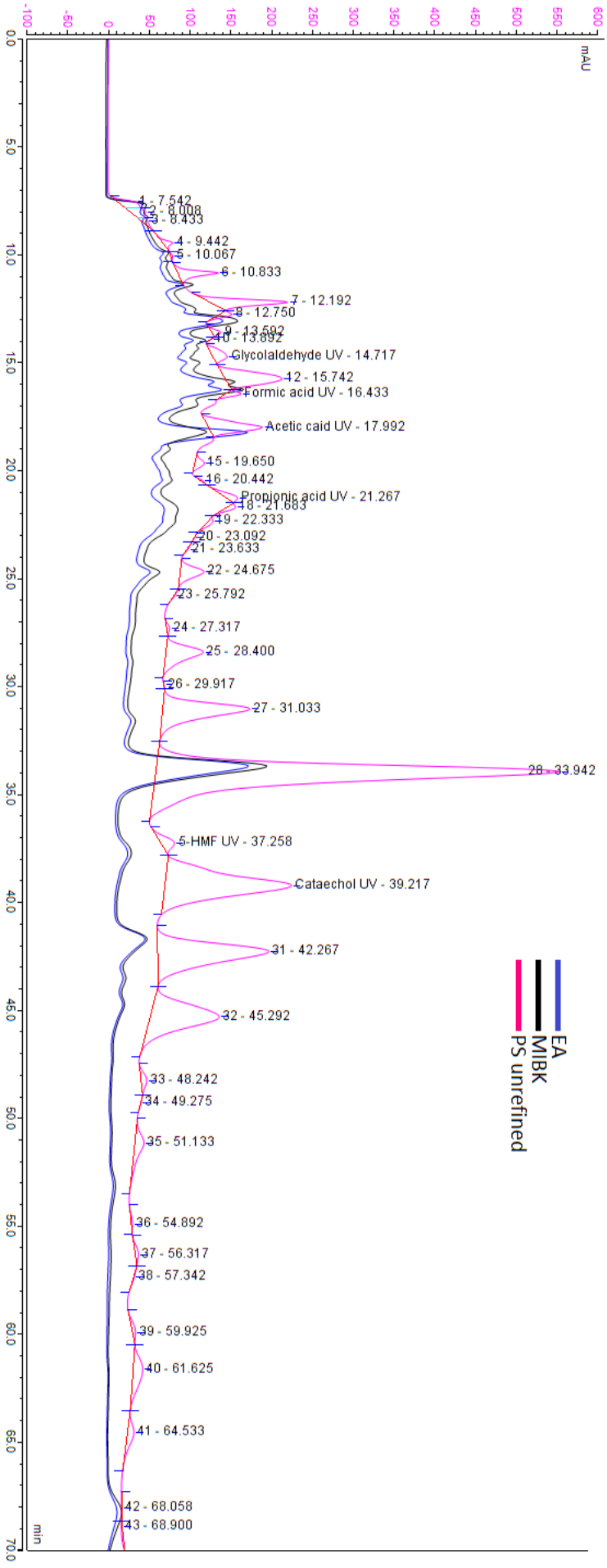
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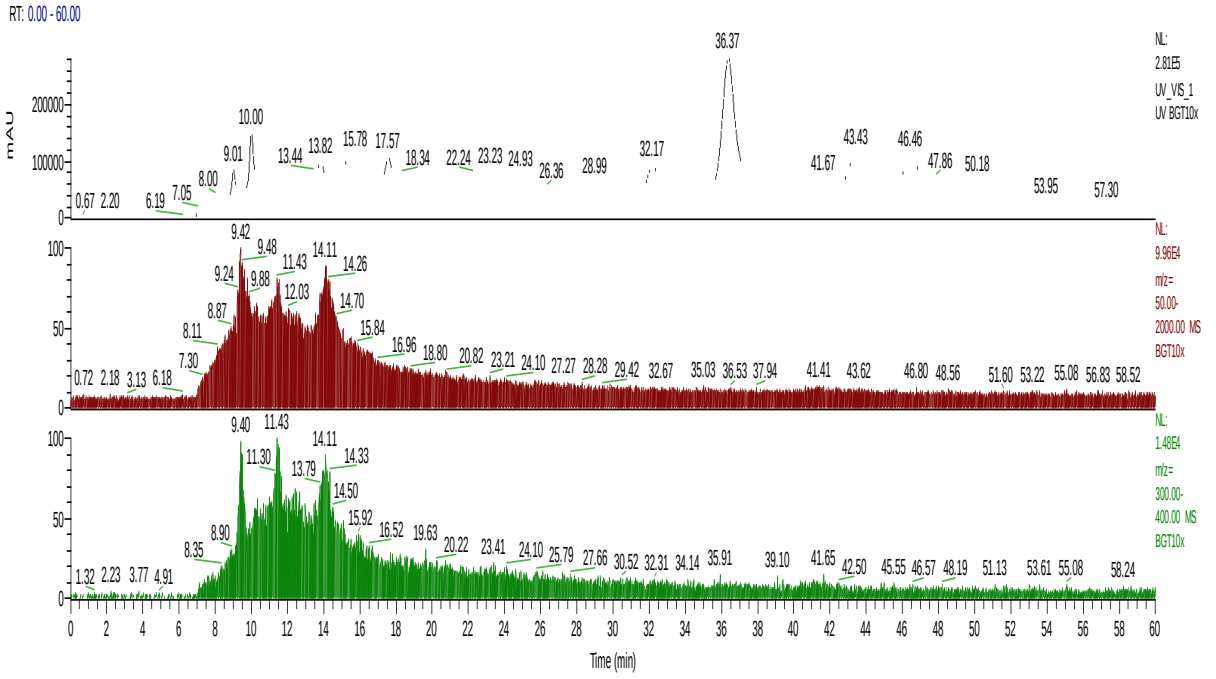
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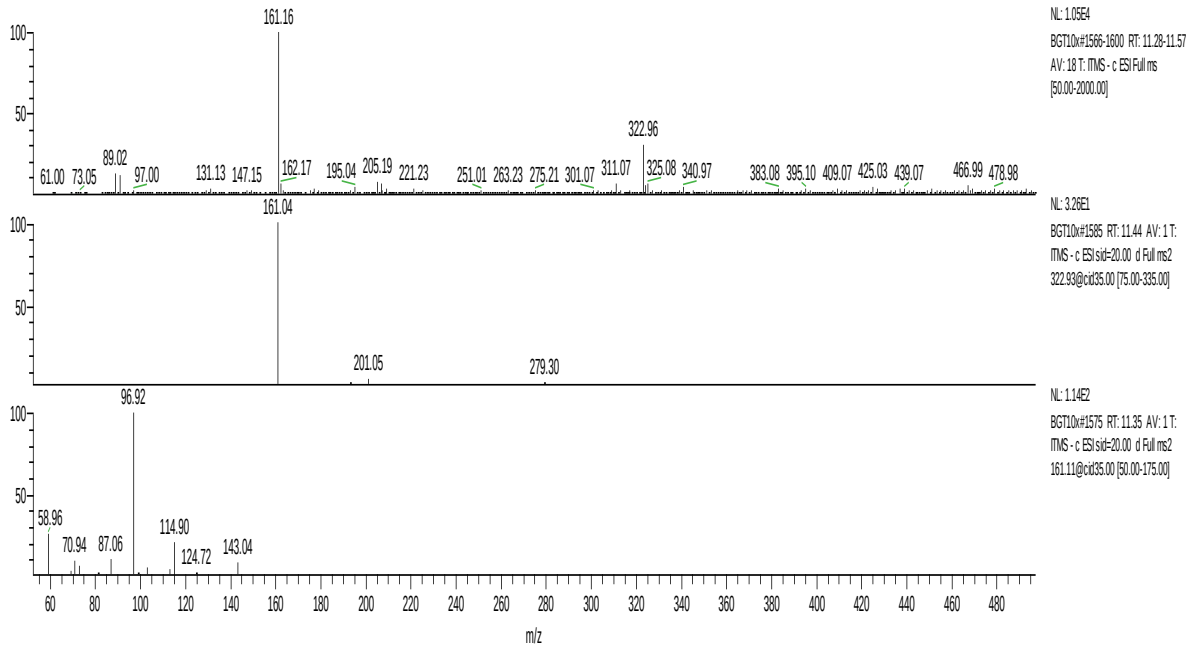
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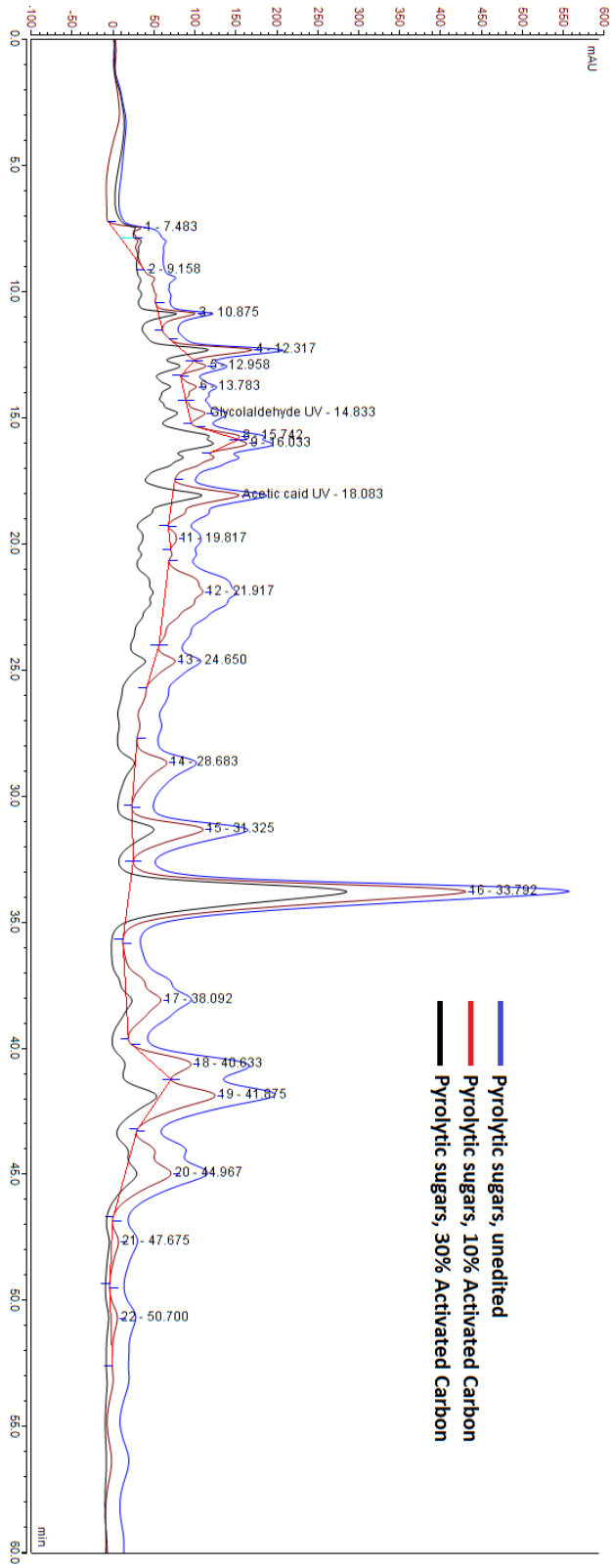
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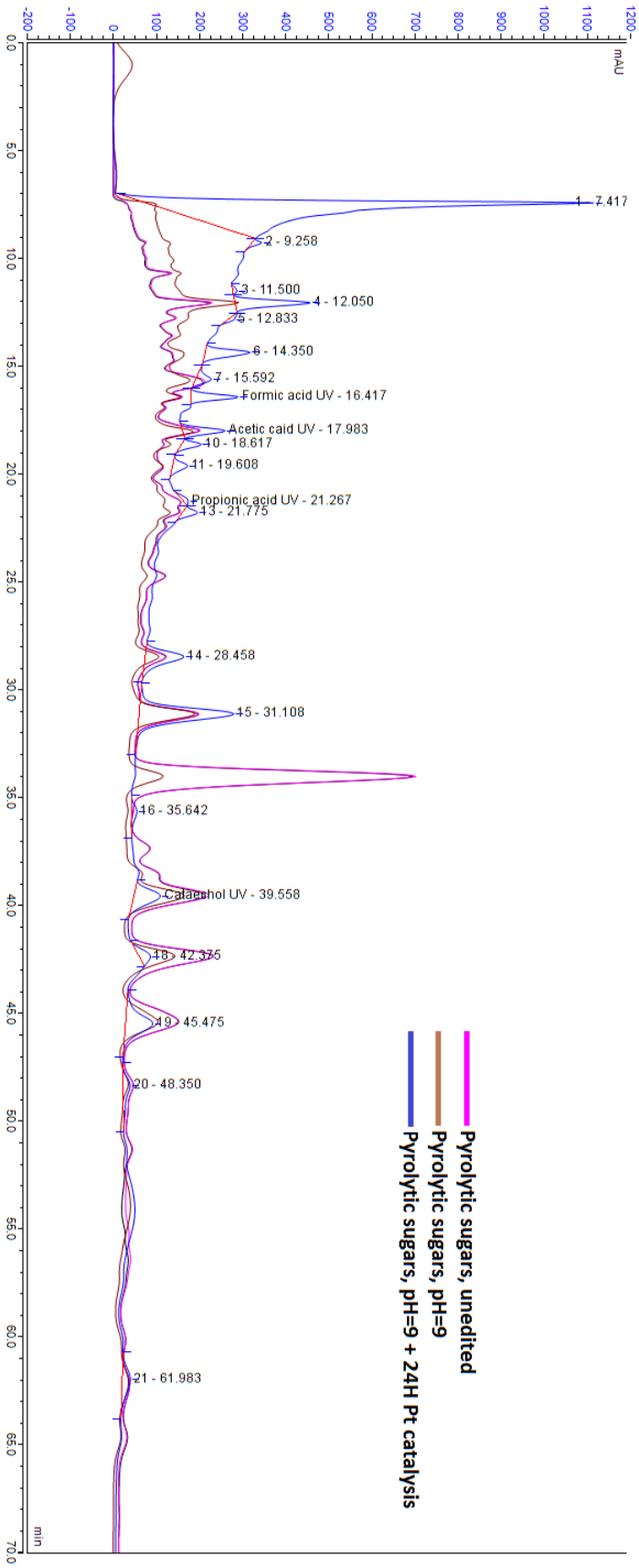
Pyrolytic sugars, 10% Activated Carbon
Pyrolytic sugars, 30% Activated Carbon

F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Pyrolytic sugars, pH=9 + 24H Pt catalysis
Pyrolytic sugars, pH=9
Pyrolytic sugars, unedited