TOWARDS IONIC LIQUID FRACTIONATION OF LIGNOCELLULOSICS FOR FERMENTABLE SUGARS

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The present study investigates wood fractionation through ionic liquid (IL) mediated pretreatment, for obtaining simple fermentable sugars, namely oligo- and monosaccharides, and in particular hexoses (and pentoses). The study focuses on softwood, Scots Pine (*Pinus sylvestris*) and Norway Spruce (*Picea abies*), exposed to ionic liquid 1-ethyl-3-methylimidazolium chloride (EmimCl).
Since both EmimCl and the monosaccharides are water-soluble and dissolve readily in similar solvents, the separation of this hydrophilic IL from sugars is difficult. Moreover, the analytics of monosaccharides released from lignocellulosics with the help of EmimCl is challenging. Sufficiently diluted samples, with low enough EmimCl concentrations, tolerated by GC sugar columns, can be also analyzed by GC.
The results obtained suggest that some IL-tolerating HPLC columns can be utilized for a quantitative determination of monosaccharides. However, frequently, these columns have low separation ability for monosaccharides and, consequently, the retention time values are very close to each other. So far, the best results on HPLC utilization were obtained with isocratic elution, using a refractive index detector and a diode array UV detector in series.

Keywords: ionic liquid fractionation, lignocellulosics, monosaccharide, bioethanol

INTRODUCTION

Today, the use of biomass in the production of chemicals and fuels is considered as a candidate solution towards a more sustainable and CO_2-neutral society. Hence, especially non-edible biorenewable feedstocks, such as lignocellulosic biomass, are highly desirable alternatives for the production of *e.g*. liquid transportation fuels, such as bio-ethanol. Indeed, the development of synthesis routes for bio-fuel production has been under intensive investigation over the past years. To produce liquid fuels *via* fermentation from the carbohydrate fraction of lignocellulosic materials, these polymeric carbohydrates – cellulose and hemicelluloses – have to be depolymerized into low molecular weight products, *e.g*. monosaccharides. A traditional way to do this is by acid- or enzyme-catalyzed hydrolysis in aqueous phase.[1] Enzyme-catalyzed processes give high selectivity towards monosaccharides but, typically, require several days to attain the desired conversion levels. Furthermore, the pretreatment of the feedstock is needed to increase its digestibility. Acid-catalyzed hydrolysis gives faster conversion, yet the control of selectivity remains difficult. Thus, it is desirable to develop a biomass hydrolysis process that eliminates the need of tedious pretreatment procedures and uses mild reaction conditions.[1]

Recently, it was discovered that ionic liquids (ILs) such as 1-butyl-3-methyl-imidazolium chloride (BmimCl), among

others, are capable of dissolving cellulose (up to 25 wt%) or a major fraction of wood, forming a highly viscous solution. Ionic liquid phase reactions have been found plausibly relevant as potential routes for the production of biofuels, cellulose and wood acid hydrolysis in ionic liquids being possible.[1] Many other ionic liquids, such as, *e.g.*, 1-ethyl-3-methylimidazolium diethyl-phosphate (Emim Dep), dissolve significant amounts of microcrystalline cellulose (up to 24 wt%), filter paper and wood flour.[2] In addition, Emim Dep – like other ionic liquids containing anions that are good hydrogen bond acceptors, such as BmimCl, AmimCl (1-allyl-3-methylimidazolium chloride) or EmimAc (1-ethyl-3-methylimidazolium acetate) to name a few –, are good solvents for cellulose and also compatible with enzymatic saccharification.[2]

It has been found out earlier that the pretreatment of lignocellulosic biomass with ionic liquids (ILs) gives rise to efficient and rapid enzymatic hydrolysis of its carbohydrates. Furthermore, a complete dissolution in ILs is not always necessary for subsequent enzymatic depolymerization of polysaccharides from lignocellulosics into monosaccharides. Instead, partial dissolution in ILs is frequently sufficient, leading to relatively high yields of monosaccharides. The disruptive forces introduced by the IL solvents as such are frequently sufficient as a pretreatment.[3]

However, a series of studies have shown that many ILs are potential solvents for polysaccharides, *e.g.* cellulose[4,5] and even wood.[5] For example, Tsukamoto *et al*. showed that polar ILs permitted to solubilize a series of polysaccharides, cellulose included, and also that 1-ethyl-3-methylimidazolium methyl phosphonate (Emim MP) is a potential candidate as a solvent for cellulose.[6] Furthermore, some ILs are able to dissolve even lignin. According to Tan *et al*., a more recent study on lignin solubility in ionic liquids has shown that up to 20 wt% Kraft wood lignin can be dissolved in 1,3-dimethylimidazolium methylsulfate, 1-butyl-3-methylimidazolium methylsulfate and 1-hexyl-3-methyl-imidazolium trifluoromethanesulfonate.[5] Lee *et al*. showed that even 40% of lignin can be extracted from wood flour with the help of ILs (in this study, 1-ethyl-3-methyl-imidazolium acetate (EmimAc) was used). On the other hand, their results also suggest that it is not necessary to remove most of the lignin to achieve a cellulose degradability exceeding 90%, as in their work >90% cellulose hydrolysis was affected by the cellulose enzyme treatment, even when the lignin content in the residual wood flour represented 60% from that of the native lignocellulosic species.[7]

An important consideration is the effect of temperature alone: *e.g.*, cellulose starts to degrade[8] at about 230 °C (≈ 500 K). Another interesting fact is that galactoglucomannans, which represent the main hemicellulose type in softwoods, can be extracted[9] with pressurized hot water from spruce at 160-180 °C. Moreover, according to Tan *et al*., the glass transition temperature of lignin is around 150 °C, so that a high temperature (also giving rise to a high pressure) is required for extracting the lignin from lignocelluloses, which occurs, *e.g.*, during chemical pulping of wood chips and of other fiber sources. The traditional methods of lignin extraction and disruption involve a wide range of industrial processes, including the widely used alkaline Kraft process, which accounts for 80% of chemical pulping. Today, the less frequently used techniques include *e.g.*, the acidic sulfite, soda, as well as various organosolv processes.[5] On the other hand, according to the studies of Lundqvist *et al*., the glass transition temperature of lignin varies[10] between about 110-170 °C, as depending on the actual pH of the cooking liquor (a pH range of 2-11 was evaluated).

EXPERIMENTAL

Objective

The objective of the present study was to investigate fractionation of wood by IL treatment, to obtain fermentable monosaccharides, hexoses (and pentoses).

Materials

Softwood – Scots Pine (*Pinus sylvestris*) and Norway Spruce (*Picea abies*) – was treated with ionic liquid 1-ethyl-3-methylimidazolium chloride (EmimCl), purchased from Merck Chemicals and used as received.

Wood

Two softwood trees were sampled from Scots Pine (*Pinus sylvestris*) and Norway Spruce (*Picea abies*). The trees were felled in Central Finland in March, 2008. The Scots Pine was 25 years old (based on the year rings counted at the sample-taking height) and 12 m high, while the Norway Spruce of the same age was 11.2 m high. The samples were taken from the height of 1.5-2 meters from both trees. It was visually determined that both trees looked healthy, free of insects and of any diseases or effects caused by fungi or other microorganisms. The samples were cut with a handsaw, to avoid any contamination from motor saw chain oil or other contaminants.

The wood samples from the tree species were immediately stored at -20 °C before being chopped and milled to sawdust and screened with a sieve, resulting in a maximum particle size of 2 mm. After grinding, the samples were prefrozen to -20 °C prior to freeze-drying. Finally, the samples were stored in a dark and dry place, at -20 °C, until used in the experiments. The samples were also seen to be free from knots and branches, as well as from any compression wood (abnormal wood, resulting from exposure to external stress).

In the present study, wood was not extracted, to see how native (dried) wood behaves as such during an IL treatment. It would be also more economical and practical, in terms of potential industrial use, if IL hydrolysis of wood as such was successful and if the presence of extractives did not result in too strong inhibition effects in later process steps.

Ionic Liquid, IL

1-ethyl-3-methylimidazolium-chloride EmimCl of 98.0% purity was purchased from Merck (Merck KGaA, Germany). Although a dry inert gas is often used as a protective shield to avoid any moisture (EmimCl is highly hygroscopic), in the present study we decided to work under ambient atmosphere, since the aim of the project was to establish potential, practical and economically feasible methods for industrial use. Even if water is also found to decrease the solubility of carbohydrates in EmimCl, some water is essential for carbohydrate conversions.[1]

Working procedures

The wood sawdust samples were prepared by exposing them to an IL-mediated thermal treatment, keeping them in an oven at a preset temperature, and submerged in EmimCl for various time intervals; depending on the experiment, a preset temperature of 80-250 °C and an exposure time interval of 0-24 h were applied.

Analysis methods

It is known that the traditional columns for monosaccharide analysis by gas chromatography (GC) and high-performance liquid chromatography (HPLC) tolerate poorly higher concentrations of ionic liquids or any salts. Furthermore, the quantitative determination of monosaccharides in ionic liquids by means of GC is difficult. On the other hand, qualitative analysis can be easily done with sufficiently diluted samples, using the traditional GC columns available for sugar analysis. Consequently, GC analyses were performed using a traditional column, but after proper dilution of samples, with a dilution ratio depending on the concentration of ILs (*e.g.*, a tenfold amount of distilled water in the IL ratio was used) while, with HPLC, an IL-tolerating column was selected, despite its lower separation ability.

Gas chromatography, GC

GC analysis was performed directly after sample silylation: the analysis method without acid hydrolysis or acid methanolysis was applied to elucidate the effect of EmimCl on monosaccharide release, instead of just analyzing the composition of the wood polysaccharide components. Xylitol was used as an internal standard (ISTD).

The monosaccharides were analyzed on a 25 m × 0.2 mm i.d. HP-1 column (film thickness of 0.11 μm), and the chromatograms were handled with the computer software Totalchrom from Perkin-Elmer. The column oven parameters were as follows: 100 °C, 8 min, raised at 2 °C/min to 170 °C and raised at 12 °C/min to 300 °C (7 min); split injector (1:20), 250 °C; FID detector, 300 °C; injection volume, 1 μL. Hydrogen was used as a carrier gas.

High-performance liquid chromatograph (HPLC)

A method utilizing a Hewlett Packard 1100 series HPLC, with a refractive index detector (RID) model HP1047 A and a diode array UV detector (DAD), was evaluated. Both detectors, arranged in series, were utilized simultaneously. An isocratic elution method was used. Both detectors are known to be appropriate for detecting mono- and polysaccharides, although RI is more common in this connection. Moreover, they are usually used with different types of columns, such as the CX-Ca one (UV: 195 nm, water: 80 °C) for monosaccharides, and the TSKpw one (UV: 195 nm, water/acetonitrile) for polysaccharides.[11] However, in the present work, especially RI performed well, as expected, even though each of them has its characteristic advantages and disadvantages.

A few different IL-tolerating HPLC columns were initially tested. The HPLC chromatogram examples found in this paper were analyzed with the Supelco LiChrospher RP-8 column, which is a reversed phase, silica-based column with octyl as carbon load, non-polar column or packing with 8 carbon hydrophobic hydrocarbon chain bound to silica. The column specifications were as follows: column length – 25 cm, particle size – 5 µm and pore size – 100 µm.

Upon HPLC method development, different analysis parameters were tested: flow rates of 0.1-1 mL/min, temperatures of 25-60 °C, injection volumes of 1-5 µL, as well as mixtures of water/acetonitrile (with and without phosphate buffer), water/methanol and water alone, and a buffer solution as rival mobile phases. The HPLC analysis chromatograms of the samples are shown in Figure 1. For this analysis, a reversed phase column (RP-C8) was used (25 °C), with a refractive index (RI) detector (35 °C) and a Diode array UV detector; the injection volume of the samples was of 1.0 µL and the flow rate of the eluent was of 0.8 mL/min. Only deionized water was applied as mobile phase (eluent), all samples being filtered through a 0.45 µm PVDF membrane syringe filter.

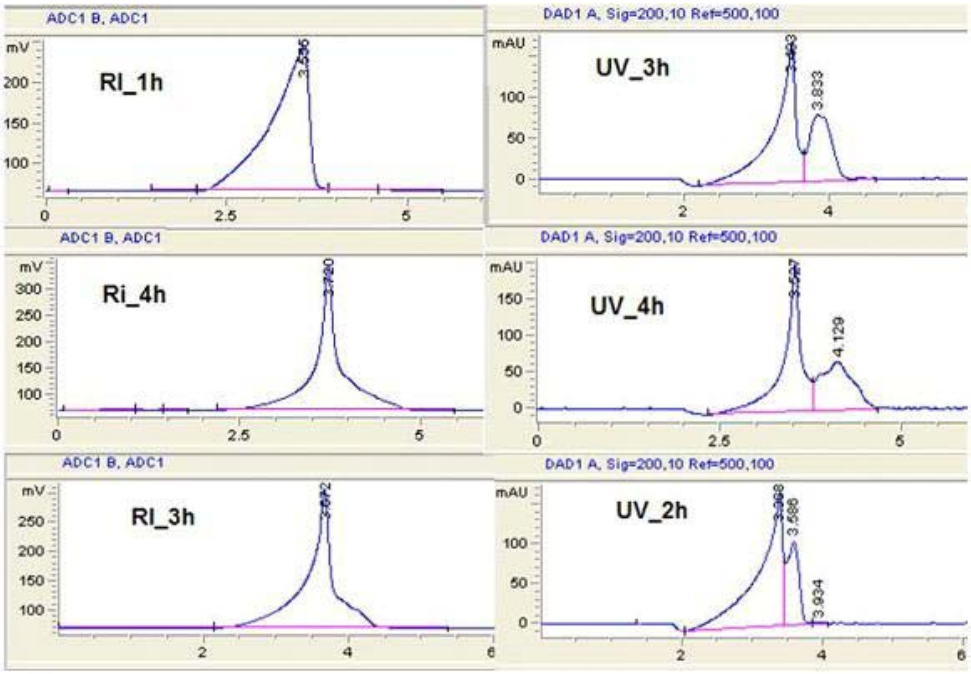

Figure 1: HPLC chromatograms of samples detected by RID and DAD. EmimCl and heat treated Norway Spruce samples (wood:IL:water – 2:5:83, water was added after heating at 100 °C for different time periods). Proper integration is not shown

RESULTS AND DISCUSSION

The results obtained suggest that some IL-tolerating HPLC columns can be utilized for the quantitative determination of mono-saccharides. However, unfortunately, these columns possess rather low separation ability for monosaccharides, since the retention time values are very close to each other.

Figure 2 introduces a GC chromatogram of a highly diluted sample of IL and of a heat-treated softwood. Originally, the sample contained 30% Scots Pine sawdust in 70% EmimCl. The sample was put in an oven with controlled temperature, between 94-104 °C, and left to react for 22.5 h. Sawdust particle size was below 2 mm. Xylitol was used as the internal standard (ISTD). The GC analysis was carried out directly on the silylated samples without acid hydrolysis or methanolysis. It can be seen that EmimCl was able to fractionate the polysaccharides of pine to monomeric sugars. As illustrated in Figure 2, the sample included the following monosaccharides: arabinose, xylose, fructose, galactose and glucose. Because of the known analytical difficulties occurring at higher IL concentrations, the samples were diluted with distilled water prior to analysis. In this case, for example, a 10% original pine concentration was too low, after dilution

giving almost exclusively the peak of the internal standard.

Even standard sugar solutions including exactly the same concentration of each monosaccharide with and without EmimCl were analyzed by GC. The traditional GC column, which was meant for sugars, was used, and the EmimCl concentration in the test samples was close to the tolerated maximum (according to the column manufacturer, around 50 ppm) to test the reproducibility and reliability of the GC analysis results when an IL component (salt) is present. The results (data not shown) showed some lower and higher chromatogram peaks, compared to the standard chromatograms, in the absence of any IL. In addition, also one extra peak appeared, which clearly demonstrates that at least higher EmimCl concentrations (near 50 ppm) hamper the reliability of the quantitative results. Consequently, the exact amounts of the different monomers were compromised. The extra peak appearing in the chromatograms is at the moment still a mystery, although it could result from, *e.g.*, complexation of the IL with sugars.

Figure 1 plots the HPLC chromatograms of six experiments, in which the pretreatment time was the only parameter. Both the diode array UV and the refractive index detectors (DAD and RID) were used here simultaneously. For DAD, only one signal "SigA" is shown, with the following detection parameters: SigA 200 nm and Ref. 500 nm. The samples were prepared from Norway Spruce sawdust by exposure to EmimCl in a 10:90 ratio and heating in an oven at 100 °C for 1-19 h, followed by a fivefold water dilution, leading to a wood:IL:water ratio of 1.6:15:83 (wt). The figure demonstrates the difference between RID and DAD: the first reacts more selectively to EmimCl and sugars, while the latter may also react with the other wood components (wider area with more easily separable chromatogram detection "peaks"). Tentatively, judging from the RI chromatograms (some of which are shown in Fig. 1), higher sugar amounts are obtained at longer treatment times (starting from 3-4 h), while 1-2 h did not seem to be a sufficient treating time for spruce. Nevertheless, further investigations are needed.

Figure 3 illustrates the HPLC chromatograms displaying D-(+)-galactose and D-(+)-xylose in deionized water with retention time values (RTs) very close to each other. It is important to note that these RTs differ from the earlier ones, plotted in Figure 1, as due to the different analysis conditions. In addition, the entire chromatograms for these species are shown separately in Figures 4 and 5. In fact, the RP-C8 column with a RI-detector gave close RTs for all monomeric sugars.

All in all, Figures 3-5 provide the calibration sample chromatograms used in HPLC analysis. Here, the flow rate applied was of 0.4 mL/min, at a sample injection volume of 1.0 μL. A reversed phase column (RP-C8) was used (25 °C), followed by the refractive index (RI) detector (30 °C). Pure deionized water was applied as the mobile phase, and all samples were filtered through a 0.45 μm PVDF membrane syringe filter.

Table 1 tabulates the average retention time values (RTs) and their standard deviations for the most common monosaccharides present in aqueous solutions: galactose (Gal), glucose (Glc), xylose (Xyl), mannose (Man), arabinose (Ara) and rhamnose (Rha), of which Xyl and Ara are pentoses, while the rest are hexoses. The RTs varied between 7.9 and 8.9 min. Xylose gave rise to two separate peaks (chromatogram), one close to the other, corresponding to retention time periods of 8.1 and 8.5 min. Under the same analysis conditions, the retention time for EmimCl remains below six min, if its concentration is below 1 wt%, and less than 7 min at a concentration lower than 2 wt%. Higher EmimCl concentrations can overlap the monosaccharide peaks, illustrating the challenges in the analysis of fermentable sugars. It should be pointed out that the retention time values given in Table 1 do not coincide in all cases with the images here provided. The variations in the retention time from one experiment to another are due to the different experimental conditions, *e.g.* a different type of column, column temperature or flow. In fact, various experimental conditions were studied, to find the optimum conditions. When IL-tolerant

HPLC columns were applied for the quantitative and qualitative determination of the monomeric sugars, the retention times of the eluated sugars can occur disturbingly near each other (Table 1 illustrates a representative example). It should be noted that columns especially designed for carbohydrate analysis cannot be applied since they do not tolerate high concentrations of salts.

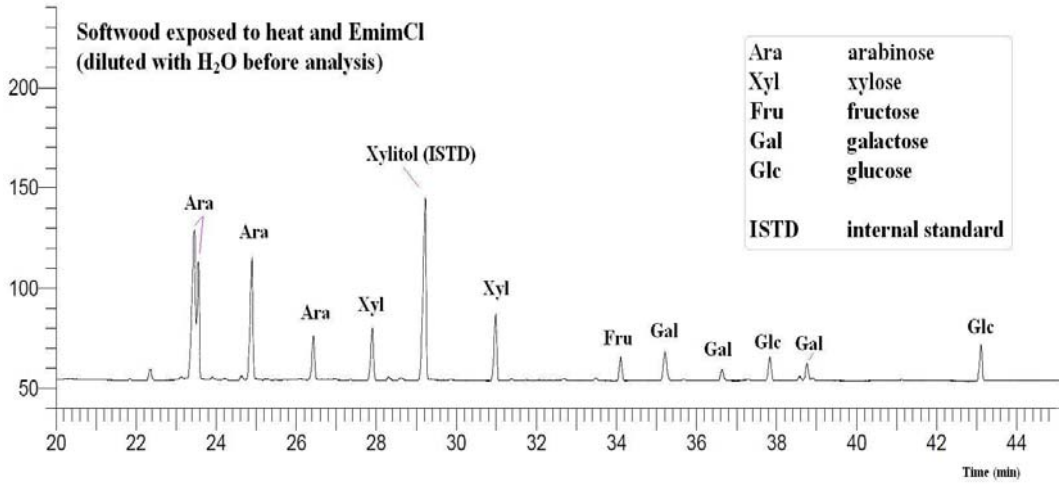

Figure 2: GC chromatogram of a highly diluted sample of IL and heat treated softwood: 30% pine was heated in EmimCl at 94-104 °C. Analysis was done without acid methanolysis (direct silylation)

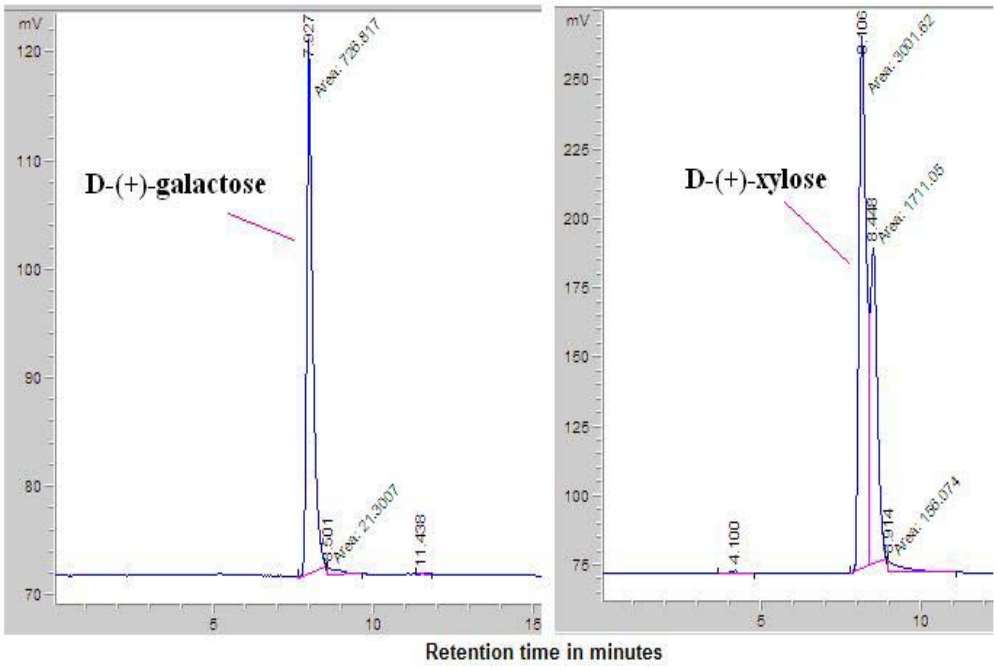

Figure 3: Example of monosaccharide elution detected by RID: observe the very close retention time values

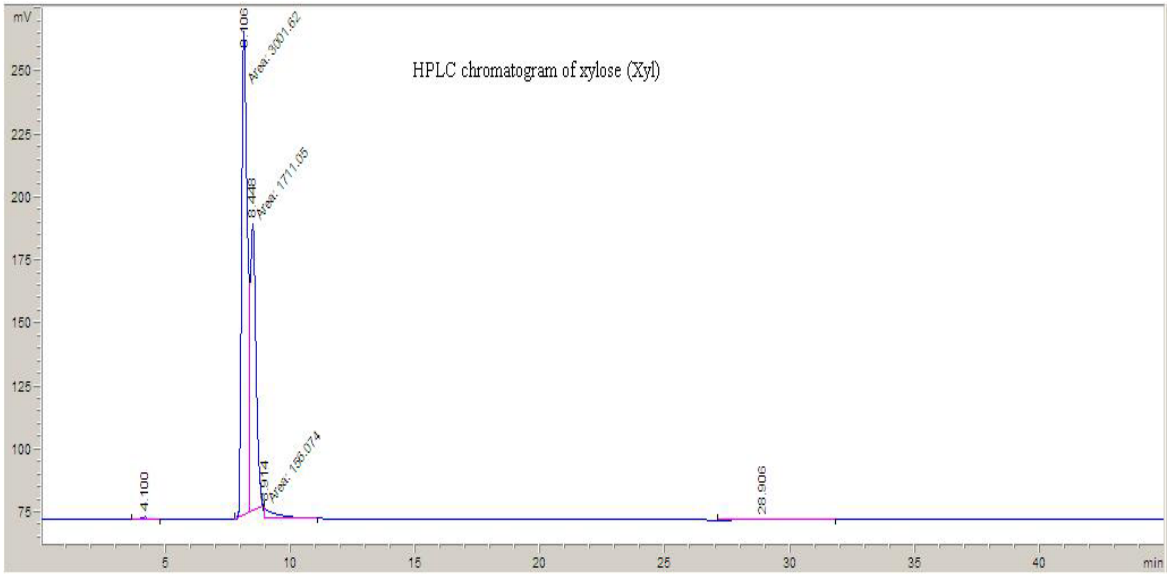

Figure 4: HPLC chromatogram of D-(+)-xylose, in deionized water (detected by RID)

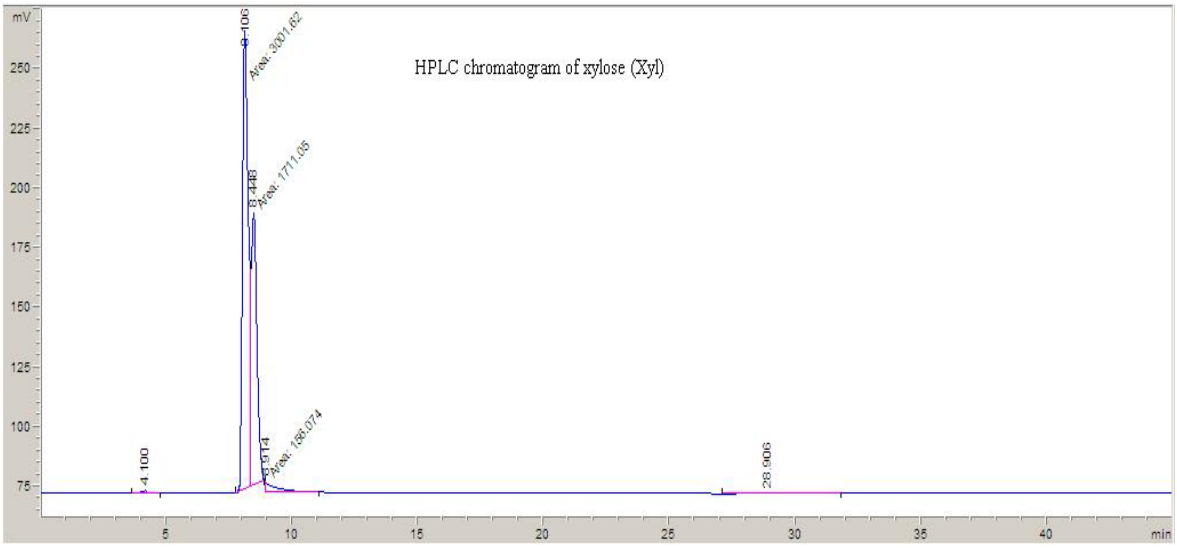

Figure 5: HPLC chromatogram of D-(+)-galactose, in deionized water detected by RID (see Fig. 4 for comparison)

Table 1
Example of HPLC retention time (RT) and standard deviations (std. dev.) for monosaccharides using an IL-tolerating HPLC column

Monosaccharide	RT, min	Std. dev., %
Galactose	7.9	0.0
Glucose	8.1	0.2
Xylose	8.1	0.0
Mannose	8.1	0.1
Arabinose	8.3	0.1
Xylose – peak 2	8.5	0.0
Rhamnose	8.9	0.3
Average	8.3	
Std. dev., %	4.1	

One solution for the analytical challenges in the HPLC analysis of carbohydrates in ILs could be the use of IL as an eluent. For example, Tsukamoto *et al*.[6] developed a successful SEC analysis of polysaccharides including cellulose, when they applied a high-pressure tolerant equipment and EmimMP as an eluent. In their study, they first examined the size exclusion effects of HPLC with EmimMP as a mobile phase, by applying standard pullulan with different molecular weights and glucose. Also, the elution peaks assigned to the standards were separated, reflecting their molecular weight. In their study, the elution time became longer with decreasing the molecular weight of the standards. Their results suggest that EmimMP might be an applicable solvent for the SEC analysis of molecular weight distribution of cellulose and other polysaccharides. In addition, they analyzed the molecular weight distribution for different types of cellulose samples: microcrystalline cellulose (MCC), filter paper pulp (FPP) and bacterial cellulose (BC). The observed sequence of the average molecular weight of the celluloses in EmimMP (where MCC<FPP<BC) agrees well with the reported data.[6]

Xylose is known to appear in its β-and α-pyranose compounds (63 and 36.5%, respectively) in an aqueous solution at equilibrium. However, this does not explain why xylose gives two peaks in the HPLC chromatogram (Figs. 2 and 4). The mutarotation phenomenon and inter-conversions of β- and α-pyranoses in water solutions concern other monosaccharides, as well, while these double peaks were not seen in their chromatograms. Furthermore, the HPLC columns are seldom able to separate, *e.g*. different pyranoses or furanoses from monosaccharides, instead, they all appear at the same retention time.

CONCLUSIONS

It was demonstrated that EmimCl alone is capable to fractionate softwood polysaccharides to mono- and dimeric sugars: hexoses and pentoses. The results of our study suggest that some IL-tolerating HPLC columns can be utilized in the quantitative determination of monosaccharides. However, these columns have a low separation ability for monosaccharides, since their retention time values are very close to each other. In this study, an HPLC equipped with refractive index and Diode array UV detectors in series, as well as isocratic elution were developed for a simultaneous quantification of ILs and of the degradation products of lignocellulosics from softwood.

Furthermore, the presence of phenolic compounds in softwood might be another reason causing challenges in HPLC analysis. Essentially, quite frequent, thorough cleaning procedures were required at least in the case of the RP-C8 column applied in this study. The mobile phase has a significant role in HPLC performance. One of the options for quantitative analysis would be to separate IL from the obtained mono- and disaccharides, and to analyze both fractions separately by conventional columns and methods. However, EmimCl separation from simple sugars is also challenging, when considering that both of them tend to dissolve in similar solvents (*e.g*., both are water soluble). However, the qualitative determination of monomeric sugars even in very diluted, IL-containing samples can be easily carried out by GC analysis, using columns meant for detecting sugars and other carbohydrates. However, the reproducibility should be carefully controlled. It would be important to determine the concentration limits for various ILs, upon which the reliability of traditional GC analysis methods can be negatively affected. Nevertheless, it should be emphasized that very low IL concentrations are a must when traditional GC analysis methods are applied. Anyway, it seems possible to carry out quantitative monosaccharide determination for highly diluted samples by means of GC.

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