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Analysis of Retention Time and Substances Released Enzymatically from Lignocellulose, Coconut Coir Treated by Alkaline, Ionic Liquid[MMIM][DMP] and Combined Method by Observing the HPLC-RI Spectra

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Abstract : The retention times and substances realesed from lignocellulose, coconut coir dust treated by alkaline, ionic liquid and combined technique, were analyzed sucessfully by observing HPLC-RI (high performance liquid chromatography equipped by refractive index detector) measurement.using Aminex HPX87P (Bio-Rad, CA) column and pure water as mobile phase. The results obtained in this work were compared to those of references reported by some authors. Average retention times ofglucose, xylose and galactose dissolved enzymatically from coconut coirwere around 13.42, 14.81 and 17.15using cellulase and xylanase, in which they were relative similar to pure sugars recorded at 13.30,14.72 and 17.70min, respectively. After conducting a comparative study with some reports published before, the peak appearing clear that was located at 7.5 min, was identified as cellobiose substance that was unknown previously in this investigation.
Key words : Cellobiose; Glucose; Galactose; HPLC-RI; Retention time; Xylose.

Introduction

Many investigations of lignocellulosic conversion into sugars, which have been conducted for years, were reported by authors around the world[1,2,3].Most studies purposed to find of alternative energies, such as bioethanol to subsitute a part of fosil based fuels[4]. Scientists that have developed lignocellulose into biofuels came to agree that prior to hydrolysis applied, the pretreatment must be conducted in order to the conversion was accelerated significantly[5]. The amount of molecules released enzymatically depended on chemical compositions of lignocellulosic substances investigated[6].

To understand types of molecules formed, especially liquid phase, from chemical reaction was needed an instrrument to split them based on their structures, polarity and atom arrangment[7]. The strongness of molecule interaction towards other substances was different, eventhough their formulasweresimilar[8]. The apparatus used to obtain an information the molecule kind, which isseparated in liquid phase, is HPLC[9]. This tool could separate substances into their components in different retention time and calculate the amount of each product obtained[10].

Many types of substances, polar and no-polar molecules, could be detected and determined by HPLC, such as fats, vitamines, acids compounds and sugars, which consisted of glucose, galactose, arabinose, xylose, sucrose, fructose that glucose was the biggest portionof among them[11,12,13,14,15].

Those works previously mentioned have not yet investigated and reported about retention timesand substances produced from lignocellulose derived from coconut coir and pretreated by alkaline, ionic liquid and their combination using HPLC-RI. This study was to evaluate the retention times and compounds hydrolyzed from lignocellulosic coconut coir dust and conductedan analysis the other substances formed during catalytic conversion by reading HPLC-RI diagram.The results obtained were compared to those have been reported by other investigators.

The steps of study were as follows: the lignocellulose was dried and milled into powder and proceeded by pretreatment using alkaline, ionic liquid and their combination. The treated- and native solids were continued with hydrolyzing them into sugars using pure cellulase and xylanase. The sugars and other substances hydrolyzed were analyzed their components and retention times by performing HPLC-RI.

Experimental

The lignocellulose used was obtained freely from coconut fiber industry in South Minahasa District, North Sulawesi Indonesia. After dried and milled becoming powders, particles were treated by using alkaline, ionic liquid, *methyl-methyl imidazole dimethyl phosphate* (MMIM)(DMP) and their combination (alkaline followed by ionic liquid/NaOH+IL). The method followed and adapted the works, which were been published previously[16,17] and then the native-, NaOH-, IL- and NaOH+IL-treated powders were converted into sugars via enzymatic hydrolysis using cellulase and xylanase, which was adapted from previous works[18].

The HPLC measurement was conducted at Analytical Instrument Laboratory located at Chemical Engineering Dept. ITB Bandung. The system configuration of HPLC employed was as follows: the pump was Isocratic HPLC pump Waters 1515 equipped by Auto sampler Waters 2707. The detector sensor to read the refracted lights that passed through sample was Refractive Index Detector Waters 2414. Meanwhile, the most important component of instrument was its column and used Aminex HPX87P (Bio-Rad, CA) and resin ionic form was hydrogen supported by sulfonated divinyl benzene-styrene copolymer 8% cross linkage. The packing materials filled inside column were size of 9 μm whose temperature was kept constant at 80^{o}C and mobile phase applied was pure water at 0.6 mL/min and he present work for HPLC measurement followed investigations as published previously[19,20].

Results and Discussion

Prior to measurement, pure sugars, which consisted of glucose, galactose and xylose were characterized to be a reference for sugar calculations and HPLC spectra is shown in Fig. 1. From data showed that pure sugars employed as standard were separated in three components, such as galactose, xylose and glucose, respectively, which were comparable with previous reports[21]. No other components showed up from HPLC spectra. The average retention times of galactose, xylose and glucose were 17.700, 15.720 and 13.300 min and if compared to HPLC using other column, the highest area was glucose and followed by xylose and galactose, which were similar to this study[22].

When sugars were converted from native-, or treated biomass (coconut coir dust) via enzymatic hydrolysis, its spectra showed the similar pattern, especially for three main components as described before. Figure 2 shows the HPLC profile of substances liberated enzymatically from native substrate using cellulase and xylanase. The retention times were 13.419 (glucose), 14.794 (xylose) and 17.128 min (galactose), which were relatively similar as described[14]. Study published that retention time glucose significantly shifted to 7.5 min if using HPLC-RI and

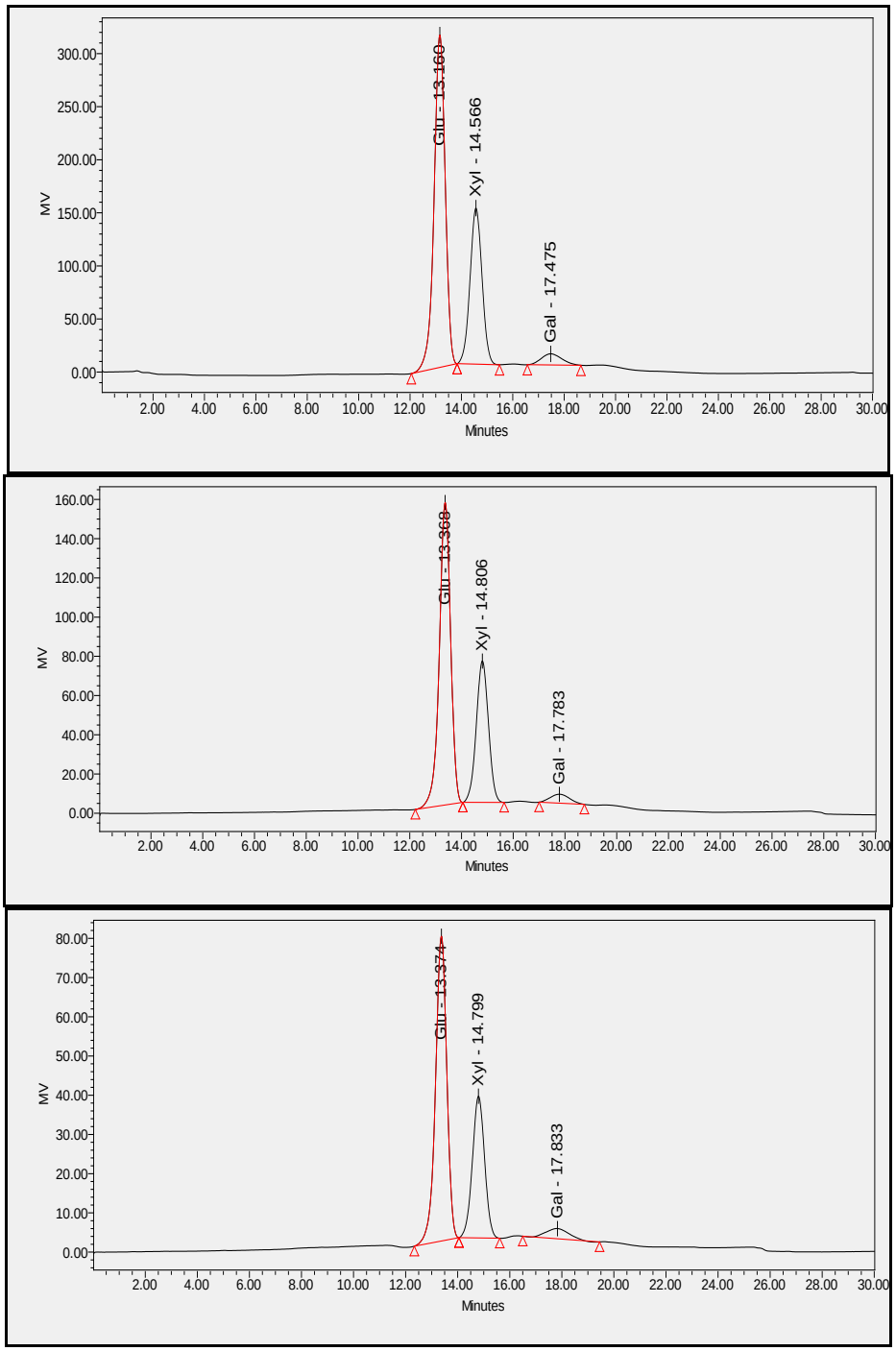

Figure 1. The separation of pure sugars (glucose, galactose and xylose) moved by pure water at 0.6mL/min and they were pressurized inside column Aminex HPX87P (Bio-Rad, CA) inserted packing materials 9µm and temperature 80^{o}C and conducted in triplicate

H_2SO_4/H_2O as mobile phase[23].There were two peaks appeared in spectra that were located at 11.325 and 7.5 min, in which the second was the highest peak and biggest area butunknown. Data also shows the area and height of three component peaks belonged to glucose, xylose and galactose.

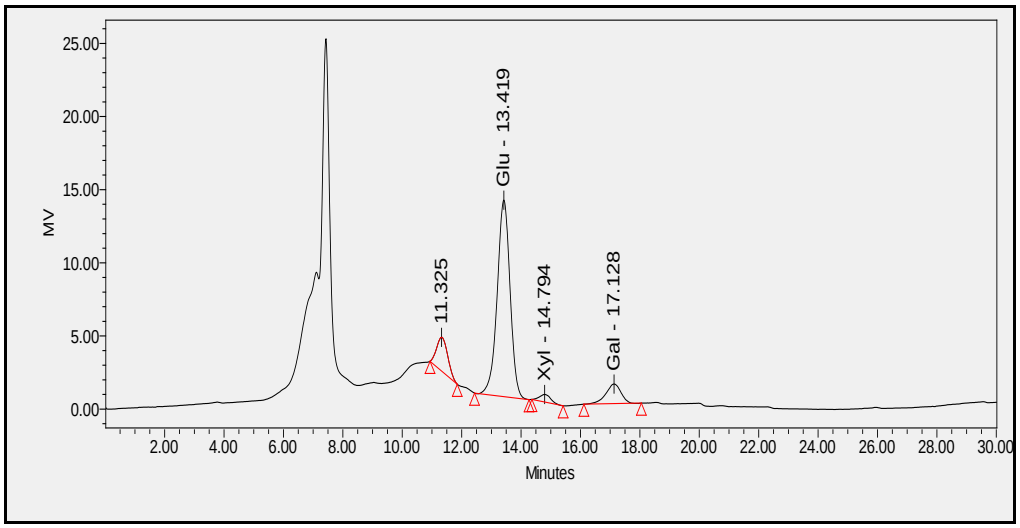

Figure 2. The pattern of HPLC spectra of sugars liberated from native lignocellulose using cellulase+xylanase.

Molecules released from 1%NaOH-treated lignocellulose (coconut coir dust) were relatively similar to those of native- and pure solids, except unknown substances as explained above. The times until spectra showed up were 17.092 min (galactose), 14.635 min (xylose) and 13.355 min (glucose), respectively[14]. However, the peak height of xylose and galactose released from NaOH-treated lignocellulose was relatively similar but almost disappeared as shown in Fig. 3. Xylose was derived from hemicellulose, whereby it was easily dissolved by alkaline[24,25].

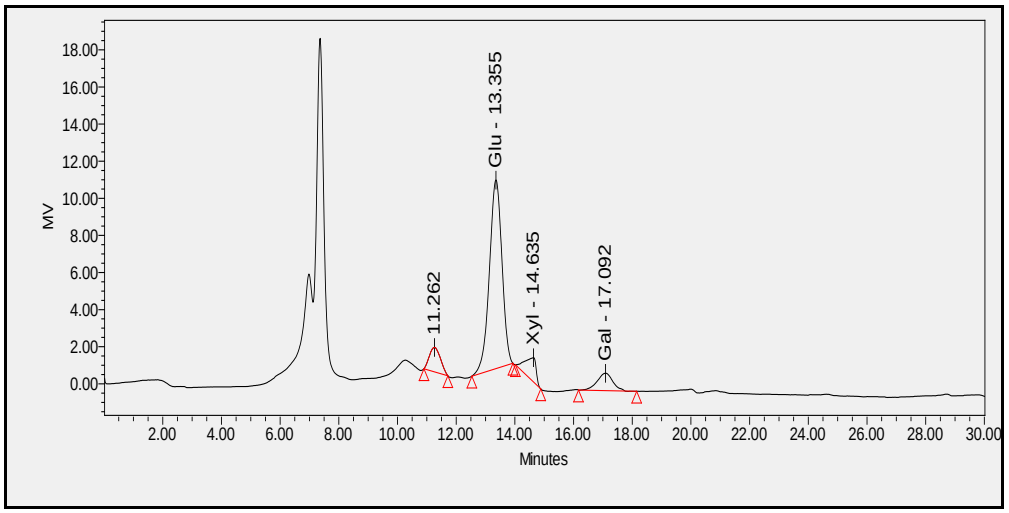

Figure 3. The profile of HPLC spectra of substances dissolved from biomass treated by 1%NaOH using cellulase+xylanase

The sugar hydrolyzed from lignocellulose, coconut coir dust was dominated by glucose[26]. The unknown substances were located at 11.262 min and 7.500 min and would be explained in the last section of discussion.

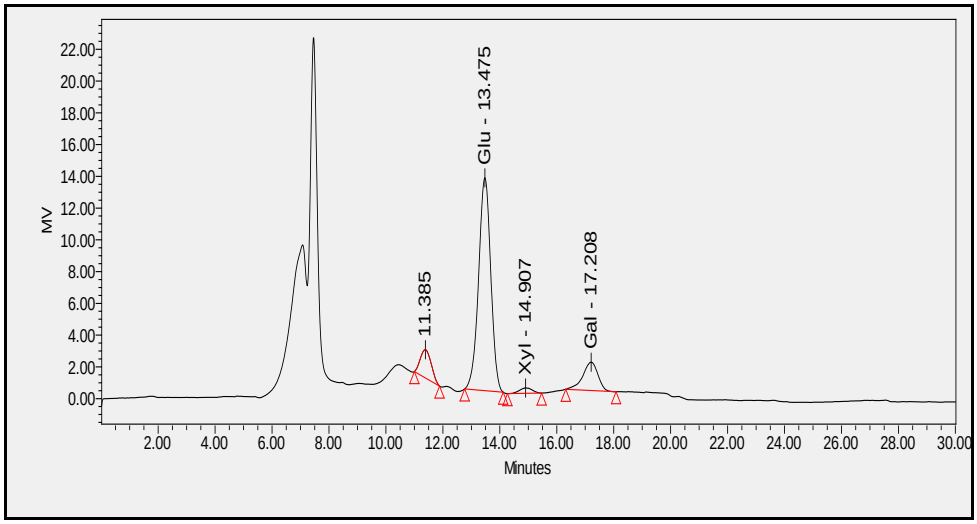

Figure 4. The patter4 of HPLC voltage vs retention time of molecules released enzymatically from 4%NaOH-treated substrate using cellulase+xylanase.

When alkaline concentration was increased from 1 to 4%, the substances liberated from treated solid gave similar pattern including their retention times but xylose peak declined significantly as shown in Fig. 4 that was comparable with Fig. 3 as shown previously.

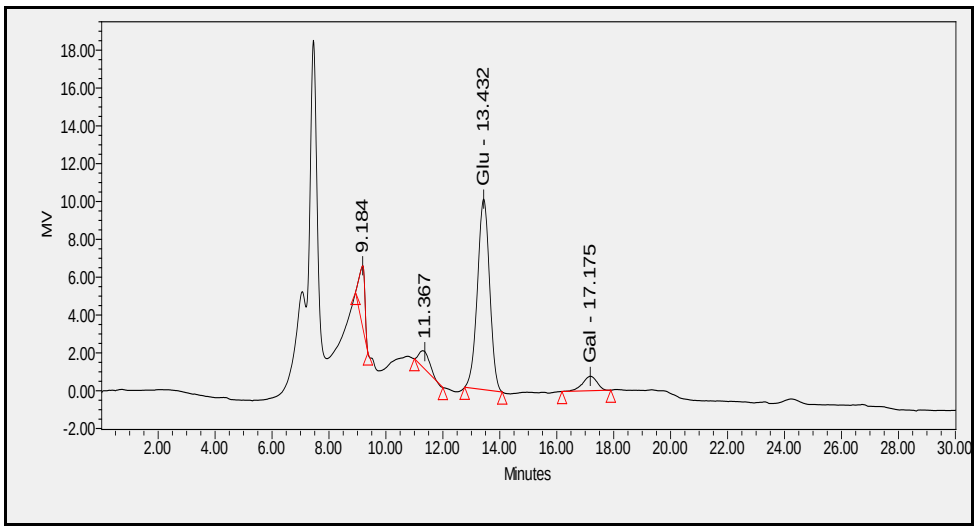

Figure 5. The HPLC profile of separated substances released from IL-treated substrate enzymatically using cellulase+xylanase

Ionic liquid (IL) was also employed for biomass pretreatment that was conducted at 120^{o}C for 15 hours. After pretreatment, IL-treated solid was preceded into enzymatic hydrolysis obtaining sugars. Fig. 5 shows the HPLC pattern of separated substances, which were dominated by sugars. The xylose peak was almost disappeared and new peak located at 9.184 min showed up. Like alkaline, ionic liquid used in this work could liquefy hemicellulose as the source of xylose[3]. There are three unknown substances whose retention times were 7.500 min, 9.184 min and 11.367 min, respectively.

The NaOH-treated biomass was continued with IL pretreatment that was symbolized as NaOH+IL-treated substrate. When that solid was hydrolyzed employing cellulase and xylanase, the HPLC spectra was more dispersed than that of other pretreatments as shown in Fig. 6.

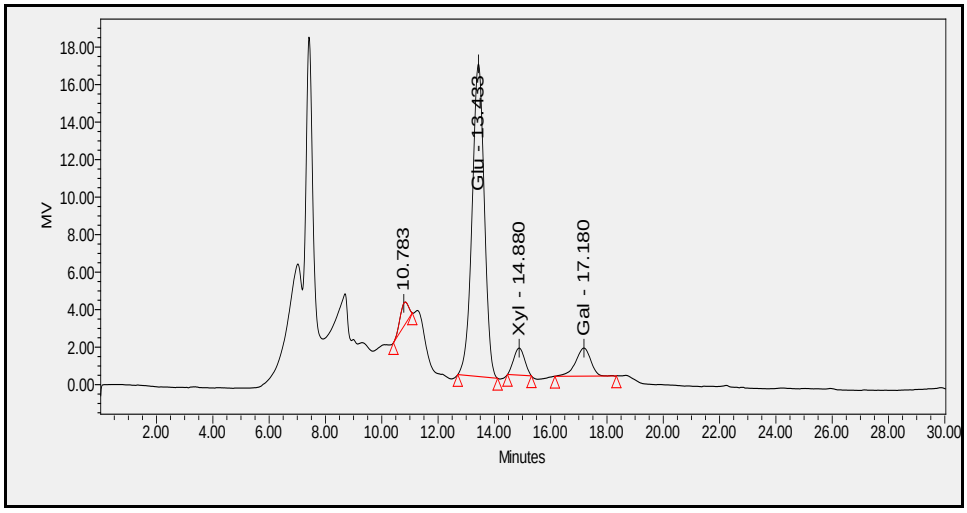

Figure 6. The HPLC spectraof separated substances released enzymatically from NaOH+IL-treated substrate using cellulase+xylanase.

All three main sugars, glucose, xylose and galactose, showed up and new peak located around 8.5 min appeared clear. The present study also employ the recycled ionic liquid (*R.IL) to treat lignocellulose. The substances released from *R.IL-treated solid was almost identical if compared to previous pattern as shown in Fig. 7.

All patterns showed that retention time of galacose was the highest of all substances that appeared in HPLC spectra followed xylose, glucose and unknown substances that were comparable with previous report[15,27]. If compared with previous work as reported by authors[28,29],present investigation did not produce more complicated sugars, like sucrose, or arabinose. By using HPLC equipped by diode detector and HPLC-RI, retention times of glucose were 17 min and 6.36 min[21,30]. With using UV detection, galactose, xylose and glucose gave retention times of 32min, 29min and 25.5min, respectively[31,32]that its sequences were comparable with this study.

Interaction strength between galactose and stationery phase, packing materials inside column is strongest of other substances. Other words state the polarity, or interaction of xylose and glucose is weaker than that of galactose but stronger than unknown substances, which were located at 11.350 min, 9.989 and 7.500 min, respectively as shown in Fig. 7 and 8. The polarity depends on the number of hydroxyl group of molecule[33].

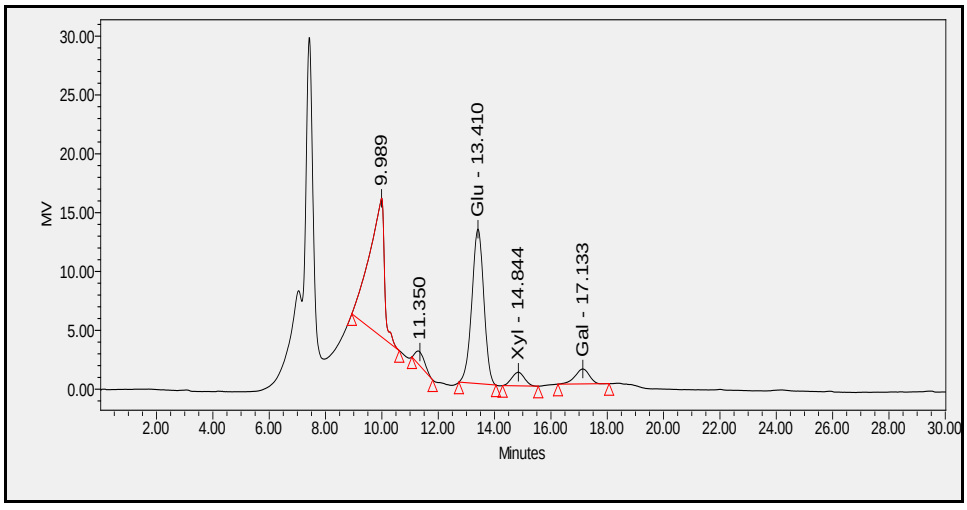

Figure 7.The HPLC profile of separated substances liberated of 1%NaOH+*R.IL-treated substrate using cellulase+xylanase.

Now, it is time to reveal what unknown substances that appeared clear in HPLC spectra for all pretreatments. It is quite difficult to identify those molecules, which freed together with monosaccharide from biomass during phase transformation, or liquefaction. Three unknown peaks emerge vividly except for pure sugars and those substances have weak electron affinities with stationery materials in HPLC column so they appear fast if compared to other components. They had low polarity whereby molecules were not attracted too strong on packing materials.

Previous authors reported that substances released from biomass, whose retention times were less than glucose, were lignin derivatives, such as furfural and 5-HMF using HPLC-RI equipped by capillary electrophoresis (CE)[34]. Meanwhile, those compounds (lignin derivatives) were not appearing if employing GC chromatogram, whereby the nost substances dissolved from lignin have retention times below those of sugars[35]. By using the similar HPLC but different detector (UV), phenolic compounds, furfural and 5-HMF showed up at higher retention times[9,27].

By conducting a comparative study between high performance anion exchange chromatography with electrochemical detector (HPAEC-ECD) and HPLC-RI, other work reported that retention times of reducing sugars and 5-hydroxymethyl-2-furaldehyde (HMF) were reversed. Retention time of 5-HMF using HPAEC-ECD analysis was at 4.5 min, while HPLC-RID was at 25.5min[36]. Other investigator found that retention time of Klason lignin converged at 26 min using HPLC-UV detection 280nm[37].

The other investigation showed that compounds 5-HMF and furfural could not detect if using HPLC-RI but appeared vivid performing HPLC-UV detector at 25.6 and 38.5 min. This work also found retention time of the other furanic compounds was located above 20 min employing UV detector[38].When HPLC-RI used for analysis, 5-HMF and furfural were located 32.5 and 48.81 min, while cellobiose, glucose and xylose 7.28, 9.10 and 9.64, respectively[39], which were very close with present study. It was also supported by old finding using HPLC-RI H_2SO_4 as mobile phase that the 5-HMF and furfural gave retention times 29 and 45 min, meanwhile cellobiose was at 7.2-7.5 min[40]. Investigators[41] discovered that sugars retention times were relatively close with present study employing HPLC-RI H_2SO_4 as mobile phase and 7.5 min (disaccharide/cellobiose), 9.0 min (glucose) and 9.8 min (xylose) but lignin derivatives could not be detected.

Pure cellobiose retention time using HPLC-RI with pure water as mobile phase was around 3.27 min as proved by authors[42] and it was supported by the other work[43].With the similar tool, investigators in different countries[43,44,45] reported clearly that retention times of many sugars and especially, cellobiose was around 7.5 min and was comparable with new works[46,47,48], which was relatively similar to other references as previously explained above.

As described above it could be explained that ionic liquid and lignin derivatives were eliminated from the suspected compounds since they did not show up from HPLC spectra. They were removed from substrate when it was washed by huge warm water. The most possible the unknown substances liberated together with simple sugars, wereother sugars, like cellobiose that has longer chain, or more than 1-2 monomers and less polarity. Retention time of cellobiose for this study was located at 7.5 min, which was near to previous reports as described above.

Conclusions

HPLC-RI is powerful to separate sugars into its components, cellobiose, glucose, xylose and galactose. Some substances liberated enzymatically from lignocellulose were not known, whereby they mostly were showed up in the range of sugars retention times. Lignin derivatives, like Klason lignin, 5-HMF, furfural and others furanic compounds dissolved during pretreatmentdid not emerge in HPLC diagram. Of course, they were removed in wash step after pretreatment conducted prior to enzymatic hydrolysis. As lignin compounds liquefied enzymatically along with sugars were possible exist but could notalso detected by HPLC-RI performed in this study. It was an indication that the instrument applied has a limitation to identify complicated substances that their retention times were much higher than that of sugars.By surveying intensive references found that retention times of substances liberated from biomass were significant different if altering either mobile phase, packing materials on HPLC-RI because their polarities were different. Tools HPLC-RI- and HPLC-UV detectors should be used together for identifying sugars substances and lignin derivatives.

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