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On-line desalting and carbohydrate analysis for immobilized enzyme hydrolysis of waste cellulosic biomass by column-switching high-performance liquid chromatography

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ABSTRACT

An innovative green column-switching high-performance liquid chromatographic (HPLC) technique was developed by coupling traditional and Pb^{2+} ion-exclusion columns to study enzyme hydrolysis components of waste cellulosic biomass. Pure water was used as the mobile phase to separate neutral polar analytes in high salt content solution. The column-switching HPLC-RI was connected on-line to the immobilized enzyme reactor for successive on-line desalting and simultaneous analysis of six carbohydrates (cellobiose, glucose, xylose, galactose, mannose, and arabinose) in the hydrolysate of waste paper and waste tree branch by incorporating the heart-cut and the elution-time-difference techniques. Six internal standard calibration curves in the linear concentration range of 0–2000 $\mu g\,mL^{-1}$ were prepared. Xylitol was used as the internal standard to give excellent linear correlation coefficients (0.9984–0.9999). The limits of detection and quantification for cellobiose, glucose, xylose, galactose, mannose, and arabinose varied between 0.12–4.88 and 0.40–16.3 $\mu g\,mL^{-1}$, respectively, with an accuracy of 90–102% and a precision of 0.1–7.8%. Cellulose and hemicellulose contents were higher in waste paper than in waste tree branch.

1. Introduction

Enormous amount of glucose is produced through plant photosynthesis each year. Glucose is not only important in food and drink industry as an essential nutrient for human being but also its fermentation products such as ethanol, lactic acid, acetic acid can play an important role for alternative fuel and chemical feedstocks in the future [1,2]. The strategy of using waste plant cellulose and hemicellulose for the production of glucose was initiated as early as 1980 with the study of ethanol production from acetic acid reduction [3–7]. Since waste paper and waste tree branch are easily collected and comprised of abundant of cellulose, hemicellulose, and lignin [8–11], waste paper and waste tree branch were selected as the substrate for the cellulase and hemicellulase co-hydrolysis to obtain glucose.

Modern high-performance liquid chromatographic (HPLC) instrument coupled with mass spectrometric (MS) detector [12–15], pulsed amperometric detector (PAD) [15–20], and refractive index (RI) detector [5–7,21–24] for direct detection has long been used for the analysis of carbohydrates. However, the success of these methods for carbohydrates analysis depends heavily on the selectivity of chromatographic column. Sometimes this problem is difficult to solve for monosaccharide analysis partly due to their similarity in structure and the same molecular mass of the same carbon number sugars. Also, the use of single chromatographic column for simultaneous separation of disaccharides and monosaccharides at the same time is not easy in most cases. Various kinds of chromatography have been used for the separation and analysis of carbohydrates such as hydrophilic interaction chromatography [13], anion-exchange chromatography [16–20,25–27], cation-exchange chromatography [6–7,21,28], ion-exclusion chromatography [12,29], ligand-exchange chromatography [15], and normal-phase chromatography [5,22–24]. The most popular method used for monosaccharide analysis is the anion-exchange chromatography. However, the mobile phase used for this type of chromatography is strongly alkaline solution which makes it only suited for analytical purpose and with no benefit for sample pretreatment or possible product recovery. In addition, the use of strong alkaline solution as the mobile phase produces the problem on environmental contamination. Therefore, we are most interested in the ion-exclusion chromatography which uses deionized water as mobile phase for separation of various carbohydrates

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with partition between the aqueous mobile phase and occluded water within the resin beads [30].

In general, salt free samples must be used for complex biological samples in order to obtain appropriate column selectivity and detector sensitivity. Methodologies such as solid-phase extraction [31,32], cation-exchange membrane desalter [25–27], Nafion cation-exchange capillary desalter [33], and centrifugal size-exclusion chromatographic method [34] have been developed for either on-line or off-line desalting. Most of them were used for oligosaccharides analysis; only the membrane desalting device was suitable for monosaccharide analysis and could be used for on-line procedure with the help of a special device. Therefore, it is mandatory to develop a simple, convenient and on-line operated desalting technique for monosaccharide analysis.

Recently, the development of column-switching technique [35–40] on chromatography allows the hyphenation of different chromatographic columns with different separation modes to promote the chromatographic system from one-dimensional (1D) to two-dimensional (2D) [41,42] or multi-dimensional (mD) system that can be used to resolve completely a wide range of analytes in complicated samples. With the cooperation of heart-cutting technique the multi-dimensional chromatographic system can even perform the on-line sample preparation for matrix elimination or desalting that greatly help the automation of the chromatographic process.

The purpose of the present study is to develop and demonstrate an efficient column-switching HPLC system that was coupled on-line to the immobilized enzyme hydrolysis system to perform the successive on-line sample pretreatment (desalting) and simultaneous sugar products analysis in the waste cellulosic biomass hydrolysate. The two coupled columns for the column-switching HPLC system were the H^+ ion-exclusion column and the Pb^{2+} ion-exclusion column and the mobile phase of the system was highly purified water that makes no secondary contamination and fulfills the requirement of the green technology.

2. Experimental

2.1. Chemicals and materials

The enzyme complex such as cellulase (EC3.2.1.4, purified from *Trichoderma reesei*), hemicellulase complex (EC3.2.1.8, purified from *Aspergillus niger*), and reagent grade D-cellobiose were purchased from Sigma (St. Louis, MO, USA). The reagent grade D-glucose, D-xylose, D-galactose, D-arabinose, D-mannose, xylitol, and $CaCl_2$ were bought from Merck (Darmstadt, Germany). The sodium alginate was obtained from Lancaster (Lancaster, England). Reagent grade NaOH and H_2SO_4 were from Showa Chemical Co. (Tokyo, Japan). Waste copy papers of A4 size were collected in the laboratory. Waste tree branches (*Cinnamomum camphora*) were collected from the campus. Ultrapure water (18.2 MΩ) was prepared first from the tap water by reverse osmosis and distillation once then treated with Barnstead ultrapure system (Dubuque, IA, USA). Membrane filter of 0.2 μm pore size and 25 mm diameter was obtained from Advantec MFS, Inc. (Dublin, CA, USA). The helium gas was purchased from San-Yin Co. (Yinggo, Taiwan).

2.2. The immobilized enzyme reactor system

The enzymatic hydrolytic reaction was performed with an automated 3 L fermentation system (Bio Top, BTF-A, Taichung, Taiwan). The combined glass pH electrode was obtained from Mettler (InPro 30301225, Switzerland). The beads of immobilized cellulase and immobilized hemicellulase were put in many small net-pockets. All of the net-pockets contain about 20–25 beads. The net-pockets were hung vertically on a rack that was specifically designed to suit the reactor's inner diameter [6,7].

2.3. The pretreatment of waste paper

The waste paper was cut into small scraps and was digested with 5 M H_2SO_4 solution at a temperature of 40 °C for 48 h. After digestion, the pH of the waste paper solution was adjusted to 7.0 with NaOH and centrifuged (Hettich Universal 30RF, Tuttlingen, Germany) for 10 min to precipitate the digested waste paper fiber. The centrifugation at 10,000 rpm was repeated several times to remove the Na_2SO_4 and other soluble materials. Then, the fiber like solution was treated with an ultrasonic disruptor (Heat Systems W-385, Farmingdale, NY, USA) at room temperature for 30 min (2–4 times) to a gel-like solution and was centrifuged again. The treated waste paper fiber was finally oven dried to become a solid form. Part of the solid was ground into powder for subsequent hydrolysis reaction and the rest of the dried waste paper solid was stored at 50 °C in an oven for later use [6,7].

2.4. The pretreatment of waste tree branch

The waste tree branch was cut into small pieces and was pulverized for 30 s. The pulverized waste tree branch was suspended in 250 mL deionized water and autoclaved for 30 min under 121 °C and 1.1 kg m^{-2} several times to eliminate most of the water soluble and highly volatile matrices. The autoclaved solution was then pretreated with the same procedure as described in the previous section with one last additional delignification step after the ultrasonic disruption. The delignification of the waste tree branch fiber was performed by using 100 mL 1% (w/v) H_2O_2 at pH 11.5 [43,44] and was orbitally shaken at a rate of 150 rpm and a temperature of 80 °C for a period of 1 h. After delignifications, the waste tree branch fiber was centrifuged and washed with distilled and deionized water several times. The treated waste tree branch fiber was dried, used, and stored in the same manner as described in previous section.

2.5. Immobilization of enzyme

The enzyme solution containing either 450 mg cellulase or 90 mg hemicellulase was separately added into 180 mL 2% (w/v) sodium alginate solution. The immobilization of cellulase and hemicellulase was performed by dripping either the cellulase or hemicellulase – sodium alginate solution into the 200 mL 0.5 M $CaCl_2$ solution using a peristaltic pump (Masterflex model 7524-10, Chicago, IL, USA). During the dripping procedure, the $CaCl_2$ solution was stirred with a magnetic stirrer at a constant rate in order to prevent the enzyme beads from sticking together and to obtain a suitable and uniform size of enzyme beads [6,7].

2.6. Waste paper and waste tree branch hydrolysis with vertically hanging immobilized enzyme reactor

Either 2.5 g pretreated paper fiber or waste tree branch fiber in one liter aqueous solution was sterilized 30 min in an autoclave and cooled down to the optimum reaction temperature of 45 °C. The immobilized cellulase pellets and immobilized hemicellulase pellets were put separately into many small net pockets and hung vertically on a rack that was specifically designed to suit the inner diameter of the reactor. Then, the whole rack was put into the 1 L fiber solution. During the hydrolysis the pH of the fiber solution was adjusted to 4.0 for waste paper fiber hydrolysis or 5.5 for waste tree branch fiber hydrolysis by using either H_2SO_4 or NaOH. The solution was stirred at a rate of 150 rpm to begin the cellu-

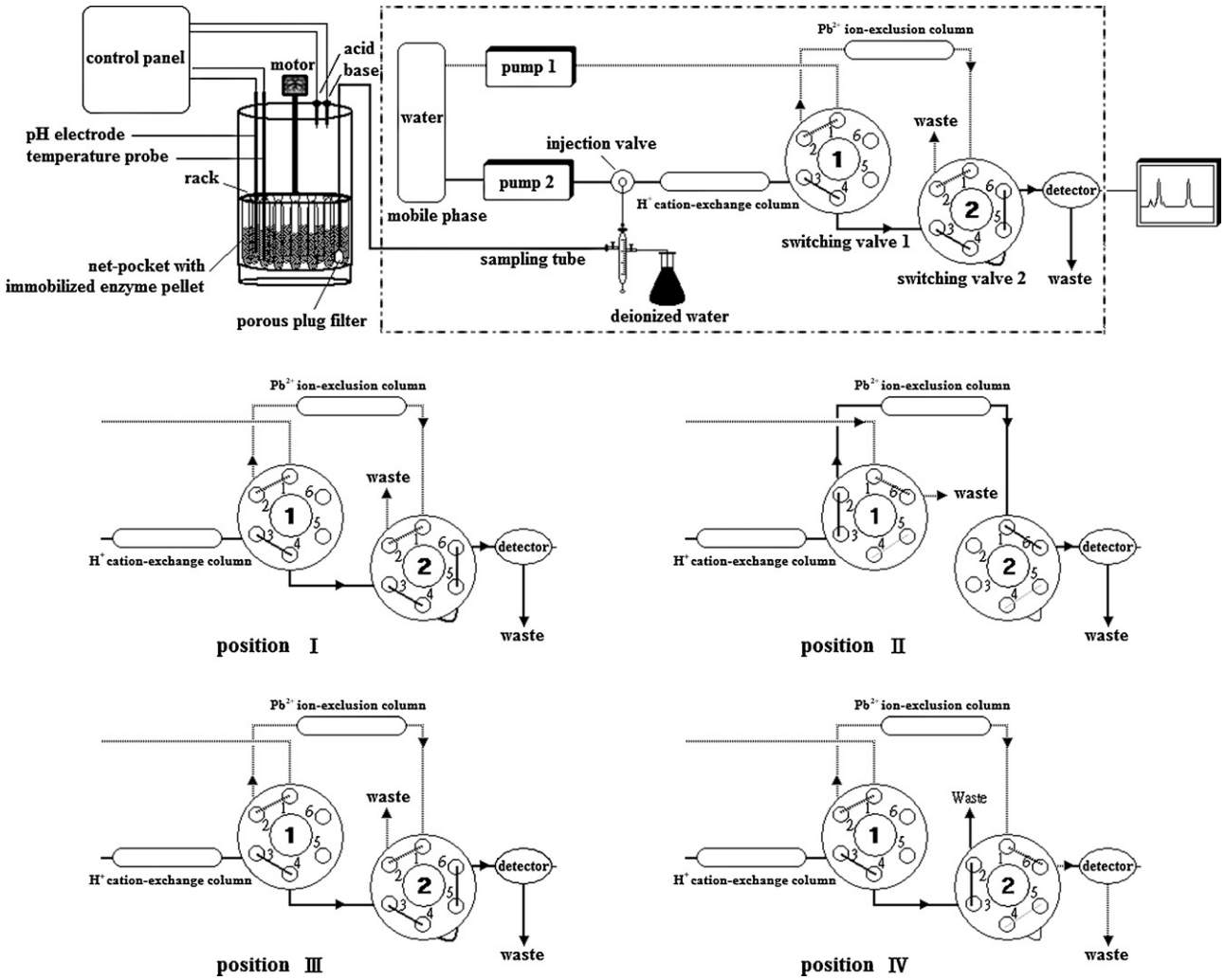

Fig. 1. The schematic diagram for the hyphenated system of the bioreactor and the column-switching HPLC (the dotted line area) through a specific designed sampling device.

lose and hemicellulose hydrolysis. The reaction period was 4 days [6,7].

2.7. Column-switching HPLC system

The HPLC system consists of two dual piston pump (Waters 515, Milford, MA, USA), an injection valve (Rheodyne 7125, Cotati, CA, USA) with a 20 μL sampling loop, a column oven (Shimadzu CTO-6A, Kyoto, Japan), a refractive index (RI) detector (Shimadzu RID-6A, Kyoto, Japan,), and a personal computer (Acer Pentium 4-2, Taipei, Taiwan) including a chromatographic data processing software (SISC, Taipei, Taiwan). The two analytical columns used for the column-switching HPLC system were the H^+ cation-exchange column (Phenomenex Rezex ROA-organic acid, 300 × 7.8 mm i.d., 8 μm, Torrance, CA, USA) protected with a guard column (Phenomenex Rezex 4 × 3 mm i.d., 8 μm, Torrance, CA, USA) and the Pb^{2+} ion-exclusion column (Supelcogel Pb, 300 × 7.8 mm i.d., 9 μm, Bellefonte, PA, USA) protected with a guard column (Supelcogel Pb, 5 × 4.6 mm i.d., 9 μm, Bellefonte, PA, USA). The switch of column was operated manually with two switching valves (Rheodyne 7000, Cotati, CA, USA) through four column-switching steps (positions) as shown in Fig. 1. The detailed operation procedures are described in Section 3.1 and the corresponding column-switching time for the four valve positions was summarized in Table 1.

2.8. Preparation of internal standard calibration curves

Six carbohydrates, viz. glucose, xylose, cellobiose, arabinose, galactose, and mannose, produced in the immobilized enzyme hydrolysate were quantitatively determined by the on-line matrix matched internal standard calibration method. Xylitol was used as the internal standard. To match the matrix, 2.5 g waste paper fiber or waste tree branch fiber, standard carbohydrates at certain concentration, plus 0.1 g of xylitol were put in 1 L deionized water and hydrolyzed with non-enzyme calcium alginate pellets as described in Section 2.6. The matrix matched standard carbohydrates solution were then on-line sampled and analyzed with the column-switching HPLC [7] system. The peak area ratio of the specific internal standard carbohydrate calibration curve was obtained by measuring the peak area of standard concentration of 0 (matrix solution), 5, 10, 15, 20, 50, 100, 300, 500, 1000, 1500, and 2000 μg mL^{-1} individually then dividing each of them by the

Table 1
The column-switching duration time for the four valve positions.

Time (min)	Valve position
0.00–11.00	I
11.00–16.10	II
16.10–20.50	III
20.50–47.00	IV

peak area of internal standard at 100 μg mL^{-1}. The best linear internal standard calibration curve with the peak area ratio versus the standard carbohydrate concentration [7,45] was obtained by the least-square regression method. The quantification of the internal standard xylitol during the immobilized enzyme hydrolysis was monitored by the on-line matrix matched external standard calibration method [7] with the same linear concentration range of 0–2000 μg mL^{-1}.

2.9. Successive on-line column-switching HPLC analysis of carbohydrate products in immobilized enzyme hydrolysate

One milliliter hydrolysis sample containing the internal standard xylitol was drawn on-line from the bioreactor with a specific designed sampling device [7] and loaded on-line to the sampling valve of the HPLC system and analyzed directly by the column-switching HPLC system described previously at certain selected reaction time intervals. During the 4-day reaction period, the initial sampling time was at 0 h, the next two sampling times were at the 4th and 8th hour, then every 8-h for the next 2 days, then every 12-h for the remaining day.

2.10. Limit of detection and lower limit of quantification of column-switching HPLC system

The limit of detection (LOD) and the lower limit of quantification (LLOQ) of the RI detection for the six carbohydrate products in the immobilized enzyme hydrolysate were obtained by using the six linear on-line internal standard calibration curves with the five lower analyte concentrations (0, 5, 10, 15, and 20 μg mL^{-1}). The best linear straight line ($y = a + bx$) of the calibration curve was obtained by the least-square regression method with Excel software. Then, the following equations were used to calculate the LOD and LLOQ.

$$s_{y/x} = \sqrt{\frac{\sum_i (y_i - y_i')^2}{n-2}} \quad (1)$$

$$\mathrm{LOD} = \frac{3s_{y/x}}{b} \quad (2)$$

$$\mathrm{LLOQ} = \frac{10s_{y/x}}{b} \quad (3)$$

In these equations, y_i is the measured peak area ratio for each individual carbohydrate standard concentration, y_i' is the theoretical peak area ratio obtained from the linear regression line, $s_{y/x}$ is the overall standard deviation of the linear regression line, b is the slope of the linear regression line, n is the number of the calibration standard used for the standard calibration curve, and $n-2$ is the degree of freedom [45]. Eq. (2) used for calculating LOD meets the definition of three times the signal to noise ratio (LOD = 3S/N) found in most instrumental analysis book [46].

2.11. Measurement precision and accuracy of the column-switching HPLC system

The measurement precision of the on-line column-switching HPLC analysis for the successive monitoring of the six carbohydrate products in the immobilized enzyme hydrolysate was estimated by calculating the percent relative standard deviation (RSD) from the five replicate measurements for each waste paper and waste tree branch hydrolysis sample taken from the bioreactor at the specific selected time interval.

The measurement accuracy of the on-line column-switching HPLC analysis was estimated from the spiked experiment wherein standard solution containing about matched amount of the six individual carbohydrate products was spiked on-line to the hydrolysate and analyzed exactly the same way as described previously. The individual percent recovery of the six spiked carbohydrate standards was calculated from the following equation. The percent recovery of the spiked carbohydrate standard reflects the accuracy of the analysis.

$$\mathrm{Recovery}(\%) = \frac{C_{\mathrm{measured\ total}}}{C_{\mathrm{hydrolysate}} + C_{\mathrm{spike}}} \times 100\% = \frac{C_{\mathrm{measured\ total}}}{C_{\mathrm{theoretical}}} \times 100\% \quad (4)$$

In the equation $C_{\mathrm{hydrolysate}}$ is the concentration of the specific carbohydrate product in the immobilized enzyme hydrolysate, C_{spike} is the measured concentration of the specific added carbohydrate standard, and $C_{\mathrm{measured\ total}}$ is the total concentration of the specific carbohydrate in the immobilized enzyme hydrolysate after spiking.

3. Results and discussion

3.1. Novel elution-time-difference column-switching HPLC technique

For the separation of the six carbohydrate standards (cellobiose, glucose, xylose, galactose, mannose, and arabinose) and the internal standard xylitol in aqueous solution, the use of single H^+ ion-exclusion column alone with deionized water as the mobile phase cannot resolve mannose, xylose, and galactose. The same situation was observed before with this column and a mobile phase of diluted H_2SO_4 [6,7]. The use of a single Pb^{2+} ion-exclusion column with distilled deionized water as the mobile phase was still not ideal, because there is a partial overlap between the peaks for glucose and for xylose and between the peaks for arabinose and for mannose.

Since the Pb^{2+} ion-exclusion column uses only mobile phase of high purity water and separates samples dissolved in highly pure water, it is not suitable for the separation of the six carbohydrate products in the high salt content enzymic hydrolysate with the Pb^{2+} ion-exclusion column alone. The salt material must be removed first from the carbohydrate products before the carbohydrate products enter the Pb^{2+} ion-exclusion column. Thus, a column-switching HPLC system was formed by coupling the H^+ ion-exclusion column parallel to a Pb^{2+} ion-exclusion column with two switching valves (Fig. 1).

In this column-switching HPLC system, the H^+ ion-exclusion column serves to separate the salt from the carbohydrates and to initially separate the carbohydrates and the internal standard. Since salts are eluted out first from the H^+ ion-exclusion column, they can be eluted to the waste container through the two switching valves following the flow direction as shown in valve position I of Fig. 1 until the first sugar (cellobiose) about comes out of the column. Then, the two switching valves were turned to valve position II (Fig. 1) that can heart-cut and deliver only the first five sugars, cellobiose, glucose, xylose, galactose, and mannose, to the Pb^{2+} ion-exclusion column for further separation. After the two switching valves were turned to valve position III which is the same as valve position I (Fig. 1), the arabinose sugar and the internal standard xylitol bypassed the Pb^{2+} ion-exclusion column and were quickly directed to the detector. In the meantime, the five sugars were still within the Pb^{2+} ion-exclusion column under elution. Therefore, arabinose and xylitol can be detected in advance owing to the elution-time-difference. The two switching valves were then turned to valve position IV (Fig. 1) to let the five completely separated sugars (cellobiose, glucose, xylose, galactose, and mannose) out from the Pb^{2+} ion-exclusion column to the detector. With this elution-time-difference technique the problem of the unresolved mannose and arabinose with the two columns connected in series was solved.

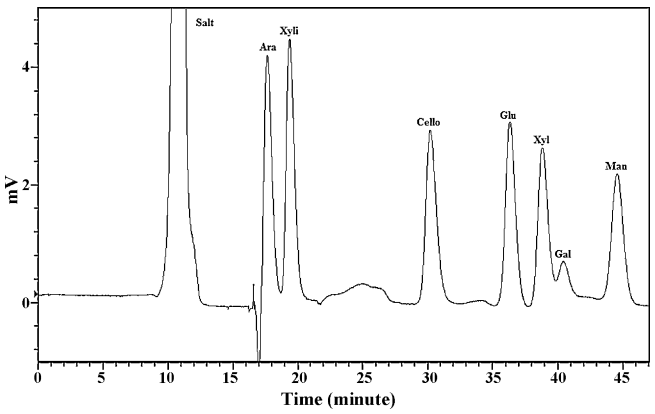

Fig. 2. The chromatogram of the column-switching HPLC shows the separation of salt, six carbohydrates, and internal standard in the matched matrix of immobilized enzyme hydrolysate with two chromatographic columns in parallel and the application of heart-cut and elution-time-difference technique. Column: Rezex ROA-organic acid; column: Supelcogel Pb; column temperature: 80 °C; mobile phase: deionized water; flow rate: 0.4 mL min^{-1}; detector: RI. Peak: Salt = salt; Ara = arabinose; Xyli = xylitol (internal standard); Cello = cellobiose; Glu = glucose; Xyl = xylose; Gal = galactose; Man = mannose. The concentration of the six carbohydrate standards and the internal standard was 100 μg mL^{-1}.

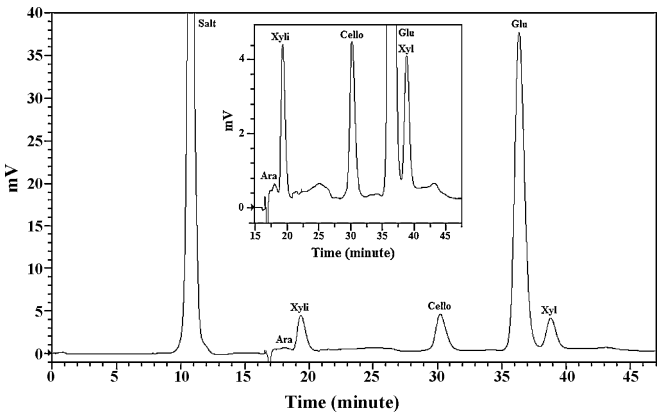

Fig. 3. The chromatogram of carbohydrate products in the immobilized enzyme hydrolysate of waste paper fiber at 32 h reaction time by the heart-cut and elution-time-difference technique coupled column-switching HPLC. Separation conditions, solute peak identification, and the internal standard concentration are the same as in Fig. 2.

Since xylose, galactose, and mannose turns out to be at one peak during the separation with the H^+ ion-exclusion column and is close to the next arabinose peak, the determination of the column-switching time for cutting the two peaks evenly with the valve position III is critical that will affect the analysis accuracy and precision of xylose, galactose, mannose, and arabinose. The optimal column-switching time found for switching the valve position II to valve position III was at 16.1 min that was determined statistically by ANOVA [45]. The exact column-switching time for each of the four valve positions is summarized in Table 1. Fig. 2 shows the chromatogram for the complete separation of salt, six sugars, and internal standard with the four sets of optimal column-switching time.

3.2. Quantification with internal standard calibration method

The linear regression results using the least-square method showed that the linear correlation coefficients (r^2) for the six internal standard calibration curves were 0.9984–0.9999. These values indicate excellent linearity for these standard calibration curves in the linear concentration range 0–2000 μg mL^{-1}. The LOD (LLOQ) for cellobiose, glucose xylose, galactose, mannose, and arabinose in the waste paper matrix were 1.24 (4.13), 1.92 (6.40), 4.96 (16.5), 6.57 (21.9), 1.48 (4.93), and 2.41 (8.03) μg mL^{-1}, respectively. The LOD (LLOQ) for cellobiose, glucose xylose, galactose, mannose, and arabinose in the waste tree branch matrix were 0.77 (2.57), 1.87 (6.23), 3.64 (12.1), 4.88 (16.3), 0.12 (0.40), and 1.44 (4.80) μg mL^{-1}, respectively. All these LODs and LLOQs show improved sensitivity for cellobiose, glucose, and xylose as compared with previous study [7].

The quantification of the internal standard xylitol was done by the on-line matrix matched external standard calibration curve method. The linear regression from the least-square method gave a best straight line equation $y = 1889.9x - 1247.8$ and a linear correlation coefficient (r^2) 0.9999. The linear concentration range of this calibration curve was 0–500 μg mL^{-1}. The LOD and LLOQ of xylitol found from five lower concentration standards of the on-line matrix matched external standard calibration curve was 0.40 and 1.33 μg mL^{-1}, respectively.

3.3. Carbohydrate product analysis for waste paper hydrolysis with on-line column-switching HPLC system

The chromatogram in Fig. 3 illustrates that salts are successfully separated from the carbohydrate products and only cellobiose, glucose, xylose, and arabinose can be found in the immobilized enzyme hydrolysate of waste paper fiber with the column-switching HPLC system. The amount of galactose and mannose in the immobilized enzyme hydrolysate of waste paper are below the LOD of the RI detector. Results in Table 2 show that the largest amount of glucose and xylose produced at the reaction time 96 h were 1739 and

Table 2
The quantitative results of the four carbohydrate products in the immobilized enzyme hydrolysate of waste paper fiber by the heart-cut and elution-time-difference technique coupled column-switching HPLC.

Reaction time (h)	Cellobiose		Glucose		Xylose		Arabinose		Xylitol	
	Conc. (ppm)	RSD (%)	Conc. (ppm)	RSD (%)	Conc. (ppm)	RSD (%)	Conc. (ppm)	RSD (%)	Conc. (ppm)	RSD (%)
0	20	0.21	6.6	0.5	n.d.[a]	–	n.d.[a]	–	96	0.2
4	172	1.8	236	0.6	42	0.2	3.3	2.0	94	3.0
8	185	0.6	431	0.2	63	1.7	3.9	4.6	93	2.5
16	205	0.4	742	0.6	102	0.6	4.5	0.9	95	0.2
24	184	0.1	917	0.2	120	0.4	4.6	0.3	94	0.1
32	162	0.3	1129	0.3	136	0.4	4.9	0.4	94	0.2
40	147	0.3	1256	0.1	144	0.1	4.3	2.7	94	0.2
48	127	0.4	1359	0.1	151	0.9	4.0	2.1	94	0.3
60	109	0.1	1476	0.1	158	0.1	3.8	0.8	94	0.1
72	97	0.1	1605	0.2	162	0.6	3.6	1.7	94	0.2
84	81	0.1	1673	0.1	165	0.3	3.5	2.2	94	0.1
96	71	0.1	1739	0.1	168	0.5	3.3	0.8	94	0.1

[a] n.d. = no detection.

Table 3
The quantitative results of the five carbohydrate products in the immobilized enzyme hydrolysate of waste tree branch fiber by the heart-cut and elution-time-difference technique coupled column-switching HPLC.

Reaction time (h)	Cellobiose		Glucose		Xylose		Mannose		Arabinose		Xylitol	
	Conc. (ppm)	RSD (%)	Conc. (ppm)	RSD (%)	Conc. (ppm)	RSD (%)	Conc. (ppm)	RSD (%)	Conc. (ppm)	RSD (%)	Conc. (ppm)	RSD (%)
0	9.2	0.1	9.9	3.6	n.d.[a]	–	n.d.[a]	–	n.d.[a]	–	95	0.7
4	44	0.4	167	1.6	12	2.4	0.7	3.5	4.4	2.0	90	0.3
8	22	0.2	224	0.7	14	1.8	0.8	5.1	3.9	2.7	91	0.2
16	12	1.0	192	1.0	15	0.3	0.8	2.7	3.3	1.7	89	2.6
24	9.0	4.4	182	0.9	15	5.0	0.9	3.2	2.9	4.5	89	4.1
32	8.3	3.8	183	0.6	16	1.6	0.7	4.5	2.9	3.1	91	0.2
40	6.2	3.9	182	1.9	16	6.2	0.7	5.3	2.6	3.3	88	1.1
48	4.4	3.8	175	2.1	14	4.0	0.6	7.8	2.3	5.1	90	0.3
60	4.6	2.8	176	1.2	11	2.3	0.5	2.7	2.2	5.7	93	0.1
72	3.3	0.8	156	1.3	9.5	1.8	0.4	1.2	n.d. [a]	–	90	0.1
84	3.2	5.3	124	2.0	8.6	0.8	n.d. [a]	–	n.d. [a]	–	89	0.3
96	n.d.[a]	–	100	1.8	7.6	1.6	n.d.[a]	–	n.d. [a]	–	90	0.9

[a] n.d. = no detection.

168 μg mL^{-1}, respectively. This result also indicates that cellulose is the principal component and hemicellulose is the minor component in the waste paper fiber. The largest amount of arabinose produced was at the reaction time 32 h (4.9 μg mL^{-1}) and subsequently its amount decreased quickly for an unknown reason.

Except for the initial reaction period the RSD values for glucose measurement were all quite low (<0.6%) after an 8-h reaction that demonstrated a high analysis precision for glucose determination with the on-line column-switching HPLC. The analysis precisions for both cellobiose and xylose were also very good (<1.8%); the RSD values were less than 0.9% after a 24-h reaction period. The approximate yield for glucose was about 70%, and the total hydrolysis sugar product yield including cellobiose, xylose, and arabinose was about 79%.

3.4. Carbohydrate product analysis for waste tree branch hydrolysis with on-line column-switching HPLC system

Only five carbohydrate products (cellobiose, glucose, xylose, mannose, and arabinose) were found in the immobilized enzyme hydrolysate of 2.5 g waste tree branch (Fig. 4). However, except for mannose which was not produced in the paper fiber hydrolysate, the amounts of all other four sugars were smaller than those produced from the hydrolysis of waste paper fiber (Tables 2 and 3). We also noticed that the reaction solution became acidic during the final 2 days. This could be due to the formation of gluconic and xylonic acids from the oxidation of glucose and xylose, respectively, in the complex waste tree branch fiber solution. The approximate glucose yield was about 9%, and the total product yield of the five sugars was about 12%.

For glucose analysis, the largest RSD value was 3.6% at the beginning of the hydrolysis and the range of the RSD values was 0.6–2.1% after the hydrolysis time of 4 h that demonstrates a very good measurement precision of the on-line column-switching HPLC system. For other sugars, the RSD values were in the range 0.1–7.8% which depends on the concentration level. In general, if the concentration is low and is close to the LOD such as mannose, the values of RSD are usually large. However, those large RSD values of arabinose were also due to the variation of baseline by switching the column.

The accuracy for the analysis of six sugars was found through the percentage recovery of the spiked experiment. The spiked amount for cellobiose, glucose, xylose, galactose, mannose, and arabinose was 5.7, 225, 17, 5.7, 2.8, and 2.7 μg mL^{-1}, respectively, that was about the amount produced for each carbohydrate product (excluding galactose and mannose) in the hydrolysate at 32 h reaction time (Table 3). The recovery rate determined for cellobiose, glucose, xylose, galactose, mannose, and arabinose was 97%, 99%, 102%, 95%, 94%, and 90%, respectively, that shows very good accuracy of the on-line column-switching HPLC system.

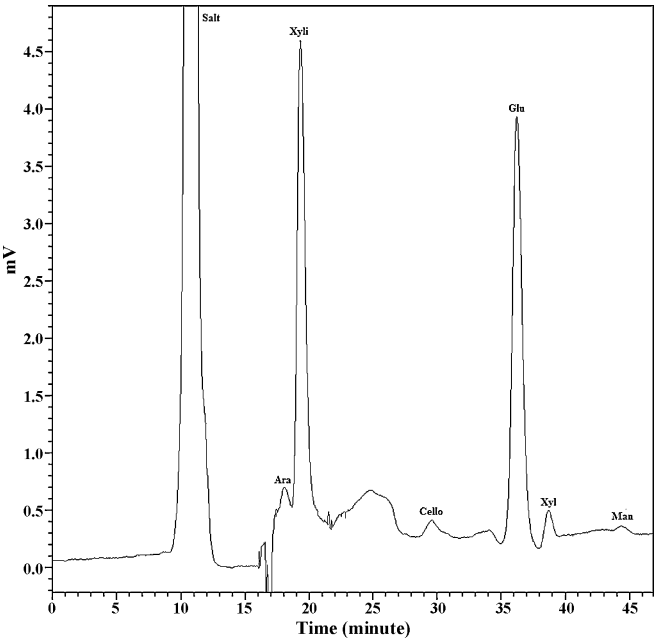

Fig. 4. The chromatogram of carbohydrate products in immobilized enzyme hydrolysate of waste tree branch fiber at 24 h reaction time by the heart-cut and elution-time-difference technique coupled column-switching HPLC. Separation conditions, solute peak identification, and the internal standard concentration are the same as in Fig. 2.

4. Conclusions

An innovative green column-switching HPLC analysis system was developed by on-line coupling of a H^+ ion-exclusion column and a Pb^{2+} ion-exclusion column through two column-switching valves. This column-switching HPLC system was successfully connected on-line to a vertically hanging immobilized enzyme reaction system with a specific designed sampling device to perform the on-line sampling, desalting, and simultaneous successive analysis of the six carbohydrate products from the co-hydrolysis of the immobilized cellulase and immobilized hemicellulase for either waste paper or waste tree branch fiber. All six carbohydrate products in the immobilized enzyme hydrolysate can be separated and analyzed efficiently with the heart-cut and the elution-time-difference technique coupled column-switching HPLC. The analytical results

demonstrated that the application of the carbohydrate products analysis with the developed on-line column-switching HPLC was accurate, precise, sensitive, time saving, and with no secondary contamination. The use of high purity water as the mobile phase fulfills the requirement of green technology.

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