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Developing a large-volume preparative method using a handmade HPLC column to fractionate dissolved organic matter

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ABSTRACT

To purify the heterogeneous natural organic matter (NOM), various experimental methods are used (e.g., XAD resin, ultrafiltration, size exclusion chromatography (SEC)). However, these methods have few limitations such as the reproducibility, the sample recovery, and the difficulty of the experimental procedure. Preparative high-performance liquid chromatography (prep-HPLC) with a handmade column was employed as a fractionation method for NOM. The method enables accommodation of a relatively large volume (up to 5–10 mL). To optimize the efficiency of the NOM fractionation system based on the molecular size of the NOM, the preparative column was packed with polymethacrylate resin in a laboratory. Quantitative evaluation of the handmade preparative column performance was conducted for several parameters including the number of theoretical plates (N), asymmetry factor (A_s), height equivalent to theoretical plates, and column resolution (R_s). The N parameter increased as the packing velocity increased, and A_s was close to 1. The optimum flow rate was selected at the highest R_s. A greywater includes complex organic matters that generated in households without fecal contamination. To verify the separation efficiency of the handmade column, the organic matters in the greywater are fractionated. The fractions were reanalyzed by an analytical HPLC system to confirm the separation efficiency of the prep-HPLC. The polydispersity (ρ) of the fractions were around 1. A comparison of the retention time of the original and fractionated samples indicates that the prep-HPLC system with the handmade column efficiently fractionated NOM according to its molecular size.

Keywords: NOM fractionation; Prep-HPLC; Handmade column; Toyopearl resin

1. Introduction

Natural organic matter (NOM) is known as a problematic material (i.e., membrane foulant) in water treatment systems [1,2] and as a disinfection by-product (DBP) precursor [3]. However, it is also a key material in aquatic ecosystems, where it serves as an energy source for microorganisms. Although NOM plays an important role in the aquatic environment, information about NOM characteristics is inadequate. Much of the resulting uncertainty is due to the heterogeneity and complexity of NOM. To obtain detailed and specific characteristics of NOM, fractionation is widely used (e.g., XAD resin, ultrafiltration, size-exclusion chromatography (SEC)).

Preparative high-performance liquid chromatography (prep-HPLC) has been employed as a purification procedure

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in chemical, food, and pharmaceutical research. Recently, prep-HPLC with a large-volume SEC column has been applied to characterize NOM using various detectors [4–7]. These studies were conducted under different prep-HPLC operating conditions because the system operating condition depends on the column properties. With the prep-HPLC system, various types of columns are available, but eluent type, flow rate, and sample injection volume should follow the instructions provided by the column manufacturer. Although these instructions guarantee analytical efficiency of the column, they can be a limiting factor for designing an experiment that satisfies the criteria of certain research questions. High price of column is another difficulty. High-resolution preparative column prices are almost 10,000 USD, but the durability of the column is weak because the injection of the high concentration of organic matters easily occurs column clogging or contamination. Thus, some researchers have made preparative columns suitable for their purposes in their laboratories to conduct specific experiments [8–10].

Although numerous papers have been published about prep-HPLC using handmade columns, there are only a few studies evaluating column-packing efficiency [9,10]. In most studies, separation efficiency of the manually packed columns was estimated graphically, by comparing the chromatograms made with the packed column and a ready-made column. However, quantitative evaluation of the performance of a packed column is important because the calculated values help to diagnose the column-packing state and operating condition of the system. Column performance is represented by several parameters, which include the number of plates (N), asymmetry factor (A_s), the height equivalent to theoretical plate (HETP), and column resolution (R_s). These parameters show the specific conditions of the packed column, but are influenced by the system operating conditions. These include the composition of eluents, flow rate, column length, and particle size [13]. Thus, it is necessary to evaluate a manually packed column using numbers calculated in relation to the operating conditions.

In this study, column packing procedures for a preparative SEC system were developed, and its separation efficiency was investigated. The objective of this study was to evaluate the separation performance of the prep-HPLC system with a handmade column using quantitative methods. Four different experiments were conducted to optimize the operating conditions of the prep-HPLC system using a manually packed column with quantitative evaluation methods. The different experiments addressed the influence of packing velocity on column performance, the interaction between polymethacrylate resin and sample, eluent flow-rate optimization, and performance estimation of the prep-HPLC using an analytic column.

2. Materials and methods

2.1. Sampling and sample preparation

Greywater samples were used in this study to evaluate the performance of the developed prep-HPLC system. A greywater includes complex organic matters that generated in households without fecal contamination. The greywater included wastewater from kitchen and bathroom sinks, showers, and washing machines. The water samples were collected from a small village (Namwon, South Korea) in December 2014. After sampling, the water was filtered through a 0.45-μm cellulose acetate membrane (Advantec, Japan). Dissolved organic carbon (DOC) was measured using a total organic carbon analyzer (TOC-V CPH, Shimadzu, Japan). The DOC concentration of the greywater was 8.9 mgC/L, and pH was 6.4. Because the injected sample was diluted due to diffusion of the sample in the preparative column, the sample was concentrated to about 100 mgC/L DOC by a rotary evaporator (N-1110S-W, Eyela, Japan) to increase the sensitivity of the prep-HPLC system. The concentrated sample was also filtered through a 0.45-μm cellulose acetate membrane. Samples were stored in the dark at 4°C until analysis.

2.2. Column packing procedure

Toyopearl resin is one of the general packing materials for SEC columns. It is composed of hydroxylated polymethacrylic polymers, and hence, surface hydroxyl groups induce minimal non-specific adsorption [14]. Because this resin is available in bulk, columns of different sizes can be devised by researchers in their laboratories [7,8]. Moreover, the stable physical and chemical characteristics (pH range: 2–14, pressure range: <19.7 atm) of the resin enable the HPLC system to operate under a variety of conditions.

Stainless steel columns of two sizes (250 × 20 mm and 500 × 20 mm) were packed with Toyopearl resin (HW50S, Tosoh Bioscience, Japan) according to the instruction manual (Fig. 1). To remove fine particles in the bulk (<35 μm), the resin in the shipping container was suspended following mixing with a phosphate buffer in a 1,000-mL graduated measuring cylinder (Duran, Germany). After 5–7 h, when the resin had settled, the supernatant was discarded. This step was repeated three times to minimize the amount of fine particles, which induce backpressure problems in the column by clogging. The settled resin was carefully poured into each column, which was about 50% full of phosphate buffer, along the inner wall to prevent the formation of air bubbles. Once the column was filled with resin, the phosphate buffer was pumped through the column after connecting the column to an LC binary pump (NS2001p, Futecs, Korea). Separation efficiency of a handmade column is related to its packing velocity, so there is potential for different column-packing states [9]. According to the packing instructions of the manufacturer, Toyopearl resin

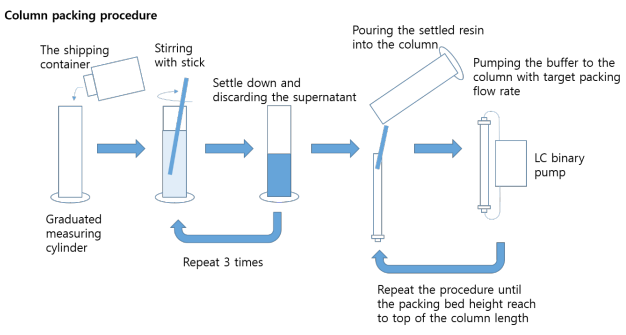

Fig. 1. Schematic diagram of the column packing procedure.

was packed into the columns at a higher flow rate (4 and 5 mL/min) than the targeted operating flow rate (2 mL/min). The flow rate was increased slowly to match the packing flow rate (4 and 5 mL/min). After about 30 min, each column was opened, and the packing bed height was checked. Then, the cavity formed by packing of the resin was refilled with more resin. This procedure was repeated until the cavity was completely removed. Finally, the column was connected to the prep-HPLC system and rinsed with 500 mL of phosphate buffer.

2.3. Evaluation of column-packing efficiency

The effectiveness of the packing procedure was tested using 5% acetone. From the chromatogram of 5% acetone, the number of theoretical plates (N), the asymmetry factor (A_s), and the HETP were calculated. The number of theoretical plates (N) means the number of sections in the column through which an injected sample should pass. Thus, the separation performance of the column relates to this number, with a larger number indicating higher separation efficiency. The number of theoretical plates was determined using the following equation [15]:

$$\mathrm{N} = 5.54\left(\frac{t_R}{W_{1/2}}\right)^2 \quad (1)$$

where t_R is retention time, and $W_{1/2}$ is the peak width at 50% of the maximum peak (Fig. 2).

The HETP is another significant indicator for column separation efficiency. This number is calculated by dividing the column length by the number of plates (N):

$$\mathrm{HETP} = \frac{L}{N} \quad (2)$$

where L is the length of the resin, and N is the number of theoretical plates. Hence, the column separation efficiency is better if the HETP is smaller.

The empirical asymmetry factor (A_s) is b/a. Here, "a" is the peak width (left half) at 10% of peak height, and "b" is the peak width (right half) at 10% of peak height (Fig. 2). The ideal value for A_s is 1, and the acceptable range is from 0.8 to 1.4. This number estimates the tightness of column packing.

The eluent flow rate significantly affects the resolution of a column (R_s). As R_s explains peak broadening and the selectivity of the column for the injected solutes, column resolution can be a better column-performance parameter than plate count N. Therefore, the optimum flow rate was selected after calculation of R_s depending on flow rates:

$$R_s = \frac{2(V_{R2} - V_{R1})}{W_{b1} + W_{b2}} \quad (3)$$

where V_R is the peak retention time of two eluting solutes (subscripts 1 and 2), and W_b is the peak width formed by intersection of the tangents to the curve inflection points with the baseline. Here, W_b can be obtained by $W_b = 1.72\ W_{1/2}$ [16].

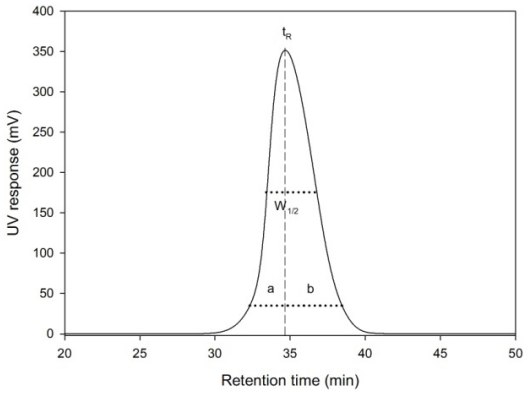

Fig. 2. Parameters for calculation of the number of theoretical plates (N) and asymmetry factor (A_s). t_R = retention time, $W_{1/2}$ = the peak width at 50% of the maximum peak, 'a' and 'b' = the peak width (left half, right half) at 10% of peak height.

2.4. Preparative HPLC

A prep-HPLC system (JAI-LC-9201, JAI, Japan) with a handmade column was employed, along with a ultraviolet (UV) (UV detector 3702, JAI, Japan) and an refractive index (RI) detector (RI detector 50s, JAI, Japan). Two columns of different sizes (250 × 20 mm and 500 × 20 mm), packed with Toyopearl resin, were used to fractionate NOM samples. The wavelength of the UV detector is 254 nm. The operating flow rate of the eluent was 2 mL/min, but different flow rates (3 and 4 mL/min) were also applied to optimize the flow rate. A phosphate buffer (2.4 mM sodium phosphate, 1.6 mM disodium hydrogen phosphate) was used as an eluent. In addition, 96 mM sodium chloride was added into the eluent to increase the ion strength. In the SEC, a strong ionic strength is required to reduce the interactions between the column resin and NOM. Various salts, such as sodium sulfate, sodium nitrate, and sodium chloride, are available to increase the ionic strength, but NaCl was selected according to the previous study [17]. In the paper, the ionic strength influences the analytical result until the ionic concentration is 0.1 M. Thus, 96 mM sodium chloride was used for achieving 0.1 M total ionic strength (2.4 mM sodium phosphate + 1.6 mM disodium hydrogen phosphate + 96 mM sodium chloride = 0.1 M total ionic strength). The ion strength of every injected sample was adjusted to about 10 μS cm^{-1} by adding sodium chloride. A gastight syringe was used to inject 5 mL samples.

The column separation efficiency was calculated using polyethylene glycols (PEGs; 400, 600, 1,000, and 4,600 MW). In many SEC studies, PEG and poly(styrene sulfonate) (PSS) were used as a molecular weight standard solution for system calibration. It has been reported that the characteristics of PSS are more similar to NOM than those of PEG [18,19]. However, the PEG solution was selected in this study because of the unwanted interaction between PSS and Toyopearl resin [18]. Because PEGs have no UV-detectable chromophore, an RI detector was connected in series after the UV detector to check the response of the PEG.

2.5. Analytical HPLC-SEC

The number-averaged molecular weight (M_n), weight-averaged molecular weight (M_w), and polydispersity (ρ) for fractions were measured using an analytical HPLC system with a silica-based Protein-Pak 125 column (7.8 × 300 mm, Waters, USA) and a UV detector (SPD-10AVP, Shimadzu, Japan). A phosphate buffer was used as an eluent. The flow rate of the eluent was 0.7 mL/min, and the injection volume was 200 μL. PSSs (210; 1.8, 4.6, 8, and 18 KDa) were used as standards for the molecular weight calibration curve. Values for M_w, M_n, and ρ were calculated using the following equations:

$$M_w = \sum_{i-1}^{n}\left(h_i \cdot M_i\right) / \sum_{i-1}^{n} h_i \quad (4)$$

$$M_n = \sum_{i-1}^{n} h_i / \sum_{i-1}^{n}\left(h_i / M_i\right) \quad (5)$$

$$\rho = M_w / M_n \quad (6)$$

where h_i and M_i are the height of the chromatogram and the molecular weight at eluted volume i, respectively [18].

3. Results and discussion

3.1. Influence of packing velocity on column performance

In the case of a manually packed column, the packing velocity is an important factor because it affects the packing condition of the column. A packing velocity that is too high causes a high-density packing condition by which resins can be damaged. On the other hand, a packing velocity that is too low causes inefficient separation performance. In this study, two packing velocities (4 and 5 mL/min) were used to determine their influence on column performance. The targeted packing velocities were selected to consider the expected operating eluent flow rate and the column internal pressure. General eluent flow rates are from 0.5 to 1 mL/min in analytical HPLC, but in prep-HPLC, the flow rates are from 2 to 5 mL/min because the preparative column has a larger diameter and longer length. Thus, the range of the operating flow rates of the handmade column were designed from 2 to 5 mL/min. Because the packing velocity should be higher than the expected operating eluent flow rate, the velocities were selected from 4 to 6 mL/min. However, the maximum velocity was decided to be 5 mL/min due to the limitation of the column internal pressure. The application of pressure was recommended from 0.5 to 3 bar by the resin manufacturer, but the column internal pressure reached to 5 bar when the packing velocity was 6 mL/min. Therefore, 4 and 5 mL/min were chosen as the packing velocities to find the optimum packing velocity.

After packing the column, 5 mL of 5% of acetone was analyzed at a flow rate of 2 mL/min. The number of theoretical plates (N), asymmetry factor (A_s), and the HETP were calculated for each column size (Table 1). Both columns show the same pattern (their HETP and A_s values are reduced at 5 mL/min packing velocity). It means that the higher packing velocity induced higher packing density of the column under the applicable pressure limit. The increase of N explained that a solute should pass more theoretical stages, which relate to the separation efficiency. Then the calculated A_s value at 5 mL/min packing velocity (250 × 20 mm: 1.7 and 500 × 20 mm: 1.3) verified that the resin is evenly packed in the column. Thus, column performance increases with increasing packing velocity.

Table 1
Column efficiency in relation to operating factors

Column size (mm)	Packing velocity (mL/min)	N (plates)	HETP[a] (cm/plate)	A_s[b]
250 × 20	60	577	0.043	2
	75	855	0.029	1.7
500 × 20	60	847	0.059	2.4
	75	1,837	0.027	1.3

[a]Reported HETP of Toyopearl packing columns ranged from 0.031 to 0.044 cm/plate [21].
[b]Acceptable A_s number ranged from 0.8 to 1.4.

According to Kato et al. [12], a final packing pressure range of 0.5–1.0 atm is optimal, and the optimum packing velocity of the 300 × 22 mm and 600 × 22 mm columns is 0.7–1 and 0.4–0.5 mL/min, respectively. However, in our system, the final packing pressures were 2 atm at 4 mL/min, and 3 atm at 5 mL/min. The difference in the results of both studies for optimum packing velocity and pressure might be explained by the use of different pumps. Kato et al. used a peristaltic pump to supply the solvent, and the maximal operating pressure of the pump was only 2.1 atm. In contrast, in this study, an LC binary pump with 250 atm of maximal operating pressure was employed. Because packing density relates to packing pressure, the relatively low packing pressure used in Kato et al.'s study might have caused a different optimum flow rate. Yamamoto et al. [21] reported that the HETP values in the Toyopearl columns ranged from ~0.031 to ~0.044 cm/plate. Thus, the packed columns at 5 mL/min of packing velocity were found to have acceptable packing states (0.027–0.029 cm/plate), when comparing the HETP values of Yamamoto et al. and this study. After packing the column, a calibration curve was defined using PEGs (400, 600, 1,000, and 4,600 MW) to confirm the column separation efficiency in relation to the molecular weight (Fig. 3). The retention times were proportional to the molecular weights of the PEGs (250 × 20 mm: R^2 = 0.9796 and 500 × 20 mm: R^2 = 0.9951).

3.2. Eluent flow-rate optimization

5 mL of mixed 400 and 4,600 MW PEG solutions were injected to find the optimum flow rate (Fig. 4). From the results, R_s values were calculated depending on the flow rates (Fig. 5). Total analytical time of the 500 × 20 mm column was 63 min at 2 mL/min, 42 min at 3 mL/min, and 32 min at 4 mL/min. The results of the 250 × 20 mm column were 33 min at 2 mL/min, 23 min at 3 mL/min, and 18 min at 4 mL/min. The end of the analytical time was determined by the time when the negative peak of the RI detector was completed. The negative peak appears due to the difference of the RI in the eluent and injected sample solution. This phenomenon always occurs unless if the solution is exactly same with

eluent. For this reason, the total analytical time was decided by using the negative peak.

Peak areas were reduced with increasing flow rate. This result can be explained by flow-induced degradation that occurs when the mobile phase velocity is high. According to Striegel [16], it was reported that the large amount of mechanical stress to highly extended macromolecules and the generated shear rates by the interstitial medium and the pore boundaries are the degradation mechanisms. Because the PEG, which is used in this paper, is a highly extended polymer and the high mobile phase velocity induces the high shear rate, the reducing peak areas can be explained by the flow-induced degradation.

The R_s of both columns decreased with increasing the flow rate (Table 2). It can be inferred that a high flow rate reduced the time gap between large and small molecules so that the resolution decreased. Therefore, based on these results, 2 mL/min was selected as the optimum flow rate for this study.

3.3. Interactions between resin and sample

Toyopearl resin is designed to minimize the non-specific adsorption between the packed resin and NOM as the hydroxylated aliphatic groups linked to the backbone structure of the resin. However, the various mechanisms, which induce the unnecessary interaction between a sample and a packed resin, always exist in SEC system, so the empirical experiments were conducted to investigate the possibility of sample loss during operation. 5 mL of 40 mgC/L of dissolved Suwannee River NOM (SR NOM) was injected into the column at a flow rate of 2 mL/min. The whole sample, which is shown from the total chromatogram by UV detector, was collected. Then, the organic carbon mass in the collected sample was calculated by multiplying the DOC concentration with the sample volume. The recovery rate was calculated by comparing the organic carbon mass in the injected and collected samples. The recovery rate of the 500 × 20 mm column was 89.5% ± 3.1% (n = 3), and the 250 × 20 mm column showed a recovery rate of 92.2% ± 5.4% (n = 3). These results were acceptable values when the recovery rate is compared with previous studies. They showed the recovery rates from 86% to 110% [22,23]. Subsequently, different concentrations of SR

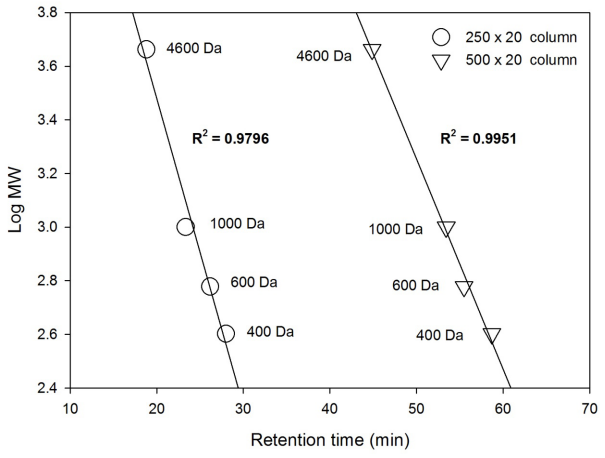

Fig. 3. Standard curve between logM_W and retention time with PEGs indifferent columns. The retention times of the injected PEGs in each columns (250 × 20 mm, 500 × 20 mm) were proportional to the column length.

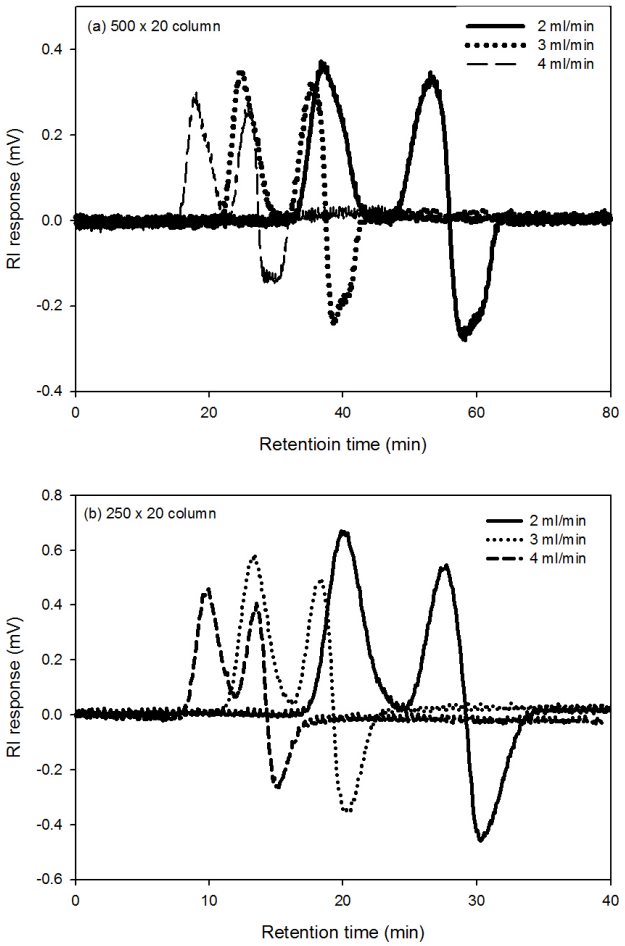

Fig. 4. Patterns of peak broadening by the different flow rate with columns (a) 500 × 20 mm (upper panel) and (b) 250 × 20 mm (lower panel).

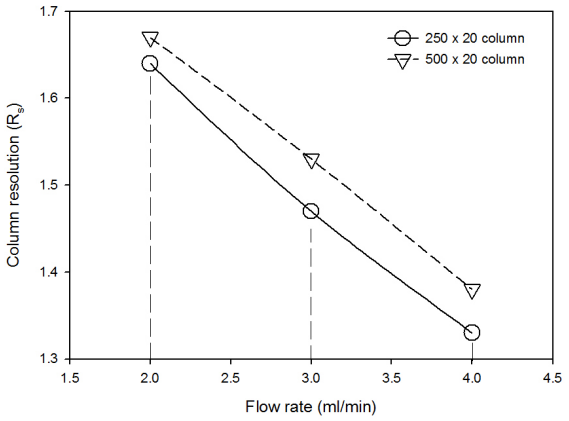

Fig. 5. Effects of flow rate on column resolution. The result in each columns showed lower flow rate was higher R_s.

Table 2
Analytical time and R_s value depending on column size

Column size (mm)	Packing velocity (mL/min)	Analytical time (min)	R_s
250 × 20	2	33	1.64
	3	23	1.47
	4	18	1.33
500 × 20	2	63	1.67
	3	42	1.53
	4	32	1.38

NOM samples were injected to verify the sensitivity of the UV detector in relation to the NOM concentrations (Fig. 6). The peak heights of the samples showed good proportionality to the DOC concentrations (250 × 20 mm: R^2 = 0.9994 and 500 × 20 mm: R^2 = 0.9998). Based on these results, the minimum detectable concentrations (2 mgC/L at 250 × 20 mm and 3.5 mgC/L at 500 × 20 mm) for separation of the system were identified, and it was concluded that most natural surface-water samples should be concentrated to achieve sufficient fractionation. When comparing the peak heights of the columns, it is apparent that all peak heights of the longer column are lower than those of the shorter column. This behavior is caused by the large inner volume of the larger column, which enables greater diffusion of the injected samples compared with the smaller column [24]. Therefore, the long column has an improved separation capacity due to its higher *N*, but the fractionated sample yield might be reduced because of diffusion.

3.4. Measuring performance of the prep-HPLC using an analytic column with greywater DOM

Greywater DOM was fractionated using the prep-HPLC system designed for this study (Fig. 7). A 5-mL concentrated sample (100 mgC/L) was injected into the 250 × 20 mm Toyopearl packing column with UV detector (254 nm). Although two columns of different sizes were packed, the 250 × 20 mm column was selected due to its shorter analytical time and similar HETP compared with the 500 × 20 mm column. Phosphate buffer was used as an eluent at a flow rate of 2 mL/min.

Fig. 8(a) illustrates the number of fractions of the injected sample depending on the peaks. The fractions were reanalyzed to verify the fraction performance, using the analytical HPLC system described in section 2.4. The R_s of the prep-HPLC system was lower than that of the analytical system, but the patterns of molecular size distribution were similar for both when the same kind of DOM sample was analyzed (Figs. 8(a) and (b)). It was also confirmed that the sample was well fractionated because the retention times at the maximum peak heights of the fractionated and original samples were identical. This was determined by comparing the chromatogram of the original sample and the combined chromatograms of the fractionated samples using the analytical HPLC system (Figs. 8(b) and (c)). To estimate the homogeneity of the fractions, the average molecular weights (M_n and M_w) and polydispersity (ρ) of the fractionated samples

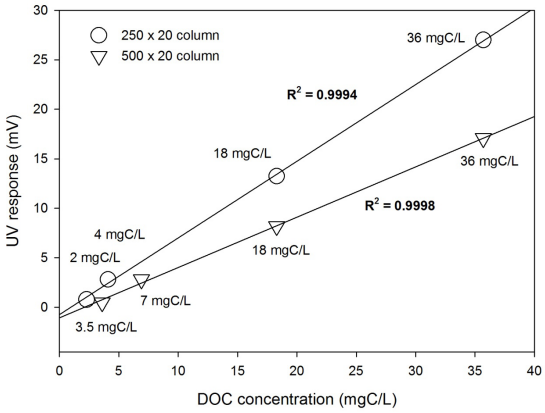

Fig. 6. Identifying the separation capacity of the two different columns through the relation between the peak intensity obtained from UV detection and the DOC concentration.

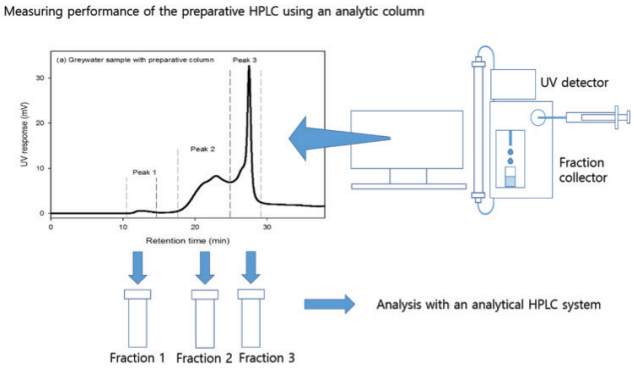

Fig. 7. Schematic diagram of the performance measuring procedure.

Table 3
Molecular weights of DOMs included in greywater samples and corresponding fractions

Sample	M_n	M_w	ρ
DOM in greywater	108	938	8.71
Peak 1	27,219	28,647	1.05
Peak 2	985	1,430	1.45
Peak 3	478	638	1.34

were determined using the analytical HPLC system (Table 3). Because a polydispersity of 1 means homogeneous polymers, the fractionation performance of this prep-HPLC system was confirmed (peak 1 = 1.05, peak 2 = 1.45, and peak 3 = 1.34). The values of the polydispersity of the fractions were similar to the reported values obtained by a ready-made column [4]. Thus, this result suggests that the prep-HPLC system with a handmade column is useful for NOM fractionation. From the result, it is verified that the reliable data can be obtained by the handmade column. The existing studies using the prep-HPLC system have the limitations about the flow rate because the ready-made column should be operated in the recommended conditions. However, the prep-HPLC system

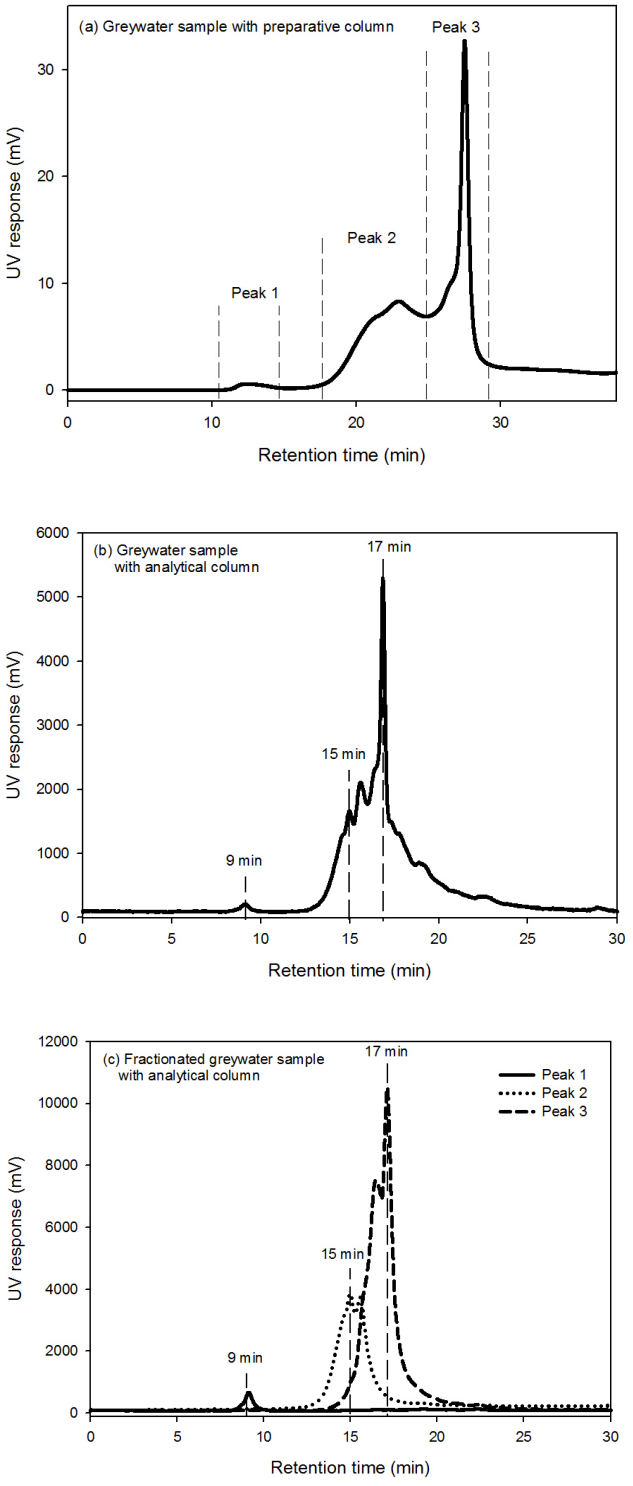

Fig. 8. Chromatograms of DOMs included in the greywater samples from preparative and analytical column;(a) greywater sample with preparative column 250 ×20 mm (upper panel), (b) greywater sample with analytical column (protein Pak 125, waters)(middle panel), (c) fractionated greywater sample with analytical column (protein Pak 125, waters)(lower panel).Chromatogram of a preparative handmade column was not exactly same with analytical one, but the preparative handmade column fractionated DOMs depending on the molecular size effectively.

with the handmade column can allow to apply various operating conditions. In this study, the injection of 5 mL sample was distinct from existing studies, which were operated with 1–2 mL sample volumes [4,22,25]. The large-volume injection of a sample is able to reduce the number of repetitions for collecting fractions. It is the major advantage to design a specific column, which can satisfy a purpose of a researcher in this study.

4. Conclusion

An economic, practical, and efficient DOM fractionation method using a prep-HPLC system with a large-volume preparative handmade column was presented. The reliable column-packing procedures were conducted to obtain the qualitative results for determining the separation efficiency of the handmade column. A stable DOM separation in terms of HETP value (0.027–0.029 cm/plate), sample recovery rate (89.5%–92.2%), and correlation between molecular weight and retention time (R^2 = 0.9796–0.9951) was observed. In addition, the calculated polydispersities of fractionated greywater DOM samples using the handmade column were nearly 1. These parameters are useful for quantitatively estimating the separation performance of the handmade column, without graphical comparisons of chromatography results between a ready-made column and a handmade column. The key information from this study is to provide the user-centered experimental design method for NOM fractionation in the aquatic environmental research field. NOM fractionation with prep-HPLC has been an effective method, but the durability of the expensive ready-made preparative column is one of the obstacles during the experiment. However, from this study, the findings about the practical column-packing method and the availability results from an environmental sample showed the alternative way to reduce the concern. Additionally, as the column is able to be made in the laboratory, various experimental attempts are allowed in terms of injection volume, eluent type, and flow rate. Therefore, the prep-HPLC system with a preparative handmade column is believed to be able to contribute to designing the various experimental conditions for NOM analysis.

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