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Molecular size fractionation of soil humic acids using preparative high performance size-exclusion chromatography

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ABSTRACT

High performance size-exclusion chromatography (HPSEC) is useful for the molecular size separation of soil humic acids (HAs), but there is no method available for various HAs with different chemical properties. In this paper the authors propose a new preparative HPSEC method for various soil HAs. Three soil HAs with different chemical properties were fractionated by a Shodex OHpak SB-2004 HQ column with 10 mM sodium phosphate buffer (pH 7.0)/acetonitrile (3:1, v/v) as an eluent. The HAs eluted within a reasonable column range time (12–25 min) without peak tailing. Preparative HPSEC chromatograms of these HAs indicated that non-size-exclusion effects were suppressed. The separated fractions were analyzed by HPSEC to determine their apparent molecular weights. These decreased sequentially from fraction 1 to fraction 10, suggesting that the HAs had been separated by their molecular size. The size-separated fractions of the soil HA were mixed to compare them with unfractionated HA. The analytical HPSEC chromatogram of the mixed HA was almost identical to that of the unfractionated HA. It appears that the HAs do not adsorb specifically to the column during preparative HPSEC. Our preparative HPSEC method allows for rapid and reproducible separation of various soil HAs by molecular size.

1. Introduction

Fractionation techniques, such as size-exclusion chromatography (SEC), adsorption chromatography [1], and precipitation [2], have been used to reduce the heterogeneity of soil humic acids (HAs), because HA is a heterogeneous mixture of natural organic macromolecules. The SEC fractionates HAs based on differences in molecular size using various soft gels with different nominal fractionation ranges [3–6]. The SEC is useful for fractionation and characterization of HAs; however, the fractionation procedure is laborious and time-consuming.

The development of high performance size-exclusion chromatography (HPSEC) allowed for rapid and reproducible size fractionation of natural organic matter. In early research, Becher et al. [7], using HPSEC fractionation of chlorinated dissolved organic matter (DOM) in marsh water, observed that mutagenic activity was associated with the low molecular weight DOM fraction. Recently, Piccolo and coworkers developed a preparative HPSEC method for HAs isolated from different sources, and characterized the separated fractions of HAs using pyrolysis-gas-chromatography/mass spectrometry, ^{1}H and ^{13}C NMR spectroscopies, and bioactivity [8–10]. These studies demonstrated the usefulness of preparative HPSEC for HA characterization.

However, there are potential problems in the application of HPSEC for humic substances. It is well known that the HPSEC elution pattern of humic substances is affected by ionic interaction with and specific adsorption to the stationary phase of the HPSEC column [11,12]. The intensity of these interactions depends on the chemical properties of the humic substances, e.g. functional group composition and aromatic structure. Since humic substances isolated from different sources show different chemical properties [13,14], the source of humic substances significantly affects the HPSEC elution pattern and fractionation.

Preparative HPSEC methods for aquatic natural organic matters were developed and validated by measuring the molecular weight distribution of separated fractions [15,16]. Egeberg and Alberts [16] show that specific adsorptions of their aquatic sample to the stationary phase are minor problem, but some aromatic standard reagents strongly interact with the stationary phase. Therefore, it is assumed that the specific hydrophobic interaction would prevent the size-exclusion separation of humic substances that have high proportion of aromatic composition. It also suggests that the optimization of HPSEC conditions for various soil HAs appears to be

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more difficult than that of aquatic natural organic matter, because some soil HAs have a significantly higher proportion of aromatic composition [14,17].

Most of the recent studies on preparative HPSEC of soil HAs have been based on the method investigated by Conte and Piccolo [18], who used a silica-based gel filtration column with 0.05 M NaCl solution as an eluent. In their method, preparative HPSEC chromatograms of some HAs showed a peak at the exclusion limit and exhibited a peak tailing [9,19]. These peaks represent non-size-exclusion effects, which could be attributed to the wide diversity of the chemical properties of soil HA. Furthermore, their method requires a long chromatography time (2–3 h) and did not taken advantage of the speed of HPSEC.

We therefore consider that there is no validated HPSEC method available for the rapid fractionation of HAs isolated from various types of soil. In particular, Melanudand (Andosol) HA, which has a significantly high proportion of aromatic composition [14], shows peak tailing in a HPSEC chromatogram [20]. We previously investigated the effects of HPSEC conditions on the elution pattern of soil HAs and developed an analytical HPSEC method for soil HAs, including Melanudand soil HA [20]. In this study, the analytical method was scaled up for the preparative HPSEC of soil HA. We demonstrated the molecular size separation of Melanudand and Dystrochrept soil HAs, and validated the method by analysis of molecular weight distribution of size-separated fractions. The main purpose of this work was to demonstrate and propose a rapid preparative HPSEC method for size fractionation of various soil HAs.

2. Experimental

2.1. Humic acid (HA)

HAs were extracted from the A horizons of Hanaore (HO; Hyogo, Japan, Typic Dystrochrept, mixed forest), Sugadaira forest (SGM; Nagano, Japan, Typic Melanudand, broad-leaved forest), and Sugadaira grassland soils (SGG; Nagano, Japan, Typic Melanudand, grassland), using the International Humic Substance Society method with some modifications [21,22]. Liquid-state ^{13}C NMR characteristics of the HAs are listed in Table 1. The SGG HA shows a significantly higher proportion of aromatic carbon and lower proportions of alkyl carbon and *O*-alkyl carbon than the HO HA. The distribution of the carbon species of the SGM HA is intermediate between that of the HO and SGG HAs.

For the preparative fractionation, 800 mg of the HA was suspended in 40 mL milliQ water and 1 M NaOH solution was gradually added to dissolve the HA. The solution (pH 7.0–8.5) was shaken slowly under nitrogen overnight. Following adjustment of the solution volume to 45 mL with milliQ water, 15 mL of 40 mM sodium phosphate buffer (pH 7.0) and 20 mL of acetonitrile were added to the solution. Consequently, the HA concentration was 10 mg mL^{-1} and the composition of sample solution was the same as that of the HPSEC eluent. This solution was filtered through a 0.22 μm hydrophilic PTFE membrane filter (Omnipore, Millipore, Tokyo, Japan). The total injected mass of the HO, SGM, and SGG HAs were 3.2, 2.3, and 2.5 g, respectively.

2.2. Analytical and preparative high performance size-exclusion chromatography (HPSEC)

We previously developed an analytical HPSEC method for various soil HAs using a Waters 600E system controller, 717 plus autosampler, and 2487 dual wavelength absorbance detector (Waters, Milford, MA, USA) [20]. In short, the method is as follows: column, Shodex OHpak SB-805 HQ column (Showa Denko, Tokyo, Japan; 0.8 mm I.D. × 300 mm; 0–4000 kDa for pullulan) with a Shodex OHpak SB-G guard column; column temperature, 40 °C; eluent, 10 mM sodium phosphate buffer (pH 7.0) + 25% acetonitrile (v/v); standard, sodium polystyrene sulfonate (PSSNa); flow rate, 0.8 mL min^{-1}; injection volume, 30 μL; detection, 260 nm. Blue Dextran (2000 kDa) and acetone were used for the determination of the void volume (V_0) and total permeation volume ($V_0 + V_i$), respectively.

The preparative HPSEC was carried using the same instruments, settings, and eluent as described above, but a Shodex OHpak SB-2004 HQ column (Showa Denko, Tokyo, Japan; 20 mm I.D. × 300 mm; 0–1000 kDa for pullulan) preceded by a Shodex OHpak SB-LG guard column (8.0 mm I.D. × 50 mm) was used at a flow rate of 3.0 mL min^{-1} and a detection wavelength of 650 nm. Although detection wavelength affects the shape of the HPSEC chromatogram of humic substances [23], 650 nm was used as a detection wavelength to estimate molecular size distribution. Two milliliters of HA solution was injected into the preparative column and fractionated into 10 fractions using a fraction collector (SF-2120, Advantec, Tokyo, Japan).

An exclusion peak of the HO HA was fractionated as fraction 1, and the eluate of the main broad peak of the HO HA was fractionated equally into nine fractions based on peak area (650 nm). Since the absorbance at 650 nm of the chromatogram peak of the SGM and SGG HAs exceeded the absorbance detector limit, the collection periods for the size fractions were calculated from a refractive index chromatogram using a Waters 2410 refractive index detector at a flow rate of 1.5 mL min^{-1} (this was the maximum tolerated flow rate of the detector). The eluates of SGM and SGG HAs were fractionated according to retention times that equally divided the peak into ten areas based on the peak area (refractive index), except for an exclusion peak of the SGM HA due to the low yield.

2.3. Isolation and HPSEC analysis of separated fractions of HA

The collected fractions were evaporated to remove acetonitrile. For the estimation of the molecular weight distributions, a portion of the solution was diluted 50 times with HPSEC eluent and analyzed by HPSEC. The whole HAs were also analyzed as described previously [20]. The molecular weight at peak maximum (M_p), weight-(M_w) and number-averaged molecular weights (M_n) were calculated by Waters Millennium 32 Chromatography Manager version 3.06 software.

The residual evaporated solution was acidified to pH 1.0 with 6 M HCl and centrifuged. Precipitated HA was dialyzed in deionized water (Spectra/Por CE membrane, molecular weight cut-off = 500 Da, Spectrum, Houston, TX, USA) and then freeze-dried.

2.4. HPSEC analysis of mixed solution of the separated HO HA fractions

To validate the effects of the fractionation procedure on the molecular distribution of the HA, fractions 2–10 of HO HA were combined and the analytical HPSEC chromatogram of the mixed sample was compared with that of the whole HO HA. The freeze-dried HO HA fraction (2.5 mg) was suspended in milliQ water (2.5 mL) and dissolved with 0.1 M NaOH. After gentle shaking overnight, the solution was made up to 25 mL (0.1 mg mL^{-1}) with 10 mM sodium phosphate buffer (pH 7.0). Absorbance of the solution at 600 nm was determined using a spectrophotometer (V-530, Jasco, Tokyo, Japan), and then the solutions of fractions 2–10 were mixed so that the contribution of each fraction to the absorbance would be equal in the mixed solution. This mixed solution was diluted to 50 times with the HPSEC eluent and analyzed by HPSEC.

Table 1
Proportion of each type of carbon and aromaticity determined by liquid-state ^{13}C NMR spectroscopy in humic acids from Hanaore (HO), Sugadaira forest (SGM), and Sugadaira grassland (SGG).

Humic acid	% of carbon species (δ, ppm)					Aromaticity[b]
	Alkyl C (5–48)	*O*-alkyl C (48–110)	Aromatic C (110–165)	Carboxyl C (165–190)	Carbonyl C (190–220)	
HO[a]	25.4	28.8	24.9	16.2	3.2	0.314
SGM	19.5	17.9	41.8	17.9	2.9	0.528
SGG[a]	6.7	15.1	54.2	19.4	4.6	0.713

Analytical parameters were detailed in Watanabe and Fujitake [14].
[a] Data from Watanabe and Fujitake [14].
[b] Aromaticity was calculated by (aromatic C + phenolic C)/(alkyl C + *O*-alkyl C + aromatic C + phenolic C).

3. Results and discussion

3.1. Fractionation of the soil HA by preparative HPSEC

Preparative HPSEC chromatograms for the three soil HAs are shown in Fig. 1, along with fractionation ranges. The absorbances of the SGM and SGG HAs were over the limit of the absorbance detector, so that chromatograms do not show precise molecular size distribution around the peak maximum.

The HA samples eluted within the effective column range (between V_0 and $V_0 + V_i$) without peak tailing, as is essential for size separation without the non-size-exclusion effects [24]. Aromaticities of HO, SGM, and SGG HAs were 0.314, 0.528, and 0.713, respectively (Table 1). The aromaticity represents the hydrophobic property of the HA, which can cause hydrophobic interactions with the gel matrix and substantial peak tailing. In this case, the peak tailing of the HPSEC chromatogram for SGG HA, which had the highest aromaticity, seemed to be suppressed enough.

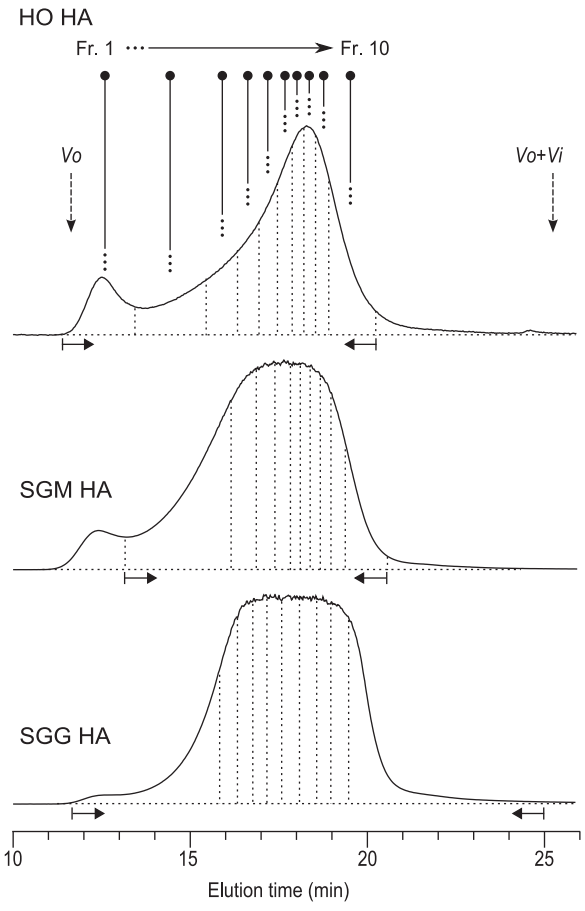

Fig. 1. Preparative HPSEC chromatograms of humic acids from Hanaore (HO), Sugadaira forest (SGM), and Sugadaira grassland (SGG) at 650 nm. Dotted lines represent fractionation periods. Column, Shodex OHpak SB-2004 HQ column with a Shodex OHpak SB-LG guard column; column temperature, 40 °C; eluent, 10 mM sodium phosphate buffer (pH 7.0) + 25% acetonitrile (v/v); flow rate, 3.0 mL min^{-1}; detection, 650 nm. V_0, void volume; $V_0 + V_i$, total permeation volume; Fr., fraction. Arrows indicate fractionation range.

The main difference between our method and previous preparative HPSEC method is the stationary phase of the column. We used the column packed with poly(hydroxy methacrylate) gel. Meanwhile, most of the studies on preparative HPSEC of HAs used a Biosep-SEC-S column (Phenomenex) [9,18,19], which consists of spherical silica gel bonded with a hydrophilic coating. The Biosep-SEC-S column was developed for the HPSEC of proteins and peptides and showed low non-specific interaction with synthetic peptides [25]. HAs might adsorb to residual silanol groups, since chemical structure of HAs should be more heterogeneous and hydrophobic than the synthetic peptides.

The peak at the exclusion limit of the HO and SGM HAs chromatograms (Fig. 1) is probably due to the lower separation range of the preparative column (0–1000 kDa for pullulan) than the analytical column (0–4000 kDa for pullulan) used in our previous study [20]. The preparative column with its narrower separation range was selected because of adequate separation of smaller molecules.

This method allowed each cycle of highly reproducible HPSEC fractionation to be completed in 30 min, thus boosting the efficiency of HPSEC preparation. Within 48 h, there was no difference between the HPSEC chromatograms. We performed HPSEC separation at very high HA concentrations (10 mg mL^{-1}). Therefore, our method is about 25- to 35-fold more efficient than the preparative HPSEC method developed by Piccolo et al. [8], which fractionated 5 mL of 0.6 mg mL^{-1} in 2–3 h.

The high concentration of HA might influence the elution pattern of HPSEC. The concentration of HA in solution should relate to its macromolecular configuration and aggregation [26,27]. In addition, a large volume injection of high concentration sample solution can give rise to differences in ionic strength between eluent and chromatographic zone. This gradient at the edge results in a specific peak at the total permeation volume ($V_0 + V_i$) [12,28].

The effect of sample concentration on the preparative HPSEC chromatogram of the HO HA was evaluated over the range of 0.02–10 mg mL^{-1}. No differences in the chromatographic pattern were observed (data not shown), and it is considered that the chromatograms are virtually independent of sample concentration. This result corresponds to the previous observations that HPSEC chromatograms of samples containing various concentrations of HA (3–10 mg mL^{-1} and 4–16 mg L^{-1}) had the same shape and elution time [29,30].

Ten fractions of each HA were collected by preparative chromatography (Fig. 1). The HA in each fraction was isolated by elimination of acetonitrile, followed by acid precipitation, dialysis, and freeze-drying. Table 2 shows the recovery of the separated HA fractions. The recovery of each fraction was significantly different, because the HA was separated based on the absorbance at 650 nm or refractive index. The relatively higher recovery of the lower

Table 2
Recovery, weight-averaged molecular weight (M_w), maximum peak molecular weight (M_p), and polydispersity of whole and size-separated fractions of soil humic acids from Hanaore (HO), Sugadaira forest (SGM), and Sugadaira grassland (SGG).

Humic acids	Recovery[a]		Apparent molecular weight[b]		Polydispersity[c]
	(mg)	(%)	M_p (kDa)	M_w (kDa)	(M_w/M_n)
HO HA					
Whole	–	–	3.52	28.2	14.9
Fr.1	–	–	163	190	70.0
Fr.2	87	2.7	47.8	82.8	13.4
Fr.3	98	3.1	21.8	31.5	2.93
Fr.4	128	4.0	12.7	16.0	1.95
Fr.5	181	5.7	8.28	10.0	2.22
Fr.6	172	5.4	5.73	6.75	1.75
Fr.7	132	4.1	4.37	5.14	1.60
Fr.8	178	5.6	3.48	4.10	1.48
Fr.9	135	4.2	2.64	3.19	1.51
Fr.10	210	6.6	2.05	2.27	1.99
Mixed	–	–	4.01	26.3	10.6
SGM HA					
Whole	–	–	3.61	12.9	5.55
Fr.1	179	7.8	34.6	58.8	4.88
Fr.2	68	3.0	17.3	20.4	1.96
Fr.3	102	4.4	11.8	13.2	2.68
Fr.4	76	3.3	8.85	9.72	3.14
Fr.5	45	2.0	7.15	7.99	2.75
Fr.6	91	4.0	6.00	6.74	2.42
Fr.7	61	2.7	4.91	5.70	2.22
Fr.8	49	2.1	4.02	4.65	2.05
Fr.9	130	5.7	3.09	3.76	1.87
Fr.10	282	12.3	1.98	2.38	1.89
SGG HA					
Whole	–	–	2.79	5.35	3.10
Fr.1	72	2.9	20.4	28.9	15.3
Fr.2	61	2.4	13.1	14.3	3.70
Fr.3	74	3.0	11.0	12.4	2.00
Fr.4	97	3.9	8.41	9.11	2.38
Fr.5	195	7.8	7.18	7.74	2.01
Fr.6	175	7.0	5.80	6.35	1.74
Fr.7	297	11.9	4.87	5.49	1.66
Fr.8	225	9.0	3.85	4.61	1.59
Fr.9	189	7.6	3.09	3.73	1.56
Fr.10	483	19.3	2.03	2.39	1.61

[a] Humic acid in each fraction was weighed after evaporation, acid precipitation, and freeze-drying.
[b] Sodium polystyrene sulfonates were used as standards.
[c] Polydispersity is the ratio of the weight-averaged (M_w) to number-averaged molecular weight (M_n).

molecular size range (later fractions) than of the higher molecular size range (early fractions) indicated that the smaller molecules have a lower relative absorbance and refractive index than larger molecules.

The total mass recoveries of the HO, SGM, and SGG HAs were 1.3 g (41%), 1.1 g (47%), and 1.9 g (79%), respectively. Significant weight loss of the HO and SGM HAs may result from adsorption of HA to the column and/or loss during the isolation procedure described above. Piccolo et al. [8] dialyzed separated fractions in dialysis tube (1 kDa cut-off) against deionized water and then freeze-dried them, attaining a mass recovery of preparative HPSEC of lignite HA of >98%. The main difference with their method was an acid precipitation procedure. The effects of weight loss on the properties of the HA were validated by analytical HPSEC of the separated fractions.

3.2. Molecular size distributions and apparent molecular weight of the whole HA and the separated fractions

Fig. 2 shows analytical HPSEC chromatograms for the whole HAs and for the separated fractions. The apparent molecular weight

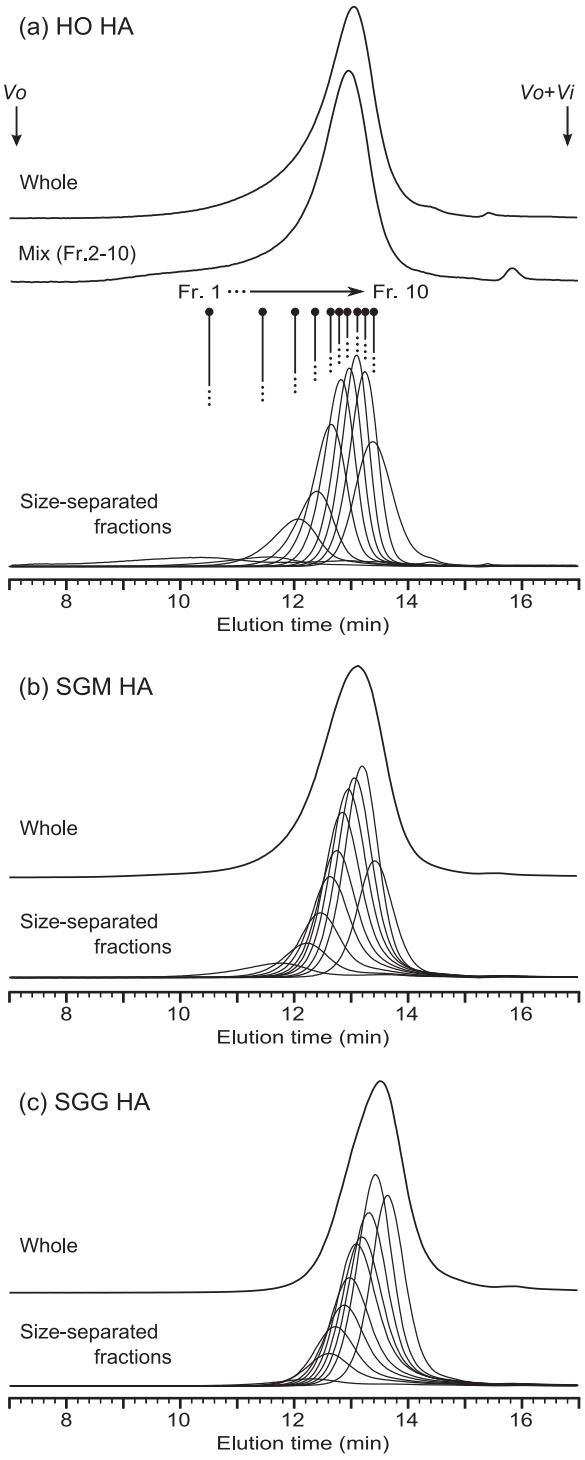

Fig. 2. Analytical HPSEC chromatograms of whole and size-separated fractions of humic acids from (a) Hanaore (HO), (b) Sugadaira forest (SGM), and (c) Sugadaira grassland (SGG) at 260 nm. The chromatogram of mixed HO humic acid, which is the mixture of fraction 2–10 of HO humic acids, is also shown. Column, Shodex OHpak SB-805 HQ column with a Shodex OHpak SB-G guard column; column temperature, 40 °C; eluent, 10 mM sodium phosphate buffer (pH 7.0) + 25% acetonitrile (v/v); flow rate, 0.8 mL min^{-1}; detection, 260 nm. V_0, void volume; $V_0 + V_i$, total permeation volume; Fr., fraction. The chromatogram of fraction 1 of HO humic acid is enlarged 5 times.

and polydispersity estimated from the analytical HPSEC chromatograms are listed in Table 2.

The whole HAs eluted as a broad monomodal distribution within the effective column range. The SGG HA eluted later than the HO and SGM HAs. Although the elution time of the peak maximum of the HO HA was similar to that of the SGM HA, the HO HA showed small shoulder in the higher molecular weight range. These results indicate that the HO HA had a larger molecular weight distribution than other HAs. This was confirmed by estimation of the apparent molecular weight of the whole HAs (Table 2). The molecular weights at peak maximum (M_p) of the whole HO, SGM, and SGG HAs were 3.52, 3.61, and 2.79 kDa, respectively, and the weight-averaged molecular weights (M_w) were 28.2, 12.9, and 5.35 kDa, respectively.

As shown in Fig. 2, the separated fractions were eluted sequentially from fraction 1 to fraction 10. The M_w of the separated fractions of HO HA decreased from 190 kDa in fraction 1 to 2.27 kDa in fraction 10 (Table 2). Similar trends were observed for the separated fractions of SGM and SGG HAs. We therefore considered that these fractions collected by our preparative HPSEC method were separated according to molecular size. Furthermore, this agreement between preparative and analytical HPSEC, with the elution order of the separated fractions, supports the supposition that our preparative HPSEC method is almost independent of sample concentration.

Apart from fraction 1, the size-separated fraction has a sharper and narrower peak than the whole HA (Fig. 2). The chromatogram of fraction 1 shows substantial peak tailing, similar to that seen following the reinjection of excluded fractions of humic substances [7,15]. The excluded fraction may contain large aggregates formed by self-association of low molecular size substances at high concentrations [7].

The narrower peaks of size-separated fractions compared to the whole HA corresponded with lower polydispersity, which is an indicator of the heterogeneity of humic substances. As shown in Table 2, the polydispersity of the HO HA size-separated fractions, at 1.51–2.93, was significantly lower than the whole HA, at 14.9, except for fraction 1. The high polydispersity of fraction 1 was due to the peak tailing. The decrease in polydispersity of size-separated fractions was also observed for the SGM (from 5.55 for the whole HA to 1.87–4.88 for fractions 1–10) and for the SGG HAs (from 3.10 for the whole HA to 1.56–2.38 for fractions 3–10). Consequently, our method was able to reduce the heterogeneity of the soil HAs.

To further validate this preparative method, fractions 2–10 of HO HA were mixed together and analyzed by HPSEC. Fraction 1 of HO HA was ignored, because the yield was significantly lower than that of the other fractions. The analytical HPSEC chromatogram of the mixed HO HA was nearly identical to that of the whole HO HA (Fig. 2), indicating the similarity in the molecular size distribution of the mixed and the whole HO HAs. This suggests the absence of specific adsorption of the HO HA on the preparative HPSEC column and of significant effects on the chemical properties of the HO HA during size fractionation. Additionally, similar chromatograms of the mixed and the whole HO HAs at 260 nm also show no significant difference in the preparative HPSEC chromatograms at lower wavelength (260 nm) and higher wavelength, since the mixed HO HA was fractionated and mixed based on absorbance at higher wavelength (650 and 600 nm).

Table 1 shows that the mixed HO HA sample exhibited higher M_p (4.01 kDa) and lower M_w (26.3 kDa) and polydispersity (10.6) compared to the whole HO HA (Table 1). These differences would be because of the absence of larger and smaller molecules eluting before and after the fraction 2–10. In particular, the M_w and the polydispersity (i.e., M_w/M_n) tend to be strongly influenced by high and low molecular weight molecules [31].

4. Conclusion

The proposed preparative HPSEC method was applied to three soil HAs (HO, SGM, and SGG HAs) with different chemical properties, and was evaluated by analytical HPSEC of the separated fractions. The negligible peak tailing indicated that the HAs eluted without the non-size-exclusion effects. Although recovery was low (HO HA; 41%, SGM HA; 47%, SGG HA; 79%), the preparative HPSEC allows for the rapid and efficient fractionation of soil HA.

Analysis at low concentrations showed that the apparent molecular weight of the separated fractions decreased sequentially from fraction 1 to fraction 10. The analytical HPSEC chromatogram of the mixed HA, which comprised fractions 2–10 of HO HA, was almost identical to that of the whole HA. These results suggest reasonable separation of the soil HAs and absence of significant effects on the chemical properties of HA during the preparative HPSEC.

The polydispersity of the size-separated fractions was lower (approximately 1.5–3.1) than that of the whole HAs (3.10–14.9), except for fractions 1 and 2. Therefore, the preparative HPSEC method used in this study is useful in reducing of the heterogeneity of soil HA. ^{1}H NMR spectroscopy demonstrates differences in the chemical structure of the size-separated fractions and usefulness of our method for detailed characterization of soil HA (see Supplementary data). The detailed characterization of size-separated soil HA would contribute to the elucidation of the genesis and functions of soil HA, since it is conceivable that molecular weight is a fundamental property of HA and has significant effects on interactions of HA with minerals, organisms, and xenobiotics [32].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2011.07.030.

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