Humic Acid Molecular Weight Estimation by High-Performance Size-Exclusion Chromatography with Ultraviolet Absorbance Detection and Refractive Index Detection

Jianzhong Song*
State Key Lab. of Organic Geochemistry
Guangzhou Institute of Geochemistry
Chinese Academy of Sciences
Guangzhou 510640, PR China
and
Dep. of Environmental Sciences
Rutgers, The State Univ. of New Jersey
New Brunswick, NJ 08901

Weilin Huang
Dep. of Environmental Sciences
Rutgers, The State Univ. of New Jersey
New Brunswick, NJ 08901

Ping'an Peng
State Key Lab. of Organic Geochemistry
Guangzhou Institute of Geochemistry
Chinese Academy of Sciences
Guangzhou 510640, PR China

Baohua Xiao
Yingjun Ma
Dep. of Environmental Sciences
Rutgers, The State Univ. of New Jersey
New Brunswick, NJ 08901

High-performance size-exclusion chromatography (HPSEC) with ultraviolet absorbance detection (UVAD) has been widely utilized to estimate the molecular weight (MW) and MW distribution of humic acids (HAs). The MW estimated by UVAD was inherently inaccurate, however, because UVAD set at 254 nm only detects limited HA components, and the molar absorptivities of different HA constituents are not equal. The objective of this study was to evaluate the refractive index detection (RID)-based HPSEC method for quantifying the MW of HAs. Five HA samples were quantified with both UVAD and RID. The chromatograms obtained on the two detectors showed that the RID/UVAD response ratios were consistently >10, indicating that RID is more sensitive for the detection of HAs. The chromatograms obtained with RID had three peaks compared with two peaks shown on UVAD chromatograms because RID detected a late-eluting peak (F_3) that was not shown on the UVAD chromatograms. Comparison of the RID/UVAD response ratio showed that the highest MW HA fraction (F_1) and lowest MW HA fraction (F_3) have higher RID/UVAD response ratios, whereas the medium HA fraction (F_2) had a lower RID/UVAD response ratio. These suggested that F_1 and F_3 may have relatively lower contents of UV-sensitive bonds such as C=C double bonds than F_2. Compared with HPSEC-UVAD chromatograms, the HPSEC-RID chromatograms resulted in higher weight-averaged MWs, lower number-averaged MWs, and higher polydispersivity for the tested HA samples. This study indicated that RID is less selective than UVAD for detection of structurally highly heterogeneous HA molecules and is thus better for characterizing the MW distribution of HA molecules.

Abbreviations: HA, humic acid; HPLC, high-performance liquid chromatography; HPSEC, high-performance size-exclusion chromatography; MW, molecular weight; PHA, Pahokee peat humic acid; PSS, polystyrene sulfonates; RID, refractive index detection; SEC, size-exclusion chromatography; UV, ultraviolet; UVAD, ultraviolet absorbance detection.

Molecular properties such as functionality and the MWs or sizes of HAs are key properties controlling HA reactivities in both natural and engineered aquatic systems (Chiou et al., 1987; Karanfil et al., 1996; Piccolo et al., 1996, 1998). Prior studies used several techniques for determining the MWs of HAs including ultrafiltration (Buffle et al., 1978), field flow fractionation (Giddings et al., 1987), vapor pressure osmometry (Figini and Marx-Figini, 1981), low-angle x-ray scattering (Wershaw and Pinckney, 1973), and HPSEC (Chin et al., 1994; Piccolo, 2001; Abbt-Braun et al., 2004; McDonald et al., 2004; Li et al., 2004; Fong and Mohamed, 2007; Wang et al., 2009). Among these techniques, HPSEC is the most convenient and is widely used. High-performance size-exclusion chromatography separates dissolved HA macromolecules according to their sizes, or more specifically, their hydrodynamic molecular volume. Molecular weights are typically estimated using synthetic polymers of known MW as external standards, assuming no chromatographic difference between polymers and HAs. Uncertainty in such

Soil Sci. Soc. Am. J. 74:2013–2020
Published online 29 Sept. 2010
doi:10.2136/sssaj2010.0011
Received 7 Jan. 2010.
*Corresponding author (songjzh@gig.ac.cn).

MW determinations is inevitable, however, due to the difference in polymer standard interactions with water and background electrolytes (Conte and Piccolo, 1999; von Wandruszka et al., 1999; Zhou et al., 2000; O'Loughlin and Chin, 2001; Her et al., 2002).

In recent years, HPSEC equipped with UVAD has been widely used due to various advantages such as small sample volume, minimal pretreatment, the availability of equipment, and the ease and speed of analysis (O'Loughlin and Chin, 2001; Zhou et al., 2000; Her et al., 2002, 2008; Hur et al., 2006; Fuentes et al., 2007; Espinoza et al., 2009). Several prior studies have reported much improved MW estimations for HAs and fulvic acids (FAs) by HPSEC. O'Loughlin and Chin (2001) examined the effect of the UVAD wavelength on the determination of the MW distribution of both HAs and FAs. They found that both the number-averaged (M_n) and weight-averaged (M_w) MWs of the humic substances increased as a function of the wavelength preset on the detector. Zhou et al. (2000) showed effects on the M_n, M_w, and polydispersivity (ρ, a measure of the sample heterogeneity) by the definition of the low-MW (LMW) cutoff. They recommended that either 2% of the maximum chromatogram height or MW = 50, whichever is higher, be the LMW cutoff and that 1% of the maximum chromatogram height be the high-MW (HMW) cutoff. Her et al. (2002) studied variations in MW distributions estimated by HPSEC with both UVAD and dissolved organic C (DOC) detection. Their results indicated that UVAD is not an adequate detector for quantitative analysis of MW estimation but rather can be used for limited qualitative analysis. They believed that the estimation of MW with UVAD is inherently inaccurate because ultraviolet (UV) absorbance at 254 nm detects only limited components (mostly π-bonded molecules) of natural organic matter (NOM), and the molar absorptivity of highly different NOM constituents may not be equal. Instead, MWs measured utilizing online DOC detection is a better presentation of NOM MWs.

This study was designed to characterize the MW distribution of HAs with HPSEC equipped with both UVAD and RID. Unlike UVAD that detects primarily the response of C=C double bonds, RID is less specific than UVAD. Prior studies have shown that RID is applicable for quantifying humic substances (Conte and Piccolo, 1999; Piccolo et al., 2001; von Wandruszka et al., 1999). Von Wandruszka et al. (1999) showed that the elution profiles recorded with both RID and UVAD were significantly different. We initiated this study to quantify and compare the apparent MW sizes and MW distribution with both RID and UVAD for typical HA samples. The information provided is useful for characterizing molecular sizes of NOM isolated from water, soils, and sediments.

Table 1. Elemental compositions and atomic ratios of the five humic acid (HA) samples.

Sample	Elemental composition				Atomic ratio	
	C	H	N	O	H/C	O/C
	% (w/w)					
Pahokee peat HA	52.7	5.39	3.15	33.8	1.23	0.48
Purified Aldrich HA	57.6	5.11	0.87	34.2	1.06	0.45
Canadian peat HA	49.6	4.78	2.66	34.3	1.16	0.52
Sandy soil HA	43.3	4.03	2.74	23.4	1.12	0.41
Kearny marsh sediment HA	52.9	5.98	2.49	25.6	1.36	0.36

MATERIALS AND METHODS

Chemicals

All inorganic chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, MO) or Merck Chemicals Ltd. (Gibbstown, NJ) in analytical grade or higher. Six polystyrene sulfonates (PSS) with MWs of 910, 3610, 6530, 14,900, 32,900, and 63,900 Da were purchased from Polymer Standard Service USA (Warwick, RI). These polymeric organic macromolecules were used as the standard materials having known MWs.

Humic Acids

A total of five HA samples were used in this study. Aldrich HA is a commercial humic acid in Na salt obtained from Sigma-Aldrich Chemicals (Milwaukee, WI). It was further purified with multiple steps of repeated pH adjustment, dissolution, precipitation, and centrifugation to remove ash, base-insoluble humin, and acid-soluble fulvic acid following a procedure reported by Swift (1996). The other four HA samples were extracted from Pahokee peat, Canadian peat, a sandy soil, and marsh sediment. Pahokee peat HA, originally collected from Pahokee, FL, was obtained from the International Humic Substances Society (St. Paul, MN). The Canadian peat sample was collected from the northern Great Plains on the eastern slope of the Rocky Mountains foothills and was kindly provided by Dr. Zicheng Yu of Lehigh University (Yu et al., 2003). The sandy soil was collected from a paddy field in the suburban area of Guangzhou, China (Song et al., 2002). The marsh sediment was collected from a marsh near Kearny, NJ.

The procedures described by Swift (1996) for isolation and purification of HA from soils and sediments were followed exactly. In brief, peat, soil, or sediment samples were first treated with 0.1 mol L^{-1} HCl (1:10 w/w), and sequentially extracted several times with 0.1 mol L^{-1} NaOH under a N_2 atmosphere, with each extraction lasting for 24 h. After extraction, the aqueous solution was separated from the solid by centrifugation at 10,000 rpm. The supernatants of all extractions were combined and acidified with 6 mol L^{-1} HCl to pH 1 to 2 for precipitating HA. After centrifugation, the HA precipitate was redissolved with a minimal volume of 0.1 mol L^{-1} KOH under a N_2 atmosphere and KCl was added to obtain a K^+ concentration of 0.3 mol L^{-1}. After removal of fine insoluble particles by centrifugation, the HA supernatant was acidified and the HA precipitate obtained was treated with 0.1 mol L^{-1} HCl + 0.3 mol L^{-1} HF solution for 24 h, dialyzed (Spectra/Por 3 dialysis tubes, 1000 MW cutoff, Spectrum Laboratories, Rancho Dominquez, CA) against distilled water until free of Cl^-. They were then freeze-dried and stored in brown glass bottles.

The purified HA samples were analyzed using an elemental analyzer (Elementar Vario EL, Elementar Analysensysteme GmbH, Hanau, Germany) following a standard high-temperature (900°C) combustion procedure. The results are listed in Table 1.

High-Performance Size-Exclusion Chromatography

The size-exclusion chromatography (SEC) was conducted on a high-performance liquid chromatography (HPLC) system (Agilent 1100, Agilent Technologies, Santa Clara, CA) coupled with a UV absorbance detector (Agilent G1315B) and a refractive index detector (Agilent G1362A). Ultraviolet absorption was recorded at $\lambda = 254$ nm based on previous studies (O'Loughlin and Chin, 2001; Zhou et al., 2000; Her et al., 2002; Hur and Schlautman, 2003; Janoš, 2003; Perminova et al., 2003; Li et al., 2004; Espinoza et al., 2009). The refractive index detector was connected to the UV absorbance detector outlet and the signals of both detectors were simultaneously processed with Agilent Chemstation software. A Biosep-Sec-2000 column (300 by 7.8 mm, Phenomenex, Torrance, CA) with a guard column of the same packing material (300 by 7.8 mm, Phenomenex) was used for SEC. The mobile phase used was a phosphate buffer (0.002 mol L^{-1} K_2HPO_4 + 0.002 mol L^{-1} KH_2PO_4) at pH 6.8 and with ionic strength adjusted to 0.1 mol L^{-1} with 0.1 mol L^{-1} NaCl solution. The HPLC was set at a flow rate of 1 mL/min and sample injection volume of 25 μL. Duplicate injections showed <3.5% of difference in peak areas of a given sample or standard on chromatograms obtained with both UVAD and RID.

Humic acid solutions at 300 mg/L were used for HPSEC analysis. The solutions were prepared by dissolving the purified and dried HA samples in 0.1 mol L^{-1} NaOH. After 24 h, the solution pH was adjusted to 6.8 with 1 mol L^{-1} HCl buffered with a phosphate buffer solution. The solutions were then filtered with a 0.2-μm cartridge (polytetrafluoroethylene) filter. The final HA solutions had exactly the same background electrolytes, solution pH, and ionic strength as the mobile phase of SEC. All five PSS standard solutions were prepared similarly. Blue Dextran (2000 kDa, Sigma-Aldrich, St. Louis, MO) served as a void volume probe and methanol (HPLC grade) as a permeation volume probe. We used PSS as the standards because they can be detected simultaneously with both UVAD and RID and they have charge densities mostly similar to HAs isolated from soils and sediments even though the structural similarity between PSS and HAs is debatable (Zhou et al., 2000). Indeed, the HA MW data determined with PSS as the standards were found to be close to those determined with vapor pressure osmometry and field-flow fractionation (Zhou et al., 2000).

High-Performance Size-Exclusion Chromatography Data Treatment

The data sets of retention time vs. known MWs for PSS standards and acetone with the two different detector systems were used to establish calibration curves for the HPSEC systems. Calibration equations, with $r^2 > 0.99$, were obtained for UVAD:

$$\log \mathrm{MW} = -3.0398 R_t + 4.6982 \quad [1]$$

and RID:

$$\log \mathrm{MW} = -4.4776 R_t + 7.7791 \quad [2]$$

where MW is the apparent molecular weight and R_t is the retention time.

These calibration equations were used to calculate the MWs of the tested HAs. The baselines of the chromatograms varied among the HA samples due to tailing. The baseline was set as 0 at 2% of the maximum chromatogram height in accordance with Zhou et al. (2000). The chromatograms were used to calculate the molecular characteristics of the HAs, including M_n, M_w, and ρ, which were determined using

$$M_w = \frac{\sum_{i=1}^{n} (h_i \mathrm{MW}_i)}{\sum_{i=1}^{n} h_i} \quad [3]$$

$$M_n = \frac{\sum_{i=1}^{n} h_i}{\sum_{i=1}^{n} (h_i / \mathrm{MW}_i)} \quad [4]$$

$$\rho = \frac{M_w}{M_n} \quad [5]$$

where h_i and MW_i are the height of the chromatogram and the MW of a HA sample corresponding to the ith retention time, respectively.

RESULTS

Ultraviolet Absorbance Detection and Refractive Index Detection Chromatograms

Figure 1 presents the HPSEC chromatograms obtained with UVAD and RID for the five HA samples. It is clear that the chromatograms obtained with UVAD and RID for each of the HA samples are very different. The chromatograms obtained with UVAD have a bimodal distribution of MW with relatively lower intensities whereas the chromatograms obtained with RID have three peaks with greater intensities. The bimodal distribution patterns shown in the HPSEC UVAD chromatograms are consistent with the observations reported by Li et al. (2003, 2004). According to the UVAD chromatogram of PHA, the first peak, designated as $F_{1,UVAD}$, corresponds to MWs ranging from 25,000 to 100,000 Da and may be structurally characterized by aliphatic functional groups (Li et al., 2003). The second peak ($F_{2,UVAD}$) has a higher intensity than F_1 and corresponds to the MWs ranging from >70 to 25,000 Da. According to Li et al. (2003), $F_{2,UVAD}$ can probably be characterized structurally as aromatic functional groups.

According to Fig. 1, the chromatograms obtained with RID have greater intensities with three distinguishable peaks, as divided in each of the chromatograms by two dashed vertical lines. The $F_{3,RID}$ peak, the lowest MW fraction on the RID chromatograms, was not detected with UVAD. As presented in Fig. 1 for PHA, the first peak ($F_{1,RID}$) has the lowest intensity among the three peaks and corresponds to MWs ranging from 25,000 to 100,000 Da. The second peak ($F_{2,RID}$) has the highest intensity and corresponds to MWs ranging from 300 to 25,000 Da, while the third peak ($F_{3,RID}$) has a moderate intensity and corresponds to MWs ranging from 70 to 300 Da. As seen from Fig. 1, the other four HA samples have similar chromatographic patterns. The quantitative properties of the chromatograms obtained for the five HA samples with the two different detectors are summarized in Table 2 and the similarities and differences are detailed below.

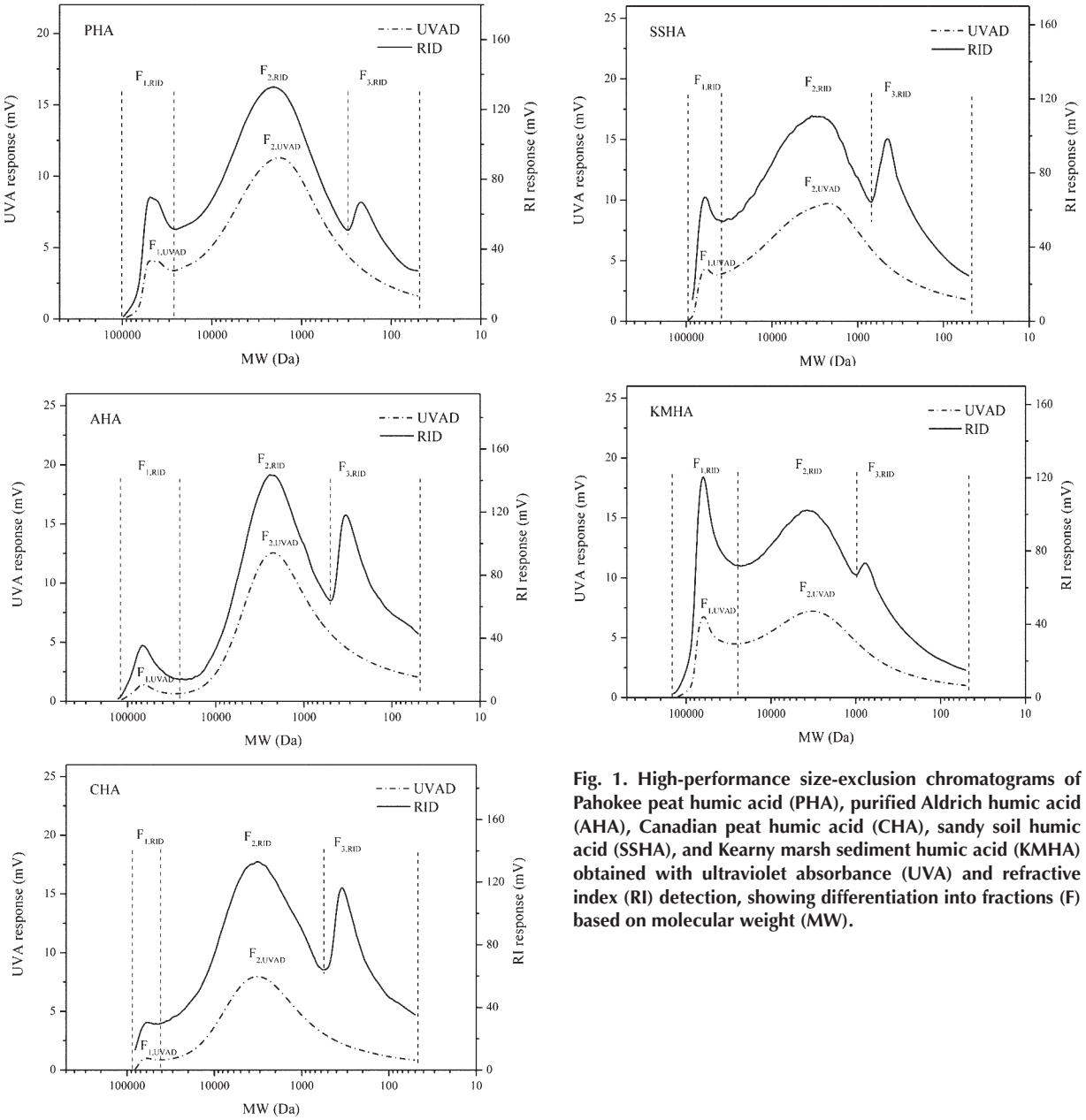

Fig. 1. High-performance size-exclusion chromatograms of Pahokee peat humic acid (PHA), purified Aldrich humic acid (AHA), Canadian peat humic acid (CHA), sandy soil humic acid (SSHA), and Kearny marsh sediment humic acid (KMHA) obtained with ultraviolet absorbance (UVA) and refractive index (RI) detection, showing differentiation into fractions (F) based on molecular weight (MW).

Molecular Weight Distribution and Polydispersivity

Table 2 lists the M_n, M_w, and ρ data and standard deviations calculated for the total HPSEC chromatograms and the peak fractions against the PSS calibration curves. As shown in Table 2, the two different detectors yielded statistically different molecular properties for each of the HA samples. For example, the peat humic acid PHA has an $M_{n,UVAD}$ value of 662 Da and $M_{w,UVAD}$ value of 6764 Da, but the calculated $M_{n,RID}$ and $M_{w,RID}$ values are 579 and 7994 Da, respectively. The resulting ρ value is 13.80 for PHA based on the RID chromatogram, which is statistically much higher than the 10.22 calculated from the UVAD chromatogram.

According to the HPSEC UVAD chromatogram of PHA, the HA sample is divided into two fractions. The $F_{1,UVAD}$ represents the high-MW subunit, which has M_n and M_w values of 40,601 and 43,222 Da, respectively. The $F_{2,UVAD}$ fraction represents the low-MW subunit, which has M_n and M_w values of 643 and 3823 Da, respectively. The high-MW subunit of PHA determined by UVAD has very low polydispersivity (ρ = 1.06) and the low-MW subunit has a relative high polydispersivity (ρ = 5.94), but both ρ values are statistically lower than the 10.22 of the bulk HA molecules (Table 2). The relative peak area of the low-MW HA subunit is 92.4%, much greater than the high-MW HA subunit (7.6%), indicating that the low-MW HA subunit dominates the PHA molecules.

As shown in Fig. 1, the PHA determined by RID has three fractions or subunits. The high-MW subunit ($F_{1,RID}$) has M_n and M_w values of 40,131 and 43,440 Da, respectively. The medium-MW subunit ($F_{2,RID}$) has M_n and M_w values of 1480 and 4565 Da, respectively. The low-MW subunit ($F_{3,RID}$) has M_n and M_w values of 125 and 159 Da, respectively. A similar low-MW fraction was reported by von Wandruszka et al. (1999) with no quantitative or semiquantitative details. The ρ values determined by RID are 1.08, 3.08, and 1.27 for the high-, medium-,

and low-MW subunits, respectively. These values are all statistically lower than the ρ value (13.80) of the bulk PHA (Table 2). The relative peak area of the medium-MW subunit is 74.1%, higher than the low- (15.3%) and high-MW subunits (10.6%).

Similar trends can be found for the other four samples. As shown in Table 2, the UVAD method yielded statistically greater M_n values and smaller M_w values than the RID method for each of the five HA samples. The M_w values calculated from the UVAD chromatograms fall in a range of 4469 to 13,961 Da, which is narrower than the commonly reported hundreds to millions of Da for soil HAs (Stevenson, 1994). The M_n values calculated from UVAD chromatograms varied within the range of 539 to 914 Da. The ρ value calculated for the high-MW subunits ($F_{1,UVAD}$) was in the range of 1.03 to 1.12, statistically significantly lower than the 4.91 to 9.27 values calculated for $F_{2,UVAD}$ (Table 2). The relative peak area of $F_{1,UVAD}$ is 2.1 to 18.8%, indicating that the low-MW subunit ($F_{2,UVDA}$) dominates.

Each of the five RID chromatograms of the five HA samples shows three subunits (Fig. 1). The high-MW $F_{1,RID}$ fraction has M_n and M_w values of 40,131 to 57,403 and 43,440 to 59,052 Da, respectively. The medium-MW $F_{2,RID}$ fraction has M_n and M_w values of 1480 to 3541 and 3328 to 8307 Da, respectively, and the low-MW $F_{3,RID}$ fraction has M_n and M_w values of 125 to 231 and 159 to 429 Da, respectively. The ρ values of the three subunits are 1.03 to 1.17 ($F_{1,RID}$), 1.96 to 3.08 ($F_{2,RID}$), and 1.27 to 1.85 ($F_{3,RID}$). The relative peak area of the $F_{2,RID}$ fraction is 53.4 to 74.1%, indicating that $F_{2,RID}$ is dominant.

Our data are comparable to those published in the literature. For example, the M_w values of the two peat humic acids (PHA and Canadian peat HA) measured by HPSEC UVAD are 6764 and 5525 Da, respectively. These M_w values are slightly lower than that of a mixture of peat HA and fulvic acid reported in Perminova et al. (2003). The sandy soil HA had an $M_{w,UVAD}$ value of 9097 Da, which is comparable with the values of 6.1 to 9.0 kDa reported by Perminova et al. (2003) for soil HAs.

DISCUSSION

The observed differences between the chromatograms obtained with two detectors for each HA sample can be attributed to the differences in the detection theory between UVAD and RID. It is known that the HPSEC UVAD method uses a UV absorption spectrophotometer as the detector for quantification of organic macromolecules and that the absorption of UV or visible radiation corresponds to the excitation of outer electrons (Skoog and Leary, 1992; Her et al., 2002; Wolfender, 2009). When an atom or molecule absorbs energy, electrons are promoted from their ground state to an excited state. The energies of the various types of molecular orbitals differ significantly. The electronic transitions among certain energy levels can be brought about by the absorption of radiation through σ–σ*, *n*–σ*, *n*–π*, and π–π* transitions (Skoog and Leary, 1992). A UV detector often detects transitions of *n* or π electrons on organic molecules to the π* excited state. The energies required for such transitions result in absorption peaks in an experimentally convenient spectral

Table 2. Molecular properties (number-averaged and weight-averaged molecular weight [M_n and M_w, respectively], polydispersivity [r], and chromatogram peak area) of the five humic acid (HA) samples and three fractions from the highest (F_1) to the lowest (F_3) molecular weight; RID detected a late-eluting peak (F_3) that was not shown on the UVAD chromatograms.

Sample†	Total			F_1			F_2				$F_{3,RID}$			
	M_n	M_w	ρ	M_n	M_w	ρ	Peak area	M_n	M_w	ρ	Peak area	M_n	M_w	ρ	Peak area
							%				%				%
							Ultraviolet absorbance detection								
PHA	662 ± 14‡	6,764 ± 152	10.22 ± 0.41	40,601 ± 1652	43,222 ± 1180	1.06 ± 0.01	7.6 ± 0.1	643 ± 29	3823 ± 130	5.94 ± 0.19	92.4 ± 0.1				
AHA	539 ± 10	4,469 ± 70	8.29 ± 0.20	54,154 ± 2746	60,518 ± 1822	1.12 ± 0.04	3.3 ± 0.1	521 ± 38	2562 ± 112	4.91 ± 0.17	96.7 ± 3.0				
CHA	749 ± 27	5,525 ± 223	7.37 ± 0.28	56,240 ± 1727	57,693 ± 998	1.03 ± 0.01	2.1 ± 0.1	742 ± 26	4428 ± 148	5.97 ± 0.13	97.9 ± 0.2				
SSHA	706 ± 31	9,097 ± 235	12.89 ± 0.41	56,912 ± 1975	58,608 ± 1278	1.03 ± 0.02	5.6 ± 0.2	667 ± 32	6184 ± 432	9.27 ± 0.43	94.4 ± 1.4				
KMHA	914 ± 24	13,961 ± 694	15.27 ± 0.65	45,803 ± 948	51,238 ± 2003	1.12 ± 0.02	18.8 ± 1.0	746 ± 26	5331 ± 167	7.15 ± 0.10	81.2 ± 2.5				
							Refractive index detection								
PHA	579 ± 17	7,994 ± 361	13.80 ± 0.65	40,131 ± 1111	43,440 ± 1368	1.08 ± 0.01	10.6 ± 0.4	1480 ± 20	4565 ± 111	3.08 ± 0.04	74.1 ± 1.3	125 ± 3	159 ± 2	1.27 ± 0.05	15.3 ± 0.7
AHA	398 ± 14	5,899 ± 191	14.81 ± 0.74	48,547 ± 1617	57,459 ± 2718	1.18 ± 0.05	6.6 ± 0.2	1699 ± 61	3328 ± 88	1.96 ± 0.08	60.6 ± 1.3	152 ± 7	223 ± 8	1.46 ± 0.04	32.8 ± 1.3
CHA	477 ± 10	6,126 ± 111	12.84 ± 0.15	57,403 ± 2315	59,052 ± 2067	1.03 ± 0.03	3.0 ± 0.1	2252 ± 73	6346 ± 184	2.82 ± 0.12	67.5 ± 0.5	165 ± 1	245 ± 3	1.49 ± 0.01	29.5 ± 1.1
SSHA	576 ± 17	9,380 ± 398	16.27 ± 0.81	52,975 ± 2283	55,446 ± 2068	1.05 ± 0.02	8.1 ± 0.3	3541 ± 87	8307 ± 475	2.35 ± 0.05	57.6 ± 2.8	219 ± 3	399 ± 2	1.83 ± 0.02	34.3 ± 0.6
KMHA	852 ± 26	15,533 ± 620	18.22 ± 0.46	43,521 ± 1758	50,853 ± 1841	1.17 ± 0.02	23.4 ± 0.7	3287 ± 44	6575 ± 289	2.00 ± 0.08	53.4 ± 2.1	231 ± 5	429 ± 15	1.85 ± 0.03	23.2 ± 0.6

† PHA, Pahokee peat HA; AHA, purified Aldrich HA; CHA, Canadian peat HA; SSHA, sandy soil HA; KMHA, Kearny marsh sediment HA.

‡ Mean ± standard deviation of three measurements for each sample.

region (200–700 nm) (Skoog and Leary, 1992). Both transitions require the presence of unsaturated C–C bonds (i.e., double or triple bonds in HA) to provide π orbitals. Because HA macromolecules are structurally highly heterogeneous, each HA molecule may have different function groups and a different content of C=C double bonds, possessing different molar absorptivity (ε). The MW determined based on the chromatograms of a UV absorbance detector (often set at 254 nm) is thus related to the HA molecules with relatively high ε rather than the bulk HA components. The inherent inaccuracy and hence underestimation of MWs by the UVAD method are unavoidable (Chin et al., 1994; Li et al., 2003, 2004; Her et al., 2002; O'Loughlin, and Chin, 2001; Perminova et al., 2003).

A refractive index detector is a common detector in HPLC and is very useful for detecting organic compounds that do not adsorb in the UV range and do not fluoresce (Conte and Piccolo, 1999; von Wandruszka et al., 1999; Piccolo et al., 2001; Chávez-Servín et al., 2004; Kamiński et al., 2004; Chen et al., 2007; Wolfender, 2009). It detects changes in the refractive index as liquid samples pass through the sample cell. A liquid or mobile phase often has a much lower refractive index than macromolecules of larger sizes. Macromolecular solutes eluted from a SEC column can cause sharp changes in the refractive index. A refractive index detector is thus an ideal detector for fast and reliable acquisition of HPLC data. It is especially suitable for quantification of non-UV-absorbing substances such as carbohydrates, lipids, and polymers (Chen et al., 2007; Chávez-Servín et al., 2004; Kamiński et al., 2004). As shown in this study and others (Conte and Piccolo, 1999; von Wandruszka et al., 1999; Piccolo et al., 2001), RID is very reliable for quantifying HAs because its response to the HA concentration is more universal than UVAD, whether aromatic vs. aliphatic structures or C–C single vs. double bonds of HA.

Our data indicate that RID is less selective and more sensitive than UVAD for the detection of structurally highly heterogeneous HA molecules. The chromatograms presented in Fig. 1 show that the RID responses are much higher than the UVAD responses for each of the HA samples, indicating that RID is more sensitive for the detection of organic molecules. To better compare the chromatograms obtained with UVAD vs. RID, the response ratios (RID/UVAD) are plotted against MW in Fig. 2. It shows that the RID/UVAD response ratios are consistently higher in the highest (F_1) and the lowest MW fractions ($F_{3,RID}$) for all five HA samples. The medium-MW HA fraction (F_2) has a low RID/UVAD response ratio. For example, PHA has a RID/UVAD response ratio of >15 for MW >25,000 and <300 Da. This suggests that the PHA macromolecules with MW >25,000 and <300 Da have relatively lower molar absorptivity of UV at the wavelength of 254 nm. As discussed above, this low UV absorptivity is probably related to lower contents of C=C double bonds. According to Li et al. (2003), the HA fractions with larger MWs contain more aliphatic carbons, whereas the fractions with smaller MWs have more aromatic structures. Apparently, the aliphatic C chains have lower contents of C=C double bonds that have lower UV absorptivity and hence a lower response to UVAD. Conversely, the aromatic HAs (equivalent to F_2) have greater contents of C=C double bonds that have a higher UV absorptivity and a stronger response to UVAD. It is noted that $F_{3,RID}$ also has a higher response ratio, which may result from a non-aromatic, low-MW HA component (von Wandruszka et al., 1999).

The apparent MWs determined for the HAs appear to be correlated with the elemental composition of the bulk HAs. Figure 3 shows that the relative peak areas of F_1 on both UVAD and RID chromatograms increase as a function of H/C atomic ratios. A regression procedure yields two linear correlations of the F_1 peak area with the H/C atomic ratios, with $R^2 > 0.75$. Such correlations may suggest that the greater MW HA may have higher contents of aliphatic C. Figure 4 indicates that the relative peak areas of $F_{2,RID} + F_{3,RID}$ or $F_{2,UVAD}$ increase as a function of the O/C atomic ratio. These linear correlations indicate that

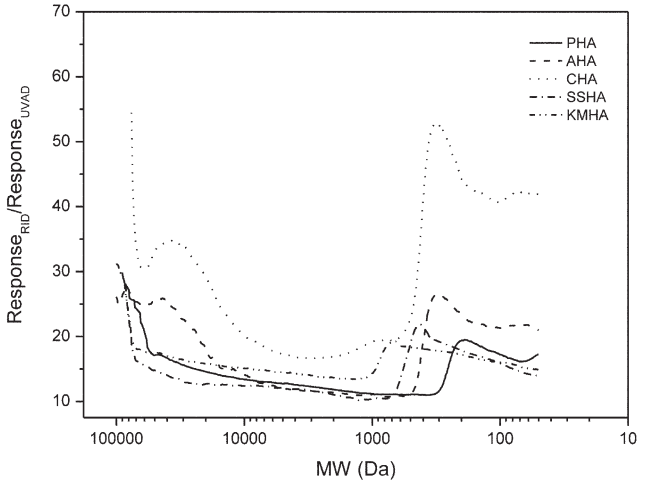

Fig. 2. Changes in the refractive index detection/ultraviolet absorbance detection (RID/UVAD) response ratios for Pahokee peat humic acid (PHA), purified Aldrich humic acid (AHA), Canadian peat humic acid (CHA), sandy soil humic acid (SSHA), and Kearny marsh sediment humic acid (KMHA) as molecular weight (MW) increases.

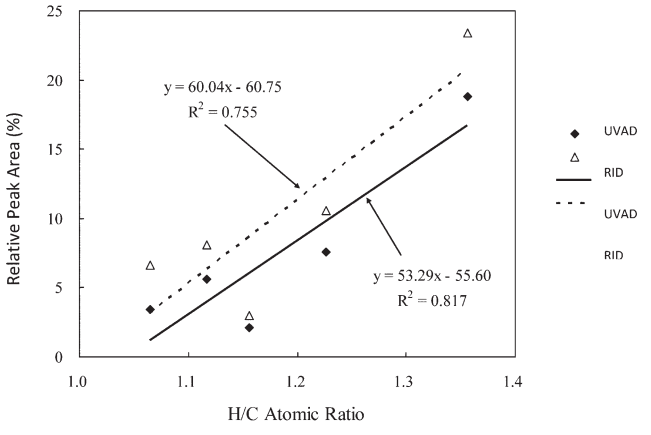

Fig. 3. Changes in the relative peak area of the high-molecular-weight subunit (F_1) as a function of the H/C atomic ratio for the five humic acid samples using ultraviolet absorbance detection (UVAD) and refractive index detection (RID).

the low-MW subunits may have more O-containing functional groups.

CONCLUSIONS

Both UVAD and RID used for quantifying HAs eluted from HPSEC were shown to result in very different chromatograms for each of the five HA samples. In general, RID has much stronger responses than UVAD, as the RID/UVAD response ratios are consistently >10. All HPSEC RID chromatograms have unique, late-eluting peaks of HA molecules having several hundreds of Daltons, whereas HPSEC UVAD failed to detect this fraction. The RID/UVAD response ratios are higher in the highest and the lowest MW fractions and lower in the medium-MW HA fraction for all five HA samples. These observations are consistent with the fact that HAs with larger MWs (F_1) are more aliphatic than those with smaller MWs (F_2). This larger MW HA fraction may have relatively lower contents of UV-sensitive bonds such as C=C double bonds. The lowest MW HA fraction ($F_{3,RID}$) also has a higher response ratio, which may result from non-aromatic, low-MW HA components. Compared with the HPSEC UVAD chromatograms, the HPSEC RID chromatograms yield higher weight-averaged MW, lower number-averaged MW, and higher polydispersivity. This study indicates that RID is less selective than UVAD for detection of structurally highly heterogeneous HA molecules.

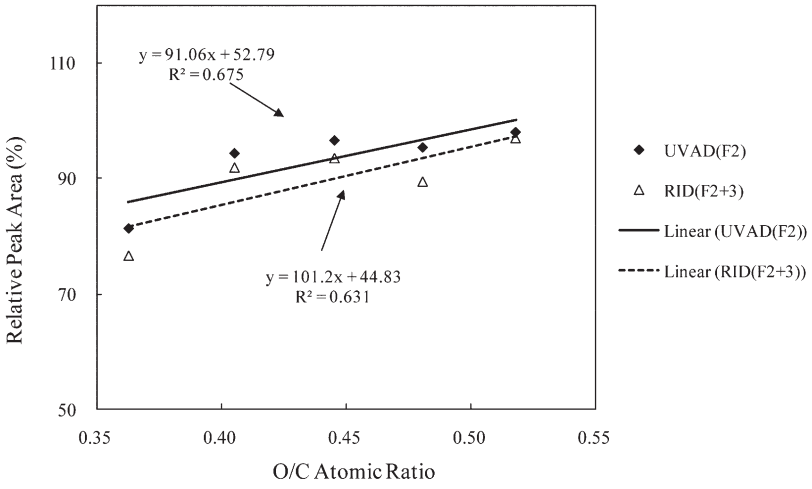

Fig. 4. Changes in the relative peak area of the low-molecular-weight subunit (F_2 or F_{2+3}) as a function of the O/C atomic ratio for the five humic acid samples using ultraviolet absorbance detection (UVAD) and refractive index detection (RID); RID detected a late-eluting peak (F_3) that was not shown on the UVAD chromatograms.

ACKNOWLEDGMENTS

We Thank Three anonymous reviewers for their valuable comments and suggestions on the original manuscript. The funding for this work was provided by the National Natural Science foundation of China (Grants 40975090, 40830745, and 40505026), Chinese Academy of Sciences, and the Multistate Project (W-1082) from the USDA Cooperative State Research, Education, and Extension Service. This is contribution is no. IS-1202 from GIGCAS.

REFERENCES

Abbt-Braun, G., U. Lankes, and F.H. Frimmel. 2004. Structural characterization of aquatic humic substances: The need for a multiple method approach. Aquat. Sci. 66:151–170.

Buffle, J., J.P. Deladsey, and W. Haerdi. 1978. The use of ultrafiltration for the separation and fractionation of organic ligands in fresh waters. Anal. Chim. Acta 101:339–357.

Chávez-Servín, J.L., A.L. Castellote, and M.C. López-Sabater. 2004. Analysis of mono- and disaccharides in milk-based formulae by high-performance liquid chromatography with refractive index detection. J. Chromatogr. A 1043:211–215.

Chen, J., Z. Wang, J. Wu, Q. Wang, and X. Hu. 2007. Chemical compositional characterization of eight pear cultivars grown in China. Food Chem. 104:268–275.

Chin, Y., G. Aiken, and E. O'Loughlin. 1994. Molecular weight, polydispersity, and spectroscopic properties of aquatic humic substances. Environ. Sci. Technol. 28:1853–1858.

Chiou, C.T., D.E. Kile, T.I. Brinton, R.L. Malcolm, J.A. Leenheer, and P. MacCarthy. 1987. A comparison of water solubility of organic solutes by aquatic humic materials and commercial humic acids. Environ. Sci. Technol. 21:1231–1234.

Conte, P., and A. Piccolo. 1999. Conformational arrangement of dissolved humic substances. Influence of solution composition on association of humic molecules. Environ. Sci. Technol. 33:1682–1690.

Espinoza, L.A.T., E. Haseborg, M. Weber, and F.H. Frimmel. 2009. Investigation of the photocatalytic degradation of brown water natural organic matter by size exclusion chromatography. Appl. Catal. B 87:56–62.

Figini, R.V., and M. Marx-Figini. 1981. On the molecular weight determination by vapour pressure osmometry: 3a. Relationship between diffusion coefficient of the solute and noncolligative behaviour. Macromol. Chem. Phys. 182:437–441.

Fong, S.S., and M. Mohamed. 2007. Chemical characterization of humic substances occurring in the peats of Sarawak, Malaysia. Org. Geochem. 38:967–976.

Fuentes, M., R. Baigorri, G. González-Gaitano, and J.M. García-Mina. 2007. The complementary use of ^{1}H NMR, ^{13}C NMR, FTIR and size exclusion chromatography to investigate the principal structural changes associated with composting of organic materials with diverse origin. Org. Geochem. 38:2012–2023.

Giddings, J.C., P.S. Williams, and R. Beckett. 1987. Fractionating power in programmed field-flow fractionation: Exponential sedimentation field decay. Anal. Chem. 59:28–37.

Her, N., G. Amy, D. Foss, and J. Cho. 2002. Variations of molecular weight estimation by HP-size exclusion chromatography with UVA versus online DOC detection. Environ. Sci. Technol. 36:3393–3399.

Her, N., G. Amy, J. Sohn, and U. Gunten. 2008. UV absorbance ratio index with size exclusion chromatography (URI-SEC) as an NOM property indicator. Aqua 57:35–44.

Hur, J., and M.A. Schlautman. 2003. Using selected operational descriptors to examine the heterogeneity within a bulk humic substance. Environ. Sci. Technol. 37:880–887.

Hur, J., M.A. Williams, and M.A. Schlautman. 2006. Evaluating spectroscopic and chromatographic techniques to resolve dissolved organic matter via end member mixing analysis. Chemosphere 63:387–402.

Janoš, P. 2003. Separation methods in the chemistry of humic substances. J. Chromatogr. A 983:1–18.

Kamiński, M., R. Kartanowicz, and A. Przyjazny. 2004. Application of high-performance liquid chromatography with ultraviolet diode array detection and refractive index detection to the determination of class composition and to the analysis of gasoline. J. Chromatogr. A 1029:77–85.

Karanfil, T., J.E. Kilduff, M.A. Schlautman, and W.J. Weber, Jr. 1996. The influence of dissolved oxygen. Environ. Sci. Technol. 30:2195–2201.

Li, L., W. Huang, P. Peng, G. Sheng, and J. Fu. 2003. Chemical and molecular heterogeneity of humic acids repetitively extracted from a peat. Soil Sci. Soc. Am. J. 67:740–746.

Li, L., Z. Zhao, W. Huang, P. Peng, G. Sheng, and J. Fu. 2004. Characterization of humic acids fractionated by ultrafiltration. Org. Geochem. 35:1025–1037.

McDonald, S., A.G. Bishop, P.D. Prenzler, and K. Robards. 2004. Analytical chemistry of freshwater humic substances. Anal. Chim. Acta 527:105–124.

O'Loughlin, E., and Y. Chin. 2001. Effect of detector wavelength on the determination of the molecular weight of humic substances by high-pressure size exclusion chromatography. Water Res. 35:333–338.

Perminova, I.V., F.H. Frimmel, A.V. Kudryavtsev, N.A. Kulikova, G. Abbt-Braun, S. Hesse, and V.S. Petrosyan. 2003. Molecular weight characteristics of humic substances from different environments as determined by size exclusion chromatography and their statistical evaluation. Environ. Sci. Technol. 37:2477–2485.

Piccolo, A. 2001. The supramolecular structure of humic substances. Soil Sci. 166:810–832.

Piccolo, A., G. Celano, and P. Conte. 1996. Adsorption of glyphosate by humic substances. J. Agric. Food Chem. 44:2442–2446.

Piccolo, A., P. Conte, and A. Cozzolino. 2001. Chromatographic and spectrophotometric properties of dissolved humic substances compared with macromolecular polymers. Soil Sci. 166:174–185.

Piccolo, A., P. Conte, I. Scheunert, and M. Paci. 1998. Atrazine interactions with soil humic substances of different molecular structure. J. Environ. Qual. 27:1324–1333.

Skoog, D.A., and J.J. Leary. 1992. Principles of instrumental analysis. 4th ed. Saunders College Publ., Philadelphia.

Song, J., P. Peng, and W. Huang. 2002. Black carbon and kerogen in soils and sediments: 1. Quantification and characterization. Environ. Sci. Technol. 36:3960–3967.

Stevenson, F. 1994. Humus chemistry: Genesis, composition, reaction. 2nd ed. John Wiley & Sons, New York.

Swift, R.S. 1996. Organic matter characterization. p. 1011–1069. *In* D.L. Sparks (ed.) Methods of soil analysis. Part 3. SSSA Book Ser. 5. SSSA, Madison, WI.

von Wandruszka, R., M. Schimpf, M. Hill, and R. Engebretson. 1999. Characterization of humic acid size fractions by SEC and MALS. Org. Geochem. 30:229–235.

Wang, L., F. Wu, R. Zhang, W. Li, and H. Liao. 2009. Characterization of dissolved organic matter fractions from Lake Hongfeng, southwestern China Plateau. J. Environ. Sci. 21:581–588.

Wershaw, R.L., and D.J. Pinckney. 1973. The fractionation of humic acids from natural water systems. J. Res. U.S. Geol. Surv. 1:361–366.

Wolfender, J.-L. 2009. HPLC in natural product analysis: The detection issue. Planta Med. 75:719–734.

Yu, Z., I.D. Campbell, C. Campbell, D.H. Vitt, G.C. Bond, and M.J. Apps. 2003. Carbon sequestration in western Canadian peat highly sensitive to Holocene wet–dry climate cycles at millennial timescales. Holocene 13:801–808.

Zhou, Q., S.E. Cabaniss, and A.M. Patricia. 2000. Consideration in the use of high-pressure size exclusion chromatography (HPSEC) for determining molecular weights of aquatic humic substances. Water Res. 34:3505–3514.