Journal of Chromatography, 259 (1983) 499–503
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 15,587

Note

High-performance size exclusion chromatography of aquatic humic substances

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(First received November 8th, 1982; revised manuscript received December 4th, 1982)

The chemical nature of aquatic organic material is a subject of much interest to environmental scientists. Many rivers and lakes in the southeastern United States are stained yellow-brown from these materials, as a result of intensive leaching of surrounding terrestrial watersheds. Most of these water-soluble materials are polymeric, complex organic acids generally classified as humic substances[1,2]. Aquatic humus is generally considered to be the colored organic component in natural waters but it should be noted that lesser quantities of the precursor and degradation constituents of the dynamic humic molecule will also be found in its presence. Because humic substances can affect a variety of chemical, physical, and biological reactions in natural waters, there has been an increased effort to characterize this complex material.

The macromolecular nature of these materials makes them suitable candidates for analysis by size exclusion chromatography, and estimates of the avarage molecular weight (MW) of humic substances derived from soil and aquatic origin have been the subject of many papers[1–5]. Literature values for MW of these substances range from a few hundred to a few hunderd thousand daltons; most of higher values were generated by conventional gel filtration chromatograpy using various Bio-Gel and Sephadex gels. Investigations have shown that gel–solute interactons such as charge-exclusion and adsorption of aromatic compounds can cause accelerated or retarded transport of humic materials through gel columns leading to erroneous estimates of MW, and several eluents have been proposed to control these effects[6–8]. Major disadvantages of these soft-type gels are poor resolution and long analysis time.

Recent advances in modern bonded stationary phases allow rapid size separation of aqueous polymers[9] but to date, only a few papers have appeared where high-performance size exclusion chromatography (HPSEC) has been applied to humic substances[10,11]. However, size separations of humic material on these new stationary phases indicate that charge exclusion remains a problem. The results presented here demonstrate the importance of extraneous factors such as charge exclusion and aromatic adsorption in modern size exclusion chromatography and illustrate the rapid size analysis of aquatic humic materials in a new stationary phase.

0021-9673/83/$03.00

EXPERIMENTAL

Separations employed a Perkin-Elmer liquid chromatograph composed of a Series 2 solvent-delivery system, Rheodyne 7125 injector (20-μl loop), LC 100 column oven, and LC 75 variable-wavelength detector. All separations were performed at 0.5 ml min^{-1} solvent flow-rate, with the column thermostated at 30°C and the wavelength set at 200 or 254 nm. The column was a Waters μBondagel E125 which has a 10-μm packing of a polyether moiety bonded to silica. According to the manufacturer, the 125 Å pore size results in a fractionation range of $2 \cdot 10^3$ to $5 \cdot 10^4$ daltons.

All chemicals were reagent grade or better, and distilled deionized water was obtained from a Milli-Q system (Millipore). Two solvent systems were investigated: distilled deionized water (DDW) (pH 5) and 0.1 *M* sodium acetate with 0.1 *M* sodium sulfate adjusted to pH 5.0 with acetic acid. Determination of void and total permeation volumes (V_t) was achieved using blue dextran and glycine, respectively. Calibration curves were produced by injection of microgram quantities of selected compounds dissolved in DDW.

Aquatic humus was collected from Lake Mize, a highly colored lake near Gainesville, Florida. Characterization of these materials as aquatic humus was done by elemental analysis and infrared and ultraviolet–visible spectroscopy and is summarized in Table I. Esterification of carboxyl groups was achieved with boron trichloride methanol (Applied Science Labs., State College, PA, U.S.A.), a reagent that is used to methylate fatty acids. Aquatic humus contains mostly carboxyl and phenolic functional groups and our goal was to esterify only the carboxyl groups to avoid water insolubility. Approximately 1 μg of methylated or unmethylated humic material was injected on to the column for analysis.

RESULTS AND DISCUSSION

Standard calibration curves for the two eluents were constructed with globular proteins (69,000–13,700 daltons) and were both nearly linear; however, elution volumes of the standard compounds differed significantly in the two eluents (see Fig. 1).

TABLE I

PHYSICAL AND CHEMICAL CHARACTERISTICS OF LAKE MIZE AQUATIC HUMUS

Parameter	*Results*
Elemental analysis	C, 48; H, 6; N, 2, O, 44%*
Dissolved organic carbon	38 mg l^{-1}
color (absorption at 420 nm)	350 chloroplatinate units
E_4/E_6 (ratio of absorbance at 465 nm to 665 nm)	9.7
Infrared analysis (major bonds)	3400 cm^{-1} (H-bonded OH) 1700 cm^{-1} (C = O of COOH; (C = O ketonic) 1580 cm^{-1} (Ar C = C; H-bonded C = O) 1375 cm^{-1} ($COO^- Na^+$)

* By difference.

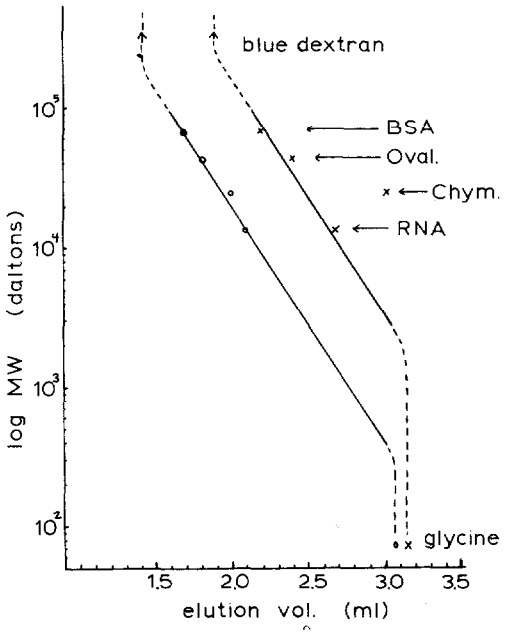

Fig. 1. Standard calibration curves for the μBondagel E125 column with two different eluents (BSA = bovine serum albumin; Oval = ovalbumin; Chym. = α-Chymotrypsinogen; RNA = ribonucleic acid). ○, DDW, pH 5; ×, 0.1 *M* sodium sulfate and 0.1 *M* sodium acetate, pH 5.

The low-ionic-strength DDW apparently resulted in charge repulsion between the solutes and stationary phase. Incomplete coverage of the silica surface with bonded phase may expose active sites that can interact with ionic solutes, resulting in ionic exclusion and premature elution. The high-ionic-strength mobile phase (0.4 *M*) suppressed ionic charge on both the solute and the silica surface and allowed full permeation of the pores. It is also possible that saturation of the polyether group with sodium ions allowed the stationary phase to act as a cation exchanger.

With DDW as the eluent, bacitracin A (1411 daltons) and vitamin B_{12} (1355 daltons) both eluted before their predicted volumes (1.73 and 2.01 ml, respectively) because of ionic exclusion. Using the high-ionic-strength eluent, bacitracin and vitamin B_{12} eluted after the total permeation volume (3.78 and 7.72 ml, respectively). Apparently the aromatic character of these compounds caused adsorption and retarded elution. Adsorption of aromatic compounds on Sephadex gels has been reported[6] but Saito and Hayano[10] did not observe this effect on another HPSEC column. Since humic substances are ionic and have aromatic character it is important to understand these effects when estimating MW by HPSEC.

Fig. 2 shows that methylated and unmethylated aquatic humus had different elution volumes with DDW, but both methylated and unmethylated humic materials behaved similarly with the high ionic strength eluent. The elution volume of unmethylated humic material in DDW implies that the material had a MW of *ca.* 20,000 daltons; the methylated material eluted in the total permeation volume, indicating a MW of 2000 daltons or less. Methylation of solute ionic groups (mostly carboxylic)

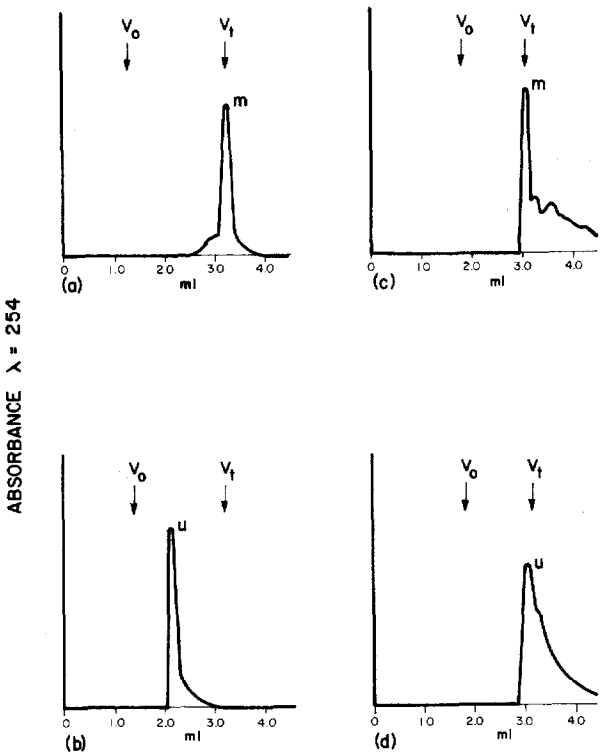

Fig. 2. Chromatograms of methylated (m) and unmethylated (u) aquatic humus on the μBondagel E125 column; (a) and (b) with DDW (pH 5) and (c) and (d) with 0.1 *M* sodium sulfate and 0.1 *M* sodium acetate (pH 5).

eliminated the problem of ionic exclusion and yielded the predicted results. Schmidt *et al.*[12] showed that a similar stationary phase (LiChrosorb Diol) required eluent ionic strengths greater than 0.2 *M* to achieve accurate size separation and high recovery of proteins. It should be noted that aquatic humus eluted as a narrow peak indicating that most of this material has a small MW range. The peaks that eluted after the total permeation volume possibly are due to "salting out" of humic materials[7] or adsorption of the aromatic moiety of humic molecules[6].

These last facts make it difficult to interpret our results in terms of the MW of aquatic humus. The humic material could be 2000 daltons or less or they could be high-MW materials that are adsorbed and coincidently elute at V_t. We believe the former to be true for several reasons. First, with DDW as the eluent, the highly aromatic compound vitamin B_{12} eluted before its predicted volume indicating that adsorption was minor. Under the same conditions, unmethylated humic material eluted before V_t because of ionic exclusion while methylated humic material eluted at V_t suggesting that ionic exclusion was controlled. Also, a recent study[13] used colligative methods to measure MW and concluded that aquatic humus was *ca.* 1000 daltons.

On the other hand, we have observed that high ionic strength eluents cause some aromatic compounds to elute as broad peaks presumably caused by the slow

kinetics of the adsorption–desorption process. Methylated and unmethylated humic material produced tailing peaks which suggests that adsorption may be occurring. Nevertheless, in all cases, the major peak did not elute after V_t. We feel that the possibility is small that three of our four chromatograms would have the major peak elute at V_t if adsorption of aquatic humus was significant.

These results demonstrate the importance of selecting the proper eluent for accurate size separation of humic substances on HPSEC columns. Furthermore, the experiment suggests that the average molecular size of aquatic humus is less than or equal to 2000 daltons which is consistent with a more recent study[13].

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