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Determination of molecular size distributions of humic acids by high-performance size-exclusion chromatography

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ABSTRACT

High-performance size-exclusion chromatography (HPSEC) was applied to the study of humic acids and a method for the rapid determination of reliable molecular size distributions of these substances was developed. Using commercial HPSEC columns, in conjunction with a neutral saline solution as eluent, molecular size distributions of four samples of humic acids of various origin and nominal average molecular weights were obtained. Measurements were independent of the operating conditions and based on differences in the molecular sizes of the humic acids examined.

INTRODUCTION

Classical gel permeation chromatography (GPC) is widely used for the determination of molecular size distributions of natural humic substances [1]. However, although its use is now fairly well accepted, many problems remain to be solved. These problems concern adsorption phenomena, inter- and intramolecular solute interactions, etc., which hinder the acquisition of meaningful molecular size distributions [2]. Moreover, when GPC is performed in "classical" columns working at moderate pressures, the use of soft or semi-rigid gels hampers the production of well resolved chromatographic peaks in a reasonably short time. One of the commonest types of stationary phase utilized for GPC is Sephadex gel, which has usually been used in connection with alkaline inorganic [3,4] and organic [5] eluents or buffers (*e.g.*, Tris, pH 9) in order to avoid the occurrence of extra adsorptive phenomena [2,6,7]. Even though this gel is nowadays extensively used, some doubt have arisen regarding its general applicability [8] and the absence of adsorption of the sample on the gel stationary phase [6].

High-performance size-exclusion chromatography (HPSEC) is a relatively new technique, widely utilized for the characterization of synthetic organic polymers. Owing to the use of short, high-performance columns and sophisticated apparatus, very reproducible chromatograms can be rapidly obtained. HPSEC has not often been applied to the study of humic "polymer". The few examples in the literature are confined to the analysis of humic matter of particular origin (aquatic humic and fulvic acids), having peculiar solubility characteristics [9–11].

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In this work, HPSEC was applied to the study of humic acids of various origins. Using commercial high-performance size exclusion columns and a neutral salt solution as eluent, a reliable, fast and reproducible method was developed. Molecular size distributions of humic substances were obtained, avoiding the use of strongly alkaline buffers which could, in some instances, affect the characteristics of the substances being studied. Further, the method permits the calculation of nominal average molecular weights which can be utilized for relative comparisons among humic acid samples.

EXPERIMENTAL

The humic acids (HA), analysed as sodium humates, were samples of various origins, either produced by dry-phase coal oxidation ("regenerated" HA) or extracted from natural substrates. Regenerated HA were extracted from two coals of different rank, Sulcis sub-bituminous coal (HAS) and North Dakota lignite (HALG), oxidized under dry conditions in a pressurized fluidized bed as reported previously [12].

The "natural" HA samples were first a leonardite-derived humic acid (HAL) sample, kindly supplied by Professor Visser of Laval University (Quebec, Canada) and second HA extracted from a worm compost (HAW) (supplied by Quadriflor, Sassari, Italy) according to the International Humic Substances Society (IHSS) standard procedure. Their production and characteristics are described elsewhere [13].

Table I reports some analytical data for the samples analysed. The most important structural differences among the products consist in their aromaticity, which is related to their origin. Coal-derived humic acids (HAS and HALG), as described previously [12], are the most aromatic products because they are derived from the oxidation of the coal organic structure, which involves the consumption of most of the aliphatic part [14]. Leonardite-derived humic acids show a similar aromaticity and HAW are the most aliphatic products [13]. This is clearly shown by the values of the H/C atomic ratio, calculated from the compositional data, which are inversely related to sample's aromaticity and are reported in Table I.

The HPSEC apparatus consisted of a Perkin-Elmer (P.E.) Series 410 pump system, coupled with an autosampler (P.E. ISS-100) and equipped with a refractive index (RI Refractomonitor) and a variable-wavelength UV detector (P.E. LC-90, 262 nm) in series. The whole system was controlled by a P.E. 7700 computer. The evaluation of chromatograms was performed using the P.E. GPC-6 software.

TABLE I

ANALYTICAL CHARACTERISTICS OF THE HUMIC ACIDS STUDIED

Elemental composition is on a dried and ash-free (d.a.f.) basis.

Sample	Composition (wt.-%)					H/C
	C	H	N	S	O (by difference)	
HALG	62.9	3.2	1.3	0.4	28.5	0.61
HAS	64.0	3.0	2.1	5.2	25.8	0.56
HAL	64.2	3.5	1.5	0.7	30.0	0.65
HAW	54.7	4.3	4.5	1.1	35.4	0.94

Chromatographic runs were carried out on solutions of different concentration obtained by dissolving 10 mg of humic substance in 0.1 *M* sodium hydroxide solution and diluting to final values of 0.3–1.0% (w/v). Before injection, solutions were filtered through a Millipore filter (0.45 μm). As eluents, 0.03 and 0.05 *M* sodium nitrate solutions in triply distilled water (Millipore, Milli-Q water system) were used. The column system consisted of three Shodex columns, having different "nominal" molecular weight ranges, coupled in series, and thermostated at 30°C in a Waters (TCM) thermostating system.

The columns utilized were Shodex Ionpak S-802s (range 0–5000), Shodex Ionpak S-803s (range 5000–50 000) and Shodex Ionpak S-804s (range 50 000–500 000). The gel phase inside the columns consisted, according to manufacturers specifications, of cross-linked sulphonated polystyrene–divinylbenzene copolymer. The flow-rates were varied in the range 0.6–1.0 ml/min and sample concentrations in the range 0.3–1% (w/v).

The theoretical limits of the chromatographic system (total and the void volumes, V_t and V_0, respectively), and a linear calibration fit were established using polysaccharide (PL polymer) standards having different molecular weights. The total volume was determined with glucose. Polysaccharides were utilized because they were perfectly compatible with the column stationary phase and eluent system, giving very precise (average correlation coefficient 0.997) calibration graphs. The chromatographic characteristics of the experimental peaks were reported both in terms of retention volumes and K_{av}, according to Laurent and Killander [15]. The latter represents the fraction of the volume of the gel that is available for the substance. It represents a very useful and straightforward way of comparing results independently of flow or system geometry. K_{av} is defined as

$$K_{av} = (V_e - V_0)/(V_t - V_0)$$

where V_e is the elution volume, V_0 the void volume and V_t the total volume of the gel bed.

A semi-quantitative analysis of various molecular weight distributions was performed and average nominal molecular weights were calculated, referring raw chromatographic data to the polysaccharide linear calibration fit and using GPC software. In this way, indicative molecular weight values, useful for relative comparisons among samples analysed in the same experimental conditions, were obtained.

RESULTS AND DISCUSSION

Reliability of the chromatographic system

Owing to the peculiar structure of humic molecules, which are very prone to interact with each other and with most of the gel phases normally used in GPC, it is necessary to avoid, or minimize, coulombic effects and either reversible or irreversible adsorption phenomena. All of these extra-permeation factors, which depend on sample concentration and the nature and concentration of the eluent, produce distortions in the chromatogram and lead to the elution of sample peaks outside the theoretical range of permeation [2]. To overcome these problems, most reported

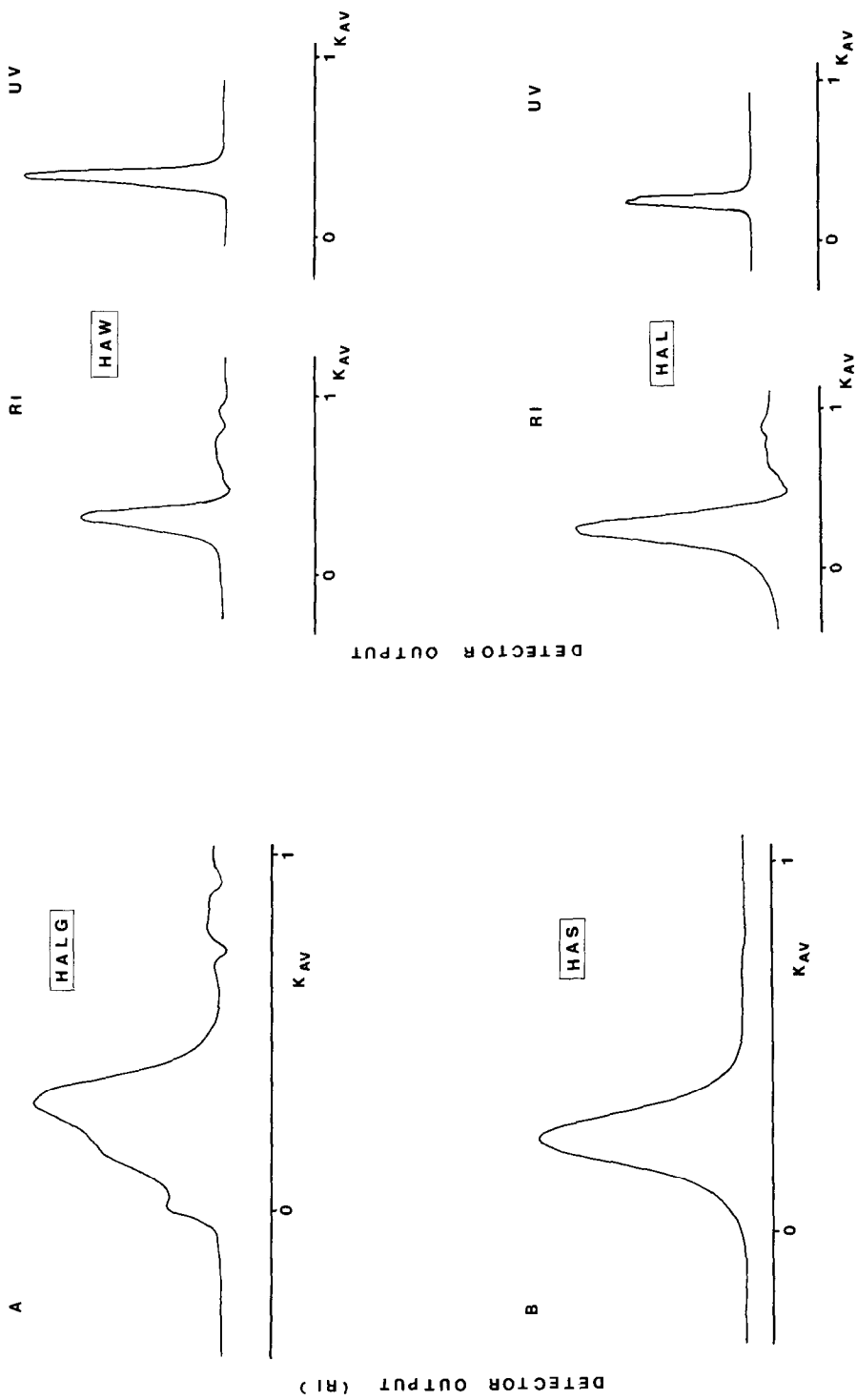

Fig. 1. Elution patterns of two humic acids. Flow-rate, 0.6 ml/mn; sample concentration, 1% (w/v).

Fig. 2. Comparison between the RI and UV elution patterns of worm compost and leonardite-derived humic acids.

classical GPC methods make use of alkaline buffers of high ionic strength and pH [6,7]. In this work, owing to the nature of the stationary phase utilized (sulphonated polystyrene), we found that the use of a strongly alkaline solution was not required and a neutral salt solution of suitable concentration allowed the determination of the chromatographic pattern of sodium humates, very rapidly and efficiently, without any detectable extra phenomena due to sample–sample or sample–gel interactions. The use of salt solutions of a certain ionic strength is necessary to avoid unwanted expansion of the charged acidic groups of the "humic molecule" and to produce a coiled form of the humic "polymer". Both phenomena increase the size of humic acids and produce a shift of the peaks obtained in the chromatograms towards lower elution volumes (or K_{av}) corresponding to larger nominal molecular weight values [16,17].

In this work sodium nitrate solutions of 0.03 and 0.05 *M* were tested as HPSEC mobile phases in order to minimize the above-mentioned effects. Some comparative preliminary tests performed with both solutions on regenerated humates (HALG) showed slight differences in K_{av}. However, using 0.05 *M* sodium nitrate solution, the K_{av} values obtained were slightly higher. Further, this higher concentration was found to be the best compromise between sample (sodium humate) solubility and the ability of humic molecules to form a fully coiled conformation in solution. In fact, as found by Ghosh and Schnitzer [16], such a concentration seems to be the threshold value at which natural humic molecules coil completely.

In an ideal gel permeation experiment, when the separation is based only on molecular size differences of the molecules constituting the sample, the chromatographic pattern must be independent of sample concentration and it must fall within the theoretical column range. Additionally, the elution volume should also be independent of flow-rates and the overall initial sample should be fully recovered at the end of the column. In this work, the absence of reversible and irreversible adsorption and the reliability of the conditions used for the determination of molecular size distribution (0.05 *M* sodium nitrate) of humic acids were tested for all the humic samples examined for the occurrence of the whole chromatogram within the theoretical limits calculated with the polysaccharide standards and by determining the effect on the chromatographic pattern of the operating parameters (sample concentration and flow-rate). The absence of irreversible adsorption was revealed indirectly by detecting variations among the peak areas of subsequent replicates, by evaluating the system efficiency (based on a standard with molecular weight of 48 000) after running the system for several months and by performing some overnight elutions of the system with 0.1 *M* sodium hydroxide solution.

Influence of the operational variables on chromatogram shape and precision of measurements

Fig. 1 shows typical chromatograms for two regenerate humate samples (HALG and HAS), obtained with 0.05 *M* sodium nitrate solution. The patterns were obtained by means of the refractive index detector. This detector is not based on molar absorptivities; its response depends on the sample concentration and it allows the detection of a larger number of chemical species than a UV detector. It should be used when molecular size distributions of polydisperse humic "polymers" have to be evaluated from the raw chromatogram.

Fig. 2 shows some comparative examples of chromatographic patterns obtained

TABLE II

ELUTION VOLUMES AND K_{av} VALUES OBTAINED FOR HALG UNDER DIFFERENT OPERATING CONDITIONS

HA (%, w/v)	Flow-rate (ml/min)			
	0.6		1.0	
	V (ml)[a]	K_{av}[a]	V (ml)[a]	K_{av}[a]
0.3	15.84 ± 0.04	0.210 ± 0.005	15.99 ± 0.03	0.224 ± 0.003
0.6	15.83 ± 0.02	0.209 ± 0.003	16.02 ± 0.02	0.227 ± 0.002
1.0	15.80 ± 0.01	0.206 ± 0.001	15.99 ± 0.03	0.224 ± 0.003

[a] Values are averages of at least three determinations, with standard deviations.

by the two detectors (RI and UV), and no large differences are present. However, some low-molecular-weight peaks (at high K_{av}) not detectable with the UV detector are evident when the RI detector is utilized. These non-UV-absorbing species, probably inorganic impurities in the samples, were not subsequently accounted for in the calculation of the average molecular weights. As shown in both Figs. 1 and 2, all the peaks fall within the void and total volume of the system, thus indicating that no evident adsorptive phenomena between the sample and the stationary phase are acting. The elution volumes and the respective K_{av} values of major peaks detected in all the chromatograms, obtained under various operating conditions, are reported in Tables II and III.

As further confirmation of the reliability of the chromatographic conditions utilized, all the measurements showed excellent repeatability and the elution patterns were almost always identical, as shown in Fig. 3, where the chromatograms of several replicates of HAS are shown. For HALG (Table II), within the same set of replicates, for the same flow-rate and concentration, the highest percentage standard deviation from the mean of the retention volumes is 0.3% at 0.6 ml/min and 0.2% at 1 ml/min. These values reach 2.4% and 1.3% when the standard deviations of K_{av} are considered. Almost the same values are obtained for HAL, HAS and HAW, as reported in Table III.

The effect of sample concentration was evaluated in the range 0.3–1% (w/v). Fig. 4 shows, as an example, the elution patterns obtained at 0.6 ml/min; the corresponding chromatograms at 1 ml/min were almost identical. In addition to the

TABLE III

ELUTION VOLUME AND K_{av} VALUES OBTAINED FOR HUMIC ACIDS EXTRACTED FROM SULCIS COAL, LEONARDITE AND WORM COMPOST

Flow-rate, 0.6 ml/min; concentration, 1% (w/v).

HA	V (ml)[a]	K_{av}[a]
HAS	20.11 ± 0.05	0.257 ± 0.007
HAL	20.40 ± 0.05	0.289 ± 0.004
HAW	20.68 ± 0.02	0.358 ± 0.002

[a] Values are averages of at least three determinations, with standard deviations.

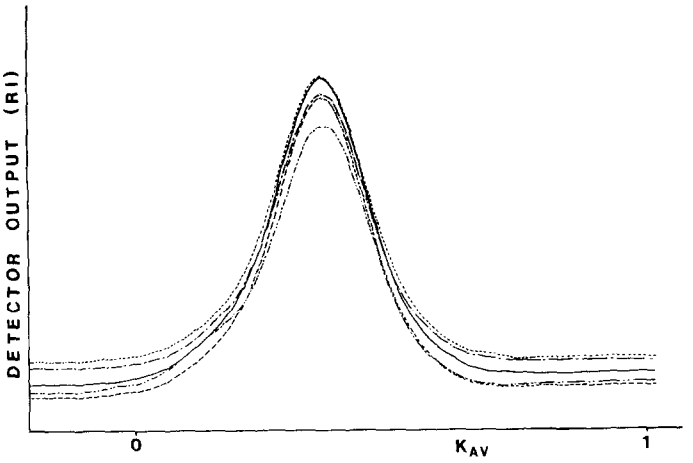

Fig. 3. Comparison among the elution patterns of five humic acid (HAS) replicates. Flow-rate, 0.6 ml/min; sample concentration, 0.6% (w/v).

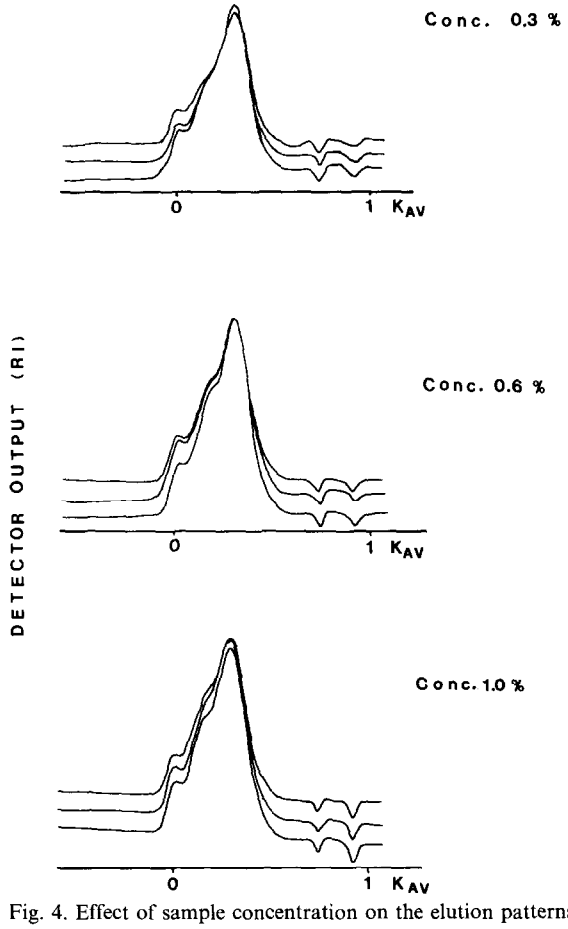

Fig. 4. Effect of sample concentration on the elution patterns of HALG. Flow-rate, 0.6 ml/min.

above-mentioned excellent repeatability, it is evident that the chromatograms are virtually independent of sample concentration. As Table II shows, the highest percentage differences between the mean values are 1.2% at 0.6 ml/min and 0.2% at 1.0 ml/min. The corresponding K_{av} differences are 1.9% and 1.3%, respectively. These results indicate that no macroscopic effects played by sample concentrations, which change the chromatographic patterns, as reported by Swift and Posner [2] using water as eluent in classical GPC, are present.

The effect of the flow-rate on the retention volume (or K_{av}) of HA was evaluated. Flow in HPSEC, as in chromatography in general, is one of the most important parameters, affecting the height equivalent to a theoretical plate (HETP) and peak resolution. In fact, owing to the intrinsic characteristics of the separation mechanism involved in this technique, which is entropy controlled, this parameter is a good indicator of the occurrence of undesirable phenomena within the column [2].

The flow dependence of the chromatograms was studied on HALG which were both chromatographed at 0.6 and 1 ml/min. Also in this instance, as demonstrated by Fig. 5 and Table II, the variations detected in the form of the chromatograms were

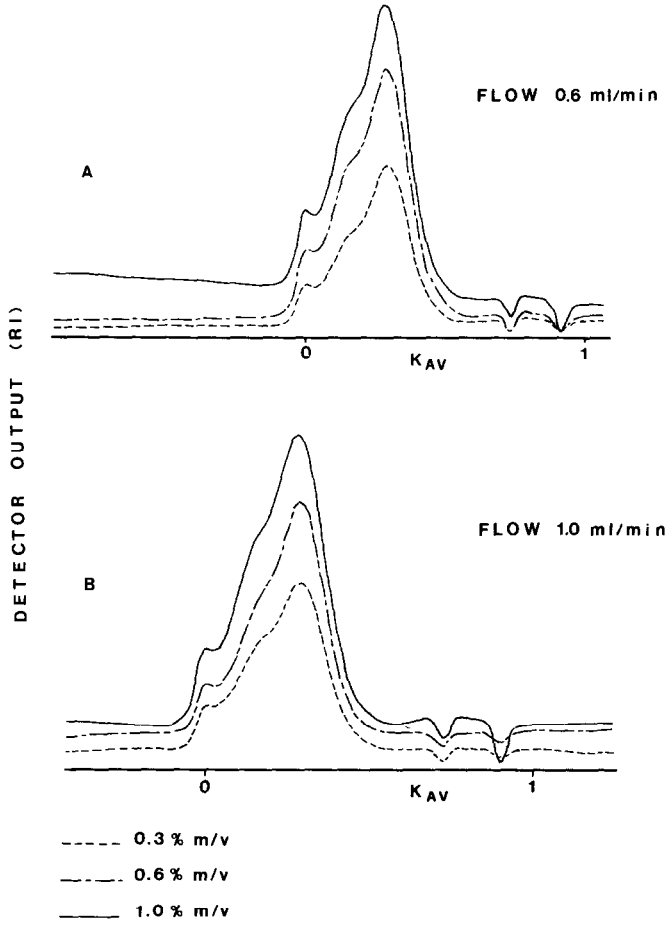

Fig. 5. Effect of flow-rate on the elution pattern of HALG.

small. The largest differences among the average retention volumes of the peaks of replicates, for the same concentrations at different flow-rates, were 1.2%, but for K_{av} they reached 9%. This variation can probably be ascribed mainly to differences in peak resolution due to band broadening related to the efficiency of the system [17], which was found to be significantly higher (18%) at the lowest flow-rate.

The absence of extra-permeation phenomena (irreversible adsorption on the column) was evaluated, as mentioned before, taking into account variations in peak areas, calculating the efficiency of the columns and "cleaning out" the overall system with an alkaline solution. Fig. 6 shows an example of the trend obtained by plotting the area counts (UV and RI) of five (HAS) replicates. The variations are very limited, and the largest differences detected between the most extreme values do not exceed 3%, suggesting that, at least under the conditions explored, there is no loss of the initial sample. The same conclusions can be drawn both from results obtained by calculating the efficiency of columns and from overnight sodium hydroxide elutions. In the first instance the efficiency of the column system, evaluated as plate number, calculated on a medium molecular weight standard (48 000) and checked at different times during several months, did not vary by more than 7%. In the second instance, no peaks were ever detected as a result of overnight alkaline elution.

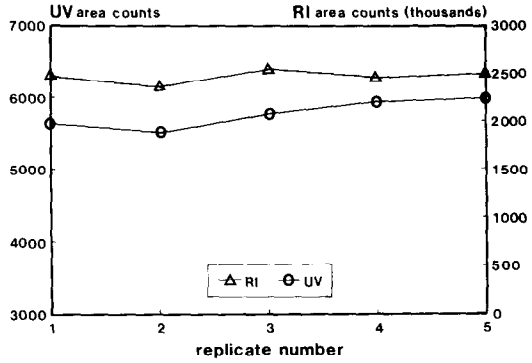

Fig. 6. Variations in area counts detected for five HAS replicates.

In conclusion, all the results indicate that the characteristics of the stationary phase utilized for the analysis are, under these conditions, virtually unaffected by the elution of humic molecules. Therefore, molecular size distributions so obtained can be considered to be related to the variations in the molecular size of the sample, thus confirming results obtained previously by comparative analyses of some ultrafiltered HA fractions [18].

Quantitative aspects

One of the most interesting possibilities offered by HPSEC, in analysing polydisperse systems, concerns the calculation of some average numerical values of molecular weights. This can be accomplished owing to the availability of extensive computerization and suitable software, which is not generally applicable with classical

GPC. Two of the most important average molecular weights that can be theoretically calculated are the number-average molecular weight (M_n) and the weight-average molecular weight (M_w), defined as

$$\bar{M}_n = \sum N_i M_i / \sum N_i \quad (1)$$

$$\bar{M}_w = \sum N_i M_i^2 / \sum N_i M_i \quad (2)$$

where N_i is the number of molecules of molecular weight M_i [17]. High-molecular-weight molecules strongly influence M_w whereas M_n is very sensitive to the low-molecular-weight components of the mixture. For polydisperse systems the value of M_w is always greater than M_n and the ratio M_w/M_n measures the breadth of the molecular weight distribution. Further details and references were reported by Yau *et al.* [17].

In Table IV, results obtained from calculations of the various average molecular weights are reported. It must be recalled that a true column calibration requires that the conformational and structural characteristics of the sample perfectly match those of the standards used, so that a unique relationship between molecular size and molecular weight can be established [17]. Unfortunately, as shown by Cameron *et al.* [19], humic substances do not behave precisely as either polysaccharides or proteins usually employed for column calibrations. Hence the absolute values obtained for these substances are necessarily nominal, and they must be used with care and only for relative comparisons.

TABLE IV

AVERAGE NOMINAL MOLECULAR WEIGHTS OBTAINED FOR THE VARIOUS HUMIC ACIDS

Flow-rate, 0.6 ml/min.

HA	Concentration (%, w/v)	$\bar{M}_n$[a]	$\bar{M}_w$[a]	$\bar{M}_w/\bar{M}_n$
HALG	1	72 273 ± 141	130 438 ± 336	1.8
HAS	1	53 585 ± 313	77 396 ± 277	1.4
HAL	1	49 622 ± 1761	79 042 ± 7312	1.6
HAW	1	42 845 ± 2701	49 345 ± 3461	1.1

[a] Values are averages of at least three determinations, with standard deviations.

Values obtained for the humic acids examined here, reported in Table IV, show differences both in the absolute values and in the ratio M_w/M_n, depending on their origin. In particular, products obtained by oxidation of lignite (HALG) have the higher average nominal absolute values. They contain very high-molecular-weight species, as indicated by their high average molecular weights (M_w). Conversely worm compost-derived HA show the lowest average values, while HA extracted from oxidized Sulcis coal and from leonardite are very similar and display, with respect to the other HA, intermediate average molecular weights.

Differences are also evident when the M_w/M_n ratio is considered. The latter, which is an indicator of the polydispersity of the macromolecular system, is high for HALG, HAS and HAW, as would be expected for products either generated by the random destruction of the parent matrix (coal) from which they are derived or extracted from a natural complex substrate (leonardite). Conversely, as shown in Table IV, humic acids extracted from worm compost denote a polyldispersity ratio close to unity. This could mean a close similarity of the molecules constituting HAW, owing to the different (biological) pathway which led to the formation of this humic acid sample.

CONCLUSIONS

Using commercial HPSEC columns with a neutral salt solution as eluent, precise molecular size distributions of different humic acids have been obtained rapidly. The conditions utilized give results that are independent of the operating parameters and thus related to differences in the molecular size of the sample. Further, using suitable software, average nominal molecular weights of humic acids useful for comparative purposes have been obtained.

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