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SIZE EXCLUSION CHROMATOGRAPHY OF HUMIC SUBSTANCES: COMPLEXITIES OF DATA INTERPRETATION ATTRIBUTABLE TO NON-SIZE EXCLUSION EFFECTS

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The use of size exclusion chromatography (SEC) to support a theory of conformational changes in humic acids induced by organic acids is reviewed. Particular attention is given to an evaluation of the role of non-size exclusion effects in SEC experiments. In this review, results from SEC studies are interpreted in terms of electrostatic, salt, and specific sorption effects that influence the behavior of the HS on the gel. That interpretation has been proven by experimental results from SEC-fractionation of HS in the absence of organic acids, and this has revealed similar interactions between the HS and the gel. The preference here is for the theory of non-size exclusion effects based on considerations of HS as natural polyelectrolytes. (Soil Science 1999;164:834–840)

Key words: Humic substances, size exclusion chromatography, non-size exclusion effects, buffer capacity, ionic strength.

SIZE exclusion chromatography (SEC) is one of the most powerful techniques for the determination of the molecular weight (MW) distribution of macromolecular compounds. The technique was developed by Porath and Flodin (1959) and applied to the analysis of humic substances (HS) by Posner in 1963. Since then, the influences of pH and ionic strength on the SEC-chromatograms of HS have remained topics of interest (Gjessing 1973; De Haan et al. 1987; Mori et al. 1987; Pershina 1987; Pershina et al. 1989; Frimmel et al. 1992; Piccolo et al. 1996; Piccolo, 1997). These studies have provided a vast amount of experimental data that have demonstrated unambiguously the crucial role of both pH and ionic strength in the SEC analyses of HS. By taking into account the acidic character of HS, the authors have tended to interpret these experimental findings as manifestations of the polyelectrolytic nature of HS macromolecules.

Recently, Piccolo and co-workers (Piccolo et al. 1996; Piccolo 1997) have provided an alternative interpretation, based on the theoretical developments of Wershaw (1986), of the results of SEC-fractionation of HS in the presence of organic acids. Wershaw considered HS as micelles of low MW acids held together by weak (hydrophobic, hydrogen, pi) bonding. Piccolo and co-workers regarded their findings on SEC-fractionation of HS in the presence of organic acids as unambiguous evidence of the micellar theory and considered the macromolecular theory to be erroneous.

The objectives of this contribution are: (i) to review critically the experimental set-up and the principles on which the interpretations of Piccolo et al. (1996) and of Piccolo (1997) are based; and (ii) to give experimental and theoretical arguments in favor of the polyelectrolyte nature of HS.

THEORETICAL BACKGROUND

Applications of SEC-fractionation on hydrophilic gels to water soluble polyelectrolytes are likely to give rise to artifacts. These arise from non-size exclusion effects, which include 'ionic exclusion' and 'specific adsorption' (Yau et al. 1979; Belenkii and Vilenchik 1983; Dubin 1988). The influences of these effects on the SEC-profiles of polyelectrolytes are shown schematically in Fig. 1.

Ionic exclusion arises from repulsive interactions between the charged analyte and the partially charged gel matrix. As a result, the

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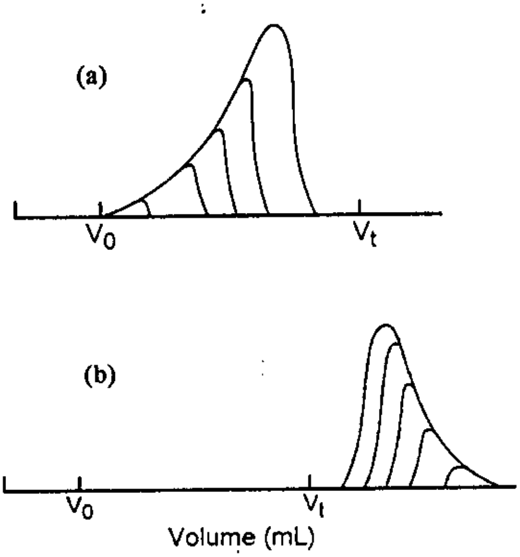

Fig. 1. Typical shapes of SEC-chromatograms of synthetic polyelectrolytes under conditions of ionic exclusion (a) and of specific adsorption (b) (after Belenkii 1998).

observed elution volume (V_e) of the analyte is much smaller (indicating much higher MWs) than would be expected purely from size exclusion effects. In the case of the highly charged analyte, V_e can be equal to the void volume of the column (V_o).

Specific adsorption occurs as a result of ion exchange adsorption and of hydrophobic or hydrogen bondings that take place between the analyte and the gel-matrix. Specific sorption causes a retardation of the analyte. Hence, the observed elution volume of the analyte is much larger (suggesting much lower MWs) than would be expected purely from size exclusion-effects. In the cases of strongly interacting analytes, V_e can reach values larger than the total pore volume of the column (V_t). Given that the size exclusion effects take place only under conditions of $V_o < V_e < V_t$ (Yau et al. 1979), elution of the analyte at the volume exceeding the total pore volume ($V_e > V_t$) indicates unambiguously an occurrence of specific sorption of the analyte on the gel.

Modifications of eluents are usually applied in order to avoid non-size exclusion effects. The hydrophobic interactions are eliminated by the addition to the eluent of hydrophobic solvents, or of surfactants that occupy the hydrophobic sites of the gel, and the hydrogen bonding effects used to suppress ionic exclusion effects (Belenkii and Vilenchik 1983). To determine a concentration of the electrolyte, which provides a complete suppression of the electrostatic effects, a series of fractionations of the same analyte are conducted with the use of eluents containing increasing concentrations of the electrolyte or buffer. Additions of the electrolyte cause a shift of the V_e values of the charged analyte to the larger values: the higher the concentration of the electrolyte, the greater is the shift in V_e that can be expected. However, as soon as a concentration of the electrolyte reaches a magnitude that provides a complete suppression of ion-exclusion interactions, its further increase has no influence on the V_e values of the analyte. Under these conditions, SEC-fractionation of polyelectrolytes does not differ from that of nonionogenic compounds, and the results can be interpreted easily.

However, applications of the modified eluents for the fractionation of samples of different compositions can give rise to other kinds of artifacts caused by the appearance of gradients at the edges of the chromatographic zone. This takes place if the volume of the injected sample is large enough to give rise to a chromatographic zone with well developed concentration and charge gradients at the edges. For example, a much higher (or lower) concentration of background electrolyte in the chromatographic zone compared with that in the eluent gives rise to the appearance of a salt-peak on the SEC-chromatogram of the polyelectrolyte (De Nobili et al. 1989). The position of the salt peak is determined by the elution volume of the low MW electrolyte. The latter is smaller than the total pore volume of the column because of repulsive interactions of the electrolyte with the gel, and it remains constant for the same electrolyte. The salt peak artifact is of particular importance for understanding the results on which we wish to comment.

It follows from the above considerations that, in order to give an adequate interpretation of the results of SEC-fractionation of polyelectrolytes, it is important to start with a determination of the possible sources of artifacts that are intrinsic to the experimental procedure chosen. When that is done, it becomes possible to provide different interpretations of the experimental results.

NON-SIZE EXCLUSION EFFECTS IN SEC-FRACTIONATION OF HS IN THE PRESENCE OF ACIDS

sign of studies using SEC results to support the micelle theory of humic acid aggregate structure (Piccolo et al. 1996). In particular, potential for non-size exclusion effects will be examined.

The design in the Piccolo and co-worker experiments included the SEC analyses of: (i) the high MW fraction (HF_1) of the HS adjusted to pH 2 using mineral and organic acids; and (ii) two nonfractionated humic materials (HS_1 and HS_2) titrated to different pH values by acetic (or formic) acids and KOH. In both cases, either 0.02 or 0.1 mol/L borate buffer (pH 9.2) was used as the eluent.

That procedure allows the localization of two possible sources of non-size exclusion effects. The first is the choice of a borate buffer for the fractionation of the HS. It is known that at alkaline pH values borate forms high MW, negatively charged complexes with HS (Schmitt-Kopplin et al. 1998). This is usually manifested in SEC-chromatograms as an appearance of a sharp peak at the void volume of the column on the 'hump' of HS (Town and Powell 1992). Such a phenomenon was also observed in the study under consideration. The authors considered it to be a high MW fraction HF_1 in the SEC experiments. The second complication in the results arises because all of the SEC fractionations of both HF_1 and HS were carried out using the modified eluent (0.02 or 0.1 mol/L borate buffer, pH 9.2) for nonequilibrated samples (pH 2, or other different pH values adjusted by different acids and KOH); some of these were at concentrations of either acids or salts equalling units of moles per L. Hence, the resulting concentration gradients of the components of the chromatographic zone influenced the SEC-fractionation of both HF_1 and the HS strongly. An example of the influence of such a system on the fractionation of the HF_1 solution adjusted to pH 2 with HCl and with acetic acid is given below.

The pH of the stock solution of HF_1 was lowered from 11.8 to 2 using various mineral and organic acids. From the data summarized in Table 1 (calculated from the data of Piccolo et al. 1996, p. 321), it follows that the concentration of HCl in the HF_1 solution at pH 2 was 0.026 mol/L, whereas that for acetic acid was 4.3 mol/L, a 200× higher factor. Because of the very low buffering capacity of dilute HCl, the pH of the chromatographic zone rose constantly as it moved along the column as a result of the mixing with the alkaline eluent. An increase to pH 4.2 in the 1 mL of injected sample occurs when mixed with 0.2 mL of 0.02 mol/L borate buffer. This follows from the expression:

$$\Delta pH = \Delta C_{HCl}/\pi \qquad (1)$$

where ΔpH is the decrease in pH following the addition of a certain amount of a strong acid (ΔC_{HCl}) to a solution of buffer capacity π. Such a small broadening of the chromatographic zone will take place readily in the upper part of the column. At pH 4.2 the strong carboxylic groups of HS dissociate, and the negatively charged large macromolecules leave the initial chromatographic zone. These enter the alkaline borate buffer, produce high MW, negatively charged complexes with borate, and appear at the void volume of the column. A similar situation is observed with the other mineral acids.

In contrast to the behavior for diluted mineral acids, it is not possible to use this method to

TABLE 1

Composition, buffer capacity and ionic strength of the solutions of HS_1 and HS_2 titrated to different pH values with CH_3COOH and KOH†

pH	[H]	c_{KOH}	c_{AcOH}	Buffer capacity	Ionic strength
	mol L^{-1}				
			CH_3COOH		
11.8	0	0.0063	0	0	0.0063
6.0	0.00001	0.0067	0.000776	0.0063	0.0126
4.5	0.00003	0.0063	0.0177	0.0094	0.0127
3.5	0.0003	0.0063	0.124	0.0152	0.0133
2.0	0.1	0.0063	9.19	0.0605	0.0326
			KOH		
3.5	0.00032	0.489	9.19	1.07	0.978
[illegible]	[illegible]	3.31	9.19	4.88	6.61

raise the pH of 4.3 mol/L acetic acid (by mixing with 0.02 mol/L borate buffer due to chromatographic broadening). The zone of the highly concentrated acetic acid spreads over the adjacent volumes of borate buffer and acidifies it. That is why the HS remain in the protonated state, sorb on the gel by hydrogen bonding, and are washed out by the alkaline eluent that replaces the chromatographic zone. As a result, the HS migrate with the zone of acetic acid until it reaches the end of the column. Thus the elution volume of HS is larger than the total volume of the column. The values $V_e > V_t$ is indicative of specific adsorption on the gel. A similar situation is observed in the concentrated solutions of the other organic acids used for the SEC-fractionation of HF_1.

Piccolo et al. (1996) preferred to interpret their data on the basis of a concept of "disruption of stereochemical arrangement" of "humic micelle-like aggregates" because of interactions with molecules of organic acids which gave rise to the formation of small micelles. In order to provide a mechanism for such an interaction, the organic acids under study were considered to behave like anionic surfactants whose aliphatic radicals penetrate into the hydrophobic interior of humic aggregates, whereas carboxylic groups "are positioned at the interfaces between the micelles and the eluent." Further, dissociation of carboxylic groups of the organic acids in the alkaline eluent were considered to give rise to repulsive forces that break down the large HS micelles into smaller ones. Piccolo et al. (1996) considered that the mechanism described above took place at pH 2. To comment on that, it should be noted that according to definition (Summ 1992), the surface activity is intrinsic to diphilic molecules that contain a large hydrophobic radical and a polar group. The critical size of an aliphatic chain that produces such a hydrophobic radical is eight atoms of C, and molecules with smaller chains do not possess surface activity. In the monocarboxylic acids used in the study, the largest aliphatic chain did not have more than three atoms (butyric acid). In addition, a shift to the larger values of the elution volume of HF_1 was observed also in the presence of formic acid (which does not have an aliphatic hydrocarbon functionality), and also in the presence of dicarboxylic acids that cannot be considered to be diphilic molecules. One of the latter–mal-

Piccolo et al. (1996) carried out another set of experiments that were aimed to prove the concept of conformational changes of HS induced by organic acids. The SEC-analyses were carried out on the nonfractionated humic materials (HS_1 and HS_2), which had additions of either acetic or formic acids and were titrated with KOH.

In order to take full account of this multifactor experiment (which, again, was conducted on the nonequilibrated samples of HS), one must have exact data not only on the pH values of the solutions (provided in Piccolo et al. (1996) but also on the concentrations of the added acids and KOH, the buffer capacity, and ionic strength of the solutions obtained. The latter parameters were calculated by the present author from the initial data provided by Piccolo et al. (1996) and summarized in Table 1 for the experiments with acetic acid.

Using this information, it is possible to follow the results of the SEC-analyses given in figures 2 and 3 in the paper by Piccolo et al. (1996). The general feature of the SEC-chromatograms of both HS solutions at pH 11.8, 6.0, 4.5, and 3.5 is the appearance of an intense sharp peak at the void volume of the column (borate complexes of HS), which is positioned on the regular hump of HS. The position of this peak, as well as of the maximum of the humic hump, remains unchanged in the pH range of 11.8 to 3.5, but it undergoes a drastic change at pH 2. At this pH, all the humic material appears at the large elution volume (equal to or larger than the total volume of the column of about 120 mL, as estimated by the present author). Piccolo and coworkers interpreted the observed phenomenon as unambiguous proof of the concept of the conformational changes of HS (disaggregation into small size micelles) induced by the organic acids. If we take into consideration the drastic change in a concentration of acetic acid in the HS solutions, which increases from 0.03 mol/L at pH 3.5 to 9.18 mol/L at pH 2 (Table 1), another explanation looks much more feasible. We are dealing here with the same phenomenon as was observed in the case of the fractionation of HF_1 at pH 2. Indeed, because of the high concentration of acetic acid, the HS are protonated, undergo specific sorption on the gel matrix, and are washed out from the gel by the alkaline eluent which reilar to those observed in the cases of specific adsorption of polyelectrolytes on the gel matrix (Fig. 1, this paper). The phenomenon has been studied comprehensively and described for SEC-fractionation of synthetic polyelectrolytes (Belenkii and Vilenchik 1983).

To follow further the transformations of the SEC-chromatograms of the HS solutions obtained at higher pH values during back titration with KOH, account should be taken of the ionic strength of the solutions to be analyzed (the last column in Table 1). The ionic strength is the main factor that influences SEC-analyses of HS at the higher pH values. Thus, the ionic strength of the HS solution back titrated to pH 3.5 with KOH is 0.9 mol/L, which is a factor 30 times higher than that of the solution at pH 2. Further titration with KOH leads to an increase in ionic strength up to a value of 18 mol/L for the solution at pH 8.5. Such an enormous concentration of salts in the sample of HS creates very strong gradients at the edges of the corresponding chromatographic zone, and that is reflected as an appearance of the salt-peak on the SEC-chromatogram. The position of the salt peak in the case under consideration is determined by the elution volume of potassium acetate, and from the chromatograms provided it can be estimated to be equal to about 110 mL. It is evident that all the chromatograms of the HS solutions prepared by back titration with KOH are characterized by the presence of the peak at about 110 mL. The intensity of the peak decreases with the increase in pH of the HS solution. Such a phenomenon is in line with the elimination of specific adsorption of HS on the gel matrix at higher pH values and with its further replacement with the pure electrostatic effects. The latter include interactions with the gel matrix of negatively charged high MW macro-ions of HS. These take place as soon as the HS leave the chromatographic zone (ionic exclusion). This process is reflected as a shift of the maximum of the humic hump to the smaller elution volumes. In addition, the low MW fraction of HS polyanions having the highest charge to mass ratio moves with the rear edge of the chromatographic zone of highly concentrated potassium acetate, which is displayed as the salt peak. In accordance with the considerations given above, refractionation of the evaporated HS sample back-titrated with KOH to pH 8.5, and.

In these latter experiments Piccolo and coworkers undertook a study of the reversibility of the "disruptions of HS aggregates" induced by organic acids. For this purpose, they rotary-evaporated acetic acid, refractionated the sample, and compared the chromatograms obtained with those of untreated HS_1 and HS_2. Because of the impact of the salts (whose concentration could only increase due to rotary evaporation), the authors never succeeded in obtaining similar chromatograms for untreated and for back-titrated samples, which is why they declared that "organic acids produce a degree of permanent alteration into the hydrophobic arrangements of humic micelles."

On the basis of the comments made above, it can be concluded that

(i) the results reported by Piccolo et al. (1996) and Piccolo (1997) can be interpreted comprehensively in terms of non-size exclusion effects characteristic of the SEC-fractionation of polyelectrolytes under nonequilibrated conditions;

(ii) the results given cannot prove a theory of conformational changes of HS induced by organic acids.

Some relevant experimental data of this author (Pershina 1987) are given below. These data provide experimentally verified arguments to refute the latter theory.

NON-SIZE EXCLUSION EFFECTS IN SEC-FRACTIONATION OF HS IN THE ABSENCE OF ORGANIC ACIDS

The experimental data of Pershina (1987) show the influence of pH and ionic strength on the SEC-fractionation of aquatic HS. Sephadex gels G-15 and G-50 were used as column packings. The gels were packed consecutively into one column: G-50 in the lower layer, G-15 in the upper layer. The height of each layer was 25 cm. The full column size was 1.4 × 50 cm, with $V_0 = 27.5$ mL and $V_t = 78$ mL. Distilled water, adjusted to the appropriate pH with NaOH and HCl, was used as eluent. The sample of aquatic HS was equilibrated with the mobile phase before the SEC-analysis. To suppress the electrostatic effects, 0.01 mol/L Tris-buffer at pH 5 and 9 peaks at neutral pH. Increases in pH are accompanied by shifts to the smaller elution volumes of the maxima of the chromatographic peaks; at pH 12.5, only one peak is observed at the void volume of the column. This is indicative of the repulsive electrostatic interactions between polyanions of HS and the gel matrix that take place at higher pH values. As the pH decreases, the elution volumes of all three peaks are shifted to the larger values, and at pH 1.8 only one broad diffuse peak, which spreads over the total volume of the column, is observed. That would indicate a strong specific sorption of HS on the gel matrix under the acidic conditions of pH. All the chromatograms were obtained in the absence of any organic acids (other than the HS), and that indicates unambiguously the crucial role of non-size exclusion effects in the SEC-fractionation of HS.

To further manifest the role of electrostatic effects, fractionations of HS were carried out using 0.01 mol/L Tris-buffer at pH values 5 and 9. As can be seen from Fig. 3, increases up to 0.01 mol/L in the ionic strength of the eluent caused a transformation into a broad unimodal of the initial trimodal distribution. The width of the peak indicates a rather large polydispersity of the fractionated material, which could be expected because of the irregularity of the structure of humic macromolecules. An increase in the pH of the Tris-buffer, from 5 to 9 at the constant ionic strength of 0.01 mol/L, caused a remarkable shift of the humic hump to the smaller values of the elution volume (Fig. 3). The effect observed can be also explained by non-size exclusion effects, namely, by the higher degree of dissociation of the polyanions of HS at alkaline pH values. This is accompanied by an increase in the repulsive interactions with the gel.

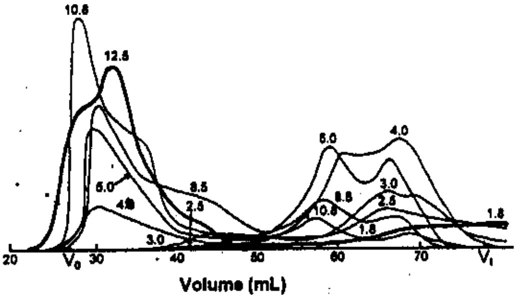

Fig. 2. Influence of pH on SEC-fractionation of aquatic

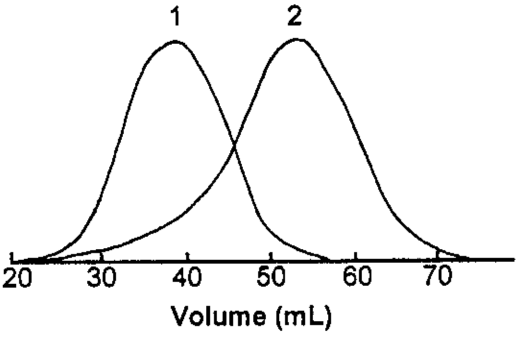

Fig. 3. Influences of ionic strength and of pH of an eluent on SEC-fractionation of aquatic HS. Eluent: 0.01 mol/L, Tris-buffer at pH 9 (1) and pH 5 (2) (Pershina 1987).

Interpretation of the demonstrated above changes in elution behavior of HS is in very good agreement with much more recent findings on the influence of non-size exclusion effects on the results of SEC-fractionation of about 80 low-MW organic acids and neutrals, as well as synthetic anionic polyelectrolytes obtained by the author of this paper, in close collaboration with the group of Prof. Frimmel of the University of Karlsruhe, Germany (Perminova et al. 1998).

CONCLUSION

The SEC-fractionation of HS is governed by non-size exclusion effects that can give results not easily interpreted, as exemplified herein by examination of the data of Piccolo et al. (1996) and Piccolo (1997). A complicated experimental design brought unexpected results, which made Piccolo and coworkers to develop a new theory of conformational changes of HS induced by organic acids. In order to accept this theory, one must acknowledge the existence of strong surface active properties for acetic (or formic) acid; that is, one must reject the theory of surface activity itself. In addition, application of this theory to interpretations of the results of SEC-fractionation of synthetic polyanions that manifest specific adsorption on the gel at acidic pH values would lead to the conclusion that polyacrylic acids are micelles of acrylic acids, polystyrenesulphonic acids of styrenesulphonic acids, etc. A strong case can be made that the experimental data reported in the original publications of Piccolo et al.

to give an alternative interpretation of the results reported in terms of the theory of non-size exclusion effects, which was developed for interpretations of the results of SEC-analysis of synthetic polyelectrolytes. That interpretation has been proven by this author by the results on SEC-fractionation of HS obtained at different pH values and ionic strengths in the absence of organic acids.

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