Frontal Gel Chromatography of a Humic Acid

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Gel chromatography has been widely applied in the study of humic acids to obtain discrete fractions or at least reduce their polydispersity. The inherent difficulties of the method, when applied to humic acids, have unfortunately not been considered in detail in most cases. Swift and Posner have recently given the first careful treatment of the subject.[1] They point out that a meaningsful separation requires that "(A) the elution volume of a substance is largely independent of sample concentration and flow rate and (B) the whole of the applied sample (*i.e.* the final peak) is eluted within the total column volume". The last point has been completely neglected by most investigators although the adsorption effects were strongly emphasized already in 1967.[2] Swift and Posner showed that only elution with alkaline buffers (pH of the order of 9) can possible fulfil the condition of negligible adsorption. Tentative molecular weight calibrations were later made using appropriate buffers[3] (one calibration necessary for each buffer).

In the study of humic acids one often has large amounts of samples of low concentrations. Therefore, frontal analysis will give a clearer insight of the complications of the fractionation procedure than zonal analysis. An example of such a frontal analysis will be given in this paper. It partly confirms the conclusions of Swift and Posner and gives at the same time some additional information about the system studied.

Experimental. The humic acid was obtained from a peat soil by pyrophosphate extraction at pH 7. The acid was precipitated with hydrochloric acid, dissolved to pH 7, dialysed and lyophilized. A 0.2 % solution in boric acid-borax buffer (0.033 M − 0.0082 M + 0.02 % NaN_3) was prepared at pH 8.5.

80 ml of this acid was fractionated by gel chromatography on Sephadex G-100 (Pharmacia Fine Chemicals, Uppsala, Sweden). Two columns (2.54 cm × 33 cm), equilibrated with buffer, were used (void volume 70 ml, total volume 150 ml); in each run 4 ml humic acid were eluted with the buffer. 10 ml fractions were collected at an elution speed of 25 ml/h. A typical elution pattern is shown in Fig. 1. Four fractions were taken out, roughly where indicated, and collected from all the twenty runs. These collected fractions are numbered 1 − 4 in the following. Small but measurable amounts of humic acid are obtained after the total volume, indicating some adsorption even at pH 8.5.

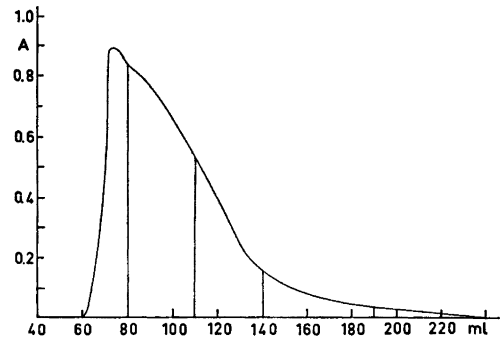

Fig. 1. Example of a zonal gel chromatographic analysis on G-100 of the humic acid. The collection of four main fractions 1 − 4 is indicated. Absorbances at 400 nm as function of elution volume.

Frontal analysis was made of each fraction on the same Sephadex G-100 column. The samples applied were large enough to give rise to a plateau level in each experiment (165 ml of fraction 1 was used, 207 ml of fraction 2, 275 ml of fraction 3 and 273 ml of fraction 4). 15 ml fractions were collected and the absorbance values at 400 nm and 500 nm measured in a 10 mm cuvette on a Zeiss PMQ II Spectrophotometer. In Fig. 2 the results obtained at 400 nm are given as histograms with tentatively drawn elution profiles. The shifts from sample to buffer are indicated by vertical lines.

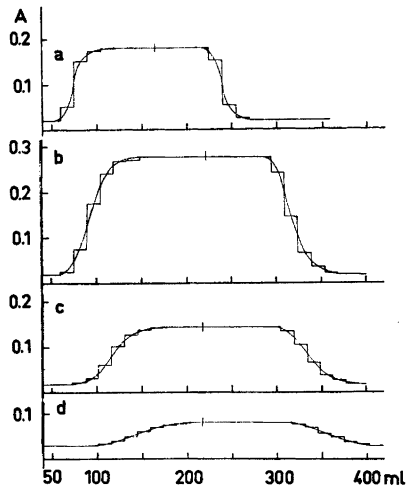

Fig. 2. Frontal gel chromatographic analysis on G-100 of the humic acid at pH 8.5. Absorbances at 400 nm as function of elution volumes. (a) Fraction 1, (b) Fraction 2, (c) Fraction 3, (d) Fraction 4.

The fractions were then recollected and dialysed against a phosphate buffer (Radiometer Type S1001) so that fractions 1–4 at pH 6.5 were obtained with the same concentrations as at pH 8.5. Frontal analyses were made in the same way as above (using 259 ml of fraction 1, 350 ml of fraction 2, 380 ml of fraction 3 and 395 ml of fraction 4). The results are given in Figure. 3.

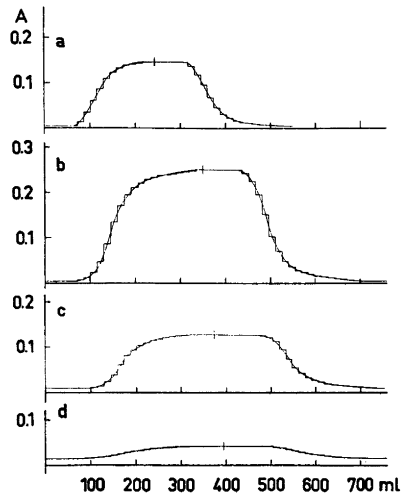

Fig. 3. Frontal gel chromatographic analysis on G-100 of the humic acid at pH 6.5. Absorbances at 400 nm as function of elution volumes. (a) Fraction 1, (b) Fraction 2, (c) Fraction 3, (d) Fraction 4.

Discussion. 1. The histograms at pH 8.5 are enantiographic within the accuracy of the experiment; the front and the back elutions do not differ appreciably. The situations of the half-values of the front and back also agree reasonably well. This means that no complicating equilibria exist in the system and that any existing adsorption must be linear. (The fact that no humic acid appears after the total volume does not itself prove the absence of adsorption; the last fractions still can be retarded.) The skewness of every histogram is in agreement with the shape of the corresponding fraction in the zonal analysis. Some of the fractions were rerun after dilution and similar patterns obtained, further confirming that a true fractionation existed.

2. At pH 6.5 the results of the frontal analyses are quite different. All fractions are appreciably retarded. The histograms are, however, still enantiographic within the limits of experimental accurary. Only two main explanations seem to be possible: the molecules have decreased in size or the adsorption (admittedly linear adsorption) is greater at the lower pH. The mere existence of a retardation cannot definitely distinguish between the two possibilities. The results show the correctness and importance of the statement by Swift and Posner that size calibrations must be made for each type of buffer.

3. The resolution achieved can easily be evaluated from the frontal analyses. When fraction 1 at pH 8.5 has passed to 90 %, fraction 2 has already passed to 37 %; and for 90 % of fraction 2, 50 % of fraction 3 has been obtained. At pH 6.5 the situation is slightly better for the resolution between fraction 1 (containing the excluded part) and fraction 2 (90 % of fraction 1 corresponds to 30 % of fraction 2), while the following resolution is much worse (90 % of fraction 2 corresponds to 80 % of fraction 3). It seems natural to explain these effects as due to a larger adsorption at pH 6.5 than at pH 8.5. Even at the higher pH the resolution is not as good as would be expected for only size separation. Some adsorption would lead to a trailing of the high-molecular fractions to overlap with the subsequent less high-molecular fractions, and would be increasingly disturbing for the last fractions as is observed. As long as the adsorption is linear it does not affect the size calibration for a given buffer but it makes the resolution of the fractionation worse.

4. The ratios A_{400}/A_{500} found for the three first fractions (the low concentrations of the fourth made the determinations very uncertain) are 2.60, 2.54, and 2.36 at pH 8.5 and 2.75, 2.62, and 2.36 at pH 6.5. The fractionation is thus reflected in the absorption spectra indicating larger similarity with the "core structure" (*cf.* Ref. 4) for the low-molecular fractions.

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2. Lindqvist, I. *Acta Chem. Scand. 21* (1967) 2564.
3. Cameron, S. R., Swift, R. S. and Posner, A. M. *J. Soil. Sci. 23* (1972) 342.
4. Lindqvist, I. *Lantbrukshögsk. Ann. 34* (1968) 2384.

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