High Performance Size-Exclusion Chromatography of Anionic Polymers in Aqueous Solutions

TONY MALFAIT, *Interdisciplinair Research Center, University of Leuven, Campus Kortrijk, 8500 Kortrijk, Belgium,* DIRK SLOOTMAEKERS, *Laboratorium voor Organische Structuurchemie, University of Leuven, Celestijnenlaan 200 F, 3030 Heverlee, Belgium,* and FRANS VAN CAUWELAERT,* *Interdisciplinair Research Center, University of Leuven, Campus Kortrijk, 8500 Kortrijk, Belgium*

Synopsis

The applicability of high performance size-exclusion chromatography was tested for anionic polymers with a new type of column. It was found that a solvent salt concentration of 0.1*M* and an elevated temperature (60°C) prevented adsorption of the polystyrene sulfonate standards on the column packing. The calibration curve and the effect of the concentration on the retention volume remained, however, column-dependent. We concluded also that the use of the column was restricted to the linear range of the calibration curve and to concentrations below 1 mg mL^{-1} for the highest molecular weight standards. The influence of the flow rate (below 1 mL min^{-1}) on the retention volume was negligible. The main cause of errors when the universal calibration technique is used originated in the experimental determination of the intrinsic viscosities of the standards. The combination of the errors on the viscosity and on the experimentally determined retention volume easily introduced an error of 15% on the determined molecular weight of the sulfated polysaccharide κ-carrageenan. The use of the universal calibration method for an exact molecular weight determination of anionic natural polymers is therefore still questionable.

INTRODUCTION

High-performance size-exclusion chromatography (HPSEC) has been widely used during the last few decades.[1,2] Contrary to the more traditional size-exclusion chromatography (SEC) this technique is far less time-consuming, and smaller amounts of sample are needed. Due to the availability of several suitable column packings and to the existence of nearly monodisperse molecular weight standards HPSEC has become of great importance for the characterization of polymers in organic solutions.[2] In the field of the water-soluble macromolecules, problems were encountered due to the adsorption of the sample and, in some cases, to the limited pH dependent stability of the packing materials.[2] Although one was able to overcome these problems with the addition of salts to the eluent that prevent adsorption of the sample and with the modification of the column particles, the majority of studies reported concerning HPSEC in aqueous solution deal with the separation and the molecular weight determination of proteins and DNA compounds.[3] A limited

*To whom correspondence should be addressed.

Journal of Applied Polymer Science, Vol. 39, 571–581 (1990)
 CCC 0021-8995/90/030571-11$04.00

number of reports[4-7] describe the determination of molecular weights of anionic polysaccharides with HPSEC. The peak position method[2] with well-characterised standards having nearly the same structure as the unknown was used in some cases. However, when such standards were not available, the universal calibration[8] provides good help. This is already very well known for traditional SEC of water soluble polymers.[9-11] In the above-mentioned references, sodium polysterene sulfonates (PSSNa) were used as calibration standards. In this paper we apply PSSNa standards for a universal calibration on a new column suitable for aqueous HPSEC. This column has been recommended for polysaccharides and anionic polymers. Problems such as adsorption, concentration effects, flow-rate dependent retention volumes, and the difficulties with the calibration procedures will be discussed.

EXPERIMENTAL

HPSEC

A HPLC equipment from Waters Assoc. was used with a R410 differential refractometer as detector. The retention times of the peaks were measured with a 740 Data Module. The system contained two in series connected 510 HPLC pumps and the flowrate (0.4 or 0.8 mL min^{-1}) was adjusted with the isocratic operation modus of an automatic gradient controller. Injections were carried out with a U6K injector. For PSSNa samples 50 μL was injected and for κ-carrageenan 100 μL. All flow connections were made as short as possible.

HPSEC was performed on ultrahydrogel linear (Waters Assoc.) columns (7.8 $\times$ 30 cm). Two different columns were used, which will be named A and B further in the text. Ultrahydrogel consists of a hydroxylated polymethacrylate packing with residual carboxyl groups. For the linear types the pore sizes were blended and the exclusion limit corresponds to a molecular weight for polyethylene oxide (PEO) of 7×10^6. The column is stable over a pH range from 2 to 12.

Viscosity

The viscosity was measured with an Ubbelhode viscometer (Scott) at 60.0 $\pm$ 0.1°C. The efflux time was calculated from the average of at least five separate measurements. The intrinsic viscosity of the solutions were determined by a linear Huggings analysis. The extrapolations were made with 5–10 different concentrations for each sample.

Materials

Sodium polystyrene sulphonate standards with narrow molecular weight distributions ($\bar{M}_w/\bar{M}_n < 1.1$) and peak molecular weights ($\bar{M}_p$) in the range of 4.6×10^3–1.2×10^6 were obtained from Polymer Laboratories (U.K.). $\bar{M}_p$ corresponds to the molecular weight values at the peak maximum in SEC and equals $\bar{M}_n$ and $\bar{M}_p$ for the standards. κ-carrageenan from the algae *Eucheuma cottonnii* and its monomer sodium neocarrabiose 4-sulfate were obtained from the Sigma Company. All sample solutions were made up with ultra pure water (Milli-Q-reagent grade water system) and filtered using 0.45 μm filters (Milli-

pore). The solvent containing 0.1M salt and 0.05% NaN_3 in Milli-Q water was filtered (0.22 μm) and degassed with a Millipore vacuum filtration apparatus.

RESULTS AND DISCUSSION

Column Conditions

SEC of polyelectrolytes in aqueous solutions is sometimes used to test the purity of the injected samples.[11-13] However, at room temperature in water PSSNa, even at concentrations below 1 mg mL^{-1}, is adsorbing on the hydroxylated polymethacrylate packing. This leads to strongly disturbed peak shapes and flow-rate-dependent retention volumes and peak areas. The decrease of the number of theoretical plates, normally above 7000, seems mainly to be due to adsorption.[2] Since adsorption on the column is usually suppressed with increasing temperature[14] and since correct molecular weight distributions of polyelectrolytes could only be obtained with a salt concentration in the eluent of at least $5 \times 10^{-2}M$,[12] HPSEC was therefore performed with the columns at 60 $\pm$ 0.5°C and with an ionic strength of the eluent of 0.1M. Adsorption was no longer observed when both conditions were used.

Calibration Methods

The Peak Position Method

A calibration curve of $\overline{M}_p$ vs. K was made for the two columns A and B (Fig. 1). K stands for the ratio between the retention volume of the standard and that of the excluded salt. It accounts therefore for the flow-rate differences. The salt exclusion was due to the Donnan effect.[12] According to the manufacturer a linear calibration curve was obtained for PEO and poly(ethylene glycol) (PEG) standards in the molecular weight range 10^2–10^6. On both columns a good linearity was observed for PSSNa in the range 18×10^3–780×10^3 only. For the lower molecular weight samples even at the lowest measured concentration, the calibration curve deviated sharply at $K \geq 0.8$. This is rather unusual since according to the SEC theory the asymptotic behavior of the curve in the region of the small molecules is expected to appear close to $K = 1$ where the exclusion of the standard equals that of the salt. The deviation from the theory was an indication for the existence of non-size-exclusion effects, e.g., such as electrostatic repulsions between the residual carboxyl groups of the packing and the negatively charged groups of the standards. As a consequence, even in 0.1M salt the calibration curve for anionic polymers was probably shifted towards lower K values. Deviations from linearity for the highest molecular weight standards ($\overline{M}_p = 1.2 \times 10^6$) will be discussed later in the text. Between both columns a systematic difference existed for the K values in the linear range. So in this region the same molecular weight will be obtained on both columns for a sample of unknown molecular weight. This is, however, not true for molecular weights above and below this region because deviations from linearity were not the same for both columns. Use of the peak position method with PSSNa for this type of column was thus restricted to the linear region. Care should also be taken when using HPSEC peak profiles as a measure for the molecular weight

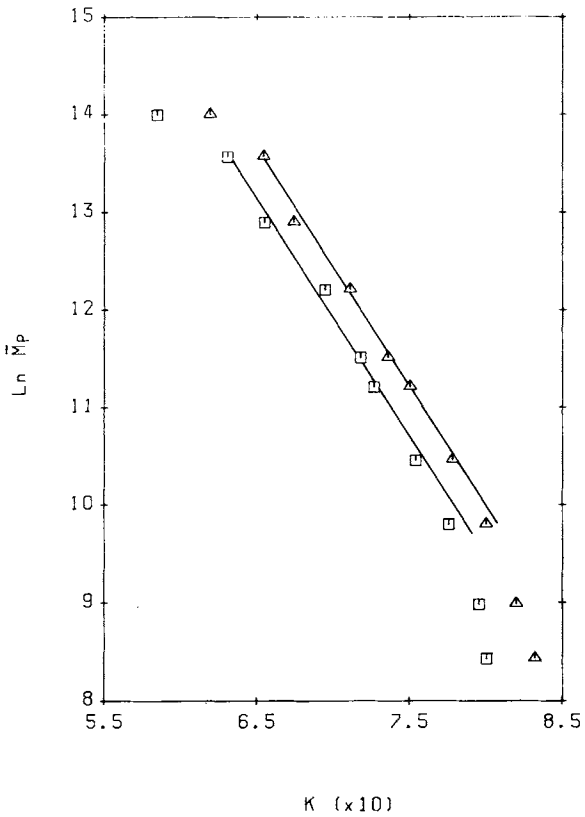

Fig. 1. Calibration curve of ln $\overline{M}_p$ vs. the retention volume ratio (K) of PSSNa for two identical HPSEC columns A (□) and B (△). All concentrations of the standards were below 0.5 mg mL^{-1}. For further experimental conditions, see text.

distribution of the injected sample. Since all the standards have a very narrow distribution ($\overline{M}_w/\overline{M}_n < 1.1$), the peak widths should therefore be comparable. However, on both columns for PSS above 2×10^5 the peak width increased with increasing molecular weight even in the low concentration range (Fig. 2). This effect was also observed for lower flow rates.

The Universal Calibration

The difference in linear range between the standards PEO and PEG, on the one hand, and PSSNa, on the other hand, can also be partially due to the very well-known fact that the hydrodynamic volume, rather than the molecular weight, is the determining factor for the elution volume in SEC experiments.[8] Due to the ionic repulsions between the charges for polyelectrolytes[15] and to the restricted flexibility of the chains, e.g., for polysaccharides,[16] these polymers are more expanded than other random coil polymers and therefore eluted earlier for the same molecular weight. For an absolute determination of the molecular weight, the use of the peak position with polymer standards of a different chemical nature compared to the unknown seems therefore doubtful. In a universal calibration of random-coil polymers the log of the hydrody-

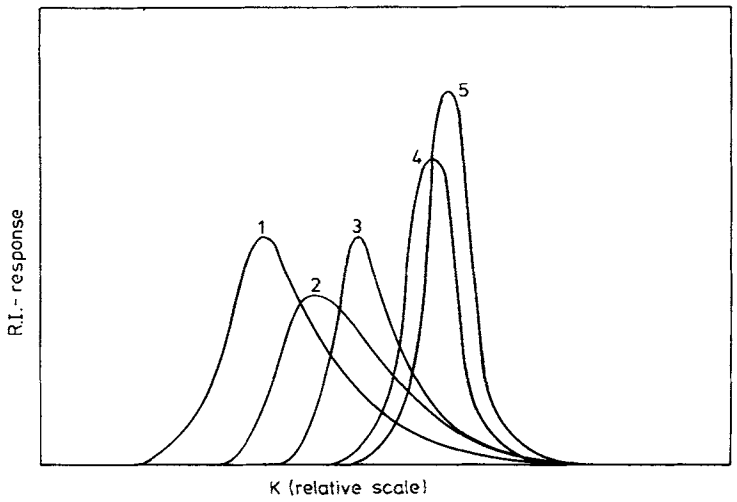

Fig. 2. Refractrometric traces of PSSNa at 0.25 mg mL^{-1} for the molecular weights; (1) 1.2×10^6; (2) 780×10^3; (3) 400×10^3; (4) 200×10^3; (5) 100×10^3.

namic volume (v_h) is plotted against K. It can be shown[17] that the log v_h is proportional to the log of the product $[\eta]M$, where $[\eta]$ is the intrinsic viscosity. For narrow molecular weight, standards in a given solvent at fixed temperature $[\eta]$ can be easily calculated when the constants of the Mark–Houwink relation ($[\eta] = kM^a$) are known. However, the constants a and k are only known for PSSNa at room temperature.[18] Since the column was used at 60°C, $[\eta]$ and the constants k and a were determined experimentally. The values found for PSSNa were $a = 0.80$ and $k = 2.4 \times 10^{-5}$. The value a is larger than that mentioned by Takahashi et al.[18] ($a = 0.68$) at 25°C

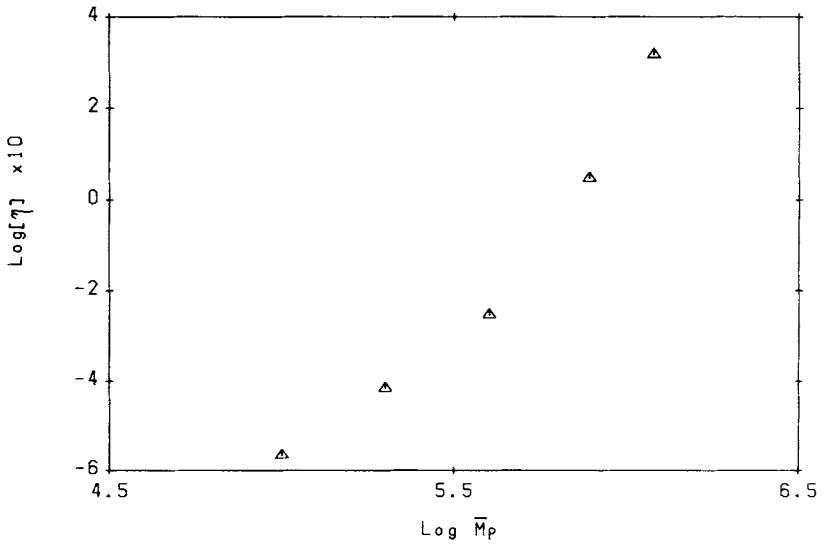

Fig. 3. Logarithmic plot of the intrinsic viscosity $[\eta]$ and the peak-molecular weight for PSSNa at 60°C.

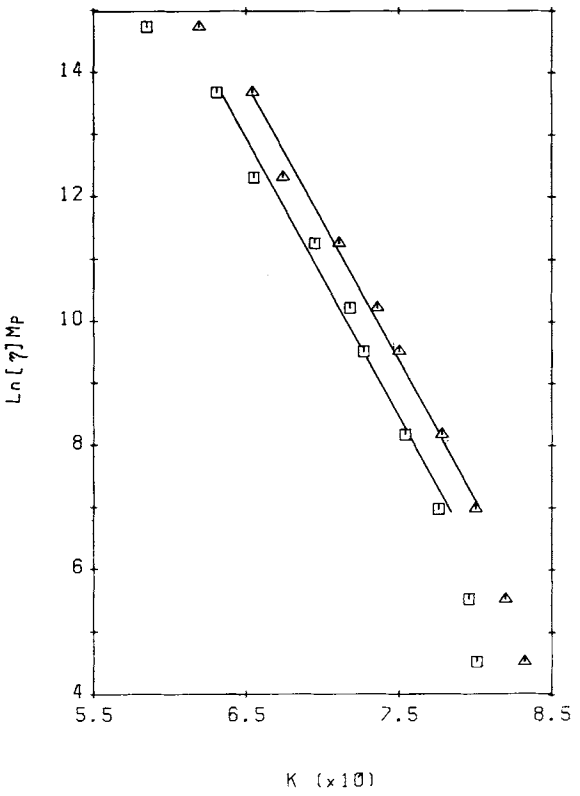

Fig. 4. The universal calibration curve: plot of the hydrodynamic volume vs. K. Conditions identical to those in Figure 1.

and 0.1M. At elevated temperatures the solvent becomes better so that a increases, which is in accordance with the theory.[19] However, the linearity between the points of the logarithmic plot of the Mark–Houwink relation was poor (Fig. 3). Due to the very low viscosities of PSSNa at 60°C, experimental errors were large, especially for the lower molecular weights. A more accurate determination of the constants a and k was limited by the lack of higher molecular weight standards, which were used in the reference cited above. On the other hand, it has been shown theoretically[20] that even in a good solvent a is not always a constant for a larger range of molecular weights. It is clear that a great source of errors in the estimation of molecular weights with SEC can arrive from the determination of $[\eta]$. For the universal calibration curve, experimentally determined intrinsic viscosities were used for the five highest molecular weights and the calculated viscosities for the lower molecular weights (Fig. 4). It can be seen from Figure 4 that the deviation of linearity for the molecular weights around 1×10^6 decreased compared to that observed for the peak position method. This was due to the high intrinsic viscosity of the highest molecular weight standard (Fig. 3). However, the increase in viscosity could not completely account for the deviation in linearity for the highest molecular weight. Moreover, a difference between both columns remained.

Concentration Effects

Large errors can also be introduced when the column is overloaded. The increase of the peak width and the retention volume with increasing concentrations for traditional SEC is sometimes called the concentration effect.[21] The origin of this effect is complex. The influence of the concentration on the retention volume has been attributed by Janca to a combination of several factors such as a decrease in hydrodynamic volume of the polymer with increasing concentration,[22] to viscosity phenomena in the interstitial volumes,[23] and to secondary exclusion.[24] The solvent plays also an important role.[25] It is a very well-known fact that the retention volume is concentration-independent in θ solvents. Furthermore, concentration dependencies of the distribution coefficient due to polymer–gel interactions have been observed.[26] It can be seen from Figure 5 that for HPSEC the concentration effect increased with increasing molecular weight. However, there was a strong column dependency, which is in agreement with the literature.[27] Although for $\bar{M}_p = 1.2 \times 10^6$ on column A peak shapes were irreproducible already around 2 mg mL^{-1}, for column B no appearance of shoulders and secondary peaks were observed even up to 5 mg mL^{-1} (Fig. 6). For the lower molecular weight samples no peak distortion was seen in the used concentration range. For SEC it was pointed out that, above a certain limiting value of the specific viscosity, independent of the sample type, the reproducibility was strongly decreased.[28] We could not find such a value since the concentration effect differed from one column to another. This is also found for the concentration dependent K values.

Compared with column A where strong peak distortions were observed around 2 mg mL^{-1} (Fig. 5), changes in K values occurred at concentrations at least twice as high for the column B (Fig. 6). Previously it was pointed out for

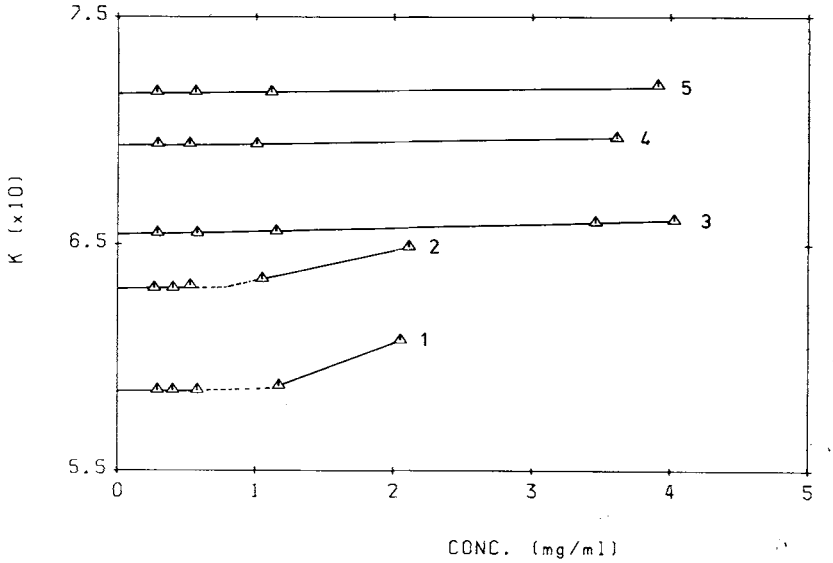

Fig. 5. Dependence of the retention volume ratio upon the concentration PSSNa injected for M_p =: (1) 1.2×10^6; (2) 780×10^3; (3) 400×10^3; (4) 200×10^3; (5) 100×10^3. Flow rate = 0.8 ml mL^{-1}; column A.

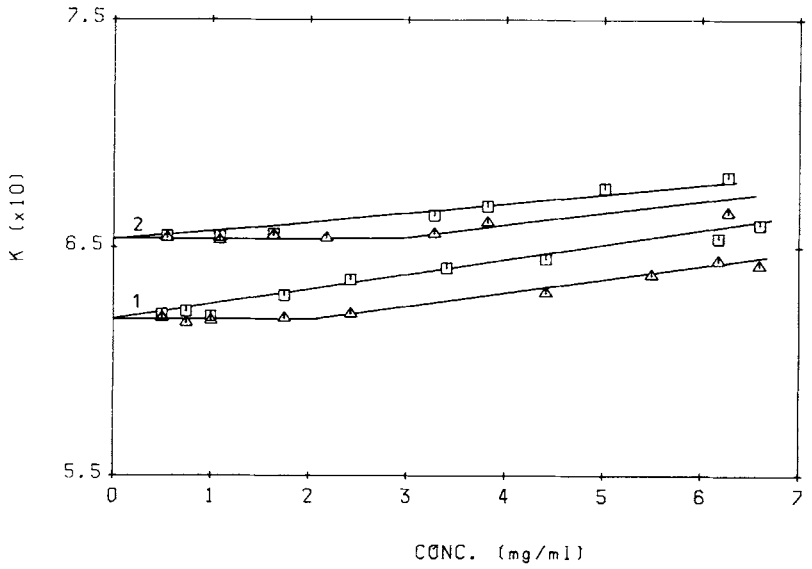

Fig. 6. Dependence of the retention-volume ratios (K) upon the concentration PSSNa injected and the flow rate for a second column. M_p =: (1) 1.2×10^6; (2) 780×10^3. (△) 0.8 mL min^{-1}; (□) 0.4 mL min^{-1}. Column B.

SEC with PSSNa samples that the elution volumes remain constant until a critical concentration. The inflection point corresponded with the critical overlap concentration (c^*), where the volume occupied by the chains equals that of the total solution volume. In our HPSEC experiments the inflection point depends on the column. Moreover, the flow rate also determines the concentration dependency (Fig. 6). This was only carried out on column B where no peak distortions were observed since on column A, due to the appearance of secondary peaks, uncertainties were too large at large concentrations. At 0.4 mL min^{-1} the inflection point was found at lower concentrations and the slope of the increase was larger in comparison with 0.8 mL min^{-1}.

However, below 1 mg mL^{-1} no significant influence of the flow rate was observed. Moreover, the flow rate had no influence on the peak width of the standards for all concentrations studied. The dependence of the concentration effect on the flowrate has already been observed previously.[29] It was thought that changes occur in the distribution coefficient between the polymer and the gel pores at high flow rates. At 0.8 mL min^{-1} the molecules would have less time to permeate in the gel so that they are eluted earlier compared to 0.4 mL min^{-1}. This effect increases with increasing concentrations. Since the diffusion depends on the molecular weight, this would also explain why the influence of the flow rate on the concentration is determined by the molecular weight.

The universal calibration concept implies infinite dilution.[22] To reduce errors introduced by concentration effects, a linear extrapolation of the retention volume to zero concentration is therefore normally used. As can be seen from Figure 6 for both molecular weights only at 0.4 mL min^{-1} a linear fit of all points seemed reasonable. The intersection at zero concentration

corresponded within experimental errors with the value measured at the lowest concentration. At higher flow rate it is obvious that a linear fit cannot be made since it would result in a K value that is much lower than the experimental values. At sufficiently low concentrations the flow rate had no influence on the experimentally determined retention volumes. The values at the lowest concentration were therefore used for the universal calibration curve.

Unknown Sample

Contrary to the well-defined PSSNa standards natural polymers, such as polysaccharides, have broad molecular weight distributions. κ-carrageenan, extracted from red algae, is a sulfated polysaccharide that is very much used in the food industry.[30] For a rapid analysis of such polymers HPSEC seems therefore suitable. Both the peak position method and the universal calibration can be used. However, the first technique which uses standards with the same structure as the unknown seems very time-consuming due to the large separation and analysis time of the carrageenan fragments.[31] We used therefore the universal calibration with PSSNa. The molecular weight determination was thus reduced to the determination of $[\eta]$ and K respectively.

Although unpurified κ-carrageenan contained K^+ and Ca^{2+}, which cause conformational changes, it is important to notice that the polymer existed in a random-coil state in our experimental conditions. It is a very well known fact that changes in the hydrodynamic volume affect both $[\eta]$ and K.

Several authors[32-34] have shown that a conformational transition of κ-carrageenan in the presence of the gel-inducing cations Cs^+ and K^+ appears only below 60°C, even for polymer concentrations above 0.1%, which was the maximum concentration used in our system.

A good linearity was obtained between all points in the Huggings plot. Extrapolation to zero concentration led to $[\eta] = 5.7$ dL/g. This value is in agreement with the value (6.6 dL/g) reported by Smidsrød[35] for a sample with $\bar{M}_w = 500 \times 10^3$ in a 0.1M salt solution at room temperature.

In addition to the concentration effect, column overloading can also occur due to the injected volume. The maximum allowed amount can be calculated from the peak width of the monomer.[36] For neocarrabiose 4-sulfate, the repeating unit of κ-carrageenan,[37] a maximum volume of 250 μL was determined in our experimental conditions. However, only 100 μL was injected on the column B which showed the smallest concentration dependency for PSSNa. In Figure 7 the effect of the concentration and the flow rate on the retention volume is given. The broad peak of carrageenan is followed by a low molecular weight fraction, which has already been previously observed,[38] and a large salt peak (not shown). The retention volume increases with increasing concentration above 0.55 mg mL^{-1}. Independent of the flow rate the resolution decreased at higher concentrations and the K values became irreproducible. Below 0.55 mg mL^{-1} the effect of the flow rate was negligible within experimental errors. For the molecular weight determination the K value of the lowest concentration was taken. This led to a relative molecular weight of 303×10^3 g mol^{-1}. Errors of 1% for the K values resulted in uncertainties of 15% for the molecular weight. Although experimental errors were great the

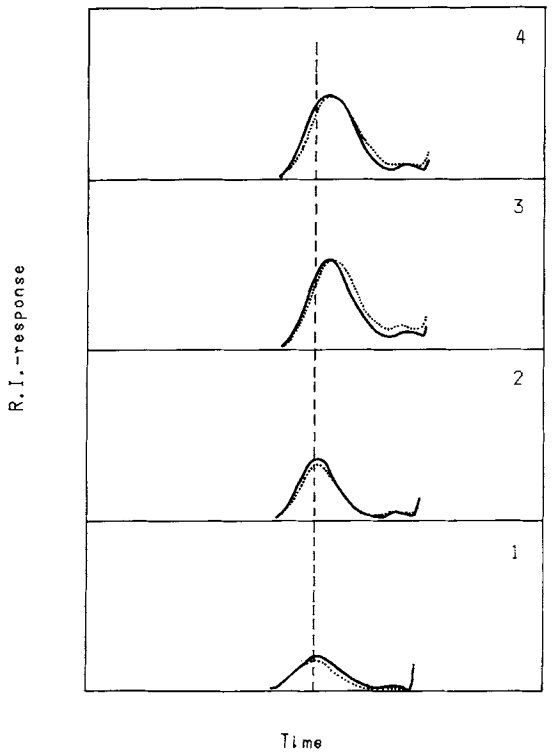

Fig. 7. HPSEC chromatograms of kappa-carrageenan in function of the concentration (mg mL^{-1}); [(1) 0.27; (2) 0.55; (3) 0.82; (4) 0.95] and the flow rate (- - -) 0.4 mL min^{-1}; (——) 0.8 mL min^{-1}].

value was still comparable with that of Slootmaekers et al.,[39] obtained from light scattering on the same sample ($M_w = 322 \times 10^3$). A more precise indication of the molecular weight from the universal calibration was impossible. However, for relative measurements of different polymer samples with varying chain lengths this technique remains quite useful.

CONCLUSION

HPSEC seems a very attractive method for a rapid analysis of anionic polymers. However, the accuracy depends on several factors. For polyelectrolytes it seems limited by the column stability and possible interactions between the packing and the sample. Although suitable solvent conditions can be found to overcome the last problem, the main sources of error lies in the experimental determination of the intrinsic viscosities, especially when the Mark–Houwink constants are unknown. Moreover, application of these constants for a large range of available standards seems doubtful. Finally other errors can be easily introduced due to column overloading, polymer orientation, and conformation.

The authors thank the Ministerie van Wetenschapsbeleid for giving a Prest-Mandaat to T. M., the Waters Chromatography Division Belgium for their excellent collaboration, and Mr. W. Noppe for technical assistance.

References

1. L. R. Snyder and J. J. Kirkland, Eds., *Introduction to Modern Liquid Chromatography*, Wiley, New York, 1979.
2. W. W. Yau., J. J. Kirkland, and D. D. Bly, Eds., *Modern Size-Exclusion Liquid Chromatography*, Wiley, New York, 1979.
3. M. T. Gilbert, Ed., *High Performance Liquid Chromatography* Wright, Bristol, 1987.
4. N. Motohashi and I. Mori, *J. Chromatogr.*, **299**, 508 (1984).
5. M. Terbojevich, A. Cosani, M. Palumbio, and F. Pregnolato, Carbohydr. Res., **157**, 269 (1986).
6. D. Lecacheux, R. Panaras, G. Brigand, and G. Martin, *Carbohydr. Polym.*, **5**, 423 (1985).
7. N. B. Beaty, W. P. Tew, and J. R. Mello, *Anal. Biochem.*, **147**, 387 (1985).
8. Z. Grubisic, P. Rempp, and H. Benoit, *J. Polym. Sci. Polym. Lett. Ed.*, **5**, 753 (1967).
9. A. L. Spatorico and G. L. Beyer, *J. Appl. Polym. Sci.*, **19**, 2933 (1975).
10. J. Desbrières, J. Mazet, and M. Rinaudo, *Eur. Polym. J.*, **18**, 269 (1982).
11. C. Rochas, A. Domard, and M. Rinaudo, *Eur. Polym. J.*, **16**, 135 (1980).
12. A. Domard, M. Rinaudo, and C. Rochas, *J. Polym. Sci. Polym. Phys. Ed.*, **17**, 673 (1979).
13. M. Rinaudo, J. Desbrières, and C. Rochas, *J. Liq. Chromatogr.*, **4**, 1297 (1981).
14. P. L. Dubin, S. Koontz, and K. L. Wright, *J. Polym. Sci. Polym. Chem. Ed.*, **15**, 2047 (1977).
15. P. J. Flory, *Principles of Polymer Chemistry*, Cornell University Press, Ithaca, NY, 1953, p. 636.
16. E. R. Morris, A. N. Cutler, S. B. Ross-Murphy, and D. A. Rees, *Carbohydr. Polym.*, **1**, 5 (1981).
17. A. Rudin and H. L. W. Hoegy, *J. Polym. Sci. A-1*, **10**, 217 (1972).
18. A. Takahashi, T. Kato and M. Nagasawa, *J. Phys. Chem.*, **71**, 2001 (1967).
19. C. Tanford, Ed., *Physical Chemistry of Macromolecules*, Wiley, New York, 1961, p. 406.
20. R. Ullman, *Macromolecules*, **14**, 746 (1981).
21. J. Janca, *J. Chromatogr.*, **134**, 263 (1977).
22. A. Rudin and R. A. Wagner, *J. Appl. Polym. Sci.*, **20**, 1483 (1976).
23. J. Janca and S. Pokorny, *J. Chromatogr.*, **148**, 31 (1978).
24. J. Janca, S. Pokorny, M. Bleha, and O. Chiantore, *J. Liq. Chromatogr.*, **3**, 953 (1980).
25. S. Mori, *Steric-Exclusion Liquid Chromatography of Polymers*, J. Janca, Ed., Dekker, New York, 1986, p. 196
26. S. Mori, Ref. 25, p. 197.
27. S. Mori, Ref. 25, p. 199.
28. G. Samay and M. Kubin, *J. Appl. Polym. Sci.*, **23**, 1879 (1979).
29. K. A. Boni, F. A. Sliemers, and P. B. Stickney, *J. Polym. Sci. A-2*, **6**, 1567 (1968).
30. G. O. Phillips, D. J. Wedlock, and P. A. Williams, Eds., *Gums and Stabilisers for the Food Industry 3*, Elsevier, London, 1986.
31. L. G. Ekström, J. Kuivinen, and G. Johansson, *Carbohydr. Res.*, **116**, 89 (1983).
32. T. H. M. Snoeren and T. A. J. Payens, *Biochem. Biophys. Acta*, **437**, 264 (1976).
33. H. Grasdalen and O. Smidsrφd, *Macromolecules*, **14**, 229 (1981).
34. C. Rochas and M. Rinaudo, *Biopolymers*, **23**, 735 (1984).
35. O. Smidrød, *Faraday Discuss. Chem. Soc.*, **57**, 279 (1974).
36. W. W. Yau, J. J. Kirkland, and D. D. Bly, Ref. 2, p. 240.
37. T. Malfait, H. Van Dael, and F. Van Cauwelaert, *Carbohydr. Res.*, **163**, 9 (1987).
38. D. Lecacheux, R. Panaras, G. Brigand, and D. Martin, *Carbohydr. Polym.*, **5**, 423 (1985).
39. D. Slootmaekers, C. De Jonghe, H. Reynaers, F. Varkevisser, and C. J. Bloys van Treslong, *Int. J. Biol. Macromol.*, **10**, 110 (1988).

Received November 28, 1988
Accepted December 16, 1988