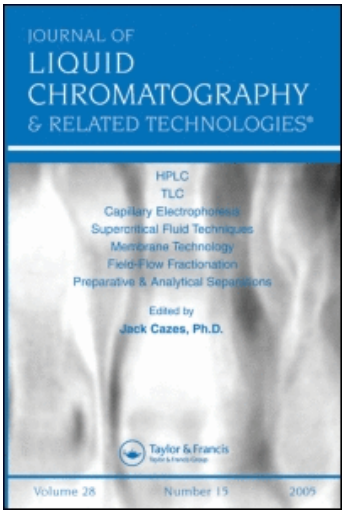

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SALT CONCENTRATION EFFECTS IN HIGH-PERFORMANCE HYDROPHOBIC-INTERACTION CHROMATOGRAPHY IN COMPARISON WITH NMR OF PROTEINS IN SOLUTION

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ABSTRACT

The effects of salt concentration on the chromatographic behavior of cytochrome C, ribonuclease A, and α-chymotrypsinogen A in hydrophobic interaction chromatography (HIC) has been examined by isocratic elutions on a Bio-Gel TSK Phenyl 5 PW column. In some cases, conformational variations were manifest chromatographically by reproducible changes in peak shape and appearance of multiple peaks as a function of sodium sulfate concentration in the mobile phase. A parallel study by proton nuclear magnetic resonance (NMR) spectroscopy on the salt concentration dependence of the spectral property of these proteins is in agreement with the possible contribution of the mobile phase composition to the observed chromatographic behavior.

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INTRODUCTION

HPLC has found in the last ten years extensive application in the high-resolution analysis and purification of many proteins and other biological macromolecules, as a result of the development of highly efficient columns.

However, proteins are sometimes eluted from HPLC columns as multiple or/and irregular-shaped peaks. This has been ascribed to several causes, including heterogeneity in the interactive sites of the stationary phase (1), aggregation (2), gradient artefacts (3), and denaturation (4). Proteins are stabilized by a combination of hydrogen bonding, electrostatic interactions, and hydrophobic interactions. The same forces are also involved in the chromatographic processes, thus conformational changes may occur induced by mobile or stationary phase or both, leading to loss of bioactivity and denaturation. The native and denaturated forms can be resolved if the kinetic process of conformational change is slow relative to the time scale of migration through the column, otherwise, a single broad peak will be obtained, which is the weighted average of the forms in equilibrium.

Hydrophobic-interaction chromatography (HIC) with weakly hydrophobic stationary phases and mostly with the use of decreasing gradients of stabilizing salts (e.g., ammonium and sodium sulfate) (5) has gained wide acceptance as a mild non-denaturating HPLC technique (6-11). Nevertheless, also in HIC proteins can yield broad or multiple peaks, depending on the chromatographic conditions and lability of the protein (12-14).

Biological activity (15), second-derivative UV spectroscopy (12), fluorescence spectroscopy (16), and circular dichroism spectroscopy (13,15,17) have been used to detect conformational variations induced by the chromatographic process.

In the last decade, nuclear magnetic resonance (NMR) spectroscopy has emerged as a powerful and versatile tool for the structural study of proteins in solution (18). Proton NMR is capable of distinguish conformational variations of proteins either by determining the whole structure through complex two dimensional methodology for complete resolution and assignment of resonances to the amino acids in the sequence

0.45-μm membrane filter (Millipore, Bedford, MA, USA) and degassed by sparging with helium before use as eluents. After each change of mobile phase composition a period of 30 min (± 0.5 min) was allowed for equilib(19), or more simply by comparing the line width of resonances in different conditions, such as pH, temperature, buffer or protein concentration (18,20). In particular, conformational variations of proteins as a function of ionic strength have been investigated by ^1H NMR (21-22).

In this paper, we examine the salt concentration dependence of the HIC behavior of ribonuclease A, α-chymotrypsinogen A, and cytochrome C and the variations occurring in the ^1H NMR spectra of these proteins in solutions containing sodium sulfate at the same salt concentrations used to perform the chromatographic experiments.

EXPERIMENTAL

Materials

The proteins ribonuclease A, α-chymotrypsinogen A, and cytochrome C were purchased from Sigma (St.Louis, MO, USA). Reagent-grade monobasic sodium phosphate, dibasic sodium phosphate, sodium sulphate, sodium hydroxide, as well as HPLC-grade water were obtained from Carlo Erba (Milan, Italy). Deuterated water (99.8%) was purchased from Stholer Isotope Chemicals (Rutherford, NJ, USA), and 2,2-dimethyl-2-silapentane-5-sulfonate was supplied by Aldrich (Milwaukee, WI, USA).

Chromatography

The experiments were performed with a Beckman (Fullerton, CA, USA) Model 342 Liquid Chromatograph, consisting of two Model 114 M solvent delivery pumps, a Model 420 system controller, a Model 340 dynamically-stirred high-pressure mixer, and a Model 163 variable wavelength UV detector. Samples were injected with a Rheodyne (Cotati, CA, USA) Model 7125 sample valve equipped with a 20 microliter sample loop. Chromatograms were obtained with a Model 5117 Omniscribe (Houston Instrument, Gistel, Belgium) strip chart recorder, or with a Shimadzu (Kyoto, Japan) Model C-R5A Chromatopac Integrator. A Bio-Gel

TSK Phenyl 5 PW column (75 x 7.5 mm) was supplied by Bio-Rad Labs. (Richmond, CA, USA). The chromatographic experiments consisted of isocratic elution at several salt concentration in the range from 0 to 1.0 M sodium sulfate. The background electrolyte was 50 mM phosphate buffer, prepared by mixing 25 mM monobasic sodium phosphate with 25 mM dibasic sodium phosphate. All solutions were filtered through a type HAration. Protein solutions (0.1 mM) were freshly made up in HPLC-grade water. A 20-μl sample size was injected. Proteins were detected by monitoring the column effluent at 280 nm.

NMR

The ^1H NMR spectra were obtained at 200.13 MHz on a Bruker (Fallanden, Switzerland) Model AC 200 Spectrometer. The spectra were run in D_2O at 294 k. Residual water signal was suppressed by pre-saturation (23), and its absorption frequency was used as internal standard and referred to external 2,2-dimethyl-2-silapentane-5-sulfonate. The stability of the water chemical shift within 2 Hz was verified after each experiment. The peaks were assigned according to Bradbury et al. (24). For each protein, either a 0.1 or a 0.05 mM solution was prepared in 50 mM D_2O sodium phosphate buffer, pH 6.9 (low-salt conditions). Part of this solution was then added with sodium sulfate up to 1.0 M concentration (high-salt conditions). Samples at 0.25, 0.50, and 0.75 sodium sulfate were obtained by mixing appropriate volumes of the low-salt solution with the high-salt solutions.

RESULTS AND DISCUSSION

The effect of salt concentration on the chromatographic behavior of ribonuclease A, cytochrome C, and α-chymotrypsinogen A was investigated by eluting these proteins under isocratic conditions with mobile phases having different salt concentrations.

Figure 1 presents the isocratic elutions of ribonuclease A with mobile phases at several salt concentrations. Different peak shapes are observed in the salt concentration range from 0.25 to 0.75 M. With the mobile phase containing up to 0.25 M sodium sulfate, ribonuclease A was eluted as a

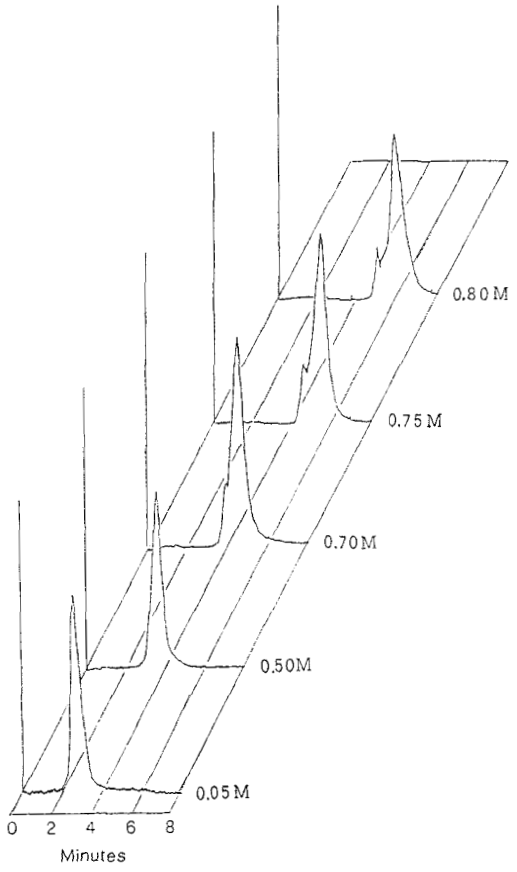

FIGURE 1. Effect of sodium sulfate concentration on the elution profiles of ribonuclease A on the Bio-Gel TSK Phenyl 5 PW column (75 x 7.5 mm I.D.). Background electrolyte, 50 mM phosphate buffer (pH 6.9); flow rate, 1.0 ml/min; detection, 280 nm, 0.05 a.u.f.s.; temperature, 21°C.

relatively sharp peak. As the salt concentration was raised, a shoulder on the upslope of the chromatographic peak was observed, and a peak splitting occurred at higher salt concentration. The peak splitting and band broadening increased with increasing the salt concentration in the mobile phase. Fractions were collected from each peak and individually rechromatographed under isocratic conditions with the mobile phase containing 0.8 M sodium

sulfate. A chromatogram similar to that reported in Figure 1 was observed. The area ratio of the two peaks were the same for both the parent protein sample and the rechromatographed peak sample. This result leads to the conclusion that the observed second peak arises from ribonuclease A itself and not from impurities. Several examples of similar two-peaks separations have been previously observed in HIC of proteins and in most cases has been ascribed to conformational changes occurring on the column (14-16). The main factors found to effect the conformational variations of proteins in HIC are the stationary phase hydrophobicity (14,16), the contact time between the protein and the stationary phase (14, 16), the temperature (12-14,16), the pH (14), and the mobile phase composition (14).

The effect of salt on the conformational stability of proteins in aqueous solution is a function of both the ionic species present and their concentration. Proton NMR spectroscopy is well suited to investigate aspects of protein structure in solution which may be affected by solvent composition.

Figure 2 presents the ^{1}H NMR spectra of ribonu-clease A in solution at various salt concentration ranging from 0 to 1.0 M sodium sulfate. By increasing the salt concentration, two variations are observable in the NMR spectra, both involving the broadening of related lines. The broadening of signals due to aromatic residues (6.5-7.5 ppm), i.e. aromatic residues are less exposed to the solvent by increasing the salt concentration. In the meantime resonances at 0.8 ppm, due to methyl groups in the proximity of aromatic residues undergo the same broadening. This indicates that hydrophobic portions of the protein stiffen by increasing the salt concentration. Broadening of signals due to the methyl groups of serine residues indicates that also these hydrophilic residues are involved in the stiffening, whereas the polar arginine and lysine residues (signals at about 3 ppm) do not show any variation.

The salt concentration dependence of the retention behavior of α-chymotrypsinogen A and cytocrome C on the HIC column under isocratic conditions is illustrated in Figure 3 as plots of logarithmic retention factor against the salt concentration in the mobile phase. At salt concentration higher than 0.5 M, α-chymotrypsinogen A was strongly retained and did not elute from the HIC column. The plot of the logarithmic retention factor as a

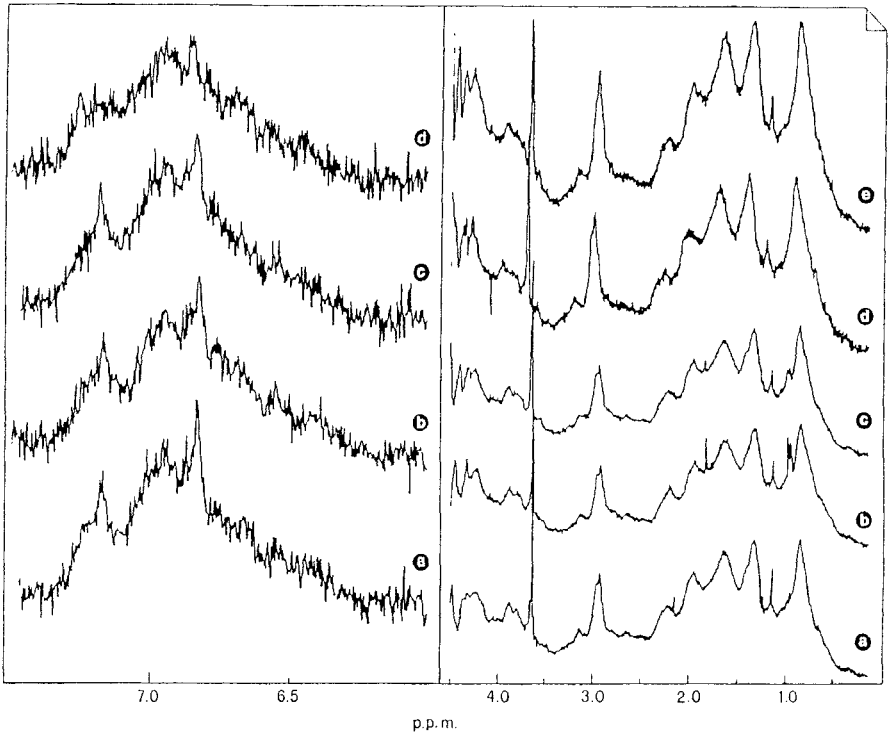

FIGURE 2. ^1H NMR spectra at 200.13 MHz (1600 scans) of a 0.1 mM solution of ribonuclease A in 50 mM D_2O sodium phosphate buffer (pH 6.9), containing no salt (a), and 0.25 M (b), 0.50 M (c), 0.75 M (d), and 1.0 M (e) sodium sulfate.

function of salt concentration in the range from 0.2 to 0.5 M yields a straight line having a positive slope, the magnitude of which, according to the theory (25-27), is expected to be proportional to the exposed hydrophobic surface area of the protein. Thus, the slope of the plots reported in Figure 3 may provide a scale of the hydrophobic character of proteins (27).

The ^1H NMR spectra of α-chymotrypsinogen A in solutions containing salt concentrations in the range from 0 to 0.75 M sodium sulfate are reported in Figure 4. ^1H NMR signals, already broad at low salt concentration, show a further broadening by increasing salt concentration.

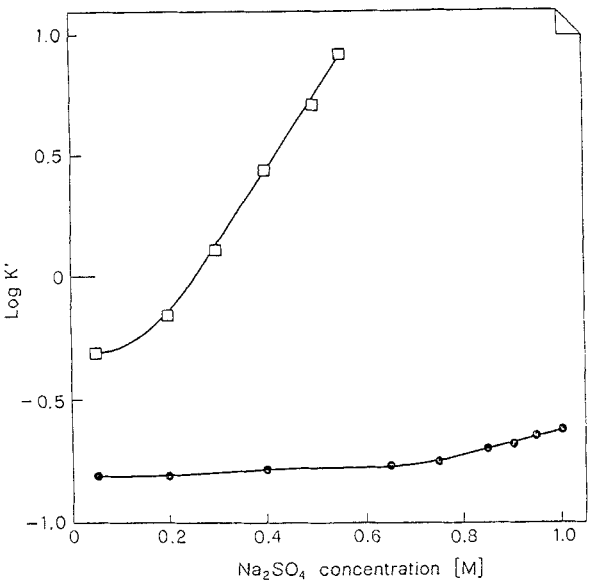

FIGURE 3. Plots of logarithmic retention factor of cytochrome C (•) and a-chymotrypsinogen A (□) against sodium sulfate concentration in the mobile phase. All conditions as in Figure 1.

The presence of large unresolved peaks does not allow any precise NMR assignment. A possible interpretation of this behavior is the formation of aggregates. The self-association characteristics of α-chymotrypsinogen A in aqueous solutions have been reported by many authors and have been related to changes in pH, ionic strength, temperature, or specific nature of the medium (28-31). Furthermore, aggregation would be consistent with the observed appearance of an opalescence in both 0.1 and 0.05 mM protein solutions containing sodium sulfate at concentration higher than 0.75 M, which did not allow NMR measurement, and with the highly hydrophobic character of α-chymotrypsinogen A, as it can be inferred by the retention behavior in HIC depicted in Figure 3.

Cytochrome C is the less hydrophobic protein among the other investigated here and was weakly retained on the phenyl column. Under

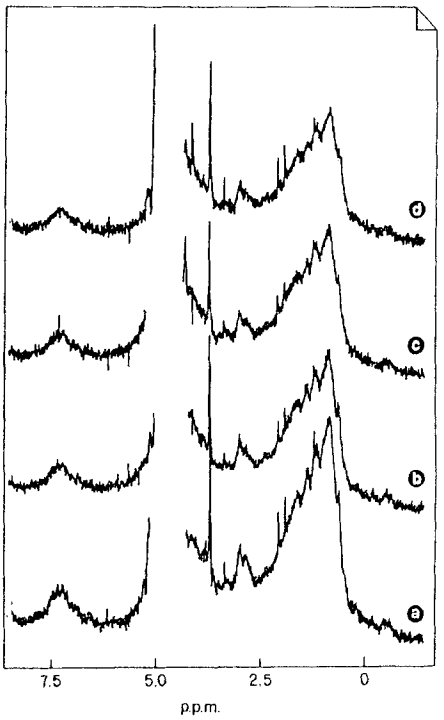

FIGURE 4. ^1H NMR spectra at 200.13 MHz (3200 scans) of a 0.05 mM solution of a-chymotrypsinogen A in 50 mM D_2O sodium phosphate buffer (pH 6.9), containing no salt (a), and 0.25 M (b), 0.50 M (c), and 0.75 M (d) sodium sulfate.

isocratic conditions, an increase in salt concentration lead to a small increase in retention time (see Figure 3), and no noticeable variations in peak shape. The ^1H NMR spectra of cytochrome C were also little effected by the salt concentration in the solutions. Figure 5 shows that besides the broadening of the signals in the range from 6 to 8 ppm, relative to aromatic residues, there are no significant variations of the NMR spectra of cytochrome C with increasing salt concentration.

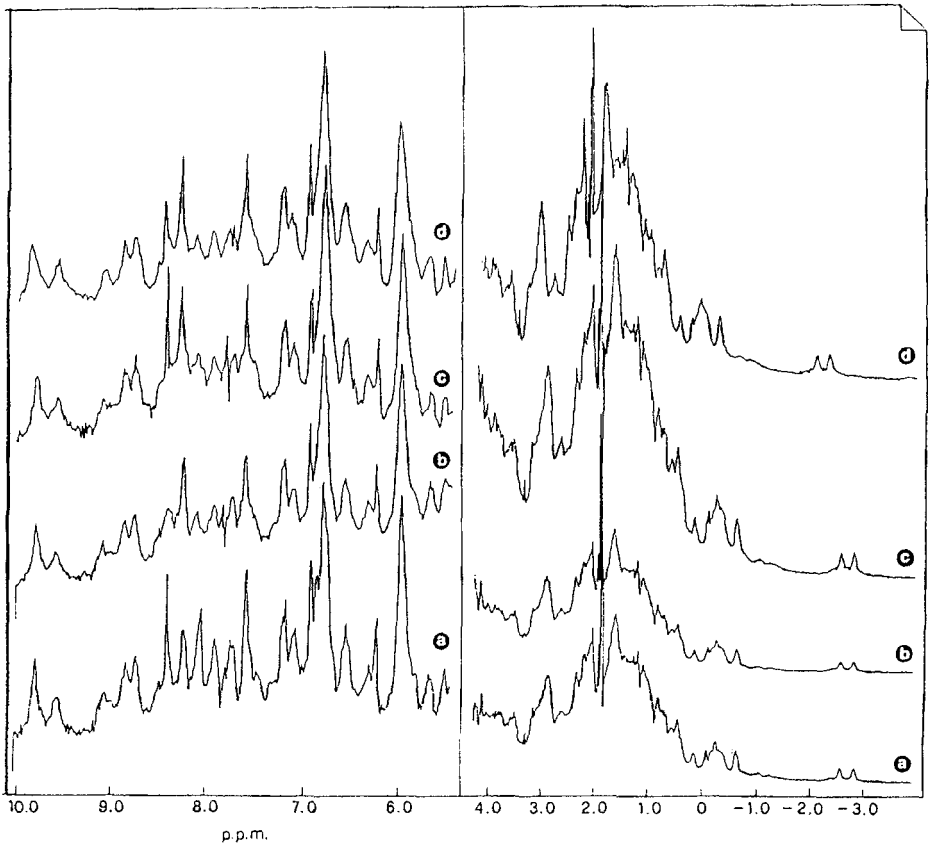

FIGURE 5. ^1H NMR spectra at 200.13 MHz (1600 scans) of a 0.1 mM cytochrome C solution. Conditions as in Figure 4.

CONCLUSIONS

This paper represents an attempt to correlate the salt concentration dependence of the ^1H NMR spectra of proteins in aqueous solutions to the possible contribution of the mobile phase effects on the chromatographic behavior of these biopolymers in HIC. Although it is restricted to a limited number of experiments and proteins, it shows that ^1H NMR may represent a

strategy to investigate variations in the protein structure due to solvent composition, which can be related to the mobile phase mediated chromatographic behavior of these biopolymers.

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REFERENCES

1. W.S. Hancock, J.T. Sparrow "The separation of Proteins by Reversed-Phase High-Performance Liquid Chromatography" in High-Performance Liquid Chromatography - Advances and Perspectives, Cs. Horváth ed., Academic Press, New York 1983, pp. 50-85.

2. M.E. Co-Sarno, M.A. Tapang and D.G. Luckhurst, J. Chromatogr., 296: 105-113, (1983)

3. L. Fausnaugh, E. Pfannkoch, S. Gupta, F. Regnier, Anal. Biochem., 137: 463-472, (1984)

4. S.A. Cohen, K. Benedek, Y. Tapuhi, J.C. Ford, B.L. Karger, Anal. Biochem. 144: 275-284 (1984)

5. T. Arakawa, S.N. Timasheff, Biochemistry, 21: 6545-6552 (1982)

6. Y. Kato, T. Kitamura, T. Hashimoto, J. Chromatogr. 266: 49-54 (1983)

7. D.L. Gooding, M.N. Schmuck, K.M. Gooding, J. Chromatogr. 296: 107-114 (1984)

8. J.P. Chang, Z. El Rassi, Cs. Horváth, J. Chromatogr., 319: 396-399 (1985)

9. N.T. Miller, B. Feibush, B.L. Karger, J. Chromatogr., 316: 519- 536 (1984)

10. W.R. Melander, D. Corradini, Cs. Horváth, J. Chromatogr. 317: 57-85 (1984)

11. D. Corradini, M.T. Giardi, A. Massacci, J. Chromatogr. 395: 523-529 (1985)

12. S.L. Wu, K. Benedek, B.L. Karger, J. Chromatogr. 359: 3-17 (1986)

13. R.H. Ingrahm, S.Y.M. Lau, A.K. Tanaka, R.S. Hodges, J. Chromatogr., 327: 77-92 (1985)

14. S.L. Wu, A. Figueroa, B.L. Karger, J. Chromatogr., 371: 3-27 (1986)

15. J. Withka, P. Moncuse, A. Baziotis, R. Maskiewicz, J. Chromatogr., 398: 175-202 (1987)

16. M.G. Kunitani, R.L. Cunico, S.J. Staats, J. Chromatogr., 443: 205-220 (1988)

17. A.F. Drake, M.A. Fung, F. Simpson, J. Chromatogr., 476: 159-163 (1989)

18. O. Jardetzky, G.C.K. Roberts, NMR in Molecular Biology, Academic Press, New York, 1981.

19. C. Redfield, C.M. Dobson, Biochemistry, 27: 122-136 (1988)

20. I.D. Campbell, "An NMR View of Preotein Structure", in NMR in Biology, R.A. Dwek, I.D. Campbell, R.E. Richards, R.J.P. Williams, eds, Academic Press, London, 1977.

21. P.G. Hartman, G.E. Chapman, T. Moss, E.M. Bradbury, Eur. J. Biochem., 77: 45-51 (1977)

22. L. Cellai, A. Segre, H. Heumann, Biochem. J., 207: 175-177 (1982)

23. J.K.M. Saunders, B.K. Hunter, Modern NMR Spectroscopy, Oxford University Press, Oxford, (1987), pp. 241-249.

24. E.M. Bradbury, P.D. Cary, C. Crane-Robinson, P.L. Riches, E.W. Johns, Eur. J. Biochem., 26: 482-489 (1972)

25. W.R. Melander, Cs. Horváth, Arch. Biochem. Biophys., 183: 200-215 (1977)

26. W.R. Melander, Z. El Rassi, Cs. Horváth, J. Chromatogr. 469: 3-27 (1989)

27. A. Katti, Y.-F. Maa, Cs. Horváth, Chromatographia, 24: 646-650 (1987)

28. J.C. Nichol, J. Biol. Chem., 243: 4065-4069 (1968)

29. M.N. Tung, R.F. Steiner, Eur. J. Biochem. 44: 49-58 (1974)

30. H. Reggio, J.C. Dagorn, J. Cell Biol. 78: 951-957 (1987)

31. A.A. Ismail, H.H. Mantsch, P.T.T. Wong, Biochem. Biophys. Acta, 1121: 183-188 (1992)

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