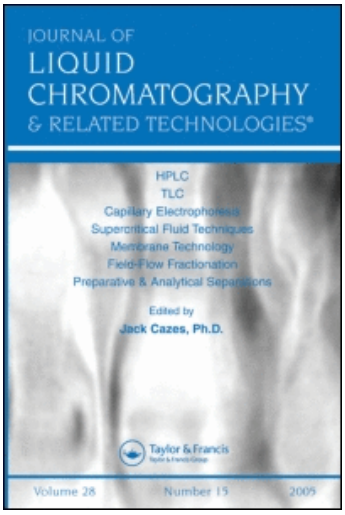

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Issaq, Haleem J. , Janini, George M. , Atamna, Ibrahim Z. , Muschik, Gary M. and Lukszo, Jan(1992) 'Capillary Electrophoresis Separation of Small Peptides: Effect Of pH, Buffer Additives, and Temperature', Journal of Liquid Chromatography & Related Technologies, 15: 6, 1129 — 1142
To link to this Article: DOI: 10.1080/10826079208018854
URL: http://dx.doi.org/10.1080/10826079208018854

JOURNAL OF LIQUID CHROMATOGRAPHY, 15(6&7), 1129-1142 (1992)

CAPILLARY ELECTROPHORESIS SEPARATION OF SMALL PEPTIDES: EFFECT OF pH, BUFFER ADDITIVES, AND TEMPERATURE

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ABSTRACT

The separation of dipeptides and small peptides by various modes of capillary electrophoresis was investigated in order to identify the best separation conditions and to compare the different charge-to-size parameters used in correlating peptide migration. For a series of equally charged polyalanines the best linear correlation was obtained when the electrophoretic mobility was plotted against $q/(MW)^{2/3}$. Deviations from linearity with other peptides are due to an imprecise charge calculation procedure. The best separations were achieved at low pH ($\sim$2.5) when a large metal ion such as Zn^{++} was added to the buffer. Under these conditions, peptides are positively charged and differences in charge are maximized. The separation of peptides at pH 2.5 improved as temperature was decreased. A set of five 9-residue peptides with no significant difference in charge-to-size ratio were separated at pH 7.0 with a buffer composed of 50 mM Tris + 50 mM DTAB.

INTRODUCTION

Peptide mapping of proteins by chemical and enzymatic methods provides valuable analytical and sequence information. For example, dipeptidyl aminopeptidase (DAP) is known to have a broad specificity in catalyzing the removal of dipeptide units, in consecutive order, from the unsubstituted termini

of the peptide chain (1,2). The separation of dipeptides and small peptides is, therefore, important as an aid in protein sequencing. It also serves as a model for investigating the separation mechanisms of more complex peptides.

The separation of dipeptides and small peptides has traditionally been achieved by using gas chromatography (3), ion-exchange chromatography (4), size-exclusion chromatography (5), and high-pressure liquid chromatography (6-10). Size-exclusion techniques are suitable for group separations of long- chain peptides and proteins (5). Currently, HPLC is the most commonly employed separation technique. Issaq and Radlon evaluated the use of beta-cyclodextrin and reversed-phase C-18 columns (6). Grushka and co-workers used a tripeptide-bonded phase (7,8). Lundanes and Greibrokk evaluated four different reversed-phases (9) while Molnar and Horvath separated dipeptides using non-polar stationary phases (10). In general, the best resolution of dipeptides was obtained on C-18 columns even though some peptides often coelute on such columns (6,11-13).

In recent years the power of capillary zone electrophoresis (CZE) for the analysis of peptides has been clearly demonstrated (14-19). The separation mechanism in CZE is based on the differential migration of solutes in an electric field due to differences in the solutes' charge-to-size ratios. Subtle variations in this ratio have allowed the resolution of molecules with minute differences in structure. The use of micellar buffer modifiers has provided additional separation power; this has proved to be beneficial in separating substances with similar charge-to-size ratios (20). Furthermore, the use of metal ion-supplemented buffers can resolve samples that comigrate in the absence of metal ions (21). Thus, CZE provides a valuable method that is orthogonal to HPLC and that may be used for further characterization of HPLC fractions in peptide analysis.

This study focused on separating small-chain peptides and dipeptides by using free solution capillary zone electrophoresis (CZE) with low and high pH buffers, micellar buffer modifiers and metal ion-supplemented buffers. It was found that peptide solutes are particularly suited for CZE analysis; this is

based mainly on differences in charge-to-size ratios and, to some extent, on hydrophobicity. The objective was to investigate the effect of pH, metal ions, micelles and temperature on the separation of small peptides and dipeptides in order to obtain the best separation conditions for the solute set and to compare the different charge-to-size parameters that are used for correlation of peptide migration in capillary zone electrophoresis.

EXPERIMENTAL

Peptide separations were performed by using a Beckman CZE System (Model P/ACE) equipped with a UV detector, an automatic injector, a column cartridge (50 cm x 75 μm i.d. surrounded by coolant), an autosampler and a printer. A Fisher Accumet selective ion analyzer (Model 750) was used to measure pH levels.

Peptides YPHFMPT, HFMPTNL, LTFGWCYKL, PHFMPTNLG, TPHFAPTNL, TPHAMPTNL, and YPHFMPANL were prepared in-house. All other peptide buffers and buffer additives were purchased from Sigma Chemical Company, St. Louis, MO, USA. Injections were performed using a pressure mode for 2 seconds. Buffer solutions were then degassed and filtered through 0.2 μm nylon 66 filters. Solute standards were prepared using the separation buffer in the concentration range of 1-5 mg/mL. The UV detector was set at 200 nm. Prior to use, each column was washed with NaOH and water and conditioned with the separation buffer. This procedure was repeated after each run.

RESULTS AND DISCUSSIONS

Charge-to-size parameters and electrophoretic mobility:

In this section, we discuss the theoretical background and compare the empirical approaches that were used to correlate solute electrophoretic mobility with charge-to-size parameters. When a charged particle is placed in an electric field (E) it is subjected to a force that is proportional to its effective charge (q) and the strength of the field. The translational motion of applied force is

opposed by a viscous drag that is proportional to the particle's velocity (V_{ef}), hydrodynamic radius (r), and medium viscocity (η). When the two forces are counterbalanced, the particle moves with a steady-state velocity (22):

$$V_{ef} = \mu_{ef} E \quad (1)$$

where μ_{ef} the electrophoretic mobility is given by:

$$\mu_{ef} = \frac{q}{6\pi\eta r} \quad (2)$$

Strictly speaking, this relationship (Stoke's Law) is only obeyed by spherical particles moving slowly through a nonconducting medium. For such systems, solute electrophoretic mobility is directly proportional to the charge-to-size parameter (q/r) or approximately proportional to $(q/(MW)^{1/3})$, where MW is the molar mass of the particle. However, the actual situation is more complicated when the medium is a conducting solvent and the moving particle is surrounded by an ionic atmosphere of the opposite charge. This atmosphere modifies both the particle's charge and the local electric field and gives the ion an effective radius that is different from its crystal radius. Moreover, equation 2 has to be modified for particles that are not effectively spherical (23). These complications, which are difficult if not impossible to treat theoretically, necessitated the use of models and empirical relationships. Grossman et al. (16,17) measured the mobility of 40 different peptides ranging in size from 3 to 39 amino acid residues and ranging in charge from 0.33 to 14, and fitted the results to an empirical equation of the form:

$$\mu_{ef} = \frac{D \ln (q+1)}{n^{0.43}} \quad (3)$$

where D is a constant for a constant buffer system and n is the number of amino acid residues. Rickard et al. (24) measured the electrophoretic mobility of several protein's digests and correlated their results with different charge-to-size parameters, namely: $q/(MW)^{1/3}$, $q/(MW)^{1/2}$ and $q/(MW)^{2/3}$. They concluded that the best fit as determined by the correlation coefficient of the linear, least square-fitting procedure is for μ_{ef} vs. $q/(MW)^{2/3}$. This was theoretically justified by invoking Offord's assertion that an ion moving through a conducting medium would experience a retarding force that is proportional to its surface area (25). This implied that the electrophoretic mobility would be inversely proportional to the square of the ion's radius (or inversely proportional to $MW^{2/3}$) rather than the first power of the radius, as implied by Stoke's Law. It should be noted that successful correlations using any of the approaches described above require an accurate determination of charge. A peptide's charge is highly dependent on the pH of the working buffer due to the presence of acidic and basic end groups as well as charged side-chain residues. The fundamental equation that is invariably used for the calculation of the net charge on a peptide is the Henderson-Hasselbalch equation. The use of this equation requires an accurate knowledge of the ionization constants of the amino acid residues. Since these values are not accurately known, most researchers use the pK values for free amino acids (26), assuming that the ionization of each amino acid is not affected by its nearest neighbors in the molecule. Others use corrected pK values that are approximate at best (as for example, the values given in Table 4 of ref. 24). Obtaining accurate values of the net charge of a peptide might not be critical for mobility correlation if the net charge is high (i.e., the peptide contains many acidic residues at high pH or many basic residues at low pH). However, an accurate knowledge of the ionization constants is essential for peptides with mostly non-ionizable amino acid residues.

In this work, we measured the electrophoretic mobility of a series of polyalanines with a phosphate working buffer at pH = 2.5. The Henderson-Hasselbalch equation was used to calculate the net charges. The pK of the C-

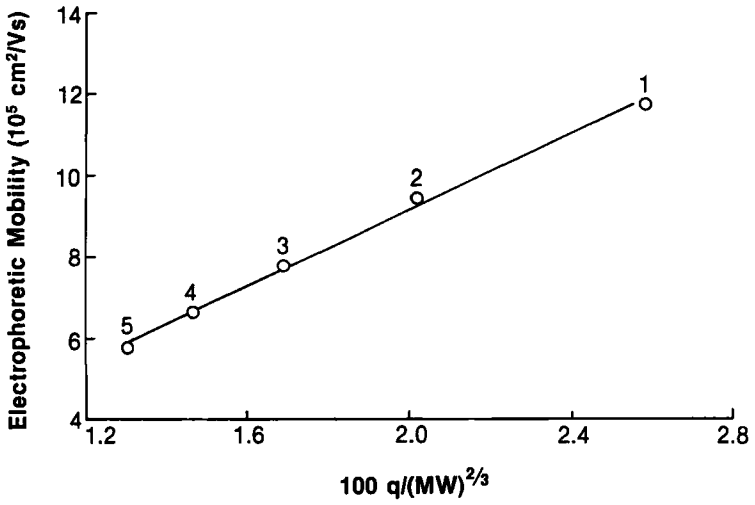

FIGURE 1. Electrophoretic mobility for dialanine and polyalanine solutes versus charge-to-size parameter. Solute: 1 = AA, 2 = AAA, 3 = AAAA, 4 =AAAAA, 5 = AAAAAA; Instrument: Beckman Model P/ACE System 2000; Column: 50 cm x 75 μm fused silica; Injection: pressure mode for 2 sec. at 0.5 psi; Buffer: 50 mM phosphate; pH = 2.50; T = 25°C; Voltage: 15 kV; electroosmotic mobility (mesityl oxide): 2.08 x 10^{-5} cm^2/Vs; Detection: UV 200 nm.

terminal residue was chosen as 3.2 and that of the N-terminal was 8.2. These values were taken from Table 4 of ref. 24. Even though these values might not be strictly accurate, it is safe to assume that net charges for members of such a system do not change with increasing chain length. The electrophoretic mobility was linearly correlated with the charge-to-size parameters: $\ln(q+1)/n^{0.43}$; $q/(MW)^{1/3}$; $q/(MW)^{1/2}$ and $q/(MW)^{2/3}$. The best fit, as determined by the largest value of the correlation coefficient, was with the charge-to-size parameter $q/(MW)^{2/3}$. Figure 1 gives a plot of μ_{ef} vs. $q/(MW)^{2/3}$ at pH = 2.5. The plot is linear with a correlation coeffieient of >0.999. This supports the fact that the best size parameter is one that is related to the analyte's surface area rather than the number of amino acid residues or the Stoke's Law radius. Figure 2 gives a plot of μ_{ef} vs. $q/(MW)^{2/3}$ at pH = 2.5 for a series of dipeptides with

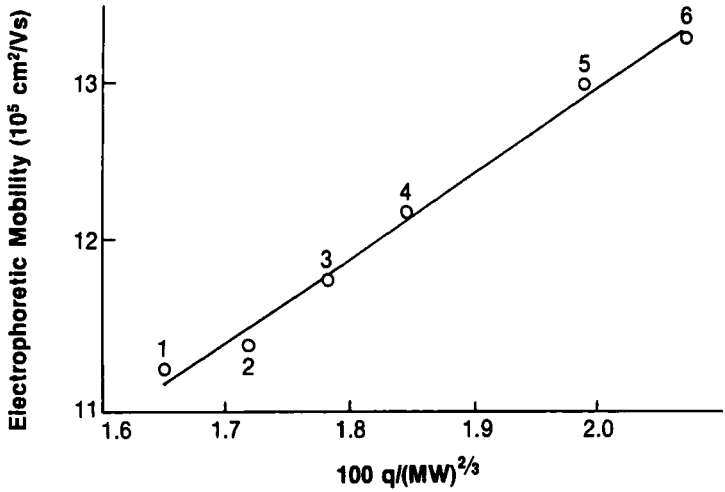

FIGURE 2. Electrophoretic mobility for dipeptides versus charge-to-size parameter. Solutes: 1 = FF; 2 = FD; 3 = FL; 4 = FV; 5 = FA; 6 = FG; Experimental conditions: As in Figure 1.

F as the N-terminal in each. The values of pK for the C-terminals were also taken from Table 4 of ref. 24. Since these values are, at best, approximate, the correlation is not as good as that shown in Figure 1.

Separation of dipeptides:

Figure 3 shows the separation of a series of dipeptide sequence isomers. This separation can only be attributed to a difference in charge as there is no appreciable difference in size between each pair of isomers. The best separation was obtained at pH = 2.5, which is close to the pK of the carboxyl group of the C-terminal. At or near this pH, small differences in pK result in maximum differences in charge and hence, maximum separation. Furthermore, the net positive charge results in both a rapid migration and a short analysis time. As the pH of the working buffer is increased (in the pH range of 3-8), differences in charge diminish and separation deteriorates. As the pH is increased further,

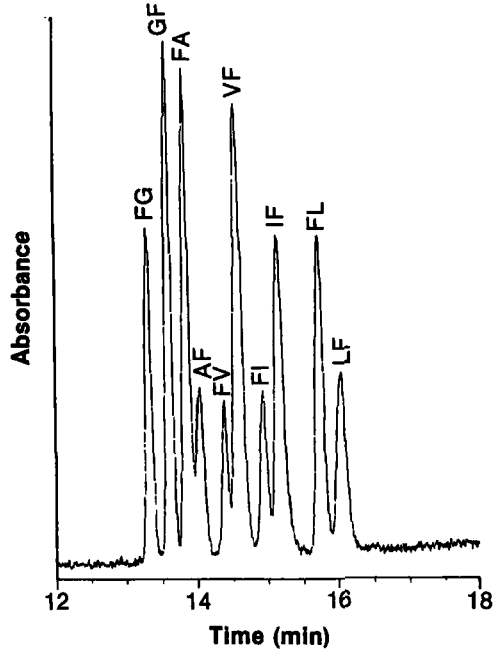

FIGURE 3. Electropherogram of dipeptide sequence isomers. Solute concentration: 1-5 mg/mL each; Buffer: 50 mM phosphate; pH=2.5; Voltage: 15 kV; Other experimental parameters: As in Figure 1.

approaching the pK range of the amino group of the N-terminal (8.5-9.5), the charge on the dipeptides turns negative and charge differences increase. Figure 4 shows an electropherogram at pH 8.2 for the same set of solutes used in Figure 3. At this pH, only seven peaks are observed for this 10-component mixture. Improved separation is obtained at even higher pH values, however, this is not recommended because fused silica column material dissolves in highly alkaline media.

Figure 5 shows the effect of temperature on the separation of a series of dipeptides with F as the N-terminal in each. The best separation was obtained at the lowest temperature studied. As the temperature was increased, the

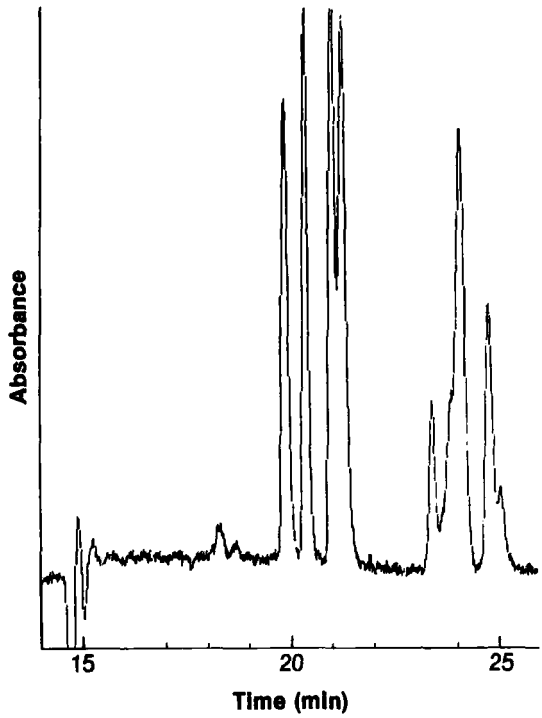

FIGURE 4. Electropherogram of dipeptide sequence isomers at pH = 8.2. Solute set: As in Figure 3; Voltage: 10 kV; Other experimental conditions: As in Figure 3.

migration times decreased. At 35°C, solutes FF and FY comigrated and at 45°C, the separation factor between neighboring peaks was further decreased.

The separation of dipeptides was also investigated using positively-charged dodecyltrimethylammonium bromide (DTAB) and negatively-charged sodium dodecyl sulfate (SDS) micelles. In comparison with neat buffers, the micelle-modified buffers resulted in broad peaks and poor separation and not all isomers were separated.

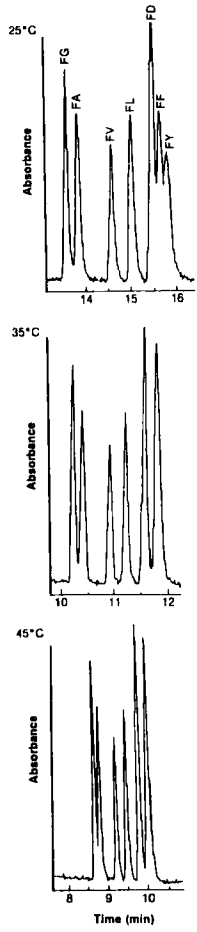

FIGURE 5. Separation of dipeptides as a function of temperature. Experimental parameters: As in Figure 3.

Separation of small peptides:

An example of short-chain peptide separation is given in Figure 6 which shows the separation of five 9-residue peptides with no appreciable difference in charge and size. Only two peaks were observed (Figure 6A) with neat buffer and the separation did not improve with increasing pH. When Zn^{++} was added to

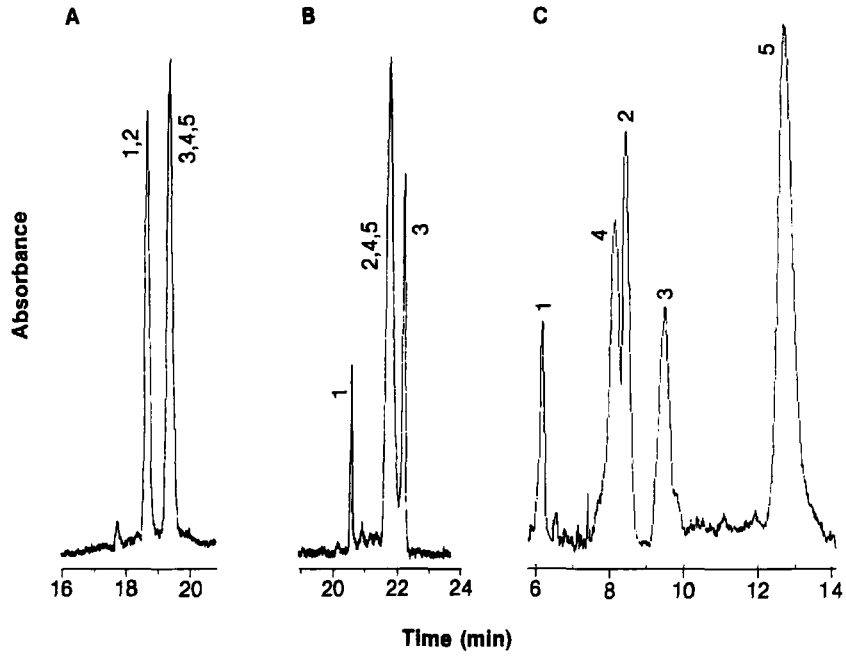

FIGURE 6. Separation of 9-residue peptides. Solutes: 1 = LTFGWCYKL; 2 PHFMPTNLG; 3 = TPHFAPTNL; 4 = TPHAMPTNL; 5 = YPHFMPANL; A: Buffer is 50 mM phosphate at pH = 2.5; B: Buffer is 50 mM phosphate + 50 mM Zn^{++} at pH = 2.5; C: Buffer is 25 mM Tris + 50 mM DTAB at pH = 7.0.

the buffer, the separation improved slightly (Figure 6B). All sample components were, however, separated at pH 7.0 with Tris buffer containing DTAB (Figure 6C). Although the use of DTAB-modified buffer did not improve the separation of dipeptide sequence isomers, the use of DTAB for this particular set of peptides resulted in a significant improvement in separation.

Metal ions such as Zn^{++} are known to coordinate with oxygen, nitrogen and sulfur atoms. The interaction of metal ions with peptides and proteins has been exploited for analytical purposes in chromatography (27). It was also shown that the addition of Zn^{++} to the buffer affected the migration behavior of peptides containing histidine (21). In this work, we observed that the addition of Zn^{++}

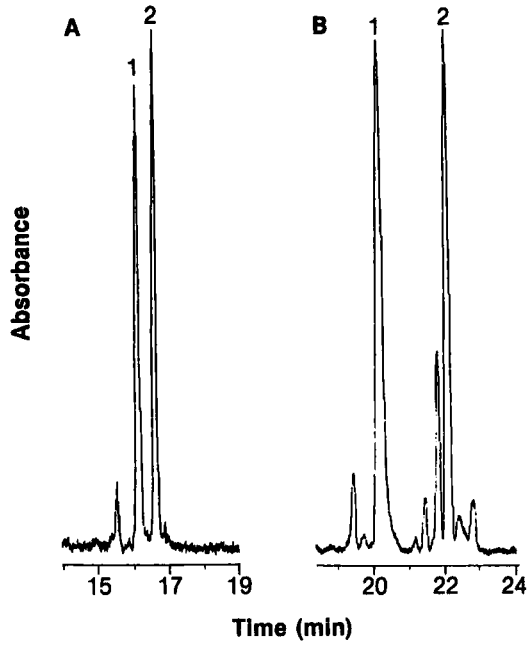

FIGURE 7. Separation of 7-residue peptides at pH = 2.5. A: Buffer is 50 mM phosphate; B: Buffer is 50 mM phosphate + 50 mM Zn^{++}; Solute: 1 = YPHFMPT; 2 = HFMPTNL; Other experimental parameters: As in Figure 3.

to the working buffer at low pH affected the migration time and improved the peak shape of peptides in general, not just peptides containing histidine. Figure 7 shows the separation of 7-residue peptides with and without the addition of Zn^{++}. The separation of the two peptides (each containing a histidine residue) shown in Figure 7A is greatly enhanced when the buffer is supplemented with Zn^{++} (Figure 7B). Several unidentified impurity peaks not separated in Figure 7A are observed in Figure 7B.

CONCLUSION

The results of this study clearly demonstrate that the migration velocity of peptide solutes is directly proportional to the charge-to-size parameter $(q/MW)^{2/3}$. For peptides that are different in charge, the best separations in the shortest analysis time are obtained at low pH values, especially when a large metal ion such as zinc is added to the buffer. For peptides with no appreciable difference in charge and size, the best separations are obtained with micellar-modified buffers. The use of micellar-modified buffers may or may not result in peptide separation, depending on the amino acid composition and hydrophobicity of the peptide solutes and on the type and concentration of the micellar additive.

ACKNOWLEDGEMENTS

Research sponsored by the National Cancer Institute, DHHS, under Contract No. NO1-CO-74102 with PRI/DynCorp. The content of this publication do not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government.

REFERENCES

1. McDonald, J.K., Callahan, P.X., and Ellis, S., Methods in Enzymology, 25, 272, 1972.

2. Callahan, P.X., McDonald, J.K., and Ellis, S., Methods in Enzymology, 25, 282, 1972.

3. Lin, S.-N., Smith, L.A., and Capriolo, R.M., J. Chromatogr., 197, 31, 1980.

4. Dizdaroglu, M. and Simic, M.G., J. Chromatogr., 195, 119, 1980.

5. Welling, G.W., Slobsema, K., and Welling-Wester, S., J. Chromatogr., 359, 307, 1986.

6. Ridlon, C.D. and Issaq, H.J., J. Liq. Chromatogr., 9, 3377, 1986.

7. Kikta, E.J. and Grushka, E., J. Chromatogr., 135, 367, 1977.

8. Fong, G. W.-K. and Grushka, E., J. Chromatogr., 142, 299, 1977.

9. Lundanes, E. and Greibrokk, T., J. Chromatogr., 149, 623, 1977.

10. Molnar, I. and Horvath, C., J. Chromatogr., 142, 623, 1977.

11. Dizdaroglu, M., J. Chromatogr., 334, 49, 1985.

12. Alpert, A.J., J. Chromatogr., 359, 85, 1986.

13. Zou, H.F., Zhang, Y.K., Dong, L.F., and Lu, P.C., Chromatographia, 31, 27, 1991.

14. Jorgenson, J.N. and Lukacs, K.D., Science, 222, 266, 1983.

15. Laver, H.H. and McManigill, D., Anal. Chem., 58, 166, 1986.

16. Grossman, P.D., Wilson, K.J., Petrie, G., and Laver, H.H., Anal. Biochem., 173, 265, 1988.

17. Grossman, P.D., Colburn, J.C., and Laver, H.H., Anal. Biochem., 179, 28, 1989.

18. Aebersold, R. and Morrison, H.D., J. Chromatogr., 516, 79, 1990.

19. Towns, J.K. and Regnier, F.E., Anal. Chem., 63, 1126, 1991.

20. Liu, J., Cobb, K.A., and Novotny, M., J. Chromatogr., 519, 189, 1990.

21. Mosher, R.A., Electrophoresis, 11, 765, 1990.

22. Wieme, R.J., In Chromatography, A Laboratory Handbook of Chromatographic and Electrophoretic Methods; E. Heftman, Ed., 3rd Ed., Van Nostrand Reinhold, New York, Chapter 10, 1975.

23. Grossman, P.D. and Soane, D.S., Anal. Chem., 62, 1592, 1990.

24. Rickard, E.C., Strohl, M.M., and Nielsen, R.G., Anal. Biochem., 197, 197, 1991.

25. Offord, R.E., Nature, 211, 591, 1966.

26. Long, C., Ed., Biochemist's Handbook, Van Nostrand, Princeton, New Jersey, 1961.

27. Porath, J., J. Chromatogr., 443, 3, 1988.