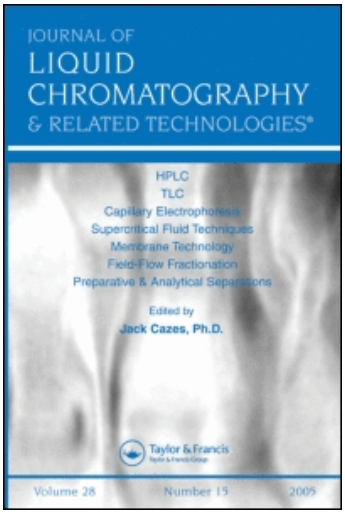


Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

Linear Multidimensional Liquid Chromatography in Protein Separation

Steven A. Berkowitz[a]
[a] J. T. Baker Chemical Company, Phillipsburg, New Jersey

To cite this Article Berkowitz, Steven A.(1987) 'Linear Multidimensional Liquid Chromatography in Protein Separation', Journal of Liquid Chromatography & Related Technologies, 10: 12, 2771 — 2787
To link to this Article: DOI: 10.1080/01483918708066825
URL: http://dx.doi.org/10.1080/01483918708066825

JOURNAL OF LIQUID CHROMATOGRAPHY, 10(12), 2771-2787 (1987)

LINEAR MULTIDIMENSIONAL LIQUID CHROMATOGRAPHY IN PROTEIN SEPARATION

Steven A. Berkowitz
J. T. Baker Chemical Company
222 Red School Lane
Phillipsburg, New Jersey 08865

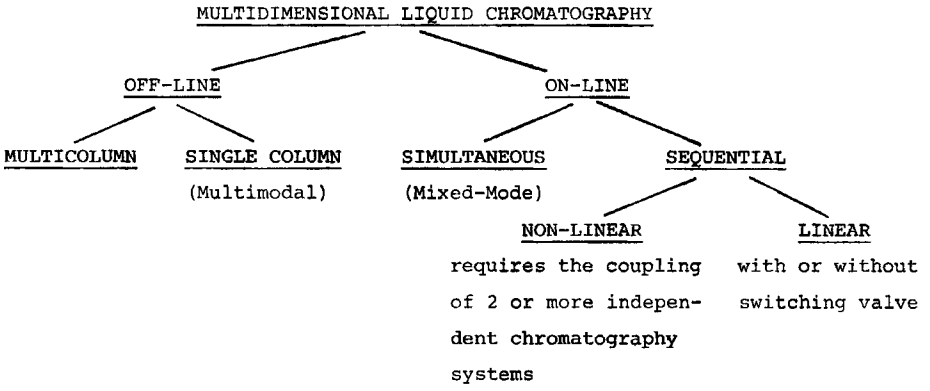
ABSTRACT

The objective of this article is to define, discuss and illustrate the concept of on-line multidimensional liquid chromatography (MDLC) in protein separation. In particular the emphasis of this paper is centered on a special form of on-line MDLC that will be referred to as linear MDLC. Examples of this technique, which involves the coupling of two or more chromatographic columns each employing a different separation mechanism, in both the analytical and preparative mode, are given in order to demonstrate its utility.

INTRODUCTION

The concept of multidimensional liquid chromatography (MDLC) suggests a number of approaches where the application of more than one physical or chemical separation (or retention) mechanism (referred to as dimension or mode) is used for the purpose of purifying a particular compound. Such techniques, which yield high resolution, are required when one attempts to isolate a particular compound from a

matrix containing many components (1). Basically these approaches can be organized into the following scheme shown below:



In protein purification it frequently occurs that the protein of interest must be separated from hundreds or thousands of other proteins, as well as other complex classes of compounds found in biological samples such as nucleic acids, lipids and carbohydrates. Since it rarely happens that one chromatographic mode is capable of removing all these contaminants a number of different chromatographic modes are required to provide the overall high selectivity needed to obtain the desired protein in a highly purified form. Classically these chromatographic steps are conducted OFF-LINE as separate and discrete steps (2). The time required to conduct these multiple chromatographic runs, especially when using conventional soft gel technology (which have significant compressibility problems and hence limitations on the speed in which samples can be separated), is very long. In addition a number of manual manipulations of the sample such as sample injection, the fractionation of the effluent from each column, the pooling of appropriate fractions, sample preparation if necessary of the pooled fractions (e.g. dialysis or sample dilution) for the next chromatographic run and the setup and equilibration of each chromatographic column further increases the purification time. This makes the purification process very tedious and exposes the protein to bacterial and enzymatic contamination which can destroy or alter its chemical and biological properties. When this is coupled with the

poor stability of many proteins the end result is a protein recovery that is low both in terms of mass and biological activity.

Attempts to solve these problems have led investigators to develop ways in which multiple chromatographic separation mechanisms can be applied to a protein mixture moving through a closed system to separate it into its various components. Such chromatographic separations, which would be conducted ON-LINE, can be accomplished in two ways: SIMULTANEOUSLY or SEQUENTIALLY. In the discussion to follow the latter approach leading to LINEAR MDLC will be the main focus of this article. Several specific examples of this technique in separating proteins will be given to illustrate its analytical and preparative capabilities. These examples will employ the coupling of two different separation mechanisms, however, extension of this coupling process to three and possibly more chromatographic dimensions is possible.

EXPERIMENTAL

Materials

Bovine brain acetone powder, mouse immunoglobulin G, horse cytochrome C (type VI), carbonic anhydrase from bovine erythrocytes, ovalbumin, whale myoglobin, egg white lysozyme, alpha-chymotrypsinogen, beta-Lactoglobulin A and horse spleen ferritin were obtained from Sigma Chemical Company (St. Louis, Missouri). Ammonium sulfate (biochemical grade), sodium acetate (HPLC grade), sodium dodecyl sulfate, SDS, (biochemical grade), BAKERBOND^{TM} PREPSCALE HI-Propyl (a 40 micron, wide-pore (275Å), silica-based hydrophobic interaction chromatography, HIC, medium) and prepacked high performance BAKERBOND HI-Propyl and polyethylenemine, PEI, (a silica-based weak anion-exchanger, WAX) 5 micron wide-pore (300Å) HPHIC and HPWAX columns respectively were obtained from J. T. Baker Chemical Company (Phillipsburg, New Jersey). A Bio-Sil TSK-250 (a silica-based size-exclusion chromatography, SEC, column) prepacked 10 micron HPSEC column was obtained from Bio-Rad Laboratories (Richmond, CA).

Chromatography

All samples were clarified by centrifugation before conducting chromatography at ambient temperature on a Model 344 chromatography

system (Beckman Instruments, Berkeley, California) consisting of two Model 110A pumps, a Model 164 UV-VIS variable wavelength detector and a Model 210A injector that was equipped with either a 0.5 or 8.0 mL sample loop. The system also employed a Rheodyne Model 7335 zero-dead volume in-line filter (located between the injector and column) containing a 0.5-micron filter. The presence of this in-line filter was critical in preventing columns from being clogged with particulate material when large volumes of apparently clear samples were injected.

RESULTS AND DISCUSSION

Simultaneous Multidimensional Liquid Chromatography (Mixed-Mode)

Although examples exist in the literature where chromatography media have been specifically designed with the idea of having more than one separation mechanism operating simultaneously (3-5) the unintentional synthesis of this type of media has been far more common. This occurs because most chromatography media contain several different types of chemical groups on the surface which can interact via different mechanisms with the appropriate sample. Hence it is not uncommon to find a given chromatography media which actually achieves a particular separation as a result of contributions from two or more retention mechanisms (6,7). This is especially true for protein chromatography since many different chemical groups exist on the protein surfaces allowing many types of interaction to take place. Hence, in some cases SIMULTANEOUS or MIXED-MODE chromatography is conducted without the user or even the manufacturer being aware. In other cases the functioning of more than one mechanism is known. In these situations an appropriate mobile phase may permit certain separation mechanisms to be eliminated or reduced allowing primarily single mode chromatography to be conducted (6-9). Such columns have been referred to as MULTIMODAL columns (7).

Although mixed-mode chromatography media can offer novel chromatographic selectivity (10) or reduce the number of columns required to purify a given protein (7) there lurks the possibility that such mixed-mode chromatography can lead to poor resolution or, even worse, irreversible binding of proteins to the column. This latter situation is illustrated in Figure 1 where calmodulin, CaM, (a

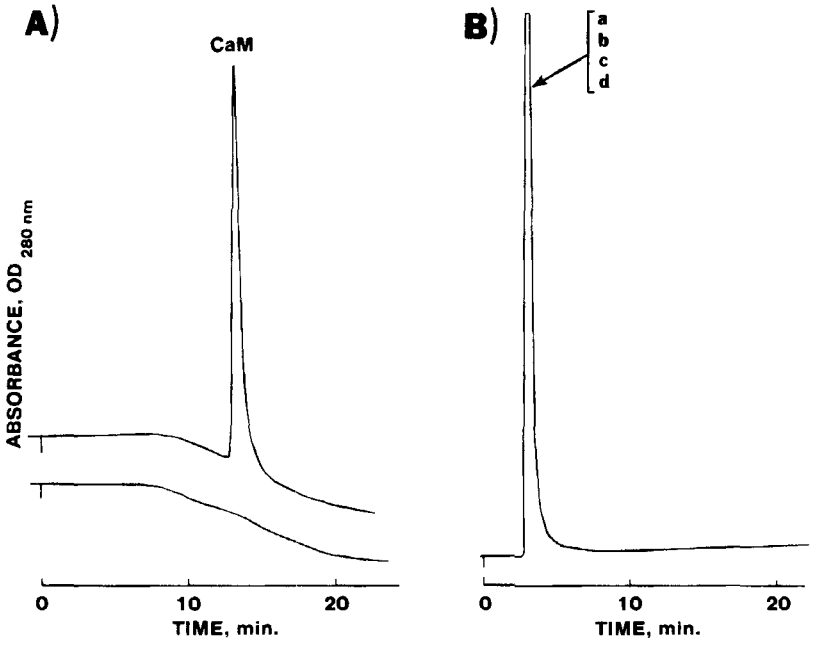


Figure 1: The mix-mode behavior of two commercially available HPWAX chromatography media. (A) The chromatography of calmodulin, CaM, on a 7.75 x 100 mm BAKERBOND PEI (upper chromatogram) and a second 4.1 x 250 mm commerically available (lower chromatogram) HPWAX column. Experimental conditions used; mobile phase: buffer A = 25 mM Tris, pH 7.0 and buffer B = 2.0 M sodium acetate, pH 7.0; linear gradient: 0% B to 100% B in 15 min. B&C) The resulting chromatography of four protein standards (a = Cytochrome C, b = Myogloblin, C = Lysozyme and d = alpha-chymotrypsinogen) using high salt on BAKERBOND PEI (B) and the other commercially available HPWAX column used in part "A" (C). Experimental conditions used; mobile phase: buffer A = 2.0 M ammonium sulfate + 25 mM potassium phosphate, pH 7.0 and buffer B = 25 mM potassium phosphate, pH 7.0; linear gradient: 0% B to 100% B in 15 min. (D) Chromatography of the same protein standards on a 4.6 x 250 mm BAKERBOND HI-Propyl HIC column. Experimental conditions used were the same as indicated in parts "B" and "C".

(continued)

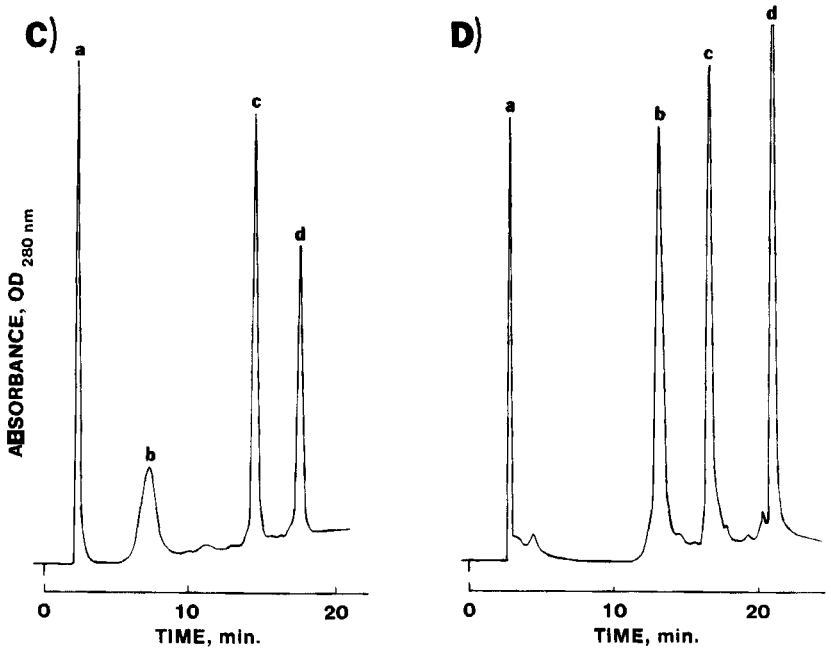


Figure 1 (continued)

calcium binding protein) was chromatographed on two commercially available high performance weak anion-exchange columns. On using the same elution conditions for both columns it is observed that in one case this protein is permanently bound to the column packing, see lower chromatogram in Figure 1A. The failure to release calmodulin from this column is due to the strong hydrophobic character of the ion-exchange media which is amplified by the presence of high salt, see Figures 1B and C. This conclusion is further supported by the similarity in the chromatography obtained with this column and a specifically designed column for conducting hydrophobic interaction chromatography, see Figures 1C and D. Since the strength of hydrophobic interactions are increased while that of ionic interactions are decreased by the addition of salt (11) at any given time during the elution gradient ionic or hydrophobic (or non-polar) or both binding mechanisms can be operating to hold a given protein, in this case

calmodulin, permanently fixed to the surface of the chromatography media. In some cases this binding could lead to the slow accumlation of material on the column which can eventually alter the chromatography properties of the column. Hence, limitations can arise in the use of mixed-mode chromatography media.

Sequential Multidimensional Liquid Chromatography (LINEAR and NON-LINEAR Forms)

In on-line Sequential MDLC, columns normally used in each discrete chromatographic step are physically coupled. Coupling can be accomplished with different levels of complexity, see Figure 2. The simplest and most economical consists of a column bank containing a LINEAR arrangement of columns having a single continuous fluid path which remains unchanged during the entire chromatography process, see Figure 2A. In this arrangement the various retention mechanisms are physically separated allowing each to interact with the sample in a sequential manner as the sample passes through each column. Chromatography conducted in such a system will be referred to as LINEAR MDLC. In LINEAR MDLC switching valves may be introduced into the system to mediate what proportion of effluent from one column is applied to the next column or to control the choice of the next column or to by-pass certain columns during the actual chromatography (9, 12, 13), see Figure 2B.

The most sophisticated and expensive form of on-line Sequential MDLC (which potentially offers the highest resolving power) is achieved by the physical coupling of two or more independent chromatography systems (14-18), see Figure 2C. In this case the effluent from the first column can be either transferred directly onto another column in a second chromatography unit, where the flow of the mobile phase is temporarily stopped during the actual transfer, or into a sample loop where it can be injected on to the next column at a later time. On-line SEQUENTIAL MDLC conducted with more than one independent fluid path, each controlled by separate solvent delivery systems which are connected at various intervals during the chromatography process will be referred to as NON-LINEAR MDLC.

In the past chromatography conducted, usually on small organic molecules, in a manner either identical or similar to that outlined

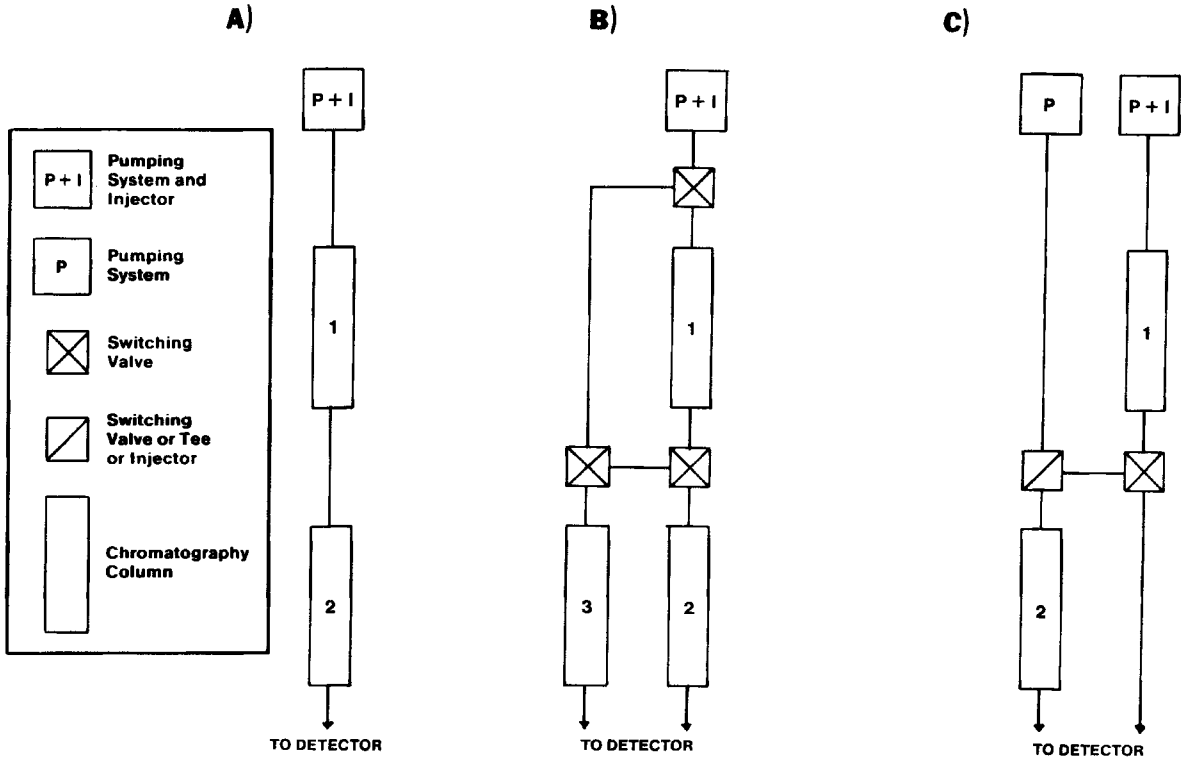


Figure 2: Hardware configurations used for (A&B) LINEAR MDLC and (C) NON-LINEAR MDLC.

above for either LINEAR or NON-LINEAR MDLC, has been referred to by the following list of names: tandem, multidimensional, multicolumn, column programming, coupled, switching, orthogonal and mode sequencing chromatography.

Requirements For Conducting LINEAR MDLC

Several requirements must be met in order to conduct LINEAR MDLC effectively. These determine the types of chromatography columns that can effectively be coupled. One requirement is that all mobile phases be chemically compatible with all chromatography media used. This could prevent, for example, the combining of resin and silica-based chromatography columns in certain situations. Another requirement, which can be eliminated by using a complex arrangement of switching values and sample holding loop (19), is that the physical properties of all media be compatible. If one attempts to combine a column packed with a rigid high performance media with a column packed with a soft or semi-hard chromatography media the chromatography can only proceed if the latter chromatography media is positioned at the end of the column bank. This is due to the pressure limitations of the soft and semi-hard gels. Since relatively high pressures are required to move the sample and mobile phase through the high performance column the positioning of a column packed with soft or semi-hard before the HPLC column would cause the soft or semi-hard media to collapse and clog the fluid path. An additional factor, which in some cases can be very important is the order in which columns are arranged in the column bank e.g. the placement of a SEC column in front of an ion-exchange column would be inferior to the reverse arrangement (see first example below). Finally, the mobile phase must not interfere with the chromatography needed to purify the protein of interest as it moves from one column to the next in the column bank. This can be illustrated by the calmodulin example described below. Here the mobile phase conditions required to elute calmodulin from the first column must allow it to bind and concentrate on the second column. If this is not achieved, the MDLC process will be destroyed. In situations where the protein of interest binds weakly the size of the sample that can be transferred may seriously be limited since the chromatographic resolution achieved in the next dimension will be

significantly degraded if the transferred sample is spread over a large area of the second column.

Examples of LINEAR MDLC

An analytical example of LINEAR MDLC is shown in Figure 3. Here a short HPWAX HPLC column was coupled to a HPSEC column as indicated at the top of Figure 3A. Chromatography on this column bank allowed all the proteins in a defined mixture to be resolved in a single chromatographic run. Initial chromatography was conducted with a mobile phase that allowed only the acidic proteins to bind to the weak anion-exchanger. The non-bound proteins passed onto the HPSEC column where they were separated on the basis of their shape and mass. Following the elution of these proteins from the HPSEC column, a mobile phase gradient was initiated to release the proteins bound to the weak anion-exchanger. As these proteins eluted they passed directly onto the HPSEC column where they were further separated on the basis of their shape and mass properties. Figures 3B and C show the chromatography results obtained when each chromatographic mode is used separately to fractionate the initial protein mixture. Clearly both modes are required to resolve all of these proteins. In this case, if each chromatographic technique were used separately, several fractions would need to be isolated and run separately in the second chromatographic dimension. The ability to physically couple the two modes of chromatography permitted the separation of all proteins in one chromatographic run.

This one-step method offers the following advantages: (A) the total time to achieve the desired separation is reduced, (B) manual manipulations and exposure of this sample to possible bacterial or enzymatic contamination (which could alter or destroy the biological activity of these proteins) is minimized, and (C) as shown in Figure 3A the proteins that eluted from the weak anion-exchanger were effectively desalted during the SEC chromatographic separation (see conductivity trace in Figure 3). This last point eliminates additional steps necessary to remove excess salts that may be undesirable in the final protein solution.

A preparative example of LINEAR MDLC is shown in Figure 4. Here several mg of highly purified calmodulin were rapidly obtained from a

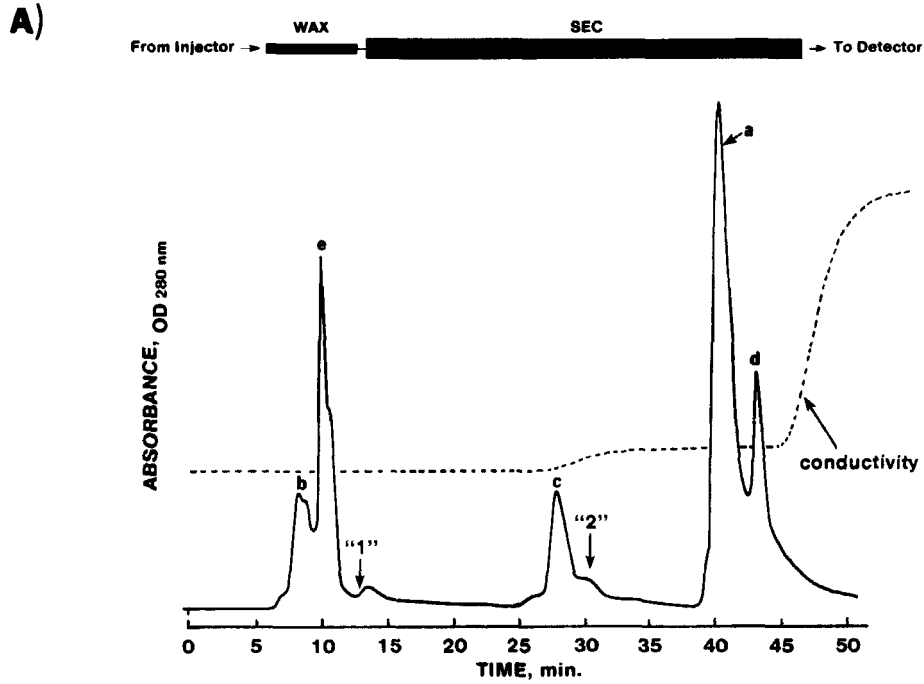


Figure 3: Analytical LINEAR MDLC. (A) LINEAR MDLC of several protein standards (a = Ferritin, b = Immunoglobulin G, c = Ovalbumin, d = beta-Lactoglobulin A and e = Carbonic Anhydrase) using a column bank (shown at the top) containing a short 4.6 x 50 mm BAKERBOND HPWAX and 7.5 x 300 mm HPSEC columns. Experimental conditions used; mobile phase: buffer A = 25 mM potassium phosphate, pH 7.0 and buffer B = 2.0 M ammonium sulfate + 25 mM potassium phosphate, pH 7.0; step gradient: "1" 0% B to 1% B and "2" 1% B to 25% B. (B) The chromatography of the same protein standards using only the HPSEC column. Experimental conditions used; mobile phase: 25 mM potassium phosphate, pH 7.0. (C) The chromatography of the same protein standards using only the HPWAX column. Experimental conditions used; mobile phase: buffer A = 25 mM potassium phosphate and buffer B = 2.0 M ammonium sulfate + 25 mM potassium phosphate, pH 7.0; linear gradient: 0% B to 25% B in 15 min.

(continued)

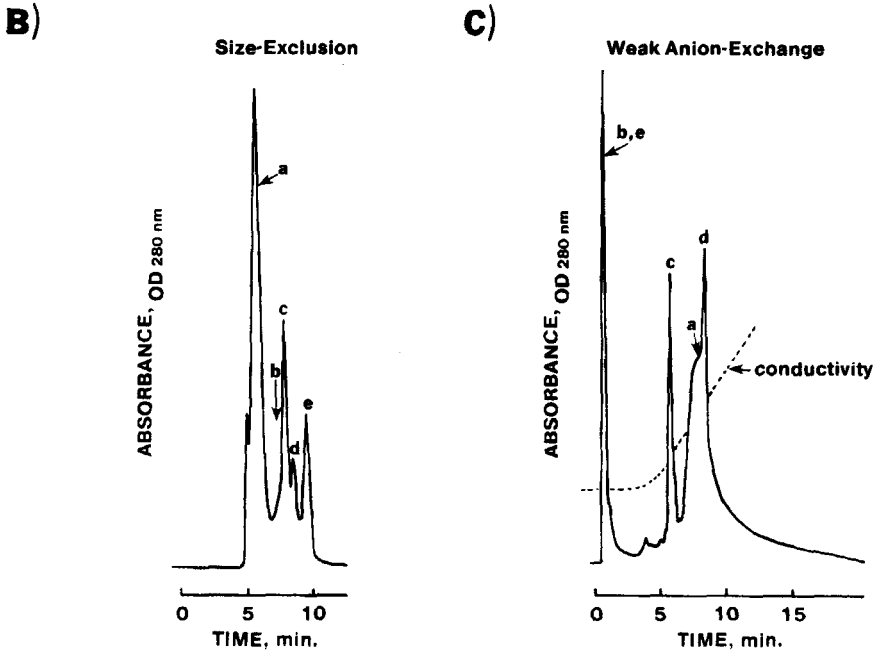


Figure 3 (continued)

crude brain extract in a single chromatographic run. In this case a column packed with 40 micron HI-Propyl HIC media was coupled to a small HPWAX column as indicated in Figure 4. 40 mL of crude brain extract (containing over 0.3 grams of protein) was loaded onto this column bank. Under the starting conditions used calmodulin bound to the HIC column. Material which did not bind to the HIC column also did not bind to the HPWAX column due to the initially high ionic strength used. Calmodulin was then eluted from the HIC column and transferred to the WAX column by conducting a high to low salt gradient. This transfer was made possible by the low ionic strength at which calmodulin elutes from the HIC column, low enough for it to bind to the weak anion-exchanger. In the process of conducting this transfer additional purification of calmodulin was achieved as indicated by the amount of UV absorbing material which is seen as flow

through (peak "B" in Figure 4). Calmodulin was finally released from the WAX column by conducting an increasing salt gradient. This resulted in further purification using the high resolution capability of the small HPWAX column. The isolated calmodulin peak was estimated by SDS electrophoresis to be better than 95% pure. As indicated in the previous example minimal manual sample handling is required and rapid purification was achieved. In this case purification was completed in a few hours instead of days required with conventional purification procedures for this protein (20,21).

CONCLUSION

As a subclass of on-line MDLC techniques LINEAR MDLC is the simplest and least expensive to implement. It offers a number of attractive advantages for improving protein purification which can result in faster purification with higher yields and with reduced expenditures of time and economic resources. In addition, protein chromatographic separations using closed systems permit easier and more effective control in maintaining a sterile environment for conducting protein purifications. Although examples exist in the literature where this type of chromatography has been performed on proteins using either conventional (22-24) or high performance (23-27) media the ability to couple both types would be very useful. Indeed, the direct coupling of a column packed with large and rigid chromatography particles to an HPLC column, as illustrated in this article, represents a general approach for combining the best attributes of both the new conventional-like low pressure and high performance biochromatographic materials now being commercialized to provide rapid, high resolving and cost effective protein purification (28). Such features should be of particular interest to those who are involved in preparative scale protein purifications.

Before concluding two important points need to be emphasized. The first is that the types of chromatographic mechanisms which can be combined to perform LINEAR MDLC are by no means limited to those specifically illustrated in this paper. The second concerns the general applicability of this technique to a wide range of proteins.

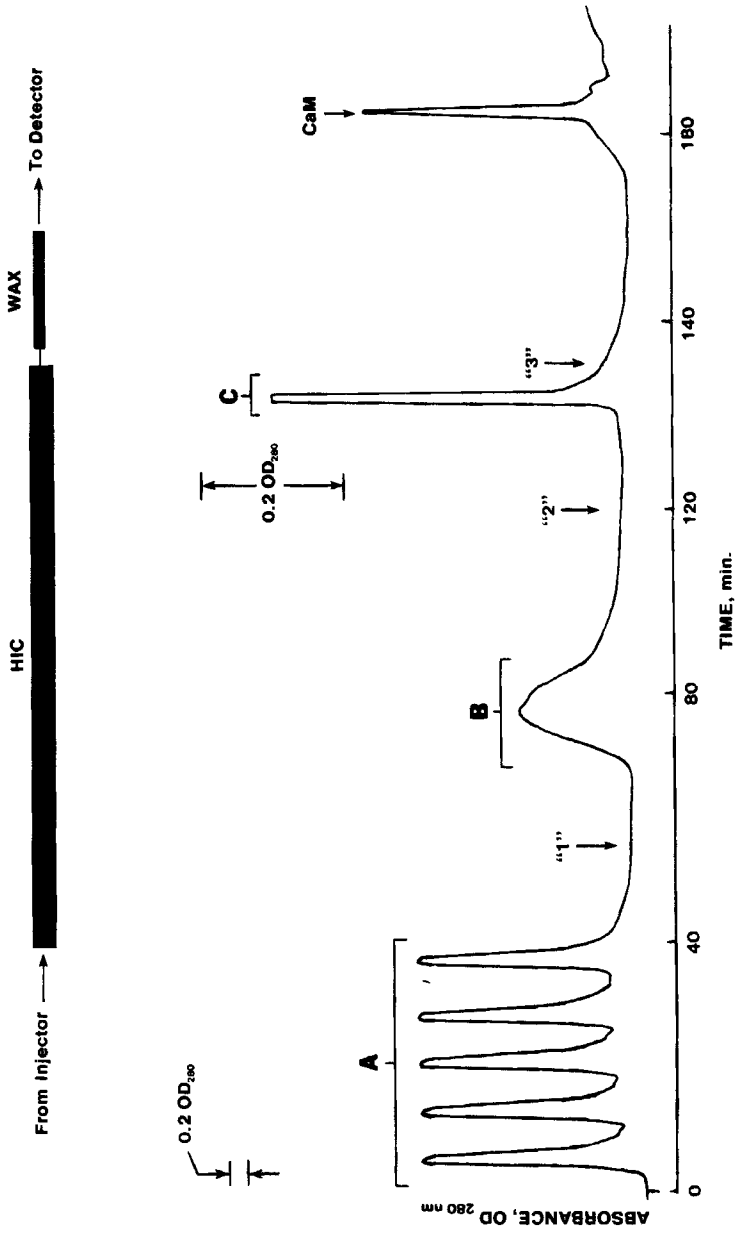


Figure 4: Preparative scale purification of calmodulin from crude bovine brain extract using LINEAR MDLC. Column bank used consisted of a 10 x 250 mm column packed with 40 micron HIC material coupled, as indicated at the top of this figure, to a small 4.6 x 50 mm BAKERBOND HPWAX column. In this example 40 mL of a crude brain extract in high salt was applied to the column bank. Peaks in region "A" are the flow through material which did not bind to the column bank during multiple sample injection. At point "1" a linear 15 min. gradient going from 0% B to 75% B was started using the following mobile phase: buffer A = 2.0 M sodium acetate, pH 7.0 and buffer B = 25 mM Tris, pH 7.0. Peak "B" is the eluted material from the HIC column which did not bind to the HPWAX column. At point "2" buffer A was changed to 2.0M sodium acetate, pH 5.0 which caused some of the material bound to the HPWAX column to be released, peak C. At point "3" a linear 15 min. gradient going from 75% B to 0% B was started in order to release calmodulin (CaM).

This latter point will depend on a number of factors including the general properties of the protein being sought, the range of chromatographic separation mechanisms which can be employed and the general cleverness of the chromatographer to manipulate experimental conditions to permit this technique to be used. Nevertheless, given the advantages of LINEAR MDLC further investigations into the use of this technique are clearly warranted.

ACKNOWLEDGMENTS

The author would like to thank Dr. L.J. Crane for her encouragement during the course of this work and for her helpful comments in preparing this manuscript.

REFERENCES

1. T. V. Raglione, N. Sagliano, Jr., T. R. Floyd and R. A. Hartwick, LC.GC, Liq. and Gas Chromatogr. Mag., 4 (4), 328. (1986).
2. R. K. Scopes, Protein Purification (Springer-Verlag, New York, 1982).
3. J. Porath and N. Farnstadt, J. Chromatogr., 51, 479, (1970).
4. R. J. Yon and R. J. Simmond, Biochem. J., 151, 281, (1975).
5. L. W. Yu, T. R. Floyd and R. A. Hartwick, J. Chromatogr. Sci., 24 (5), 177, (1986).
6. W. Kopaciewicz and F. E. Regnier, Anal. Biochem., 126, 8, (1982).
7. L. A. Kennedy, W. Kopaciewicz and F. E. Regnier, J. Chromatogr., 359, 73, (1986).
8. C. Horvath and Z. El Rassi, Chromatogr. Forum, 1 (3), 49, (1986).
9. D. H. Freeman, Anal. Chem., 53, 2, (1981).
10. D. R. Nau, Biochromatogr., 1 (2), 35, (1986).
11. J. L. Fausnaugh, L. A. Kennedy and F. E. Regnier, J. Chromatogr., 317, 141, (1984).
12. J. F. K. Huber, R. Van Der Linden, E. Echer and M. Oreans, J. Chromatogr., 83, 267, (1973).
13. J. A. Apffel, T. V. Alfredson and R. E. Majors, J. Chromatogr. 206, 43, (1981).

14. E. L. Johnson, R. Gloor and R. E. Majors, J. Chromatogr., 149, 571, (1978).
15. F. Erni and R. W. Frei, J. Chromatogr., 149, 561, (1978).
16. N. Takahashi, N. Ishioka, Y. Takahashi, F. W. Putnam, J. Chromatogr., 326, 407, (1985).
17. H. Lindblom and L. G. Fagerstam, LC, Liq. Chromatogr. HPLC Mag., 3 (4), 360, (1985).
18. S. T. Balke, Polymer News, 9, 6, (1983).
19. A. Heubner, O. Belovsky, H.-J. Grill and K. Pollow, J. Steroid Biochem., 24 (1), 207, (1986).
20. C.B. Klee and T.C. Vanaman, Adv. Protein Chem., 35, 213, (1982).
21. H. Charbonneau, R. Hice, R.C. Hart and M.J. Cormier, in Methods in Enzymology, (H.R. Means and B.W. O'Malley, eds), Vol. 102, Part G, p. 17, Academic Press, New York, 1983.
22. E. Bollin, Jr., and E. Sulkowski, J. Gen. Virol., 52, 277-33 (1981).
23. S. A. Berkowitz and J. Wolff, J. Biol. Chem., 256, 11216-23 (1981).
24. E. Rivas, N. Pasdeloys and M. LeMaire, Anal. Biochem., 123, 194-200 (1982).
25. D. N. Buchanan and J. G. Thoene, Anal. Biochem., 124, 108-16 (1982).
26. W. Kopaciewicz and F. E. Regnier, Anal. Biochem., 129, 472-82 (1983).
27. Z. El Rassi and C. Horvath, J. Chromatogr., 359, 255-64 (1986).
28. S. A. Berkowitz, M. P. Henry, D. R. Nau and L. J. Crane, American Laboratory, 19 (4), 33-42 (1987).