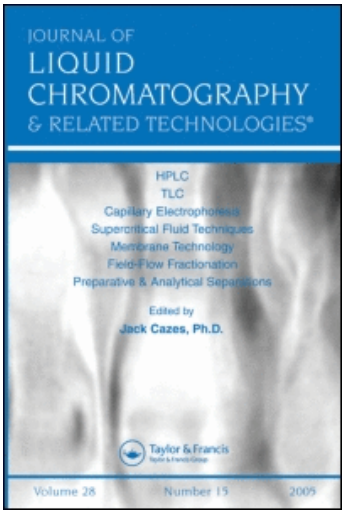

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Counting Integral Numbers of γ-Carboxyglutamic Acids Per Peptide Chain Using Capillary Electrophoresis

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COUNTING INTEGRAL NUMBERS OF γ-CARBOXYGLUTAMIC ACIDS PER PEPTIDE CHAIN USING CAPILLARY ELECTROPHORESIS

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ABSTRACT

A method for counting the number of γ-carboxyglutamic acid (Gla) residues in peptides is presented. The product mixture generated by partial decarboxylation of a peptide containing multiple Gla residues is separated by capillary electrophoresis. A mixture derived from a peptide containing n Gla residues produces an electropherogram characterized by $n + 1$ peak groups. For a given peptide, a linear relationship ($r^2 > 0.99$) is observed between the average electrophoretic mobility of each peak group and the representative number of Gla residues contained by that group. Resolution of peptide isomers is also obtained, permitting studies of the extent and sequence-specificity of decarboxylation.

INTRODUCTION

The increasing use of capillary electrophoresis (CE) in the study of peptides and proteins is driven largely by the small minimum sample size, high separation efficiency, and the charge-to-size-based separation selectivity of the technique.[1,2] CE studies aimed at elucidating the nature of the post-translational modifications to which many proteins are subject are of special interest because the structure, origin, and location of such modifications cannot be derived from genetic sequence data. These modifications are known, frequently, to affect only a subset of the protein molecules. Furthermore, the modifications may differ in a more or less subtle way among subcomponents of an otherwise homogeneous protein sample,[3] requiring the development of characterization methods that are selective to the differing structural features. An important example of a post-translational modification is the γ-carboxyglutamic acid residue [Gla; side chain $-CH_2CH(COOH)_2$], 9 to 12 of which are located in the N-terminal "Gla domain" portion of several blood coagulation proteins including prothrombin, factor IX, and others.[4,5] In 1974, Stenflo et al. employed paper electrophoresis in the earliest characterization of Gla. They identified Gla in a tetrapeptide derived from prothrombin that exhibited an electrophoretic mobility that "was too high to be explained entirely by its amino acid composition."[6] It has since been established that a vitamin K-dependent carboxylase, via a mechanism which is presently the subject of intense study, directs the *in situ* production of Gla from Glu residues at the appropriate positions in certain protein sequences.[7,8]

The characterization of Gla-containing proteins and peptides is essential to virtually any studies that involve them. Because the Gla sidechain is acid labile, however, the presence of Gla cannot be established using many routine tools, including N-terminal Edman degradation sequencing and amino acid analysis. We have developed a CE method for counting the number of Gla residues in a peptide chain. The method relies upon the charge modification resulting from the *in situ* decarboxylation of Gla, which leaves a Glu residue.[9,10] Non-Gla amino acid side chains are unaffected. A purified *n*-Gla-containing peptide is generally subject to partial decarboxylation; that is, the reaction is terminated before quantitative decarboxylation of the peptide occurs. A family of molecules having 0, 1, 2,...*n* Gla residues is produced. At moderate to high pH, Gla contains two negative charges and Glu contains only one negative charge. The product mixture of this partial decarboxylation reaction is readily separated into $n + 1$ groups of species by CE using a pH 10.0 or 12.0 sodium tetraborate run buffer. By counting the number of peak groups in the electropherogram, a determination of the number of Gla residues can be made. Knowledge of the sample concentration and molecular weight is unnecessary.

Creighton[11] was the first to point out the general usefulness of electrophoretic analysis of a partially charge-modified sample when he employed polyacrylamide gel electrophoresis to count the number of cysteine residues in protein.

The counting of integral numbers of Gla residues is facilitated by the existence of a linear dependence between the average electrophoretic mobility of peak groups and the number of Gla residues representative of each group. In many cases the isomers produced by partial decarboxylation of peptides containing two or more Gla residues can be resolved, extending the selectivity of method to include the specific sequential position of Gla residues. This paper describes the use of this method for counting integral numbers of Gla residues in peptides and for investigating the sequence-specificity of the decarboxylation reaction. The opportunity to employ capillary electrophoresis as an investigative probe of the nature of carboxylase-mediated production of Gla from Glu is also suggested.

MATERIALS AND METHODS

Materials

Capillary electrophoresis buffer solutions were prepared from sodium tetraborate and triethylamine supplied by Sigma Chemical Co. (St. Louis, MO); sodium hydroxide pellets (Fisher Scientific, Pittsburgh, PA) were employed for pH adjustment. Deionized water for the preparation of all solutions was obtained from a Hydro (Research Triangle Park, NC) dual-cartridge purification system. 50 mM HCl was prepared by dilution of the concentrated acid obtained from Fisher Scientific. Mesityl oxide was purchased from Aldrich (Milwaukee, WI), and DPCC-treated trypsin and diisopropylfluorophosphate (DIFP) were obtained from Sigma. Gla-containing peptides were prepared using solid phase methods and were purified using reverse phase HPLC. The *N*-fluoromethoxycarbonyl-γ-carboxyglutamic acid (Fmoc-Gla) required in the synthesis of these peptides was prepared via the method of Schuerman et al.[12] Bovine prothrombin fragment 1 was isolated as described elsewhere.[13,14]

Heat-Induced Decarboxylation

The method of Poser and Price[9] was used for decarboxylation of Gla residues. Approximately 10-100 μg of Gla-containing peptide was placed in an

Eppendorf tube, dissolved in about 100 μL of 50 mM HCl, and lyophilized. The dry salt was then heated (110°C) under vacuum for 15 min to several hours to achieve partial decarboxylation, or for 10 h or more for quantitative (complete) decarboxylation. The product mixture was dissolved in a minimal amount of deionized water containing 0.005 to 0.05% mesityl oxide as a neutral marker before analysis by CE. Partial tryptic digestion of bovine prothrombin fragment 1 was accomplished by incubating an aqueous, 2 mg/mL solution of the protein with trypsin in a 20:1 (w/w) ratio at room temperature. Hydrolysis was terminated after 1.2 h by addition of an excess of DIFP.

Capillary Electrophoresis

CE was performed using a home-built system similar to that described by Jorgenson and Lucaks,[15] employing a Spellman high voltage power supply (Plainview, NY). The UV absorbance was monitored with a Linear (Reno, NV) Model UVIS 200 "on-capillary" absorbance detector set at 220 nm to detect all peptides regardless of amino acid composition. Separations were performed at room temperature in a 50 μm i.d., 360 μm o.d., untreated fused silica capillary (Polymicro Technologies, Phoenix, AZ). Unless otherwise noted, capillaries were 100 cm in length (effective length 85 cm). Runs were conducted using an aqueous buffer of 12.5 to 37.5 mM sodium tetraborate (pH 10.0 or 12.0) that occasionally contained 0.22% (v/v) triethylamine. All injections were hydrodynamic and employed a reservoir height differential of 22 cm for 5 to 30 sec followed by a constant-voltage run at 10 to 25 kV. The capillary was flushed with run buffer for 5 min between runs using aspirator suction. Several minutes of flushing with 0.1 M aqueous NaOH was performed daily to regenerate the capillary surface.

Electropherograms were acquired using an Apple Macintosh computer with a Rainin MacIntegrator (Ridgefield, NJ) data collection package. Data was filtered using a 5-point binomial filter[16] available in the Igor Pro software package (WaveMetrics, Lake Oswego, OR). For the relative peak area ratio study shown in Table 2, correction was accomplished by dividing the area of each peak by the corresponding migration time.[17] The smallest peak in each group of isomeric peaks is defined as 1 area unit; the remaining peaks are normalized accordingly.

RESULTS AND DISCUSSION

A series of electropherograms acquired after progressively longer decarboxylation periods of a 1-Gla-containing peptide is shown in Figure 1.

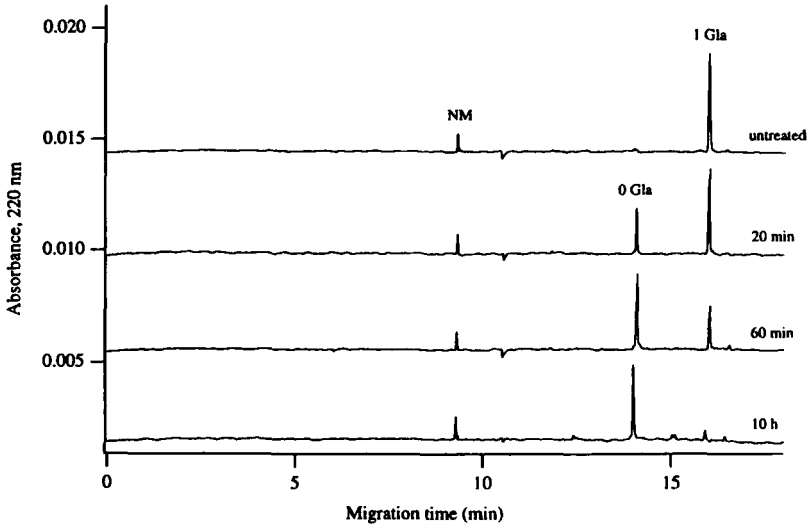

Figure 1. Electropherograms acquired after the indicated periods of decarboxylation of a peptide bearing 1 Gla residue. The peptide sequence before decarboxylation was amino-QTDγFWSKYKD-carboxyl, where γ= Gla. The removal of one carboxyl group effects a pronounced decrease in migration time. Peaks are labeled according to the number of Gla residues the peptides contain. NM indicates the neutral marker. Run buffer was 12.5 mM sodium tetraborate containing 0.22% triethylamine (pH 10.0); run voltage, 25 kV; current, 19 μA; see experimental section for additional details.

Because the high pH CE run buffer favors deprotonation of the carboxyl functionalities of Gla and Glu, the Gla-containing species contains a greater negative charge density, and subsequently exhibits a significantly greater migration time, than that of the Glu-containing product. Partial decarboxylation of a peptide containing two or more Gla residues generates a more complex mixture. The diversity of species in such a mixture results in part from the differing numbers of intact Gla residues contained by the component peptides. Due to the fundamental difference in charge of the side chain of Glu and Gla (-1 *versus* -2, respectively, at ionizing pH), high pH CE effects the separation of such a mixture into distinct groups of peaks according to the number of remaining Gla residues. Partial decarboxylation of a "parent" peptide containing *n* Gla residues will result in an electropherogram having *n* + 1 peak groups. For example, the electropherograms for a 2-Gla-containing peptide (Figure 2) show a primary separation into 3 groups based on the number of Gla residues (0, 1, or 2) remaining in each species.

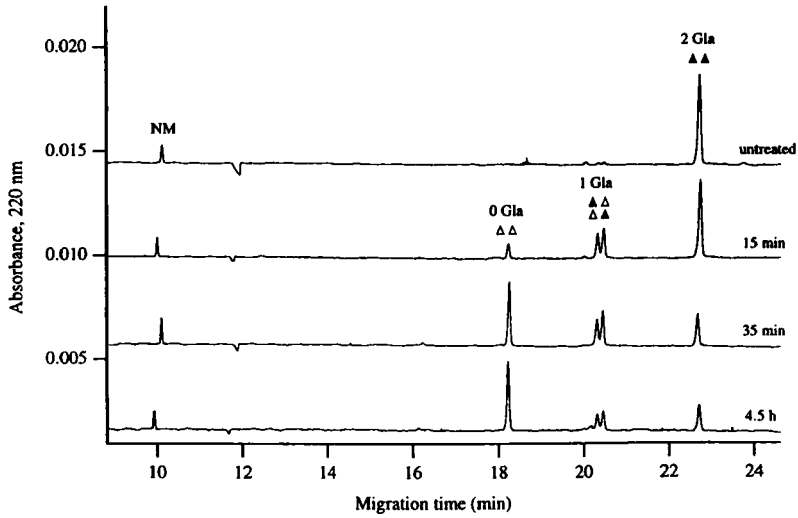

Figure 2. Separations obtained at the indicated times during decarboxylation of a 2-Gla-containing peptide of sequence N-acetyl-DAγQTDγFWSKYKYKD-carboxyl (γ = Gla). The sequence-specific Gla and Glu composition of the peptides is also indicated by icons above the peaks; filled triangles represent Gla and open triangles represent Glu. NM indicates the neutral marker. Note the resolution of the pair of peptide isomers generated by decarboxylation of one of the two Gla residues. Run buffer: 18.8 mM sodium tetraborate containing 0.22% triethylamine (pH 10.0); run voltage, 25 kV; current, 30 μA; the experimental section contains additional information.

As shown by the simple algebraic expressions in Table 1, the maximum number of groups is fixed at $n + 1$ for an n-Gla-containing peptide, but the number of peptide isomers in a given group varies as a function of both the Gla content of the parent peptide and the Gla content of the group. The electropherogram in Figure 2 exemplifies the simplest case: the doublet at about 20.5 min corresponds to the two peptide isomers produced by decarboxylation of one of the two total Gla residues present on the parent peptide. Figure 3 shows the electropherogram of a 4-Gla-containing peptide acquired after a 20-min decarboxylation. The components are separated into five groups, corresponding to 0, 1, 2, 3, and 4 Gla residues remaining per peptide. The number of peaks in each group corresponds to the number of isomers predicted using equations given in Table 1. To determine the quantitative dependence of Gla content on electrophoretic behavior, the average electrophoretic mobility *versus* number of remaining Gla residues was plotted for several partially decarboxylated multi- Gla-containing peptides (Figure 4).

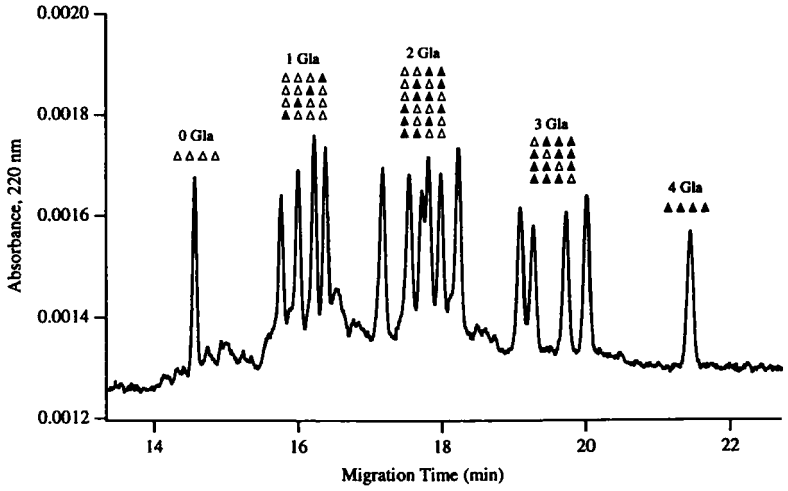

Figure 3. CE separation obtained after partial (1 h) decarboxylation of a peptide containing 4 Gla residues. The electropherogram shows separation into 5 groups (n + 1 = 5 for a 4-Gla-containing peptide). The sample was amino-AQγRIRγLNKPQYγLNRγAC-carboxyl (γ = Gla) before decarboxylation. Peak labels are explained in the caption to Figure 2. All 16 species expected from partial decarboxylation of a peptide containing 4 Gla residues are seen. Run buffer was 15 mM sodium tetraborate (pH 12.0); capillary, 60 cm long (effective length 45 cm); run voltage, 10 kV; current, 22 μA; additional details are presented in the experimental section.

Table 1

Species Produced by Partial Decarboxylation of a Peptide Containing n Gla Residues

Parameter	Algebraic Description
No. of Gla residues in parent peptide	n
Total no. of isomeric groups g	$g = n + 1$
No. of intact Gla's remaining in a given group	k
No. of isomers i in a given group	$i = n!/[k!(n-k)!]$
Total possible no. of species s generated	$s = 2^n$

The variation in the integral number of Gla residues in a partially decarboxylated sample gives rise to a corresponding variation in the charge states of the species. This incremental variation in charge state is in turn responsible for the observed incremental variation in electrophoretic mobility. The strong linear correlation ($r^2 > 0.99$) supports the assignment of decarboxylation as the source of the observed electrophoretic mobility differences. The existence of this consistent mathematical relationship can be exploited in assigning peaks in the electropherogram. For example, the presence of sample impurities may be evidenced by the appearance of one or more peaks that do not fit a linear mobility pattern. For a sufficiently pure sample, the general approach of decarboxylation followed by CE analysis provides a fast, sample-conservative count of the number of Gla residues per molecule of peptide. Such a digital count is of primary interest in the characterization of Gla-containing species including mutant proteins, genetic constructs, and synthetic peptides.

The successful separations of Gla-containing peptide isomers generated by partial decarboxylation suggest multiple opportunities for further study. In the separation shown in Figure 3, for example, the individual peaks of the quartet centered at about 38 min correspond to each of the 3-Gla-containing isomers that is generated during partial decarboxylation of a 4-Gla-containing peptide. CE analysis of synthetic versions of these decarboxylation products could be used to identify the specific isomers, providing insight into the differences in shape, and hence three-dimensional conformation, that apparently permit their successful separation. Furthermore, we believe that interests in the sequence-specificity of both heat-induced[18,19] and chemical[20-22] types of decarboxylation can be investigated using methods outlined in this report. Shown in Table 2 are preliminary data from an investigation of this sort, which suggests that the heat-induced decarboxylation of one particular 4-Gla-containing peptide is not sequence-specific under the conditions employed here. Table 2 provides the relative peak area ratios obtained as described in the Materials and Methods section for the electropherogram shown in Figure 3. The approximate equality of peak areas within each group shows there is no preferential production of any peptide isomer.

In considering the feasibility of separating Gla-containing peptide isomers, it must be noted that the theoretical maximum number of species produced from partial decarboxylation of a complete blood coagulation protein, which may contain as many as 12 Gla residues, is extremely large (e. g., 2^{10} for 10-Gla-containing prothrombin). Furthermore, the analyte/capillary wall adsorption[15] and carbohydrate microheterogeneity[23] phenomena that generally complicate CE analyses of glycoproteins would preclude resolution of partially decarboxylated blood coagulation protein mixtures.

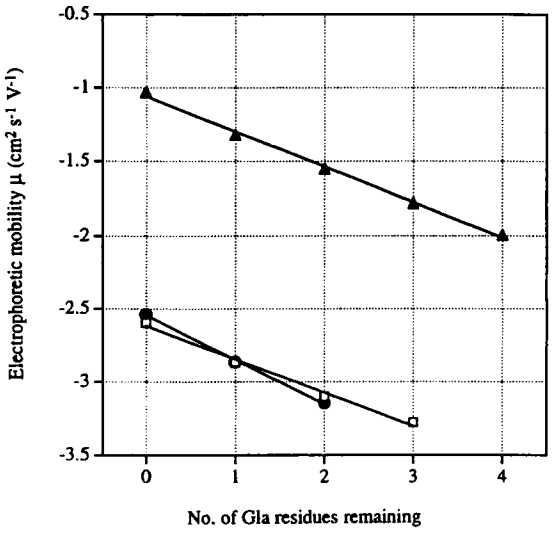

Figure 4. Plots of average electrophoretic mobility *versus* number of remaining Gla residues for three partially decarboxylated peptides that initially contained 2 Gla (circles), 3 Gla (squares), and 4 Gla (triangles) residues. The strong linear correlation ($r^2 > 0.99$ in all cases) facilitates the counting of integral numbers of Gla residues as described in the text.

Table 2

Relative Peak Areas Within Groups of Isomers Produced by Partial Decarboxylation of a Peptide Containing 4 Gla Residues

No. of Gla Residues Remaining	Relative Peak Area Ratio[a]
1	1.0 : 1.3 : 1.5 : 1.2
2	1.6 : 1.2 : 2.0 : 1.0 : 1.3[b]
3	1.2 : 1.0 : 1.1 : 1.2

[a] This data was obtained from the electropherogram shown in Figure 3.
[b] Two isomers in this group are poorly resolved, and are thus treated as a single peak, accounting for the approximately two-fold larger area seen here.

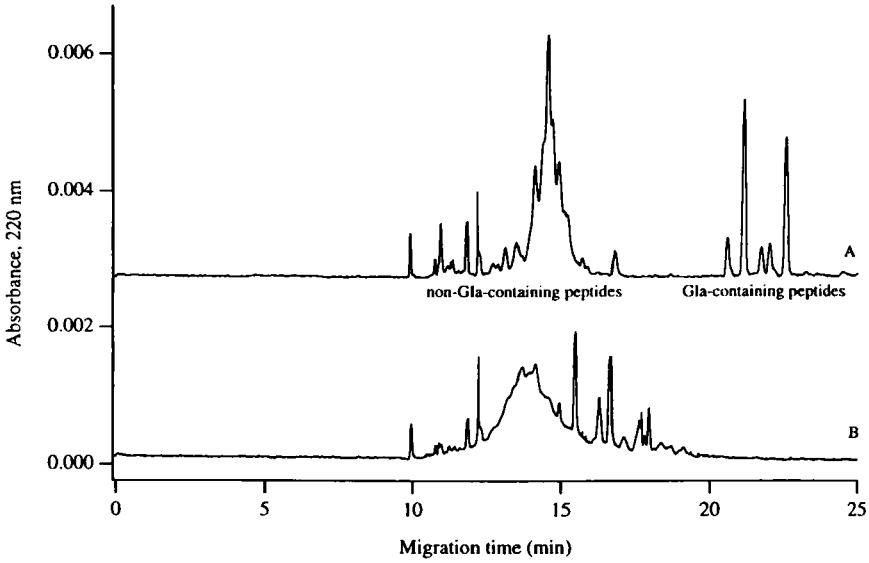

Figure 5. Electropherograms obtained after partial tryptic digestion of bovine prothrombin fragment 1, a protein containing 10 Gla residues within the N-terminal 33 amino acids of the sequence. Before decarboxylation, Gla-containing peptides are seen in the late-migrating region of the electropherogram (A). Complete decarboxylation (10 h) of Gla residues to Glu resulted in a decrease in migration time of these peptides (B). Run buffer was 25 mM sodium tetraborate (pH 10.0); run voltage, 25 kV; current, 38 μA. See main text for additional experimental details.

A primary separation into groups based strictly on the number of Gla residues remaining, even without resolution of isomers, may nevertheless be used to count the number of Gla residues in a protein. Proteolytic peptide mapping constitutes a possible approach to overcoming the limitations of analyzing intact proteins. Our results for wild type bovine prothrombin fragment 1[24] indicate that the Gla-containing peptides in enzymatic digests conveniently appear in a distinctly late region of electropherograms acquired using moderate to high pH CE run buffers (Figure 5). These studies suggest mapping as a convenient means of detecting differences in the chemical structure of Gla domains, especially the presence of uncarboxylated, mutated, or modified Gla residues that are of significance to many investigations involving blood coagulation proteins.

An application of this method that is of great biological relevance concerns the study of the nature of vitamin K-dependent, enzymatic carboxylation of Glu residues to Gla. Many investigations are aimed at determining whether or not a specific sequential order of carboxylative production of Gla exists for substrates that are destined to contain multiple Gla residues. Related studies seek to determine the number of distinct enzyme-substrate binding events required to effect complete carboxylation of one substrate molecule. Data from this work may be used to establish the processivity of vitamin K carboxylase. Complete enzymatic processivity, for example, would describe the case in which one and only one enzyme-substrate binding event occurs for production of all Gla residues in a single substrate molecule. A number of *in vitro*[25-28]and *in vivo*[29,30] investigations of both the order and processivity of vitamin K carboxylase action have appeared in the literature. Such studies invariably require characterization of samples resulting from partial or complete carboxylation of a substrate. The chemical differences in net Gla content and/or sequential position of Gla residues in these types of samples are identical to those found in the partially decarboxylated peptide samples analyzed in the research presented here. CE is apparently very well-suited for the separation of Gla-containing peptide mixtures generated during studies of vitamin K-dependent carboxylation.

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REFERENCES

1. J. P. Landers, R. P. Oda, T. C. Spelsberg, J. A. Nolan, K. J. Ulfelder, Biotechniques, **14**, 98-111 (1993).

2. C. Schoneich, A. F. R. Huhmer, S. R. Rabel, J. F. Stobaugh, S. D. S. Jois, C. K. Larive, T. J. Siahaan, T. C. Squier, D. J. Bigelow, T. D. Williams, Anal. Chem., **67**, 155R-181R (1995).

3. R. Uy, F. Wold, Science, **198**, 890-896 (1977).

4. E. W. Davie, K. Fujikawa, W. Kisiel, Biochemistry, **30**, 10363-10370 (1991).

5. J. W. Suttie, FASEB J., 7, 445-452 (1993).

6. J. Stenflo, P. Fernlund, W. Egan, P. Roepstorff, Proc. Nat. Acad. Sci. USA, **71**, 2730-2733 (1974).

7. B. R. Hubbard, M. M. W. Ulrich, M. Jacobs, C. Vermeer, C. Walsh, B. Furie, B. C. Furie, Proc. Natl. Acad. Sci. USA, **86**, 6893-6897 (1989).

8. P. Dowd, S. W. Ham, S. J. Geib, J. Am. Chem. Soc., **113**, 7734-7743 (1991).

9. J. W. Poser, P. A. Price, J. Biol. Chem., **254**, 431-436 (1979).

10. P. M. Tuhy, J. W. Bloom, K. G. Mann, Biochemistry, **18**, 5842-5848 (1979).

11. T. E. Creighton, Nature, **284**, 487-489 (1980).

12. M. A. Schuerman, K. I. Keverline, R. G. Hiskey, Tetrahedron Lett., **36**, 825-28 (1995).

13. K. G. Mann, Methods in Enzymol., **45**, 123-156 (1977).

14. J. S. Pollock, A. J. Shepard, D. J. Weber, D. L. Olson, D. G. Klapper, L. G. Pedersen, R. G. Hiskey, J. Biol. Chem., **263**, 14216-14233 (1988).

15. J. W. Jorgenson, K. D. Lukacs, Science, **222**, 266-272 (1983).

16. P. Marchand, L. Marmet, Rev. Sci. Instrum., **54**, 1034-1041 (1983).

17. K. D. Altria, Chromatographia, **35**, 177-182 (1993).

18. A. L. Gray, R. A. Hoke, D. W. Deerfield, R. G. Hiskey, J. Org. Chem., **50**, 2189-2191 (1985).

19. S. P. Bajaj, P. A. Price, W. A. Russel, J. Biol. Chem., **257**, 3726-3731 (1982).

20. S. F. Wright, P. Berkowitz, D. W. Deerfield, P. A. Byrd, D. L. Olson, R. S. Larson, G. C. Hinn, K. A. Koehler, L. G. Pedersen, R. G. Hiskey, J. Biol. Chem., **261**, 10598-10605 (1986).

21. G. A. Zapata, P. Berkowitz, C. M. Noyes, J. S. Pollock, D. W. Deerfield, II, L. G. Pedersen, R. G. Hiskey, J. Biol. Chem., **263**, 8150-8156 (1988).

22. T. Sugo, K. Watanabe, T. Naraki, M. Matsuda, J. Biochem., **108**, 382-387 (1990).

23. P. Oefner, C. Chiesa, G. Bonn, C. Horvath, J. Cap. Elec., **1**, 5-26 (1994).

24. G. M. Adams, Ph.D., University of North Carolina, 1992.

25. P. Decottignies Le-Marechal, H. Rikong-Adie, R. Azerad, Biochem. Biophys. Res. Commun., **90**, 700-707 (1979).

26. S.-M. Wu, B. A. M. Soute, C. Vermeer, D. W. Stafford, J. Biol. Chem., **265**, 13124-13129 (1990).

27. D. P. Morris, Ph.D., University of North Carolina, 1995.

28. M. E. Benton, P. A. Price, J. W. Suttie, Biochemistry, **34**, 9541-9551 (1995).

29. M. Borowski, B. C. Furie, B. Furie, J. Biol. Chem., **261**, 1624-1628 (1986).

30. D. J. Liska, J. W. Suttie, Biochemistry, **27**, 8636-8641 (1988).

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