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ABSTRACT

OPTIMIZATION OF ION-EXCHANGE CHROMATOGRAPHY WITH MULTIPLE GRADIENT INPUTS FOR PROTEIN SEPARATIONS

by
Tariq Mahmood

Gradient elution chromatography is an efficient technique for adjusting the retention of sample components during liquid chromatographic separations. However, the optimization of gradient elution is usually done by trial and error. Thus, for large scale processes this results in expensive and time consuming design and operations. Peak resolution that describes the degree of separation is a commonly used parameter for chromatographic processes. However, in processes where operating time and product dilution are of great importance, resolution alone is not adequate for describing the separation efficiency.

A new parameter "resolution optimization factor" is used for the optimization of gradient elution processes. Different gradient inputs were studied using proteins β-Lactoglobulin A/B to demonstrate the utility of resolution optimization factor. Thus, gradient profiles can be predicted which will give better separation efficiency by considering resolution as well as elution time. This is expected to lead to a systematic and rational approach that can be used to improve the efficiency of the downstream production processes, and reduce the amount of waste solvents generated in the biotechnology and pharmaceutical industries.

OPTIMIZATION OF ION EXCHANGE CHROMATOGRAPHY WITH MULTIPLE GRADIENT INPUTS FOR PROTIEN SEPARATIONS

by
Tariq Mahmood

A Thesis
Submitted to the Faculty of
New Jersey Institute of Technology
in Partial Fulfillment of the Requirements for the Degree of
Master of Science in Chemical Engineering

Chemical Engineering Committee

January 1997

APPROVAL PAGE

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To my beloved nephews and nieces
Naiem, Zain, Ussama, Iyyan, Rabbiya, Zeenat, and Sunnia

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ACKNOWLEDGMENT

I would like to express my sincere appreciation to Dr. Robert Luo for endless assistance in preparing this manuscript. He not only served as my research advisor, but also gave me support, encouragement and reassurance. Special thanks to Dr. Hanesian and Dr. Perna for actively participating on my committee.

CHAPTER 1

INTRODUCTION AND THEORY

1.1 Principles of Ion Exchange Chromatography

Chromatography is a technique in which components of a sample mixture are separated based upon the rates at which they are carried through a stationary phase by a liquid or gaseous mobile phase. The mobile phase is passed or forced over a stationary phase which is fixed in a column or on a solid surface. The components of the sample distribute themselves in the mobile and stationary phase to a different extents. Thus, the components that are not strongly held by the stationary phase move faster down the column than those which are retained by it. This difference in migration rates through the column results in discrete bands for sample components (Skoog, 1996).

Adsorption of the sample components on the stationary phase depends upon different types of interactions between the solute molecules and the ligands immobilized on a chromatography matrix. Ion exchange chromatography is based on the interaction between the charged sample molecules and the oppositely charged molecules covalently linked to a chromatography matrix (Pharmacia Catalog, 1996).

The chemical structure of biomolecules ranges widely and therefore, the separation of biomolecules inevitably depends upon those chemical structures (Belter, 1988). Ion exchange chromatography is an efficient

method for separation of biological products and has been used for purification of proteins, polypeptides, nucleic acids, polynucleotides, and other charged biomoleucles. Ion exchange chromatography has widespread application in bioseparations with high resolving power, high capacity and controllability.

In ion exchange chromatography, separation is based on the reversible adsorption of charged sample molecules to an ion exchanger (matrix) of opposite charge. This adsorption with the matrix can be controlled by pH or ionic strength of the eluting buffer. The mechanism of ion exchange chromatography can be describe in four stages. The mechanism is illustrated in figure 1.1 (Pharmacia Catalog, 1996).

a. Equilibration: The first stage of ion exchange chromatography is to bring the ion exchanger at a state where the binding of sample components will be possible. This is done by equilibrating the ion exchanger with the starting buffer in terms of desired pH and ionic strength. The ion-exchanger will be ionically associated to the counter ions from the starting buffer.

b. Sample Loading and Adsorption: In the second stage, the sample is loaded into the column and sample molecules which carry net charge will displace the counter ions on the ion exchanger and will reversibly attach to it. While the unbound substances which carry no net charge or similar charge to that of the matrix (ion exchanger) will be washed through the column.

c. Desorption or Elution: At this stage solute molecules that are attached to ion exchanger are removed by changing the elution conditions that are

unfavourable for ionic bonding. This is usually done by changing the ionic strength or pH of eluting buffer.

e. Regeneration: All the bounded impurities are eluted (washed) from the column and the ion exchanger is regenerated with the original counter ions. The mechanism of ion exchange chromatography is described in figure 1.1.

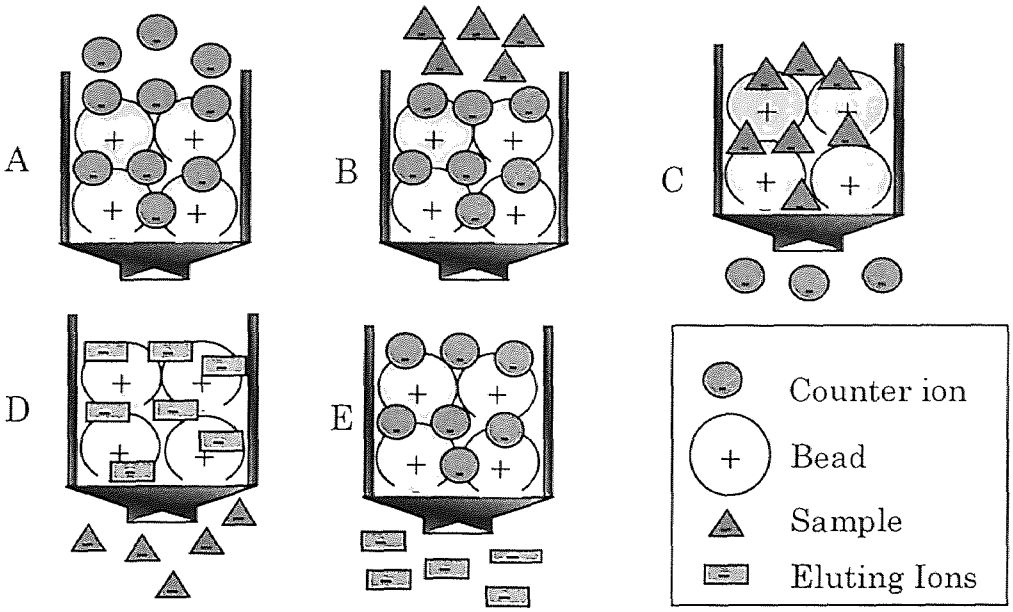

Figure 1.1. Mechanism of Ion Exchange Chromatography

A. Equilibration - counter ions are ionically bonded to the matrix

B & C. Sample Adsorption - Sample molecules displace the counter ions

D. Elution - sample molecules are displaced by ions from the eluting buffer

E. Regeneration - matrix is regenerated with the origianl counter ions

1.2 The Ion Exchanger (Matrix)

An ion exchanger consists of an insoluble porous matrix to which charged groups are covalently bound. The charged groups are associated with the counter ions from the starting buffer. These counter ion can be reversibly exchanged with other ions of the same charge.

Ion exchanger can be classified in two groups. Anion exchanger are positively charged and have negatively charged counter ions available for exchange. Whereas Cation exchanger are negatively charged with positively charged counter ion available for exchange. Cation and Anion exchangers are shown in figure 1.2 (Pharmacia Catalog, 1996).

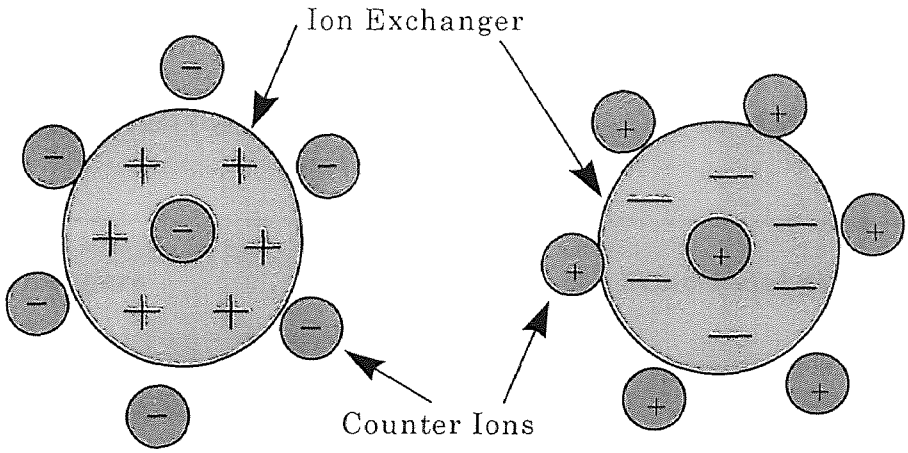

Figure 1.2. Types of Ion Exchanger

1.2.1. Charged Groups

The presence of charged groups is a basic property of an ion exchanger. The strength of the ion exchanger and its capacity is determined by the type and number of charged group attached to it. Some of charged groups used are shown in table 1.1 (Pharmacia Catalog, 1996).

Table1.1. Functional groups attached on ion exchangers.

Anion exchangers	**Functional group**
Diethylaminoethyl (DEAE)	$-O-CH_2-CH_2-N^+H(CH_2CH_3)_2$
Quaternary aminoethyl (QEA)	$-O-CH_2-CH_2-N^+(C_2H_5)_2-CH_2-CHOH-CH3$
Quaternary ammonium (Q)	$-O-CH_2-CHOH-CH_2-O-CH_2-CHOH-CH_2-N^+(CH_3)_3$
Cation Exchangers	**Functional group**
Carboxymethyl (CM)	$-O-CH_2-COO^-$
Sulphopropyl (SP)	$-O-CH_2-CHOH-CH_2-O-CH_2-CH_2-CH_2SO_3^-$
Methyl sulphonate (S)	$-O-CH_2-CHOH-CH_2-O-CH_2-CHOH-CH_2SO_3^-$

Strong ion exchangers are formed by using Sulphonic and quaternary amino groups while other groups are used to form weak ion exchangers. The degree of ionization of the charged group present characterizes the ion exchanger as a strong or weak ion exchanger. Strong ion exchangers can be completely ionized over a wide range of pH whereas the weak ion exchanger the degree of ionization is a strong function of pH. Thus pH will affect the degree of dissociation and capacity of the ion exchanger.

1.2.2. Choice of Ion Exchanger

The choice of ion exchanger depends on three factors:

a. Specific requirements of the application: The specific requirements of the application need decisions such as whether to use column or batch separation, operational scale, resolution required, optimization and required through put and economy.

b. Molecular size of the biomoleules to be separated: The exclusion limit (size of porous) of the matrix being used should be considered because it will affect the capacity of the separation system (accessibility of sample components to the charged groups).

c. Isoelectric point and stability of the sample components: Since the binding of sample components to the ion exchanger is based on the net charge opposite to that of the matrix, it is important to know the net charge of the sample components. If the solute molecules are carrying a charge opposite to that of matrix it is easy to decide which matrix to be used. However, for

amphoteric substances the net charge depends upon the pH of the buffer. The following criteria is usually used to determine which ion exchanger is to be used.

Cation exchanger is used if the sample components are stable below their isoelectric point because molecules are positively charge below their isoelectric point. Anion exchanger is used if the sample components are stable above their isoelectric point, since molecules carry negative charge above their isoelectric point (Pharmacia Catalog, 1996). Either type of exchanger can be used if the solute molecules are stable over a wide range on both sides of isoelectric point. The separation strategy for amphoteric molecules is described in Table 1.2.

Table 1.2. Separation strategy for amphoteric molecules.

Type of Ion exchanger	Cation Exchanger	Anion Exchanger
Net Charge of Molecules of Interest	Positive	Negative
Charge of Ion Exchanger	Negative	Positive
Running Conditions	pH below pI of sample molecules	pH above pI of sample molecules

1.3 Parameters Used in Ion Exchange Chromatography

The parameters which affect the separation in ion exchange chromatography are Column resolution, Capacity Factor, Selectivity, Efficiency (Skoog, 1994).

1.3.1. Column Resolution

Resolution, R_s, is a commonly used parameter for chromatographic separations. It describes the relative separation between the peaks of interest (Skoog, 1996). Resolution, R_s, of a column is a quantitative measure of its ability to separate two components.

$$R_s = \frac{t_{r,2} - t_{r,1}}{\frac{1}{2}(W_1 + W_2)} \qquad (1)$$

where $t_{r,1}$ is the retention time for component 1, $t_{r,2}$ is retention time for component 2. W_1 and W_2 is the peak width of component 1 and 2 respectively. The resolution between two peaks is shown in figure 1.3 (Skoog, 1996).

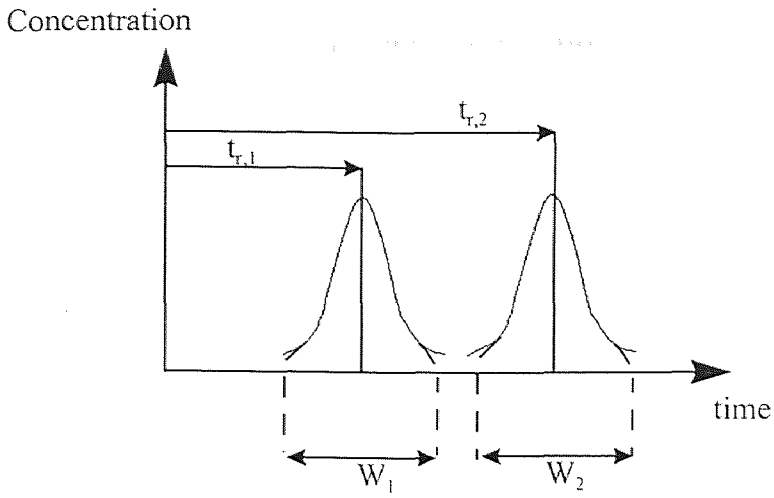

Figure 1.3. Determination of Resolution (R_s) between two peaks.

Column resolution can be related to other parameters such as the number of plates (column efficiency) in the column as well as to the capacity and selectivity factors of sample component on the column. Column efficiency, selectivity, and capacity factor are important parameter to control in column chromatography. It can be shown that column resolution, R_s, (Skoog, 1996) is given by:

$$R_s = \frac{1}{4}\sqrt{n} \qquad \left(\frac{\alpha - 1}{\alpha}\right) \qquad \left(\frac{k'_B}{1 + k'_B}\right) \qquad (2)$$

where n, α and k'_B represents the number of theoretical plates, selectivity and capacity factor of slower moving sample component respectively.

The term $\frac{1}{4}\sqrt{n}$, which represents the column efficiency is a measure of the zone broadening (peak width) of the solute in the column. The number of theoretical plates is related to plate height H by the following equation:

$$n = \frac{L}{H} = 16\left(\frac{t_R}{W}\right)^2 \qquad (3)$$

where L is the length of column packing, t_R is retention time of solute and W is peak width.

The column efficiency is largely depended on the following three factors:

1. the flow rates and sample loading (linear velocity of mobile phase influence the bandwidth and the resolution)
2. longitudinal diffusion of the solute molecules
3. column packing (evenly packed column gives good resolution).

The term $\frac{k'_B}{k'_B + 1}$, which represents the capacity factor of slower moving component is largely affected by the choice of the components of the mobile phase and their concentration. The capacity factor describes the migration rates of the sample components in the column (Skoog, 1996). The capacity factor, k'_B, can be calculated from the chromatogram by:

$$k'_B = \frac{(t_R)_B - t_m}{t_m} \qquad (4)$$

where $(t_R)_B$, and t_m represent retention times of component B and column dead time for unretained specie respectively. It is a dimensionless quantity and does not dependent on the column dimensions or the flow rate of the mobile phase. The capacity factor is the ratio of the amount of sample component in the stationary phase to its amount in the mobile phase.

The term $\frac{\alpha}{\alpha - 1}$, describes the separation selectivity of the system. Selectivity is the ability of stationary phase-mobile phase system to hold sample components to different extents. It is the measure how well the column will separate the two peaks. The selectivity, α is calculated as:

$$\alpha = \frac{k'_B}{k'_A} = \frac{(t_R)_B - t_m}{(t_R)_A - t_m} \qquad (5)$$

where $(t_R)_B$, $(t_R)_A$ and t_m represent retention times of component B, A and column dead time for unretained specie respectively. Selectivity is one of the most important parameter in ion exchange chromatography which can be manipulated in an experimental run. It depends not only on the type and number on ionic groups on the exchanger but also on the operating conditions such an pH and ionic strength of the eluting buffer. Resolution, R_s, is linear function of selectivity whereas it is a quadratically dependent on the efficiency.

CHAPTER 2

OPTIMIZATION OF ION EXCHANGE CHROMATOGRAPHY

2.1 Isocratic and Gradient Elution

Elution is a process by which the sample components are washed through the stationary phase by the movement of the mobile phase. In isocratic elution the mobile phase parameters such as ionic strength, pH and composition are kept constant (Jandera, 1984). If the sample components are differentially retarded or if all retarted substances elute under starting conditions then isocratic elution is useful. However, if the sample components differ widely in retention or if the adsorption of sample mixture is strong, elution is done by selectively decreasing the affinity of the sample molecules for the charged groups on the ion exchanger. This is achieved by changing the composition, pH or ionic strength of the mobile phase over a period of time. This process is known as Gradient Elution (Jandera, 1984).

The net charge of biomolecules depends upon the pH, thus in gradient elution by changing the pH towards isoelectric point (where no binding occurs) can desorb and elute the sample components from the column. At low ionic strength the competition for the charge groups on the ion exchanger is at minimum and thus the sample components bind strongly to the matrix (ion exchanger). However, using gradient elution, where by increasing the ionic strength gradually reduces the availability of charged groups and thus elution of sample components takes place.

2.1.1. Types of Gradient Elution Profiles

Sample components usually have different affinity towards the ion exchanger; in gradient elution by varying pH and ionic strength of the mobile phase separation can be achieved. The most common types of gradient profiles used are (Jandera, 1984) linear and stepwise gradients.

1. Linear Gradients: In linear gradient elution, the concentration of the mobile phase is a linear function of time (Jandera, 1984). Thus in ion exchange chromatography, the ionic strength of more strong eluting buffer is a linear function of time. This can be achieved by mixing starting and eluting buffer such that the volume ratio is changing linearly. Linear gradients are easier to produce and gives more reproducible results.
2. Stepwise Gradients: Stepwise gradients are produced by sequential use of strong eluting buffer at different ionic strengths. If during separation one or more components are strongly retained stepwise elution is an efficient way to elute sample components.

2.2 Optimization of Gradient Elution Chromatography

Peak resolution that describes the degree of separation is a commonly used parameter for chromatographic processes. However, in processes where operating time and product dilution are of great importance, resolution alone is not adequate for describing the separation efficiency. A new parameter "resolution optimization factor" was used for the optimization of gradient elution processes (Luo, 1996). Using this new parameter better separation

efficiency can be achieved by considering the resolution as well as elution time. Thus, an optimum gradient profile will be determined which will give the better separation efficiency (Luo, 1996).

Gradient elution chromatography is an efficient technique for adjusting the retention of sample components during liquid chromatographic separations. It is done by gradually changing conditions which are unfavorable for sample adsorption. A common approach for optimizing gradient elution processes is fine tuning of following separation conditions (Jandera, 1980):

(a) if sample components are eluted very late, use higher initial concentration of eluting buffer;

(b) to obtain better resolution use lower flow rates or increase the column length;

(c) change the solvent if components are strongly retained;

(d) variation in elution profile to achieve better resolution.

The optimization of these conditions in gradient elution chromatography is usually done by trial and error. Thus, for large scale processes this results in expensive and time consuming design operations. A common practice to optimize chromatographic processes using gradient elution is by obtaining the highest possible resolution. Which means an isocratic gradient elution of constant composition can be used to achieve highest possible resolution. But, in these types of situations speed of the separation process is often ignored. This results in processes with long operating time and dilute desired products

(Luo, 1995). Thus, the ultimate consequence of this is time consumption and expensive processes.

When ion exchange chromatography is a part of manufacturing process, conditions should be chosen which give the highest throughput with the highest product purity and yield with minimum cost. Separation efficiency of chromatographic process cannot be described solely by resolution and thus another parameter is needed for an efficient process development. A new parameter "resolution optimization factor" was used in this study. Resolution optimization factor is a function of both resolution and elution time. Whereas resolution describes the degree of separation the resolution optimization factor is a measure of separation efficiency of a process.

2.2.1. Resolution Optimization Factor

Resolution is a commonly used parameter for chromatographic separations. Resolution, R_s of a column is a quantitative measure of its ability to separate two components and is given by equation 1. In bioseparation processes where the operating time is not of primary interest resolution, R_s alone is sufficient for the gradient elution optimization. However, in cases where operational time is an important process consideration resolution alone would not give cost effective processes. Higher resolution can be achieved at the expense of longer elution time. But long operating time would result in dilute products and will cause eluting buffer as an impurity in separation process. Thus, an extra step will be required in separation scheme where highly pure product

are desired. This will increase the operating costs and might lower the product yield. Also, long operating time may be an environmental concern. For example in reversed phase chromatography, where long operation time means more use of organic solvent which would result in waste generation. Hence, resolution alone is not adequate for defining the efficiency of processes, another parameter is needed along with resolution which will take into consideration the elution time and product purity for an overall effective separation process.

In order to account for the cost in terms of time and product dilution (elution volume) a different parameter, **resolution optimization factor**, f_O, was introduced by Luo and Hsu. It is a function of both resolution and elution time. While resolution describes the degree of separation whereas the resolution optimization factor describes the separation efficiency. For a successful separation, a comparison between the desired resolution along with the yield obtained should be compared with the production time and dilution. The evaluation of these factors are done by considering the values of resolutions and average elution time. The resolution is defined earlier whereas average elution time is defined as (Luo, 1996):

$$\bar{t}_r = \frac{t_{r,1} + t_{r,2}}{2} \qquad (6)$$

where $t_{r,1}$ and $t_{r,2}$ are retention time of component 1 and 2 respectively and $\bar{t}_r$ is average elution time for both components. The local optimization factor, f_o, which deals with two adjoining peaks is defined as:

$$f_o = f\left(R_s, t_{r,i}\right) = \frac{R_s}{\bar{t}_r} = \frac{R_s}{\left(\frac{1}{2}\right)\left(t_{r,1} + t_{r,2}\right)} \qquad (7)$$

Similaly the overall optimization factor for a multicomponent process is given by:

$$F_o = \frac{n}{n-1}\sum_{i=1}^{n} \frac{R_s(1,2) + R_s(2,3) + \cdots + R_s(i, i+1) + \cdots + R_s(n-1, n)}{t_{r,1} + t_{r,2} + \cdots t_{r,i} + \cdots + t_{r,n}} \qquad (8)$$

where F_o is overall resolution optimization factor, n is the number of sample components, $R_s(i,i+1)$ is resolution between the (i)th and (i+1)th peaks, $t_{r,i}$ is the elution time of the ith peak.

This study describes the experimental application of resolution optimization factor. It will be seen that resolution as well as elution time are important parameters for separation efficiency.

CHAPTER 3

OBJECTIVES

The overall objectives of this study was to determine the resolution optimization factor by calculating resolution and average elution time. Three different gradient profiles were used to calculate resolution optimization factor. The profiles are as follows:

1. Isocratic Gradient Eltuion: These gradient were produced by keeping the ionic strength of eluting buffer constant during elution. The results obtained are discussed in Chapter 5.

2. Linear Gradient Elution: A linear profile of ionic strength for eluting buffer was used during gradient elution. The resolution optimization factor and other parameters are described in Chapter 5.

3. Multiple Gradient Inputs: A combination of linear and stepwise gradients were used and parameters calculated are discussed in Chapter 5.

CHAPTER 4

MATERIALS AND METHODS

This chapter describes the detail experimental procedure used in optimization of gradient elution chromatography.

4.1 Experimental System

In these experiments different gradient profiles were used for elution of β-lactoglobulin A and β-lactoglobulin B (Luo, 1994).

4.1.1. Experimental Apparatus

A detail experimental setup is shown in Figure 4.1. The experiments were conducted on a 1.5 x 30 cm glass column (Bio-Rad Laboratories, Hercules, CA). The packed column height was about 20 cm. The column was packed with DEAE Sepharose CL-6B (Sigma Chemical Co., St. Louis, MO). A flow adapter was used to minimize the dead space over the top of the bed in order to assure accurate gradient profiles. Gradi Frac system (Pharmacia Biotech, Bjorkgatan, Sweden) was used to generate gradient profiles. The Ultraviolet absorbance of eluent from the column was measured at 280 nm. The experimental data were collected by means of a recorder and a 386 computer with M-1101 A/D Converter (Keithley Metrabyte, Taunton, MA) using data acquisition software Scansoft.

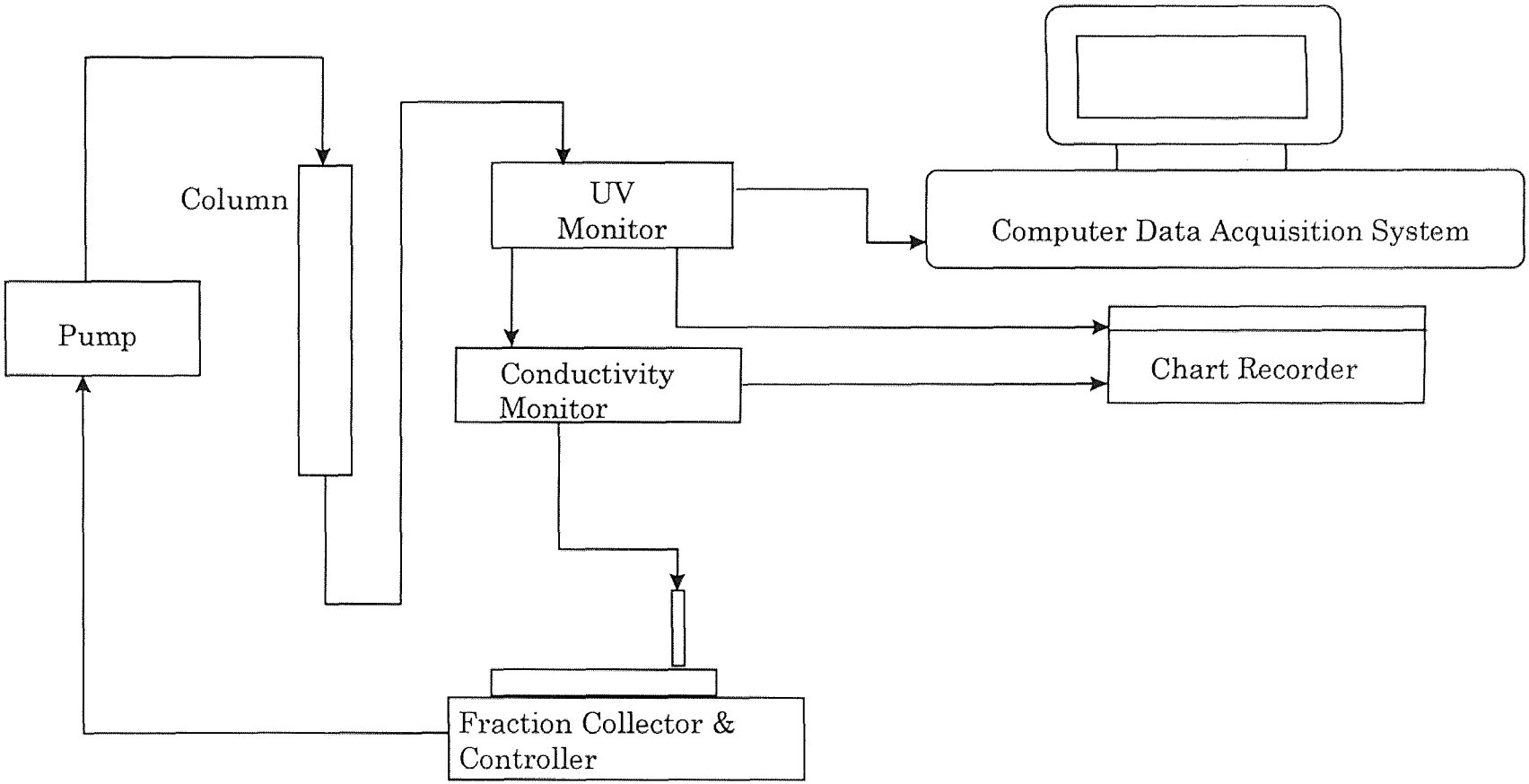

Figure 4.1. Experimental Apparatus

4.1.2. Model Proteins

β-lactoglobulin from bovine milk was used to supply the model protein for our study. It consists of two different proteins, β-lactoglobulin A and β-lactoglobulin B. The comparison of amino acid sequence between these proteins reveals that β-lactoglobulin A contains one more residue each of aspartic acid and valine, and one less residue each of glycine and alanine than does β-lactoglobulin B. A complete amino acid sequence for β-lactoglobulin A and β-lactoglobulin B is shown in Figure 4.2.

The molecular weights of these two components are about the same, 35,500. The slight difference in amino acid sequence results in very close isoelectric point (pI). The pI for β-lactoglobulin A is 5.21 and for β-lactoglobulin B is 5.34. Due to this small difference in pI the separation of these proteins using iscratic elution is difficult and thus makes a good model for gradient elution. The proteins were purchased from Sigma Chemical Company (St Louis, MO). Three times crystallized and lyophilized β-lactoglobulin (Lot. 0130) which contained approximately equal amounts of β-lactoglobulin A and β-lactoglobulin B used for gradient studies.

4.1.3. Adsorbent

A weak anion exchanger DEAE Sepharose CL-6B was used in this study. DEAE Sepharose CL-6B are macroporous bead fromed ion exchanger derived from the cross-linked agarose gel Sepharose CL-6B. The diameter of these

H-Leu-Ile-Val-Thr-Gln-Thr-Met-Lys-Leu-Asp-Ile-Glen-Lys-Val-Als-gly-Thr-Trp-Tyr-Ser-Leu-Ala-Met-Ala-Ala-Ser-Asp-Ile-Ser-Leu-Asp-Ala-Gln-Ser-Ala-Pro-Leu-Arg-Val-Tyr-Val-Glu-Glu-Leu-Lys-Pro-Thr-Pro-Glu-Gly-Asp-Leu-Glu-Ile-Leu-Leu-Gln-Lys-Trp-Glu-Asn-Asp(Gly)-Glu-Cys-Ala-Gln-Lys-Lys-Ile-Ile-Ala-Glu-Lys-Thr-Lys-Ile-Pro-Ala-Val-Phe-Lys-Ile-Asp-Ala-Leu-Asn-Glu-Asn-Lys-Val-Leu-Val-Leu-Asp-Thr-Asp-Tyr-Lys-Tyr-Lys-Lys-Tyr-Leu-Leu-Phe-Cys-Met-Glu-Asn-Ser-Ala-Glu-Pro-Glu-Gln-Ser-Leu-Val(Ala)-Cys-Gln-Cys-Leu-Val-Arg-Thr-Pro-Glu-Val-Asp-Asp-Glu-Ala-Leu-Glu-lys-Phe-Asp-Lys-Ala-Leu-Lys-Ala-Leu-Pro-Met-His-Ile-Arg-Leu-Ser-Phe-Asn-Pro-Thr-Gln-Leu-Glu-Glu-Gln-Cys-His-Ile-OH

Figure 4.2. Amino acid sequence of β-lactoglobulin A (LGA) and β-lactoglobulin B (LGB). At residues 64 and 118, LGA has aspartic acid and valine, whereas LGB has glycine and alanine.

beads ranges from 45-165 um. DEAE groups are then attached to the gel by ether linkages to the monosaccharide units to give the final ion exchange gel. The highly cross linked structure of DEAE Sepharose CL-6B gives it high chemical and physical stability. It is insoluble in all solvents and is stable in water, salt solutions and organic solvents. The working pH range for DEAE Sepharose CL-6B is between 2 to 9. The structure of DEAE Sepharose CL-6B gives them improved flow properties and prevents fluctuations in bed volume.

4.1.4. Buffer Preparation

Two buffers were used to generate salt gradients. Buffer A, a starting buffer, was 18mM TRIS/HCl, pH 7.9, I .01 M, while buffer B was 0.5 M NaCl solution. The following procedure was used to prepare these two buffers.

Buffer A, 18mM TRIS/HCl, pH 7.9:

1. 20 ml of 0.5 N HCl was added to about 500 ml of distilled water.
2. About 2.18 g of Tris Base added with continuous stirring.
3. pH of solution was checked and adjusted to 7.9.
4. Additional distilled water was added to make 1000 ml of solution.

Buffer B, 0.5 M NaCl solution:

1. 29.22 g of NaCl was added to 500 ml distilled water while stirring.
2. Solution was scaled to 1000 ml.

4.1.5. Adsorbent Preparation

1. Using Tris/HCl buffer along with a filtration device Ethanol was washed in which the adsorbent was stored.
2. Adsorbent was suspended in Tris/HCl buffer for an hour. Its pH was checked continuously to make sure that it was close to pH 7.9 of Tris/HCl buffer. If not adsorbent was suspended for an another hour.
3. If the resin is not to be used right away, it can be refrigerated at 4°C.

4.1.6. Sample Preparation

10 ml of 0.5% β-lactoglobulin A/B was prepared by the following procedure.

1. Weigh 0.05 g of protein sample using analytical balance.
2. Sample was transfer to 14 ml plastic tube. Using 1 ml pipette 10 ml of Tris/HCl buffer was added to tube.
3. Sample was dissolve with the help of mixer.
4. 0.5 % sample was then diluted to 0.025 % using another test tube.
5. Rest of 0.5% sample was stored into freezer (-20 °C)
6. Store the 0.025 % sample into refrigerator.

4.1.7. Column Packing

1. Column was packed in a continuous manner by gravity packing.
2. For 20 min column was packed with Tris/HCl buffer at flow rate of 2 ml/min.

3. Bed length should be around 20 cm.
4. Column was equilibrate with 0.5 ml/min of Tris/HCl buffer for an hour or so. The pH of column was checked to make sure that it is around the pH of Tris/HCl buffer.

CHAPTER 5

RESULTS AND DISCUSSION

5.1 Calibration

Calibration curve was made by flowing known composition of β-lactoglobulin A/B through the UV flow cell. The results obtained are shown in Table 5.1 The following procedure was used for calibration results.

1. Using 0.5% β-lactoglobulin A/B, dilutions were made at desired concentration(%).
2. 10 ml of each sample was injected without the column and data (signal in millivolts) was recorded on a disk and a chart paper.
3. A plot of millivolts versus concentration (%) is shown in Figure 5.1. The linear regression of this plot is $y = 0.0005x - 2x10^{-5}$ with a correlation coefficient of 0.998.

Table 4.1. Calibration results at different protein concentration.

Concentration (%)	Millivolts
0.001	2.0376
0.0015	3.4416
0.0025	5.2825
0.0035	7.4591

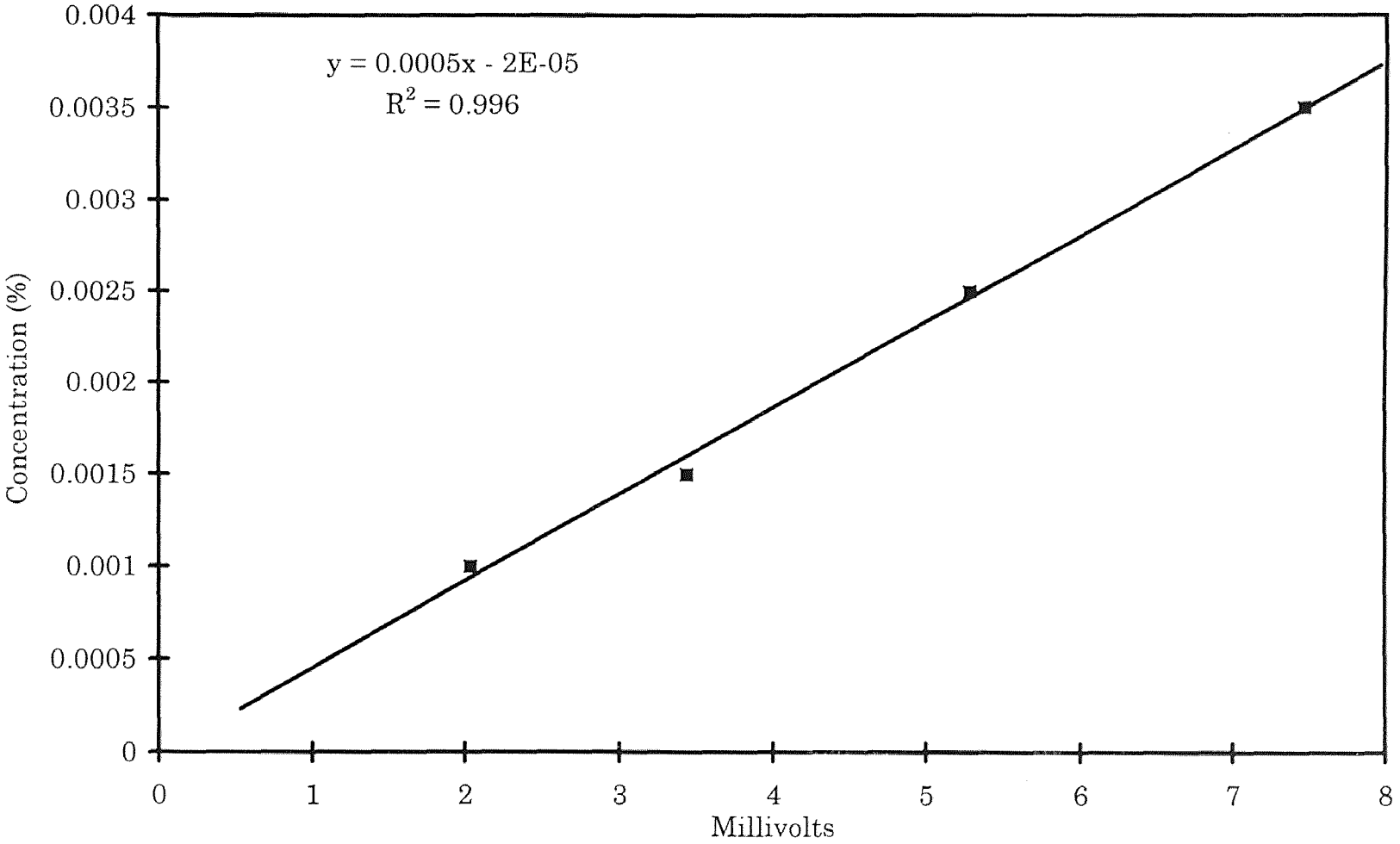

Figure 5.1. Calibration Curve

5.2 Experimental Investigations

5.2.1. Isocratic Elution

Experimental runs were made using isocratic elution, that is constant composition of eluting buffer. Data was recorded for different initial ionic strengths. For each run a plot of time versus concentration (%) was made. From these plots average elution time, $\bar{t}_r$ and resolution, R_s were calculated using equations 1 and 6 respectively. These values were used to calculate resolution optimization factor, f_o, from equation 7. The results obtained at different ionic strengths are provided in Table 5.2.

Table 5.2. Isocratic elution at different ionic strength and corresponding values of parameters:

Ionic Strength I (M)	Ave. Elution Time $\bar{t}_r$ (min)	Resolution, R_s	Res. Opt. Factor, f_ox100 (min^{-1})
0.231	216	0.780	0.361
0.240	140	0.700	0.500
0.245	110.5	0.577	0.522
0.255	94	0.455	0.484
0.280	63	0.283	0.449

In Figure 5.2, experimental peaks show the effect of ionic strength on the elution profile of β-Lactoglobulin A and β-Lactoglobulin B. The numbers

1 and 2 are used to refer different values of ionic strength. It can be seen from the figure that at lower value of ionic strength (I_1 = 0.245 M, solid line) LGB1 and LGA1 peaks took longer time to elute and also were very broad. However, by increasing the value of ionic strength to (I_2 = 0.255 M, dotted line), the LGB2 and LGA2 peaks eluted in short time and were sharp.
For the peaks in Figure 5.2, the resolution, R_s, and average elution time, $\bar{t}_r$, were calculated. By increasing the ionic strength from 0.245 M (solid line, I_1) to 0.255 M (dashed line, I_2) the resolution decreases from 0.577 to a value of 0.455 and also the elution time decreases from 110.5 minutes to 94 minutes. Similar calculations were made at other ionic strengths.

The average elution time, $\bar{t}_r$, and resolution, R_s, obtained at different ionic strength are given in Table 5.2. A plot between these two quantities is shown in Figure 5.3. As the ionic strength of eluting buffer is increased average elution time and resolution decrease. Thus, ionic strength affects both of these parameters. The objective of a separation is to obtain highest resolution in shortest possible time. The fact that resolution, R_s, and average elution time, $\bar{t}_r$, decreases by increasing the ionic strength, means that resolution and average elution time have opposing effects on separation efficiency. Thus, the question arises under what conditions will the process give a better separation efficiency.

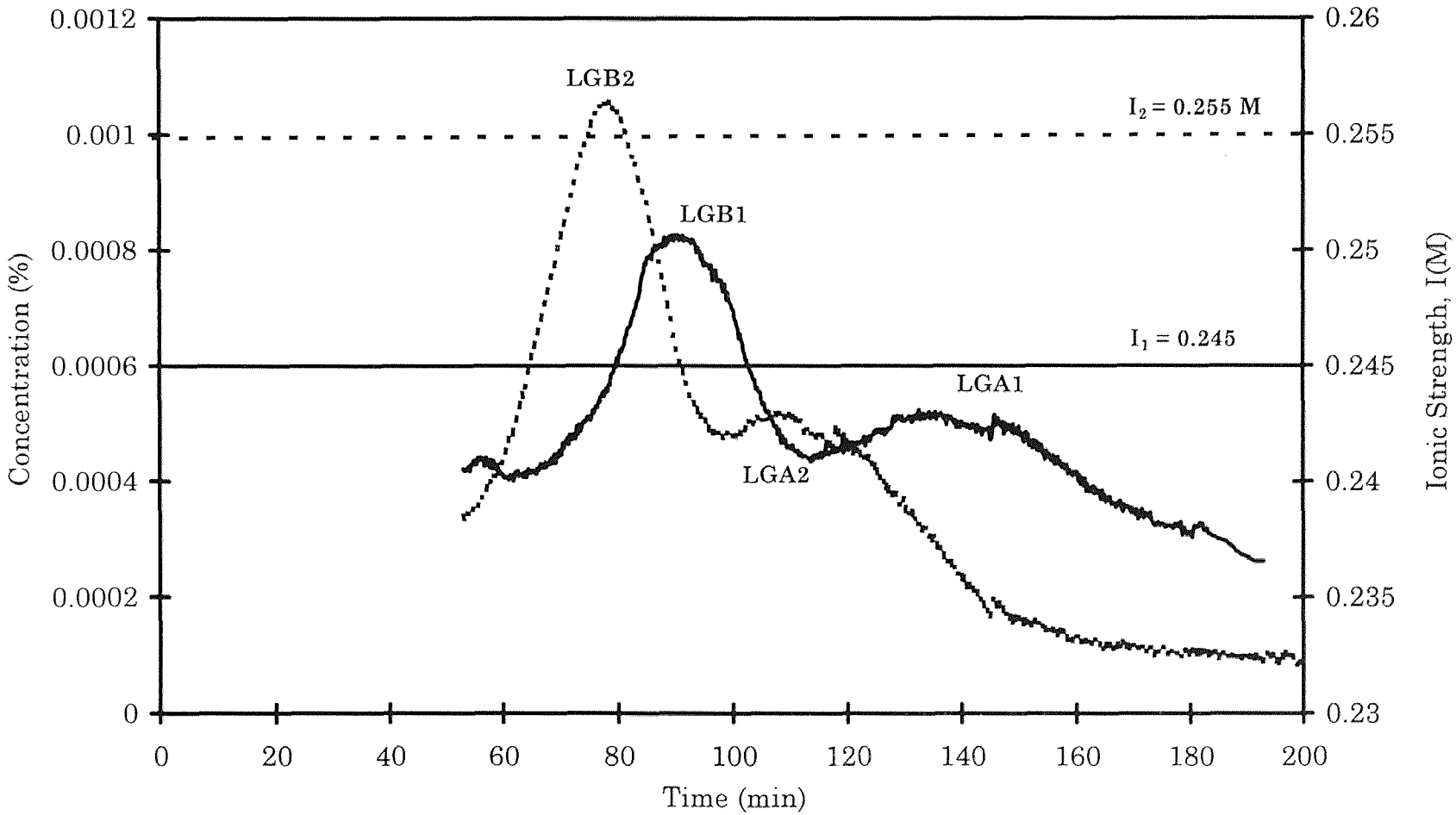

Figure 5.2. Effect of Ionic Strength on Elution Profiles of β-Lactoglobulin in Isocratic Elution

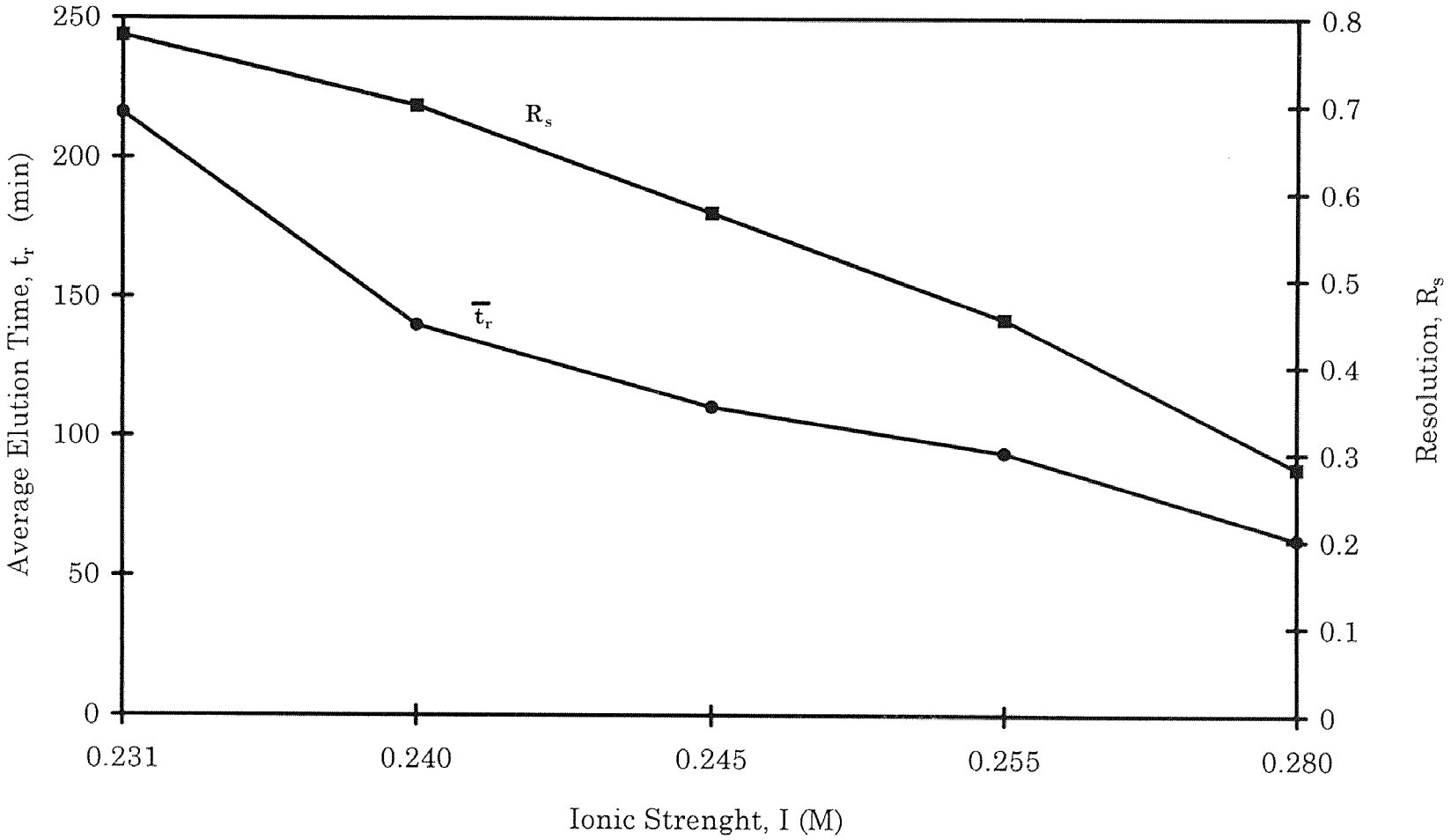

Figure 5.3. Effect of Ionic Strength on Resolution and Average Elution Time in Isocratic Elution

The combined effect of resolution, R_s, and average elution time, $\bar{t}_r$, on separation efficiency is studied by resolution optimization factor, f_o. The results obtained for resolution optimization factor, f_o, are given in Table 5.2. As the ionic strength was increased from 0.231 M to 0.280 M, the optimization factor first increases from 0.361 (min^{-1}) to 0.522 (min^{-1}) and then decreases to 0.449 (min^{-1}). That is, f_o, had a maximum value of 0.522 (min^{-1}) at ionic strength of 0.2452 M. In Figure 5.4 resolution and optimization factor are plotted against the ionic strength, I (M). As the value of ionic strength was increased the resolution decreases continuously while resolution optimization factor has a maximum value of 0.522×10^{-2} (min^{-1}) at 0.245 M. This means that under isocratic elution the system would reach maximum separation efficiency at ionic strength of 0.2452. Thus, at ionic strength of 0.245 M better separation efficiency is achieved.

5.2.2. Linear Gradient Elution

To study the effect of gradient elution on the resolution optimization factor, f_o, the isocratic elution data can give good starting conditions. Thus, an initial ionic strength of 0.2403 M with a resolution, R_s, of 0.700 from the isocratic runs was used as starting conditions for linear gradient elution. Linear gradient profiles were used and data were recorded. As done under isocratic conditions plots between time versus concentration (%) were made.

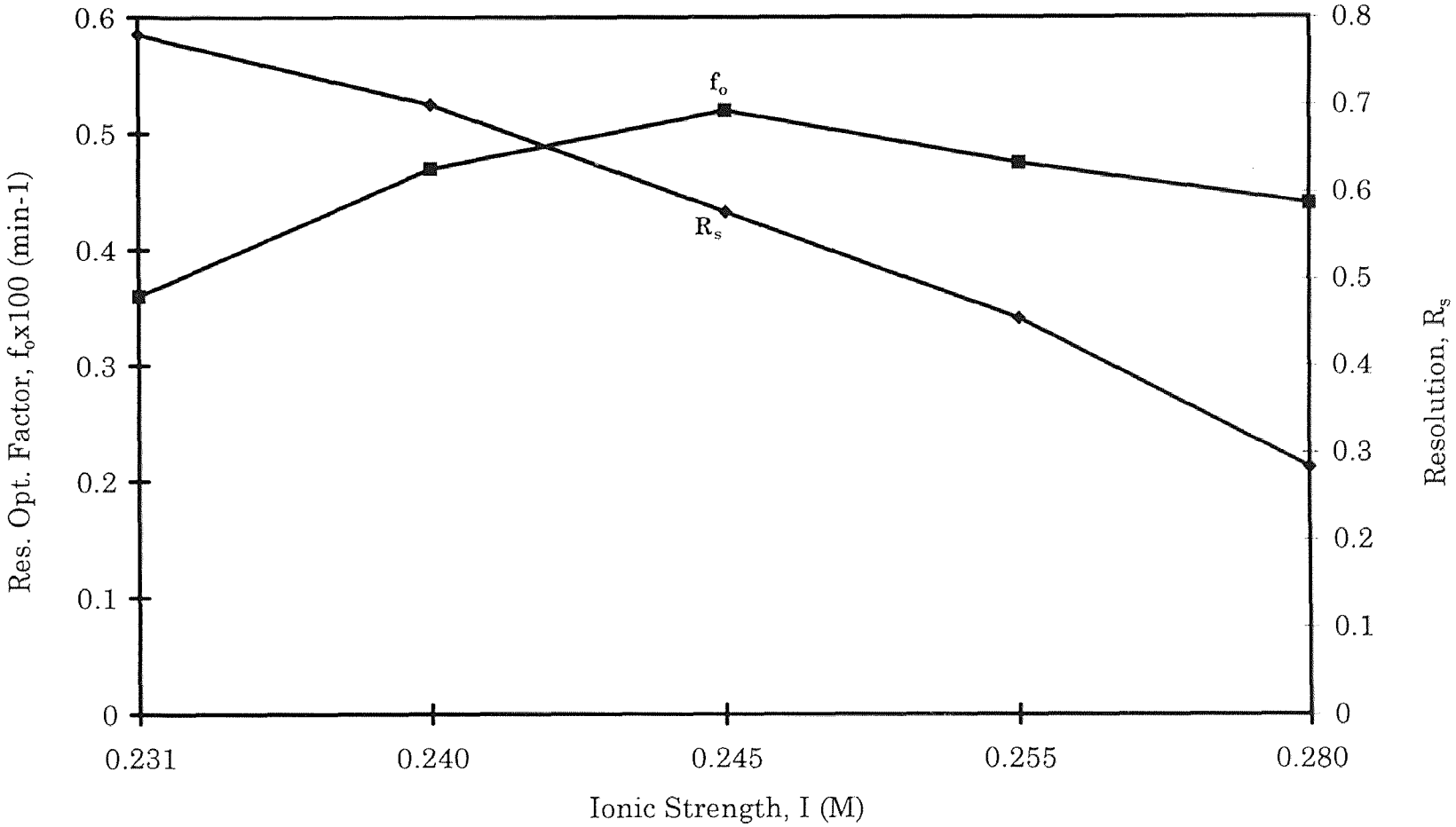

Figure 5.4. Effect of Ionic Strength on Resolution and Resolution Optimization Factor in Isocratic Elution

Using equations 1, 5 and 7, resolution, average elution time and resolution optimization factors were calculated. The results obtained are given in Table 5.3.

Table 5.3. Linear gradient slope and other corresponding parameters:

Gradient Slope $sx10^{4}$ (M/ml)	Ave. Elution Time (min)	Resolution, R_s	Res. Opt. Factor f_0x100 (min^{-1})
0.852	103	0.544	0.528
1.70	100	0.525	0.546
2.56	93.5	0.518	0.572
3.41	89	0.463	0.520
4.26	83	0.391	0.471
5.11	80.5	0.365	0.430

In Figure 5.5 experimental peaks show the effect of linear slope on the separation of β-Lactoglobulin A and β-Lactoglobulin B. As the linear gradient slope is decreased from $4.26x10^{-4}$ M/ml (dashed line, s_2) to lower value of $8.52x10^{-5}$ M/ml (solid line, s_1) better separation between β-Lactoglobulin A peak and β-Lactoglobulin B peak was acheived. In other words, by decreasing the linear gradient slope a better resolution is achieved. However, lowering the slope require longer elution time. The resolution and

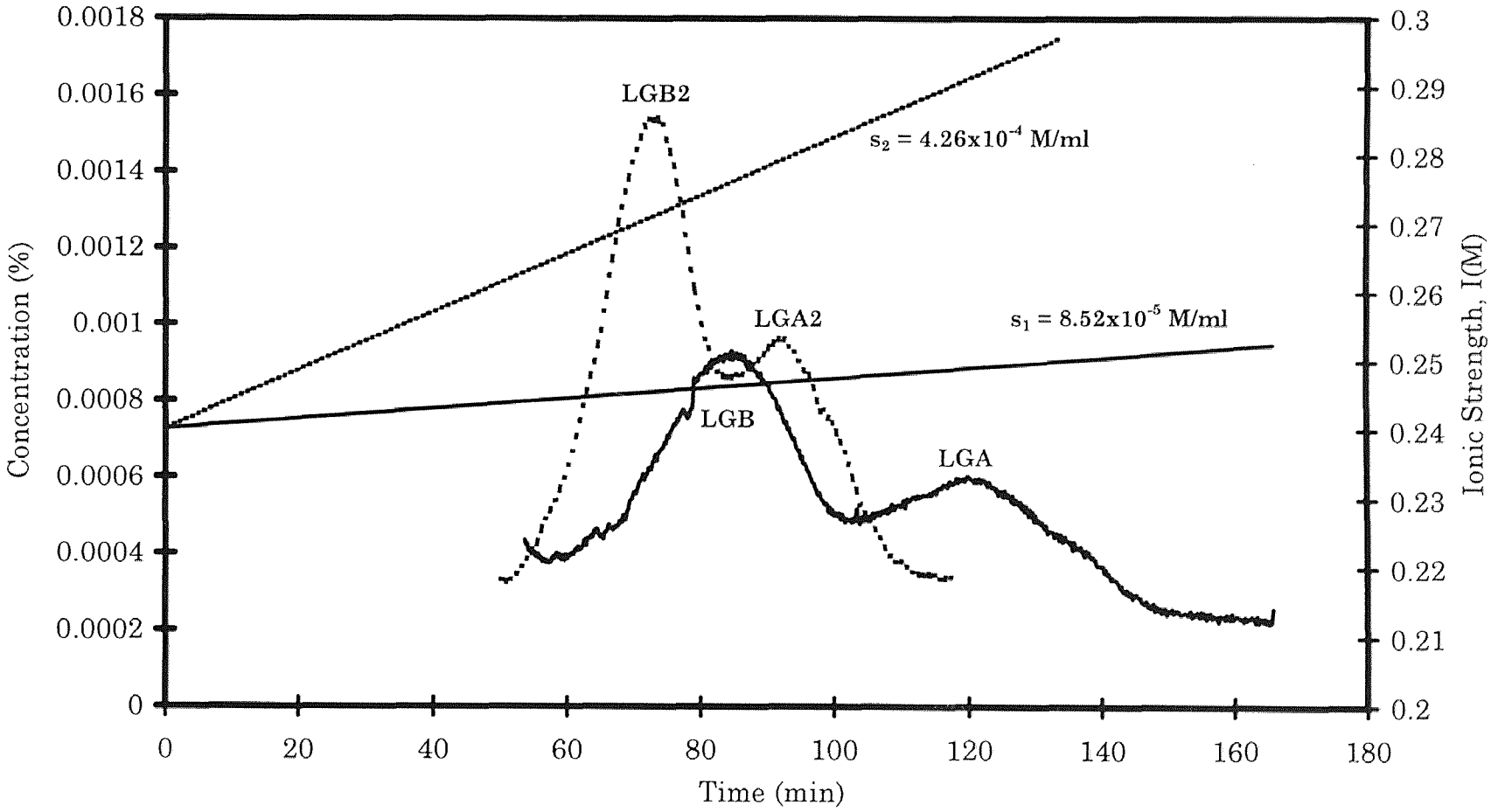

Figure 5.5. Effect of Linear Gradient Slope on Elution Porfile of β-Lactoglobulin in Linear Grdient Elution

average elution time calculated at different gradient slopes are provided in Table 5.3. and plotted in Figure 5.6.

From Table 5.3, it can be seen that as the gradient slope increases from 8.52×10^{-5} M/ml to 5.11×10^{-4} M/ml average elution time decreases from 103 minutes to 80.5 minutes, while the resolution decreases from 0.544 to 0.365. Thus gradient slope affects both resolution as well as average elution time, and both have opposite effects on the separation efficiency. In Figure 5.6, as the gradient slope, s is decreased longer elution times are need to separate β-Lactoglobulin A and β-Lactoglobulin B. That is, better resolution is achieved at the expense of longer elution time which will result in product dilution. Thus, the best kind of slope that is required will depend upon comparing the cost associated with the yield gained to longer elution times as well as the possibility of an extra separation step due to product dilution. These factors are evaluated through resolution optimization factor.

The resolution optimization factor, f_o, calculated at different linear gradient slopes for separation of β-Lactoglobulin A and β-Lactoglobulin B is given Table 5.3. Resolution optimization factor, f_o, takes into consideration the opposing effects of resolution and average elution time on separation efficiency. It will determine the best gradient slope for better resolution as well as optimum elution time to avoid an extra step for purification which results due to product dilution. In Figure 5.7, the resolution optimization factor, f_o, and resolution, R_s, are plotted against the gradient slope, s. As the

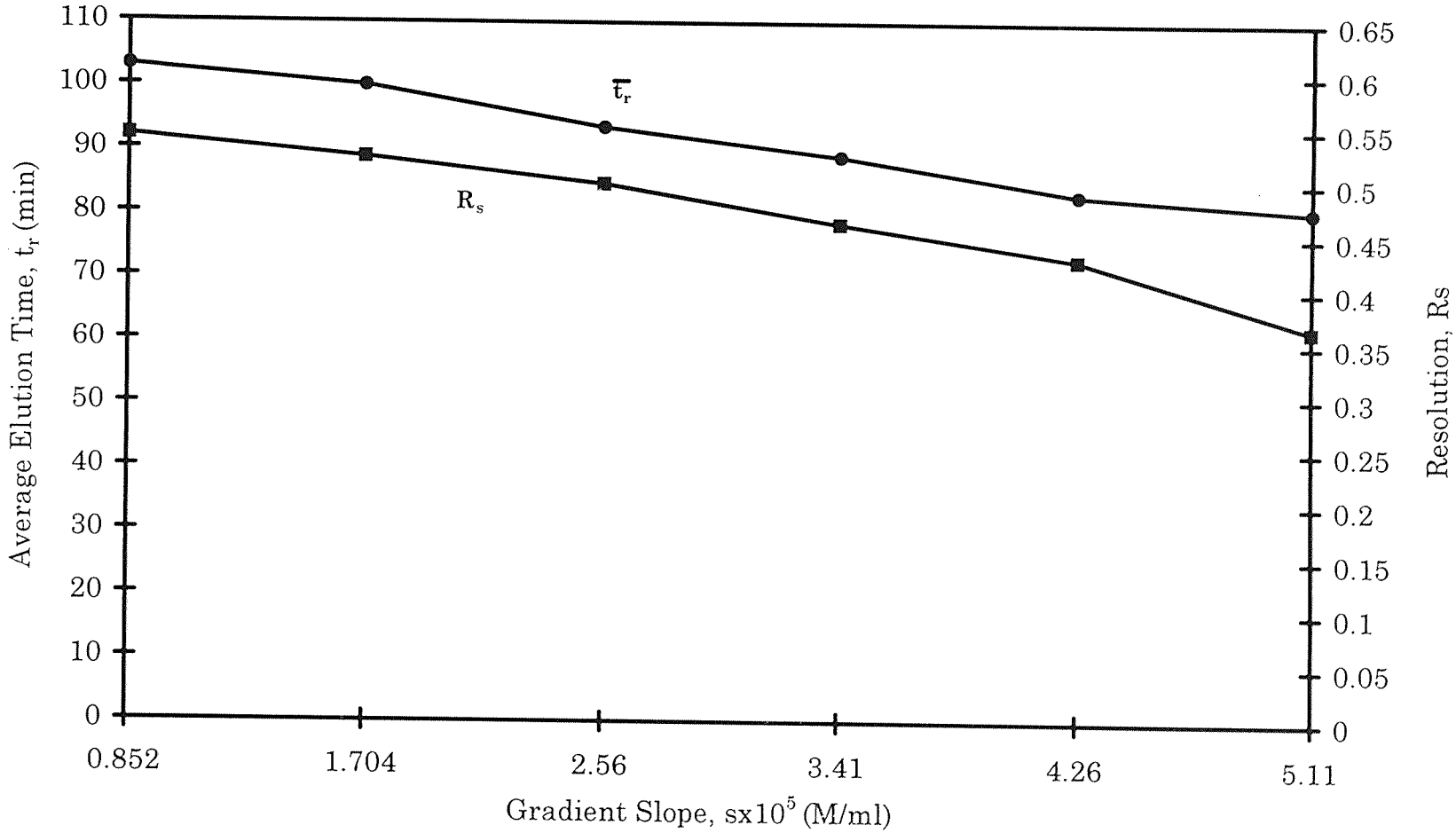

Figure 5.6. Effect of Linear Gradient slope on Resolution and Average Elution Time in Linear Gradient Elution

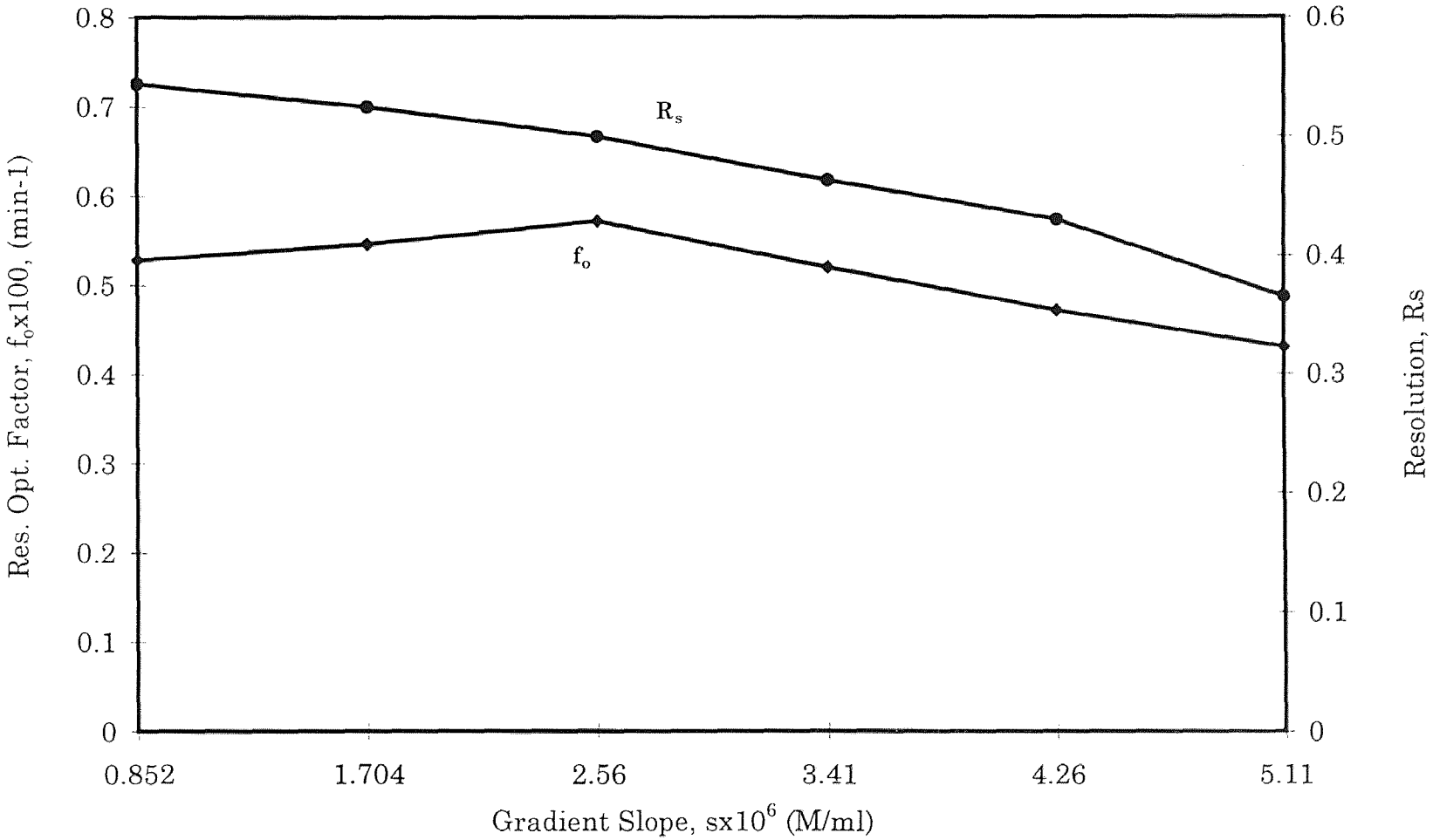

Figure 5.7. Effect of Linear Grdient Slope on Resolution and Resolution Optimization Factor

gradient slope increases from 8.52×10^{-5} M/ml to 5.11×10^{-4} M/ml the resolution, R_s, decreases from 0.544 to 0.365 whereas the resolution optimization factor first increases from 0.528 (min^{-1}) to 0.572 (min^{-1}) and then decrease to 0.430 (min^{-1}). This means that there is a gradient slope which will give us highest separation efficiency. Now we have two degrees of freedom in our separation process. If the choice is to get the highest possible resolution under given conditions ignoring the elution time, product dilution and yield, the best choice of gradient slope will be 8.52×10^{-5} M/ml. This will give the highest resolution of 0.544 with an average elution time of 103 minutes and a value of 0.528×10^{-2} (min^{-1}) for resolution optimization factor. However, if a slope of 2.56×10^{-4} M/ml is chosen it will give us highest value of resolution optimization factor and a resolution of 0.572×10^{-2} (min^{-1}) with an average elution time of 93.5 minutes. Hence, using resolution optimization factor the highest separation efficiency can be achieved.

5.2.3. Multiple Gradient Elution

In this part of study the effect of multiple gradient inputs on separation of β-lactoglobulin was studied. The effect of combination of linear gradient and stepwise increase in the ionic strength of eluting buffer on elution profile of β-lactoglobulin was investigated. The values of average elution, $\bar{t}_r$, resolution, R_s, and resolution optimization factor, f_o, were calculated from

time versus concentration (%) graphs and using equations 1, 6 and 7. The results obtained are provided in Table 5.4.

Table 5.4. Multiple Gradient Inputs and corresponding parameters.

Gradient Slope $\Delta I/\Delta V x 10^4$ (M/ml)	Ave. Elution Time $\bar{t}_r$ (min)	Resolution, R_s	Res. Opt. Factor $f_o x 100$ (min^{-1})
2.13	139	0.351	0.252
3.12	123	0.486	0.395
5.32	106	0.549	0.515
8.52	93	0.310	0.333

The elution profiles of β-lactoglobulin at $3.12x10^{-4}$ M/ml (solid line) and at $5.33x10^{-4}$ M/ml (dotted line) are shown in Figure 5.8. It consists of two parts that is the linear elution and then step change elution. The linear gradient was used from 0 to 100 minutes, and then stepwise change was made at 100 minutes. At $\Delta I_1/\Delta V=3.12x10^{-4}$ M/ml (solid line), it was observed that during the linear elution only LGB1 (β-Lactoglobulin B) was eluted while LGA1 (β-Lactoglobulin A) peak was eluted during the stepwise elution. However, when the value of $\Delta I/\Delta V$ was increased to $5.32x10^{-4}$ M/ml (dotted line) a different elution pattern was observed. That is, during the higher linear gradient elution not only LGB2 peak was eluted but some of the LGA2

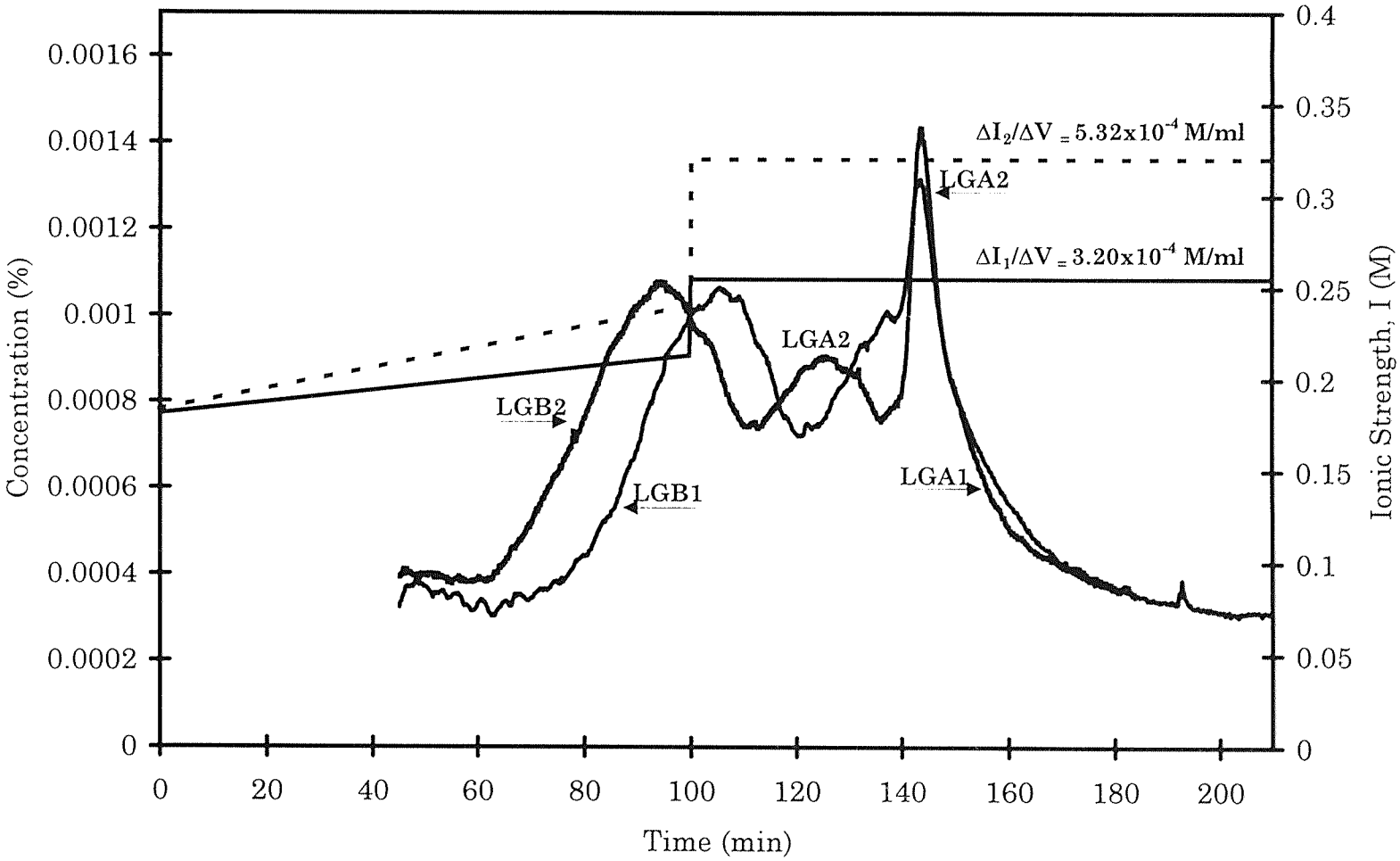

Figure 5.8. Effect of Gradient Inputs on Elution Profiles of β-Lactoglobulin in Multiple Gradient Elution

peak was observed. Due to column dead volume of about 30 minutes the concentrated portion of LGA2 peak was not observed until about 135 minutes. The resolution, R_s, average elution time, $\bar{t}_r$, and resolution optimization factor, f_o calculated at different $\Delta I/\Delta V$ are provided in Table 5.4 and plotted in Figure 5.9.

From Figure 5.9, it can be seen that as the value of $\Delta I/\Delta V$ was increased from 2.13×10^{-4} M/ml to 8.52×10^{-4} M/ml, the average elution time as expected decreased from 139 min to 93 min. However, the resolution first increases from 0.351 to 0.549 (as $\Delta I/\Delta V$ increases from 2.13×10^{-4} M/ml to 5.32×10^{-4} M/ml) and then decreases to a value of 0.310 with increase of $\Delta I/\Delta V$ to 8.52×10^{-4} M/ml. This explains that there exists an optimum combination of linear plus the stepwise gradient, which will give better separation efficiency.

This is also demonstrated by resolution optimization factor. A plot of resolution optimization factor, f_o, and resolution, Rs, versus $\Delta I/\Delta V$ is shown in Figure 5.10. As $\Delta I/\Delta V$ increases, f_o, has a maximum of 0.515×10^{-2} (min^{-1}) at 5.32×10^{-4} M/ml and then decreased to a value of 0.333×10^{-2} (min^{-1}) as $\Delta I/\Delta V$ continuously increased to 8.52×10^{-4} M/ml increases.

In multiple gradient input studies it can be observed that resolution increases as $\Delta I/\Delta V$ increases. However, continuous increase in $\Delta I/\Delta V$ results in decrease in resolution. This explains that there is an optimum value of

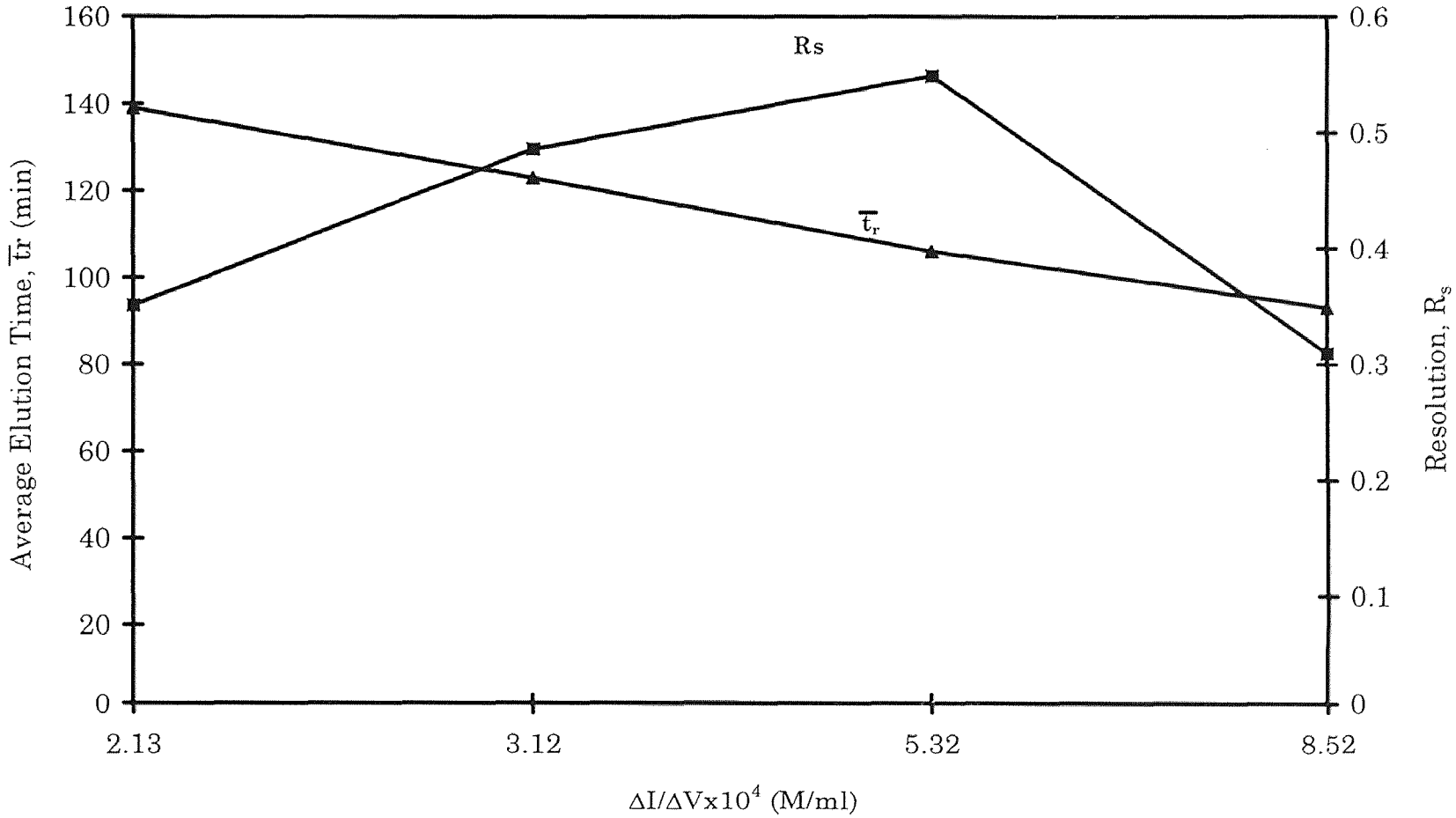

Figure 5.9. Effect of Gradient Inputs on Average Elution Time and Resolution in Multiple Gradient Elution

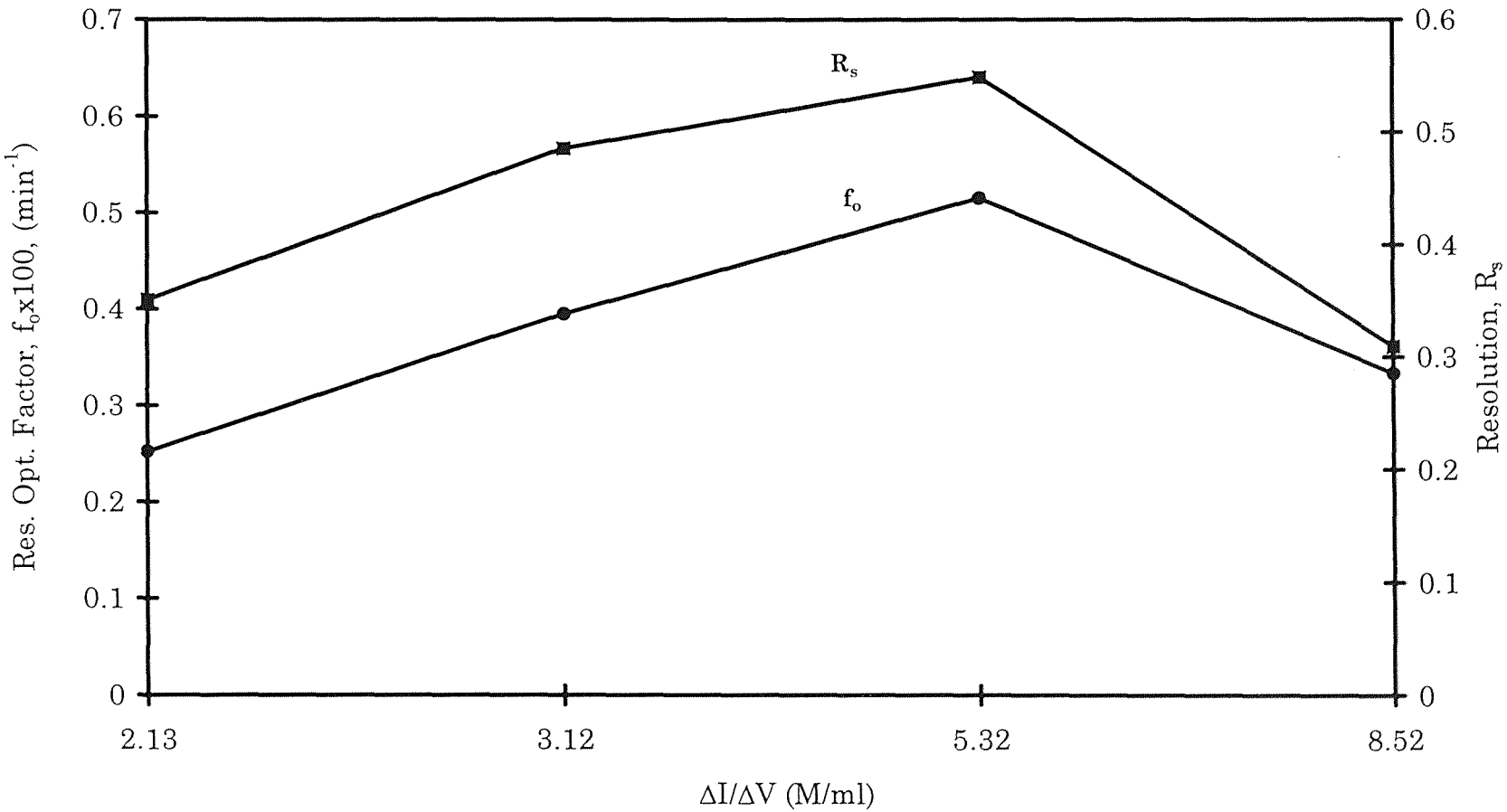

Figure 5.10. Effect of Gradient Inputs On Resolution and Resolution Optimization Factor in Multiple Gradient Elution

$\Delta I/\Delta V$ that gives us better resolution and average elution time. At this value of $\Delta I/\Delta V$ the value of resolution optimization factor is also highest thus giving us best separation efficiency. Thus, in multiple input gradient studies better resolution and better separation efficiency can be achieved if the starting conditions for elution buffer and the time at which stepwise change is made are used effectively.

CHAPTER 6

CONCLUSIONS

In processes where operating time and product dilution are of great importance, resolution alone is not adequate for describing the separation efficiency. A new parameter, Resolution Optimization Factor, f_o, was introduced. It is a function of both resolution, R_s, and average elution time, $\bar{t}_r$. Resolution optimization factor can be effectively used in gradient elution processes where operational time, product dilution and waste generation play important role on the economy of the overall process. In this work proteins β-Lactoglobulin A/B were used as a model system to conduct experiments on DEAE Sepharose column to study the effects of gradient profiles on R_s, $\bar{t}_r$ and f_o. It was observed that resolution optimization factor adequately describe the overall efficiency of a gradient elution processes by considering both resolution and average elution time.

CHAPTER 7

RECOMMENDATIONS FOR FURTHER STUDY

The effectiveness of resolution optimization factor can be tested against following changes:

1. Smaller flow rate can be used to improve the resolution of the experimental system;
2. Sample consisting of more than two component can be examined;
3. Sample component that differ in large pI point can be tested;
4. Different Ion exchanger can be studied to validate the effectiveness of resolution optimization factor in separation process.

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