

## AN ABSTRACT OF THE DISSERTATION OF

Sundar Ramanan for the degree of Doctor of Philosophy in Bioresource Engineering presented on December 14, 2000. Title: Optimization Studies in Preparative Chromatography of Biomolecules.

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Abstract approved: \_\_\_\_\_

← Ajoy K. Velayudhan

Optimization of preparative nonlinear chromatography was carried out for the first time for a biomolecule mixture. Conventional wisdom on optimization, which roots from analytical chromatography, dictates optimizing resolution in an analytical column and obtaining similar separation in a large column for isolation. Such a method of optimization significantly under uses the capacity of the column and consumes large quantities of mobile phase. Hence, in preparative chromatography, the objective function is productivity, a measure of compromise between the amount of feed that can be loaded on to the column and time. Here, we report results from optimization studies carried out on a closely related binary peptide mixture on an analytical reversed-phase column. The goal is to optimize productivity under various chromatographic modes -- nonlinear isocratic elution, gradient elution, stepwise elution and displacement chromatography. In each mode, feed mixtures at highest possible concentration (limited by solubility), for increasing feed volumes was used. Productivity was monitored for increasing feed volumes, and loading was stopped as it went through a maximum. However, in some cases, solubility limitations from one of the feed components prevented further increase in loading. Even with this constraint, high productivities

(5 — 10 g product/L stationary phase-h) were achieved. Separate experiments were carried out to measure the adsorption isotherms of these peptides over the range permitted by solubility.

Separations under nonlinear chromatographic conditions were applied to isolate commercially significant two microcystins (microcystin LR and microcystin LA) from a cyanobacterial process waste. Milligram-level loading of microcystins was obtained on a solid-phase extraction cartridge packed with 0.5 g of C<sub>18</sub> stationary phase. The separations were first carried out on an analytical column and then scaled-up to a preparative column.

We also report simple and economical process to purify phycocyanins and allophycocyanins from a cyanobacterial process waste stream for two kinds of applications food colorant and biomedical marker. A detailed design for the large-scale production of biliproteins for both applications is also presented. Economic evaluation of the process resulted in comparable costs with the current market price for food-grade product and substantially lower cost for the biomedical grade product.

**Optimization Studies in Preparative Chromatography of Biomolecules**

by

**Sundar Ramanan**

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Doctor of Philosophy dissertation of Sundar Ramanan presented on December 14, 2000

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Sundar Ramanan, Author

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My decision to continue to pursue graduate school beyond master's program although at the outset seemed like entering a tunnel with no end in sight, nor did I have any perception about the landscape when—to a certain extent, if—I get out. However, it turned out to be perhaps the best the best decision of my life yet, for I had the opportunity to work with one of the best human being I have ever come across, my advisor, Dr. Ajoy Velayudhan. I would like to thank Ajoy for not only guiding me through high level of uncertainty but also for teaching me to be a thinker. His dedication to science and research is unparalleled. I, without shame, would like to admit that I would consider myself successful when someday—hopefully in near future—I imitate his level of dedication to research. Since the unexplored terrain in my research area is vast, I have decided to stay in this field for sometime to come.

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James Tang was involved in data collection for the manuscript titled "Isolation and preparative purification of microcystin variants". Dr. Ajoy Velayudhan is the principal investigator for all the projects, and was involved in design, data analysis, and writing of each manuscript of the thesis.

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# Optimization Studies in Preparative Chromatography of Biomolecules

## Chapter 1. Introduction

Chromatography was invented as a preparative technique by a Russian botanist Mikhail Tswett (Tswett, 1906) during the early 1900's. Tswett coined the term "chromatography" meaning "writing with colors" in latin probably because he was working on the isolation of plant pigments. Coincidentally, the Russian meaning for Tswett is color. He was among the first to recognize the importance of adsorption process and the concept of differential migration, both of which are the foundations of the separation process.

Chromatography, although invented as a preparative mode, initially failed to attract researchers because the analytical methods available at that time were highly inaccurate and too slow. So this technique died slowly, only to be rediscovered four decades later as an analytical tool (Martin and James, 1952). Rapid progress was simultaneously made on the in-line detection methods and sensitivity of the detectors. During this vintage, chromatography was primarily used as an analytical technique to extract information about individual components. The separations were almost always carried out with small feed volumes with the objective of well separated Gaussian bands. If large amounts of pure components were desired, the separations were optimized for resolution in a small column and then scaled-up to a large column in a linear fashion. This method of optimization stems from the lack of fundamental understanding about

adsorption, thereby resulting in the severe under use of column capacity and excessive wastage of resources (labor, mobile phase etc.).

Here, we describe a systematic method to optimize experimental parameters in preparative and process scale chromatography. The separations were carried out under heavily overloaded conditions using various modes of chromatography (isocratic, gradient, stepwise elution and displacement). The performance of each mode was compared for increasing sample loads as measured by productivity (defined in a later section). Although, the theoretical work in nonlinear chromatography dates back to the 1950's, no experimental data is available yet, for any system of peptide or protein mixtures, that compares the efficacy of various operational modes (Guiochon, 1994). Therefore, this work will constitute the first of such kind.

## **DEFINITIONS IN PREPARATIVE CHROMATOGRAPHY**

### *(a) Purity of a product*

The purity of a product is the ratio of concentration of the desired product over the sum of concentrations of components present in the product.

$$\text{Purity} = \frac{c_1}{\sum_{i=1 \text{ to } N} c_N}$$

where  $C_1$  is the concentration of the desired component,  $N$  refers to the number of components.

### *(b) Yield*

The yield is the ratio of amount of desired component obtained at a given purity over the amount loaded on the column.

$$\text{Yield } (Y_i) = \frac{m_p}{m_i}$$

where  $m_p$  is the mass of the purified component and  $m_i$  of the same component during the feed loading step.

*(c) Cycle time*

The cycle time is the time differential between two subsequent injections. Typically, this includes the time for feed loading step ( $t_{\text{feed}}$ ), separation time ( $t_{\text{sep}}$ ) and the regeneration time ( $t_{\text{reg}}$ ).

$$\text{Cycle time } (t_{\text{cyc}}) = t_{\text{feed}} + t_{\text{sep}} + t_{\text{reg}}$$

*(d) Loading factor or feed size*

The feed size can either be represented as the feed volume ( $V_{\text{feed}}$ ) or feed mass loaded on the column. The feed mass is the product of feed concentration ( $C_i$ ) and the feed volume. Often, a dimensionless term (Loading factor,  $L_f$ ) is used as a convenient measure of sample size in preparative chromatography, and is defined as

$$\text{Loading factor } (L_f) = \frac{V_{\text{feed}} C_i}{V_{\text{SP}} \Lambda_i}$$

where  $V_{\text{SP}}$  and  $\Lambda_i$  are the stationary phase volume and column saturation capacity, respectively.

*(e) Throughput*

The throughput is defined as the ratio of amount of sample loaded on the column to the product of column volume and cycle time.

$$\text{Throughput} = \frac{C_i V_{\text{feed}}}{V_{\text{SP}} t_{\text{cyc}}}$$



*(f) Productivity*

Productivity is the ratio of amount of desired component purified at a defined purity per unit volume of stationary phase and cycle time.

$$\text{Productivity (P}_i\text{)} = \frac{C_i V_{\text{feed}} Y_i}{V_{\text{SP}} t_{\text{cyc}}}$$

The above mentioned variables are classified according to their function, and listed in Table 1.1.

Classification	Variable
Objective function	Productivity Throughput
Constraints	Purity Yield Solubility Stability of proteins (processing time)
Process parameters	Choice of stationary phase (e.g., ion exchange, reversed phase, etc.) Feed concentration Feed volume (loading) Flow rate Modulator level (e.g. salt in ion exchange chromatography and hydrophobic interaction chromatography; organic in reversed phase chromatography, etc.) Displacement (e.g., choice of displacer, concentration of displacer, etc.)

Table 1.1. Variables in preparative chromatography.

## **OUTLINE FOR THE FOLLOWING CHAPTERS**

Chapter 2 contains a systematic method to optimize separations for a binary peptide mixture under various modes by nonlinear chromatography. The purification runs were carried out on an analytical reversed phase column. The samples were loaded on the column at highest possible concentration, limited by solubility. The feed volumes were systematically increased until either the productivity decreased or the sample concentration was prohibitively high. Although, it was found that no one mode was clearly superior when either of the product was the component of interest, separations under displacement was somewhat better. However, separations by gradient elution and displacement chromatography resulted in enrichment of feed components.

Nonlinear gradient elution chromatography was applied to purify two toxins microcystin-LR and microcystin-LA from a cyanobacterial process waste. The details are provided in Chapter 3. Each step in the purification process was optimized (e.g., best solvent composition for extraction, maximum loading in the solid-phase extraction step, maximize productivity in HPLC, etc.). It was shown that better productivities were obtained by sample overloading, despite the presence of several interfering components.

Chapter 4 details a simple process to purify blue proteins (phycobiliproteins) of commercial value. Phycobiliproteins are used in the food industry as a natural colorant and as a biomedical marker in flow-cytometry. The process is designed for large-scale production, and a detailed economic analysis is also provided.

Overall conclusions are provided in Chapter 5.

## Chapter 2

### Optimization Studies in Preparative Chromatography of Chemotactic Peptides

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## ABSTRACT

Preparative chromatography is a vital process, and more often the most expensive step in the purification of valuable biomolecules. Hence, optimization of various process variables for efficient isolation of a desired product is an active area of research.

Conventional wisdom on optimization, which roots from analytical chromatography, dictates optimizing resolution in an analytical column and obtaining similar separation in a large column for isolation. Such a method of optimization significantly under uses the capacity of the column and consumes large quantities of mobile phase. Hence, in preparative chromatography, the objective function is productivity, a measure of compromise between the amount of feed that can be loaded on to the column and time.

Here, we report results from optimization studies carried out on a closely related binary peptide mixture on an analytical reversed-phase column. The goal is to optimize productivity under various chromatographic modes — nonlinear isocratic elution, gradient elution, stepwise elution and displacement chromatography. In each mode, feed mixtures at highest possible concentration (limited by solubility), for increasing feed volumes was used. Productivity was monitored for increasing feed volumes, and loading was stopped as it went through a maximum. However, in some cases, solubility limitations from one of the feed components prevented further increase in loading. Even with this constraint, high productivities (5 — 10 g product/L stationary phase-h) were achieved.

## INTRODUCTION

Preparative and process scale separations of biomolecules has been of considerable interest in recent years (Colin, 1993; Sofer and Nystrom, 1989). Chromatography remains the method of choice for purifying desired components for various applications. Of particular interest, is the purification of high value pharmaceutical compounds where the desired purity is 99.99% or greater (Wheelwright, 1991), where HPLC is routinely used. The separation is typically carried out under one of the following modes: isocratic, gradient, stepwise and displacement chromatography. Elution chromatography is one of the most widely used technique for the purification of proteins and peptides (Yamamoto et al., 1987; El Fallah and Guiochon, 1992; Jungbauer, 1994; Hu et al., 1992; Gallant et al., 1995). Displacement chromatography, an intrinsically nonlinear mode, has several advantages including the enrichment of valuable feed components. Displacement, invented by Tiselius (1943), was first used for the separation of amino acids and peptides using activated carbon adsorbents by Synge and Tiselius (1947). Concurrently, Bendall et al. (1947) reported displacement separation of amino acids on an ion-exchange column. The displacement of biomacromolecules, such as proteins, was also attempted by Hall and Tiselius (1951) with limited success, mainly due to the unavailability of efficient stationary phases. First reports on the separations of an antibiotic polypeptide was published by Poráth (1952, 1954), and oligosaccharides and branched fatty acids by Claesson (1946). After 20 years of dormancy, displacement chromatography was revived by Horváth (1985), and has since found many applications including separation of peptides on reversed-phase columns (Viscomi et al., 1988; Viscomi et al., 1991., Vigh et al., 1987; Cramer et al., 1987; Frenz et al., 1985;

Kalghatghi et al., 1992). Displacement separation of proteins on an ion-exchange system has been investigated by several groups (Peterson and Torres, 1983; Liao et al., 1987; Subramanian et al., 1988; Gerstner and Cramer, 1992) 7in recent years. A detailed discussion on the evolution, theory and applications of displacement is found in Frenz and Horváth (1988)

Optimization in preparative chromatography has received increased attention recently (Colin, 1993; Felinger and Guiochon, 1992a; Felinger and Guiochon, 1992b; Guiochon et al., 1994). Productivity is used in this study as a measure of the effectiveness of displacement in separating a binary peptide mixture. Classically, the objective in displacement chromatography is to attain the final pattern of contiguous rectangular bands of feed ahead of the displacer front. Although such final patterns give high yields, they may not result in high productivities. As pointed out by Gadani et al. (1995) and Zhu and Guiochon (1995), maximum productivity is likely to be achieved by mixed bands that are far from their corresponding final patterns.

In this paper, all common modes of chromatography were used to separate a pair of closely related peptides, n-formyl-Met-Phe (denoted by P) and n-formyl-Met-Trp (denoted by T) on a reversed-phase column using ACN as the mobile phase modulator. These peptides are implicated in bacterial chemotaxis (Marasco et al., 1984; Budrene and Berg, 1995). Solubility of P and T in the mobile phase was limited and acted as a major constraint in optimization. P was found to be weakly soluble at the low pH's used in typical reversed-phase runs. It was also observed that the solubility of P increased in the presence of T. Hence, P and T were first suspended in ACN-phosphate buffer solution; only after they were completely dissolved was TFA added. This kept the P in solution; by

contrast, adding P directly to ACN-phosphate buffer-TFA solution does not dissolve all the P. Detailed discussions on the solubility studies for this system was reported in Kim (1997).

Productivity was calculated for each feed separately using the expression:

$$\text{Productivity} = \frac{c_f V_f Y}{t_c V_{sp}} \quad (2.1)$$

where  $c_f$  and  $V_f$  are the concentration and feed volume respectively,  $Y$  is the yield at 98% purity,  $t_c$  is the cycle time and  $V_{sp}$  is the adsorbent solid volume (excluding pore volume).

The cycle time is the sum of the separation time and the time required for column regeneration. Operating parameters such as initial modulator level, gradient slope, displacer concentration and flow-rate were varied systematically to obtain optimal productivity. The consequences of changing the modulator level associated with the displacer are also reported. Commercial displacers invariably contain impurities; these impurities may affect the separation [Jen and Pinto, 1993; Shukla et al., 1998]. The effect of displacer impurities on the separation of P and T was quantified for one case.

## **MATERIALS AND METHODS**

The peptides n-formyl-Met-Phe (P) and n-formyl-Met-Trp (T) were obtained from Sigma Chemicals (St. Louis, MO, USA). Sodium monobasic phosphate and sodium dibasic phosphate was purchased from Mallinckrodt (Paris, KY, USA). HPLC-grade acetonitrile was obtained from EM Science (Gibson, NJ, USA), and sequanal-grade trifluoroacetic acid (TFA) from Pierce (Rockford, IL, USA) respectively. Water was distilled and deionized using the Milli-Q ultra-pure water system (Millipore Co., Bedford,

MA, USA). Tris[2-methoxyethoxy]ethyl]amine (TMEEA) and benzethonium chloride were purchased from Aldrich Chemicals (Milwaukee, WI, USA). Other candidates (5-nonanone, 4-heptanone, 3,4 dimethoxybenzylalcohol, n-benzylbenzamide, diethyldodecamide, heptoxybenzylchloride) were obtained from the Department of Chemistry at Oregon State University (Aldrich Chemicals, Milwaukee, WI, USA).

### *Apparatus*

All preparative separations and subsequent analysis of fractions were performed on a Waters (Milford, MA, USA) HPLC system, which consisted of a quaternary pump (Model 600), UV detector (Model 486), and an autosampler (Model 717 plus). All the units were controlled by a DEC (Nashua, NH, USA) personal computer using Waters Millennium software. The feed mixture for all preparative separations was loaded using Model 7125i injector (Rheodyne, Cotati, CA, USA). Displacement separations by different displacers were carried out on the same Nova-Pak C<sub>18</sub> column (Waters, Milford, MA, USA) with dimensions of 150 mm × 3.9 mm I.D. The average particle size of the packing was 4 μm with an average pore size of 60 Å.

### *Procedures*

*Sample Preparation:* The peptides P and T were dissolved in ACN-phosphate buffer-TFA with either 10:90:0.1 or 15:85:0.1 v/v% composition. Phosphate buffer was made by preparing a 10 mM dibasic solution to which 10 mM monobasic solution was added until the pH reached 7. For all feed preparations TFA was added after the feed was completely solubilized in the organic-buffer mixture. In all preparative runs the mobile



phase composition of feed was identical to the initial state of column, since previous work (Kim and Velayudhan, 1998) has shown that otherwise the separation quality suffered.

*Elution runs:* The column was first equilibrated with the desired modulator level (e.g., isocratic runs, ACN-phosphate buffer-TFA, 15:90:0.01). The feed containing P and T were loaded into the sample loop. The feed loading step was followed by either no change in the modulator level (nonlinear isocratic runs), a linear gradient (nonlinear gradient runs) or a step increase in the modulator level (step-wise elution). Product was collected at the UV-detector outlet as 15 or 30-second fractions.

*Displacement runs:* The reversed phase adsorbent was first equilibrated with ACN-phosphate buffer-TFA (10:90:0.1) mobile phase mixture. The feed containing P and T in the same mobile phase composition was then loaded into the sample loop. After the end of feed introduction the displacer solution was fed continuously into the column. Fractions were collected at the outlet of the UV detector in 15 or 30-second fractions.

*Column Regeneration:* After the emergence of peptides and displacer, the column was washed three times using a gradient cycle from 10% to 95% ACN in 15 min followed by equilibrating the column at 20% ACN for analysis of fractions.

However, we were also able to clean the column by stepping up to 100% ACN and washing at 2 ml/min for 10 min. Then, the mobile phase composition was stepped down to 20% ACN and equilibrated at this level for 10 min. The entire regeneration step lasted 20 min; the latter regeneration time was used for all productivity calculations. The cycle time for elution runs were also 20 min.

*Peptide Analysis:* The peptide fractions from preparative runs were diluted to fall within their calibration curves, and were analyzed using the same column, with ACN-phosphate buffer-TFA (20:80:0.1) for mobile phase under isocratic conditions. The peptides were monitored using the UV detector at 214 nm. The data from all the fractions was used to reconstruct the chromatograph.

*Displacer Analysis:* Benzethonium chloride was analyzed under isocratic conditions using ACN-phosphate buffer-TFA mixture (60:40:0.1) for mobile phase and detected at 214 nm. Benzethonium chloride was only 97% pure, and the remaining 3% impurities consisted of at least two or three different components, as seen in the frontal runs at various displacer concentrations. However, only one other peak was seen under analytical conditions. Since the impurities were just ahead of the displacer, the other peak was considered the main impurity, and quantified in the same way as benzethonium chloride.

The absorbance of TMEEA was highly nonlinear: while it is easily visible beyond 2 mg/ml, concentrations below this level gave very low peaks. This makes it difficult to analyze collected fractions. Attempts to quantify TMEEA are discussed later.

## **RESULTS AND DISCUSSION**

### *Feed properties*

The feed components (P+T), although a mixture of small molecules, exhibited several features characteristic of macromolecules. They had very limited solubility in the chromatographic mobile phase (low pH). The solubility of P was better in the presence of T. ACN/buffer solution was somewhat better in solubilizing P+T than ACN/water

solution. The presence of TFA decreased their solubility. For a detailed summary on the feed properties the reader is directed to the M.S. thesis of Kim (1997).

The retention properties ( $k'$  versus modulator plot, Figure 2.1) and the single-component isotherms (Figure 2.2) for these closely related peptides were reported in Kim and Velayudhan (1998). P and T showed converging retention behavior with increasing modulator levels.

### *Nonlinear isocratic elution runs*

The mobile phase conditions for isocratic runs were chosen based on Figure 2.1. The retention factors plot clearly indicate high selectivity at 10% ACN. However, choosing this condition for preparative runs could result in unreasonably long times leading to poor productivity values. Preparative isocratic runs carried out at 20% ACN (data not shown) resulted in significant mixing. Therefore, the modulator level of 15% ACN, which reflects the best compromise between the separation time and selectivity, was chosen for subsequent nonlinear isocratic runs. Figure 2.3 is the chromatogram for a 5 ml feed volume run. Although there is mixing between the P and T, highest productivity was achieved for P from this run. It is evident from the figure that the effluent P concentration is higher than the feed level. This is due to two effects: the "self interference" of P at high loading, and the "mutual interference" or the pushing effect of T. The productivity of T was higher at a lower loading since the fractions with highest concentrations were mixed with P for a 5 ml run. Further increase in loading resulted in decreased productivity (figures not shown). The comparison of efficacy of various modes is given in a later section.

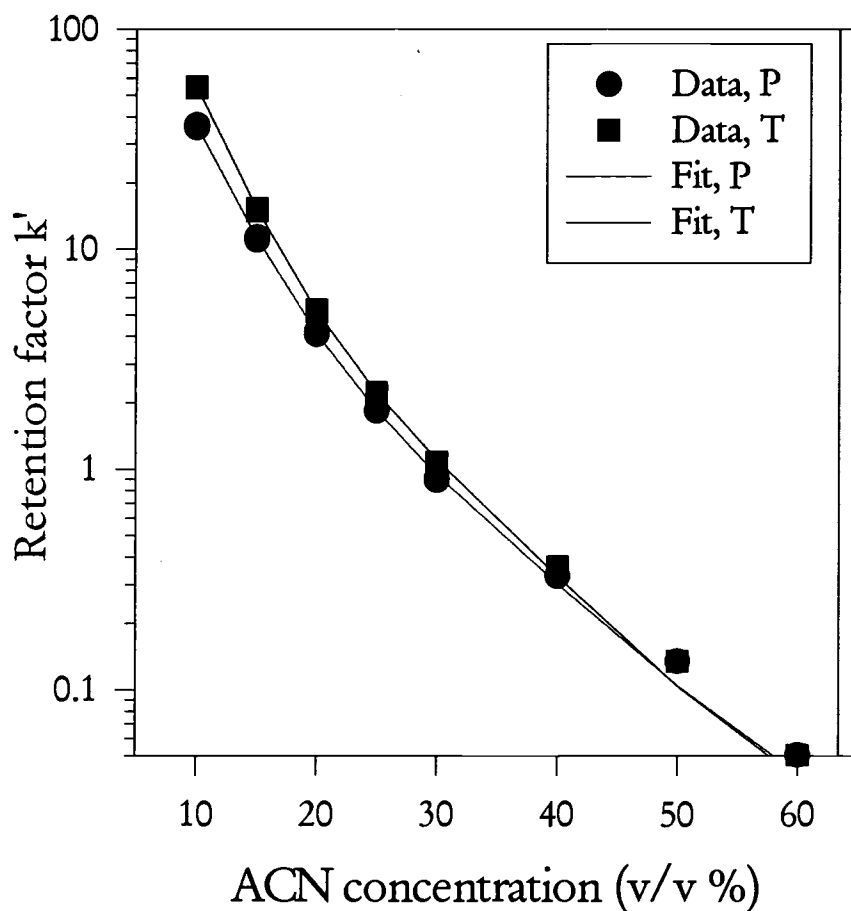


Figure 2.1. The retention factors plot for P and T as a function of ACN concentration. The data are shown in circles (P) and squares (T), and double-exponential fits to the data are shown as solid lines. *Reproduced with permission from Kim, B. and A. Velayudhan, J. Chromatogr. A, 796, 195-209 (1998).*

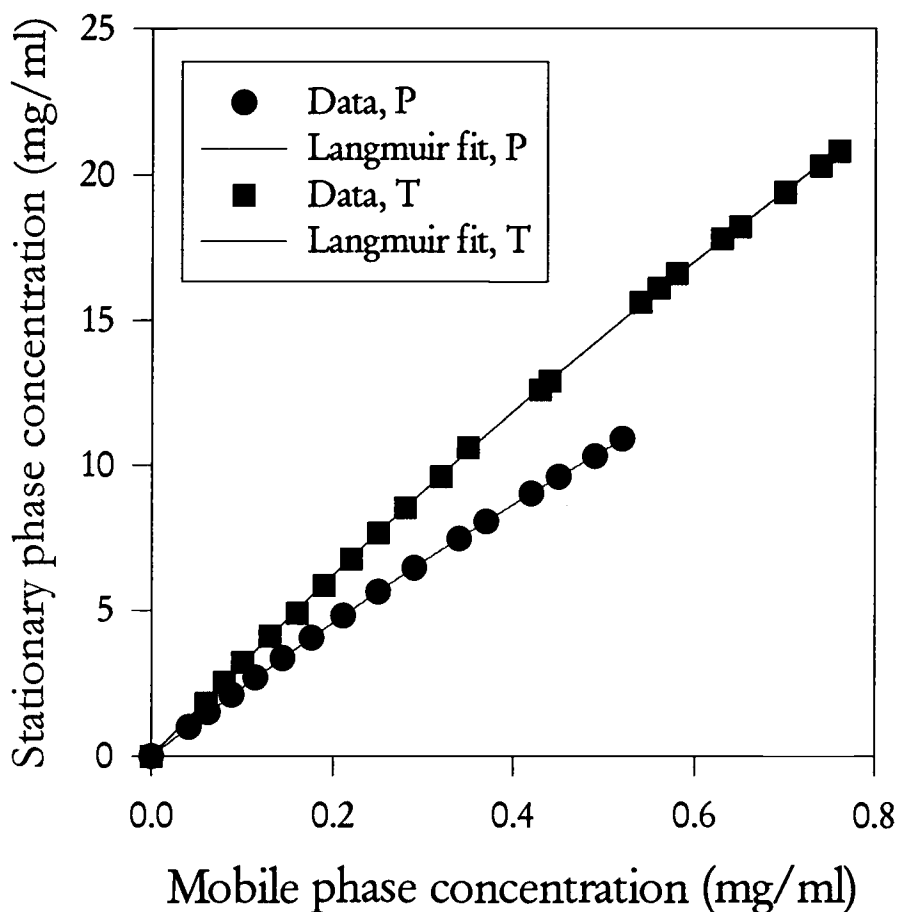


Figure 2.2. Single-component isotherms for P and T. Elution by characteristic-point (ECP) was used from nonlinear isocratic runs for each component. The chromatographic conditions were ACN-buffer-TFA (15:85:0.1). *Reproduced with permission from Kim, B. and A. Velayudhan, J. Chromatogr. A, 796, 195-209 (1998).*

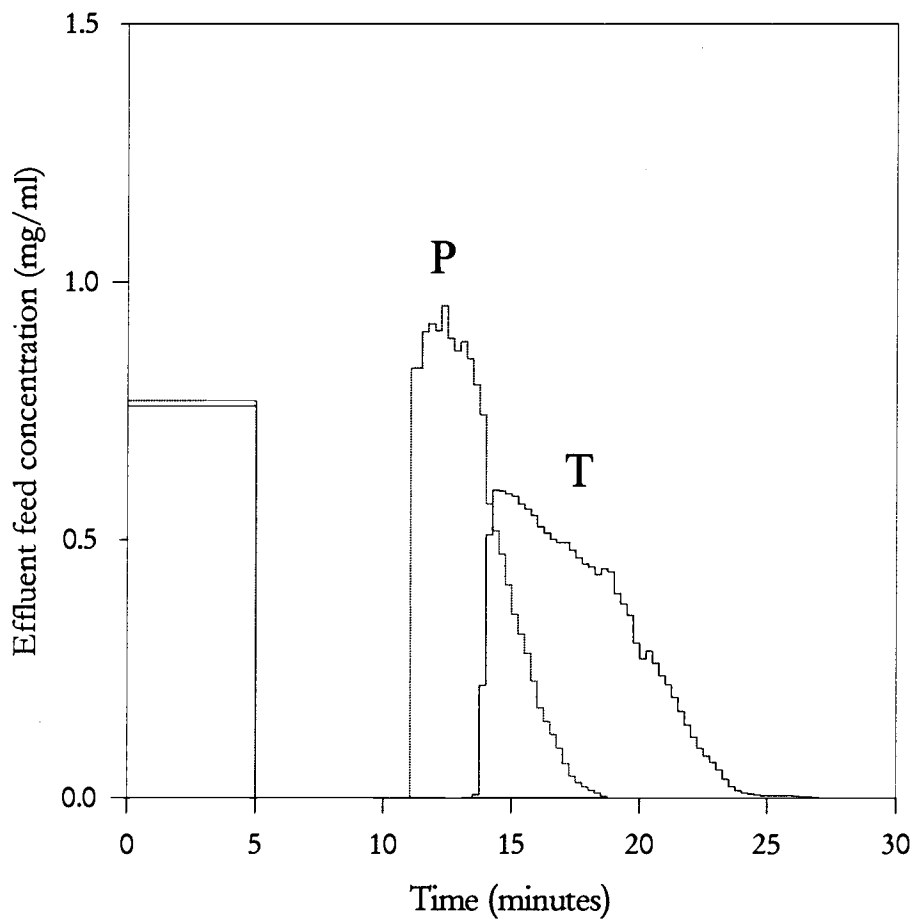


Figure 2.3. Chromatogram of preparative nonlinear isocratic elution run at ACN-buffer-TFA (15:85:0.1). Experimental conditions: feed volume, 5 ml; flow rate, 1 ml/min; fraction size, 15 sec.

### *Stepwise elution runs*

The initial modulator level for the stepwise runs was chosen as 10% ACN in order to take advantage of the higher selectivity at this modulator level. Then, the modulator level was increased to 15% ACN in 0.5 min. Figure 2.4a shows the chromatogram for a 3.4 ml feed volume run where the separation was almost complete. When the feed volume was increased to 5.0 ml, although mixing occurs, the productivity of P increased. Again, enrichment of P occurred similar to the isocratic runs. During the 5.0 ml run, P crystallized immediately after eluting from the column. Therefore, further increase in loading was not carried out in order to prevent on-column crystallization. The maximum productivity for T occurred at 3.9 ml feed loading (figure not shown). The performance of stepwise elution in comparison to other modes is given in a later section.

### *Gradient elution runs*

The important parameters in gradient elution chromatography are the initial modulator level and the gradient slope. Three kinds of gradient runs were carried out:

- (1) Low initial modulator level, steep slope (10 to 40% ACN in 20 min)
- (2) Low initial modulator level, shallow slope (10 to 40% ACN in 30 min)
- (3) High initial modulator level, steep slope (15 to 40% ACN in 20 min)

The 10 to 40% ACN in 20 min runs gave extremely concentrated bands even for 3.0 ml feed volume (figure not shown). The concentration effect was due to the phenomena called "gradient focussing", and briefly explained as follows: when a wide feed band is run under gradient conditions, the front portion of the solute band sees a lower modulator concentration while the latter portion is present at a higher modulator

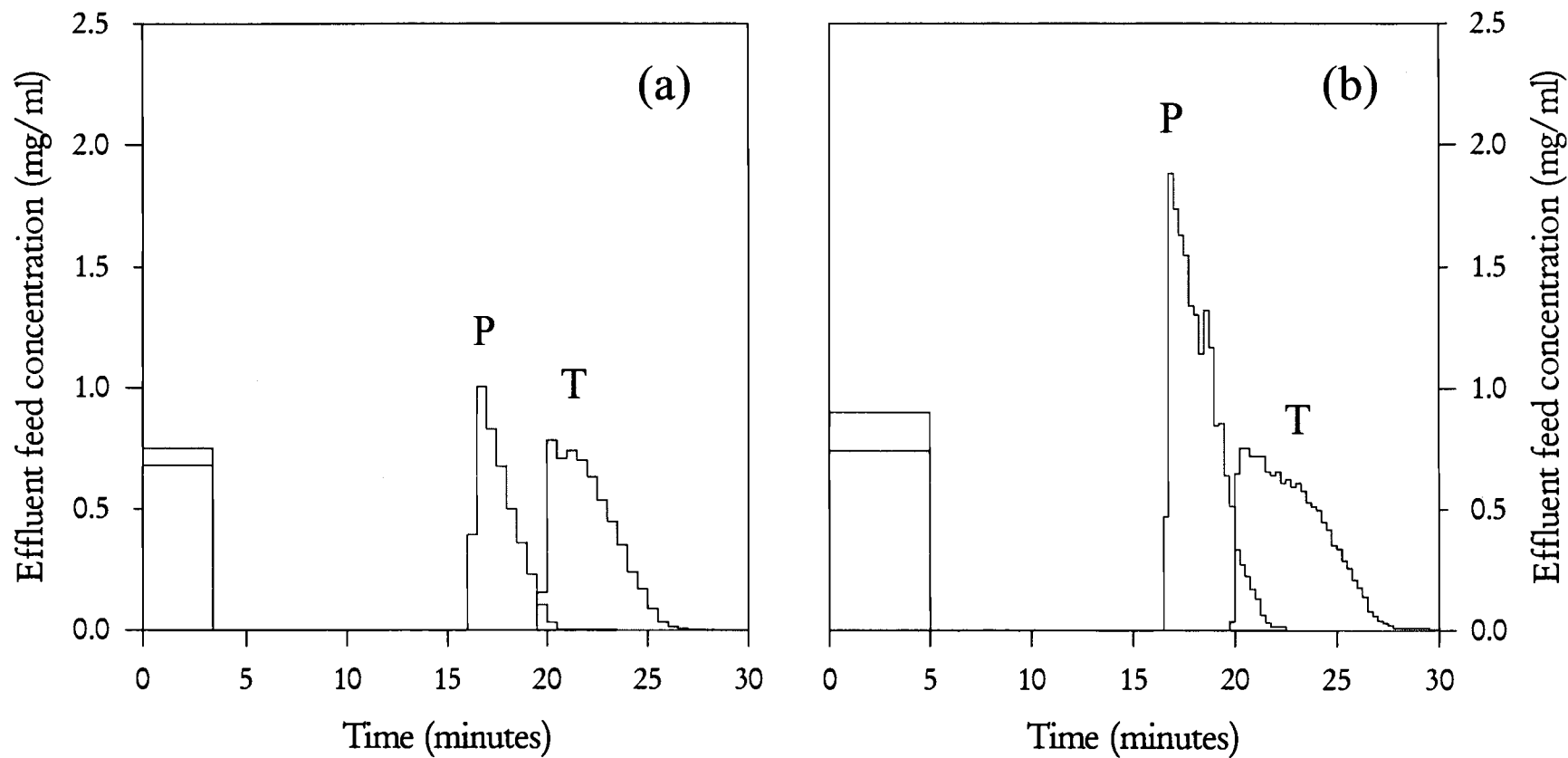


Figure 2.4. Chromatogram of preparative stepwise elution run. The feed volumes were 3.4 ml (panel a), 5.0 ml (panel b). The step from 10 to 15% ACN occurs at 0.5 min.



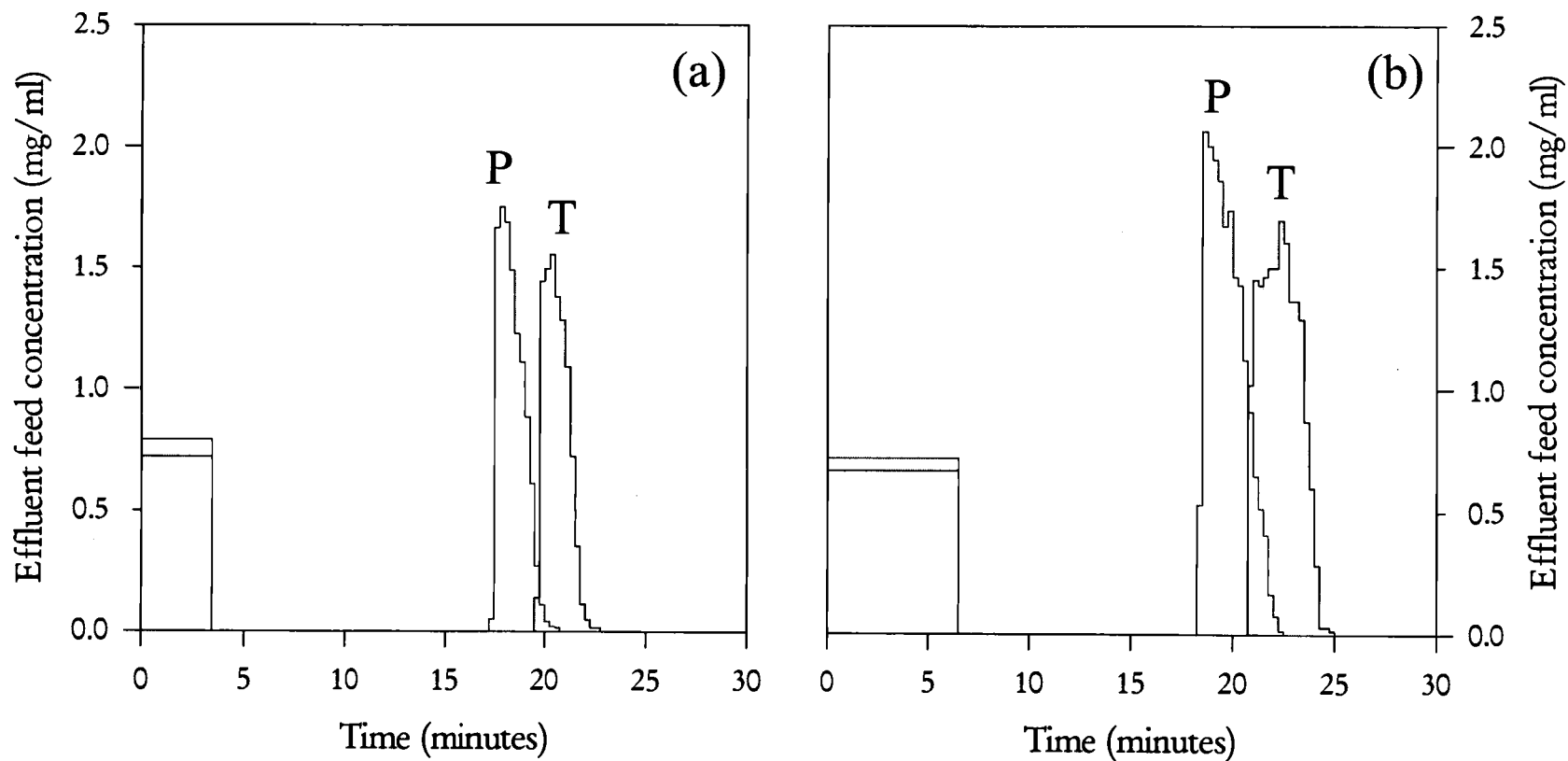


Figure 2.5. Chromatogram of preparative gradient elution run. The feed volumes were 3.4 ml (panel a), 6.5 ml (panel b). Gradient from 10 to 40% ACN in 30 minutes. All other conditions as in Figure 2.3.

level. It is known that the retention of the solute at lower modulator level is high. Therefore, the front portion of the solute band travels at a slower rate. The back portion of the solute band, which is amidst the higher modulator level, elutes faster. The combination of slower moving front portion and the faster moving back portion of the solute band causes the band to focus on one point in the gradient, hence the focussing effect. To prevent on column crystallization further increase in loading was discontinued.

Figure 2.5 shows the chromatogram for the gradient run with shallow slope. As anticipated, the maximum concentration decreased (panel a) suggesting that the focussing effect has decreased. This enabled further increase in loading, resulting in higher productivity. Gradient run carried out at high initial modulator level and steep slope resulted in an early eluting mixed bands with high concentration even for 3.0 ml feed volume. Hence, further loading was not carried out.

#### *Displacement of peptides by benzethonium chloride*

Benzethonium chloride was recently shown (Shukla et al., 1998) to be a useful displacer in hydrophobic interaction chromatography; here its potential as a displacer in reversed phase is examined. Frontal runs of benzethonium chloride in ACN-phosphate buffer-TFA (15:85:0.1) were carried out at various concentrations, and shown in Figure 2.6a. The single-component isotherm was constructed from the shocks in the frontal runs, and is shown in Figure 2.6b. Based on the single-component isotherms of benzethonium chloride and of the feed components, displacer concentrations of 7.5 mg/ml and 10 mg/ml were chosen. Displacement runs were carried out for both concentrations at increasing feed volumes until the productivity dropped for either

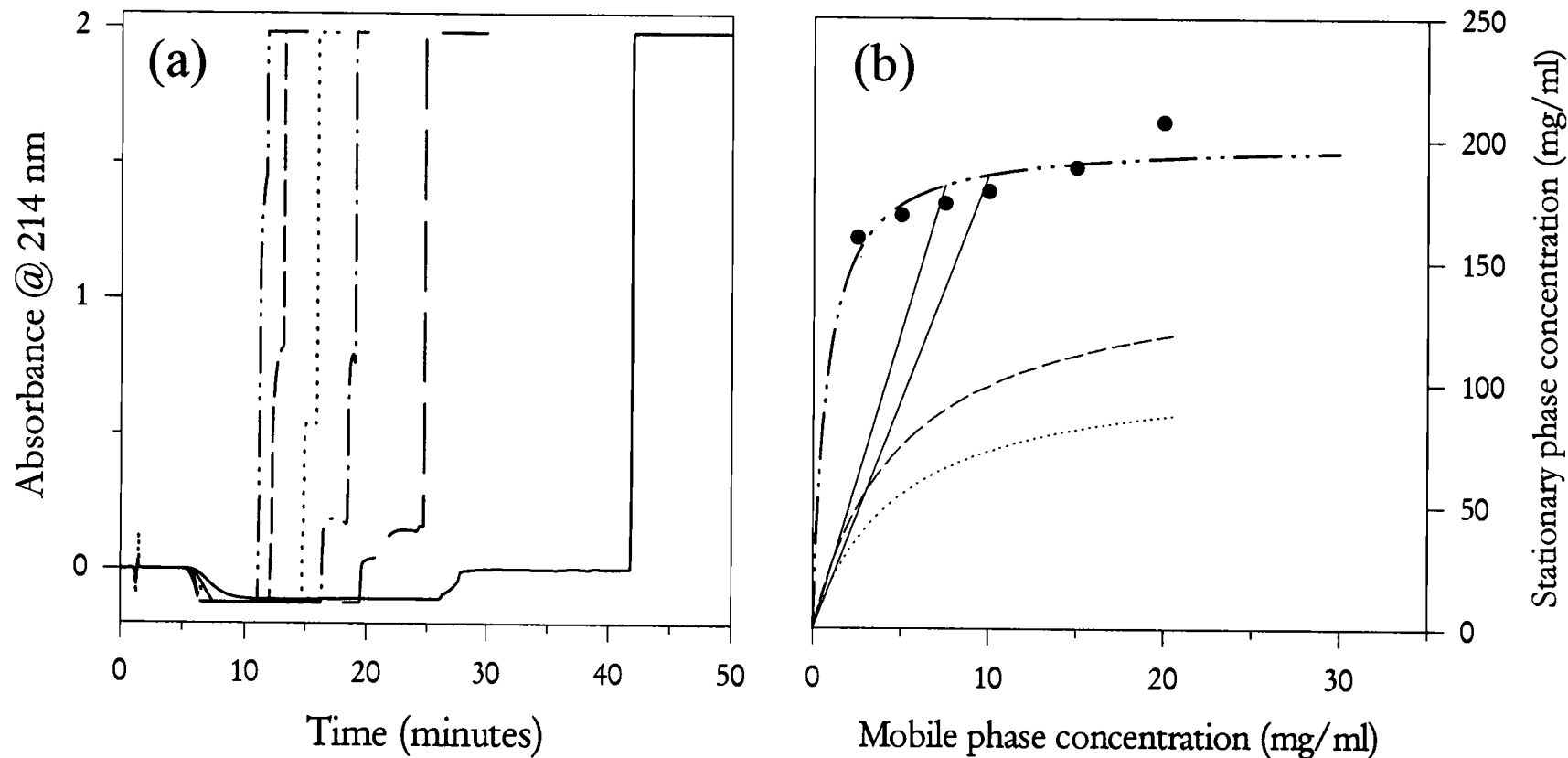


Figure 2.6. (Panel a) Frontal runs at various benzethonium chloride concentrations [2.5 mg/ml (solid line), 5 mg/ mg/ml (dots), 15 mg/ml (dash), 20 mg/ml (dash-dot-dot)]; displacer suspended in ACN-phosphate buffer-TFA (15:85:0.1). (Panel b) Single-component adsorption isotherm of benzethonium chloride (dash-dot-dot) constructed from the frontal runs in (a). Operating lines for 7.5 mg/ml and 10 mg/ml shown (solid lines). Single-component isotherms for P (dots) and T (dashes) from Kim (1997) are also shown.

component. In some cases, further increase in feed volume was not possible due to solubility constraints, even though the productivity was still increasing. From previous studies on solubility for the P+T system (Kim, 1997), it is known that P in particular had very limited solubility. Hence, when the effluent concentration of P reached 2.5 mg/ml, runs with larger feed volumes were not attempted.

The column was first equilibrated at 10% ACN followed by loading of feed containing 10% ACN. The displacement step, where the displacer was suspended in 15% ACN, started 0.5 min after the end of feed introduction. Figures 2.7a, b and c show the displacement results at 7.5 mg/ml benzethonium chloride for feed volumes of 3.9, 5.0, and 6.5 ml respectively. For convenience, the feed input into the column is also shown, starting at  $t = 0$ . The maximum concentration of P increased with increasing feed volume. In all cases, P showed a right-triangle pattern with tailing into T, suggesting that P elutes. The increase in concentration of the early fractions is then attributed to the generation of a concentrated band of P during loading ("roll-up") [Ruthven, 1984]. The amount of mixing increased steadily with loading; however, the productivity of P reached a maximum at 6.5 ml. Further increase in feed volume to 7.4 ml (figure not shown) resulted in decreased productivity since the amount recovered at 98% purity decreased. In all cases, the last fraction of T is significantly more concentrated than the others. The rectangular pattern for T was more developed for low feed volume runs (3.9 and 5.0 ml) than for higher feed volume runs (6.5 and 7.4 ml). This is because of greater mixing between P and T at higher feed volumes. The inset in all diagrams shows the quantification of the most strongly retained impurity of benzethonium chloride. The first fraction of this impurity in Figures 2.7a and 2.7b is highly concentrated. However, this

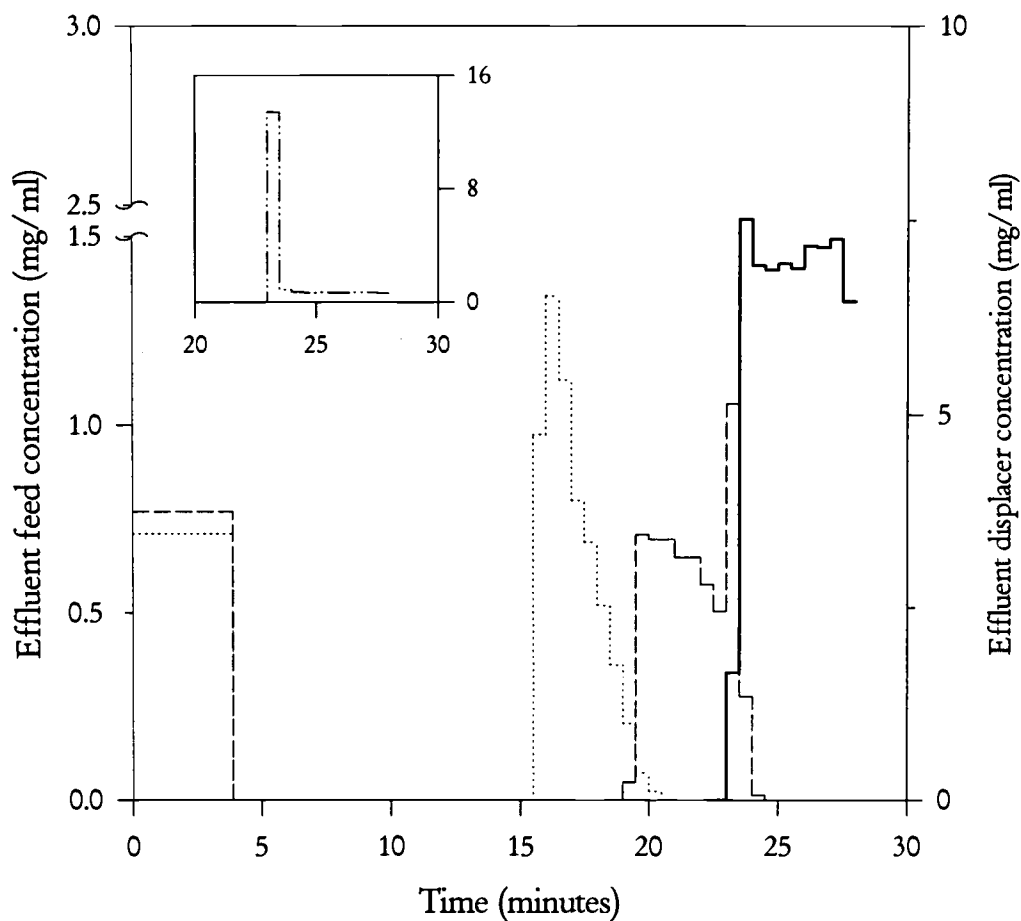


Figure 2.7a. Displacement chromatogram using 7.5 mg/ml benzethonium chloride as displacer in 15% ACN. Feed: 0.71 mg/ml P (dots) and 0.77 mg/ml T (dashes) in 3.9 ml at 10% ACN; displacer, benzethonium chloride (solid line); flow-rate, 1 ml/min; fraction size, 0.5 ml each.

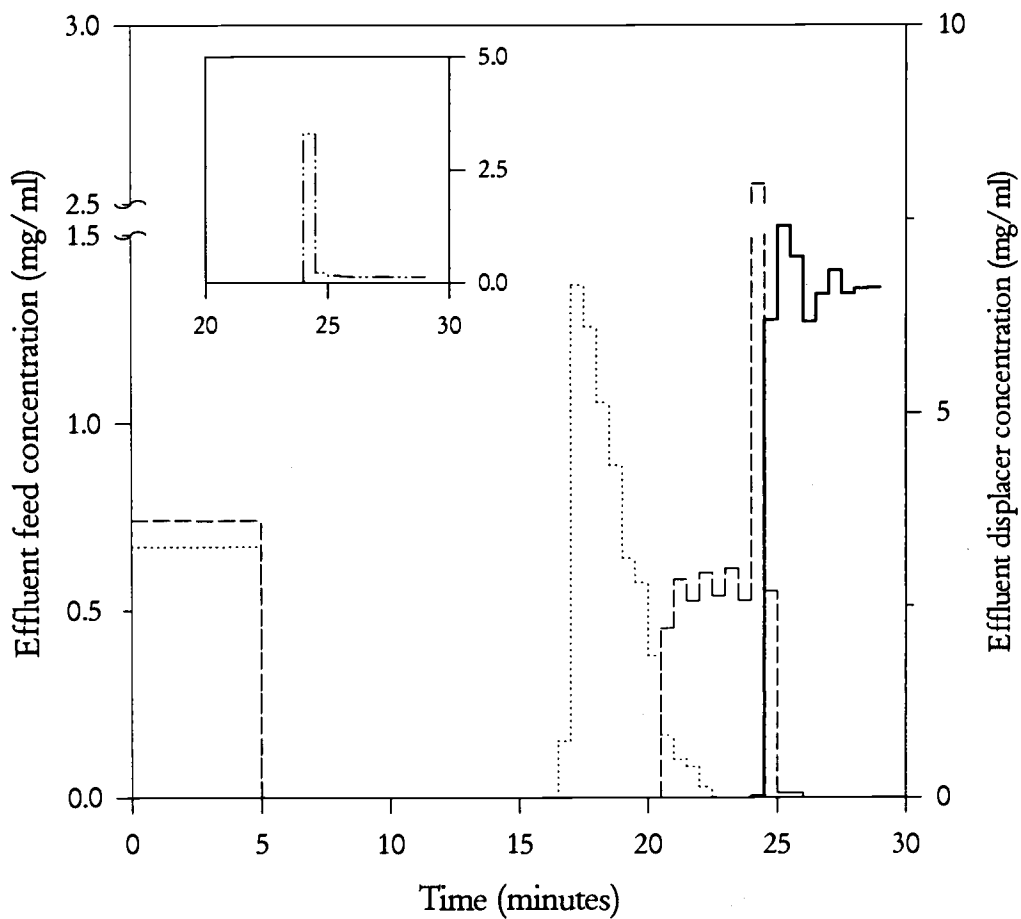


Figure 2.7b. Displacement chromatogram using 7.5 mg/ml benzethonium chloride as displacer in 15% ACN. Feed: 0.67 mg/ml P and 0.74 mg/ml in 5.0 ml feed volume. All other conditions as in Figure 2.7a.

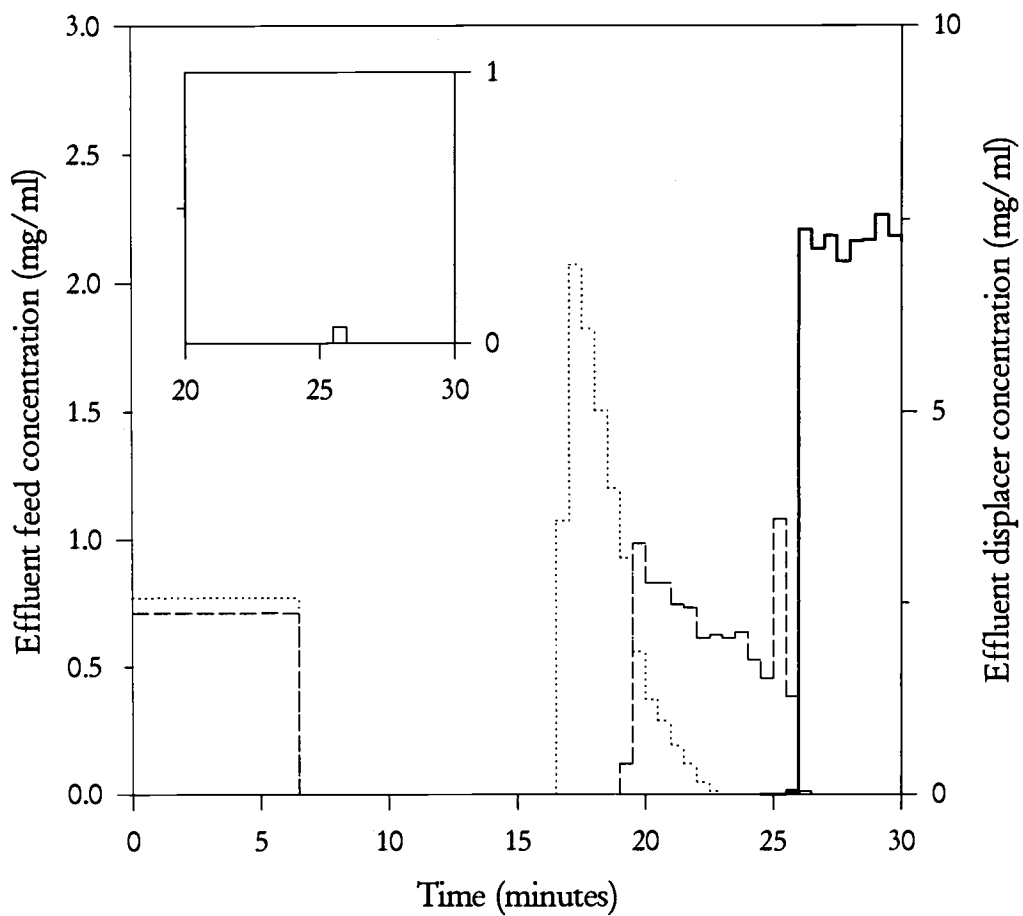


Figure 2.7c. Displacement chromatogram using 7.5 mg/ml benzethonium chloride as displacer in 15% ACN. Feed: 0.76 mg/ml P and 0.71 mg/ml T in 6.5 ml feed volume. All other conditions as in Figure 2.7a.

impurity was found to degrade quickly at room temperature in separate experiments, which could explain its low level in Figure 2.7c. Separations beyond 7.4 ml feed volumes for 7.5 mg/ml benzethonium chloride concentration were not carried out since the productivity for both P and T dropped.

Figure 2.8 shows the chromatograms for 3.9, 6.5 and 7.9 ml feed volumes for 10 mg/ml benzethonium chloride. Again, P shows similar behavior, indicating elution. However, T formed a narrow concentrated band in the 3.9 ml run. As the feed volume increased, so did the mixing, resulting in lower concentrations of T further ahead of the displacer front. Feed volumes beyond 7.9 ml were not used because of solubility constraints on P, even though productivity was still increasing. Relatively low levels of the impurity were found, which is again attributed to degradation. A detailed comparison of productivities will be made in a later section.

#### *Displacement of peptides by TMEEA*

Frontal runs of TMEEA in 15% ACN were carried out at different concentrations, and are shown in Figure 2.9a. In order to measure the linear retention ( $k'$ ) of TMEEA (so as to describe the initial slope of the single-component isotherm), gradient runs (steep and shallow) were carried out under analytical conditions, following the method of Quarry et al. (1986). However, it was found that TMEEA did not confirm to LSS behavior (i.e., linear retention did not vary exponentially with modulator level). Hence, another approach was taken: gradients of varying slopes starting at 15% ACN were performed. A double exponential fit was used to fit the relationship between  $k'$  and gradient slopes, and was then extrapolated to estimate the isocratic retention time at 15% ACN. The single-



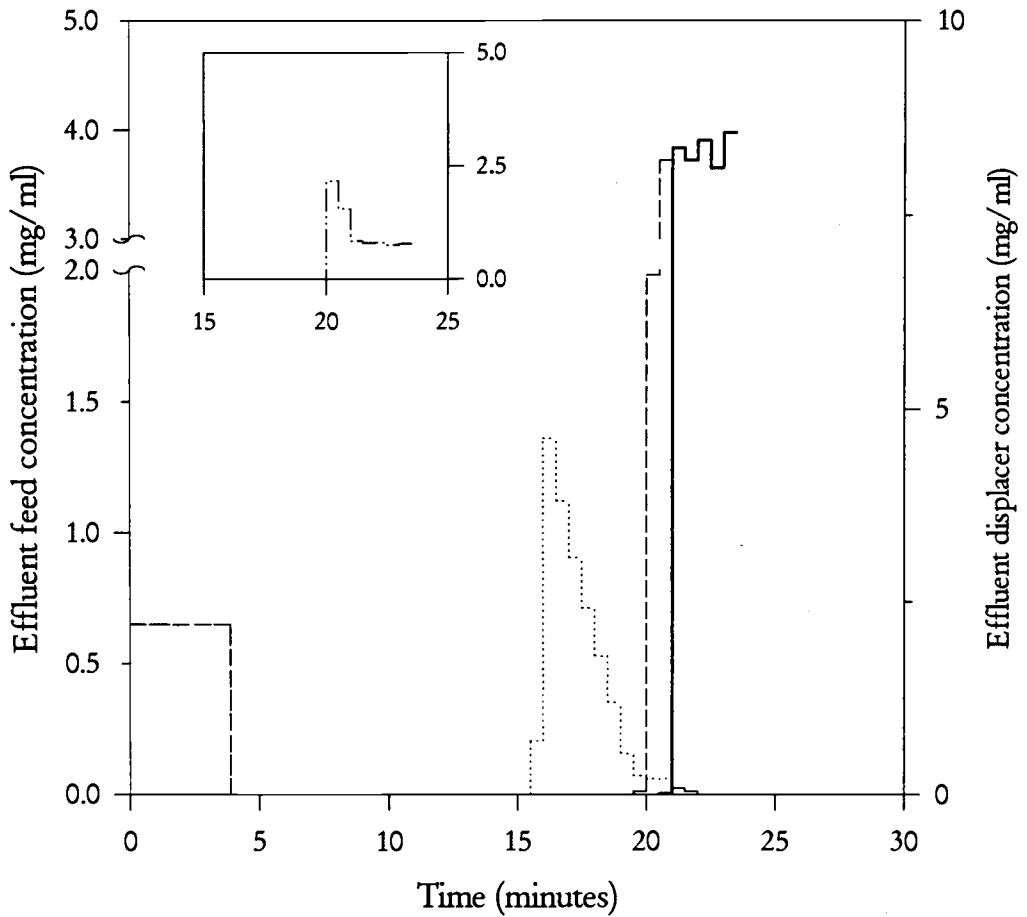


Figure 2.8a. Displacement chromatogram using 10 mg/ml benzethonium chloride as displacer in 15% ACN. Feed: 0.65 mg/ml P (dots) and 0.65 mg/ml T (dashes) in 3.9 ml at 10% ACN; displacer, benzethonium chloride (solid line); flow-rate, 1ml/min; fraction size, 0.5 ml each.

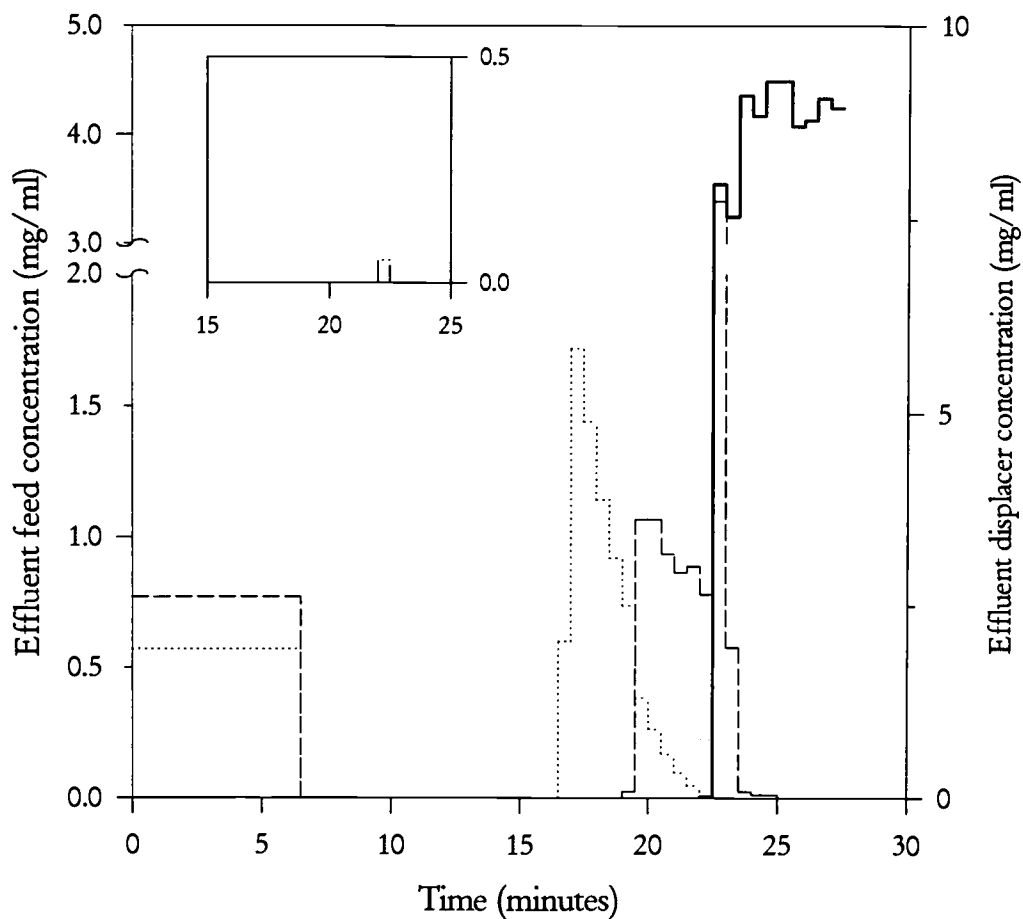


Figure 2.8b. Displacement chromatogram using 10 mg/ml benzethonium chloride as displacer in 15% ACN. Feed: 0.57 mg/ml P and 0.77 mg/ml T in 6.5 ml feed volume. All other conditions as in Figure 2.8a.

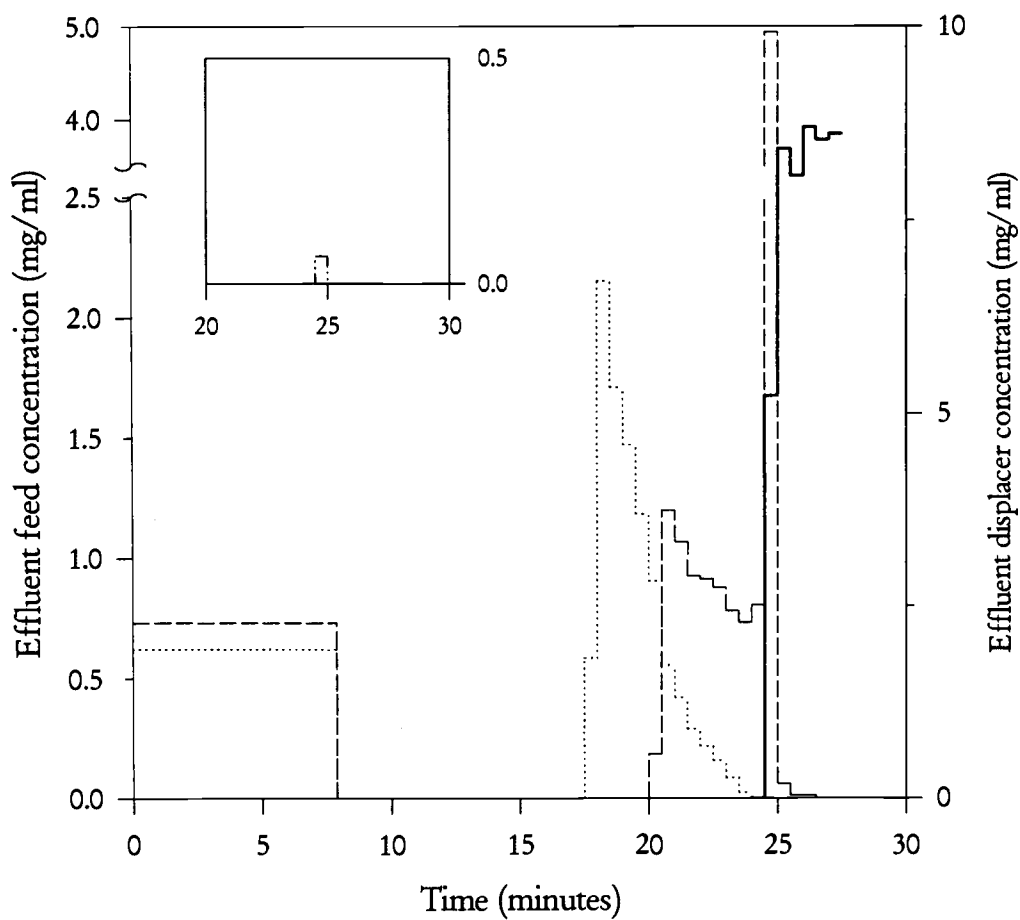


Figure 2.8c. Displacement chromatogram using 10 mg/ml benzethonium chloride as displacer in 15% ACN. Feed: 0.62 mg/ml P and 0.73 mg/ml T in 7.9 ml feed volume. All other conditions as in Figure 2.8a.

component isotherm for TMEEA was obtained by combining the retention factor estimate with the location of shocks from the frontal runs, and is shown in Figure 2.9b. The single-component isotherm was well fitted by a double Langmuir expression, shown by the dash-dot-dot curve in Figure 2.9b.

Figures 2.10a, b and c shows displacement runs for feed volumes of 3.4, 3.9 and 5.0 ml respectively. The column was initially at 10% ACN, as was the feed; the displacer was dissolved in 15% ACN. Touching-band separations with very low levels of mixing for both feeds were achieved for 3.4 ml and 3.9 ml runs. The concentrations of both peptides were substantially higher than in the feed. Although slightly greater mixing was found for the 5.0 ml run (Figure 2.10c) the productivities of both P and T increased. However, the maximum concentration of P was close to the maximum level of 2.5 mg/ml prescribed by its limited solubility. Hence the feed volume could not be further increased.

TMEEA did not absorb in UV at low concentrations, and hence its concentrations could not be estimated by direct analysis of fractions. In fact, UV absorption of TMEEA was extremely nonlinear, as can be seen from the frontal runs (Figure 2.9a). The run for 2 mg/ml was much lower than that for 3 mg/ml. The fractions collected from displacements at 3 mg/ml were diluted during analytical isocratic runs to the point where the TMEEA peaks could not be clearly identified. Hence, the location of the TMEEA shock was sought by repeating the preparative experiment with no feed in the sample loop. The fronts from these runs were overlaid on the corresponding separations, and quantified on the secondary y-axis in Figure 2.10. It is evident from all the plots that

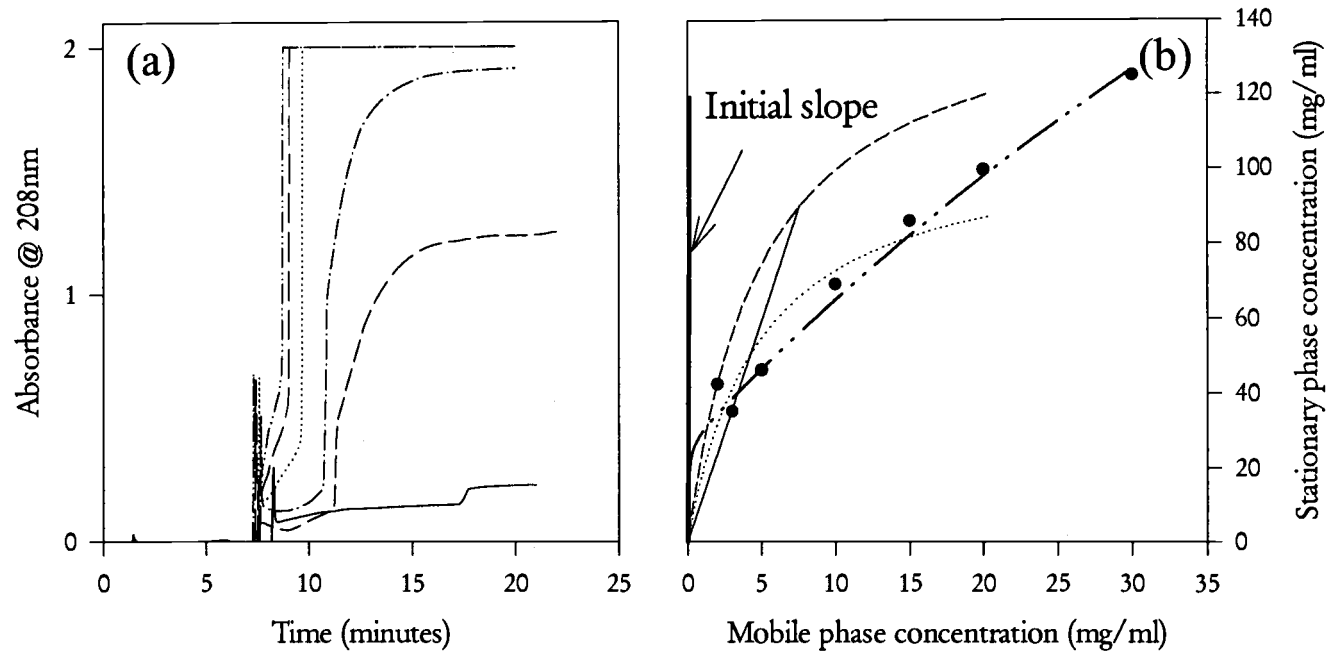


Figure 2.9. (Panel a) Frontal runs at various TMEEA concentrations [2 mg/ml (solid line), 3 mg/ml (dashes), 5 mg/ml (dash dot), 10 mg/ml (dots), 15 mg/ml (long dash), 20 mg/ml (dash-dot-dot)]; displacer suspended in ACN-phosphate buffer-TFA (15:85:0.1). (Panel b) Single-component isotherm for TMEEA (dash-dot-dot). Operating line for 3 mg/ml shown (solid line). Single-component isotherms for P (dots) and T (dashes) from Kim (1997) are also shown.

TMEEA is immediately behind T. Additionally, the rear of T was very sharp, making it likely that it is being displaced.

In this study, experiments were performed with TMEEA from two different stocks. All the experiments shown here were from an older supply of TMEEA. The frontal run for the newer TMEEA (not shown), although qualitatively similar, was quantitatively somewhat different from that of the older TMEEA. Nevertheless, upon repeating the displacement run for 3.9 ml feed volume (Figure 2.11) with the newer stock, the separations were similar. Comparing Figure 2.11 with Figure 2.10b shows that the breakthrough of P and the rear of T occur at the same location, although the amount of mixing was higher when using the newer TMEEA.

*Choice of displacer:* When choosing a displacer for reversed-phase chromatography a compromise has to be made between analytical retention, saturation capacity (which may be regarded as a measure of nonlinear retention), and solubility. In addition, since the displacer produces an enriched zone of modulator next to the displacer front (Velayudhan and Horvath, 1995) complex retention changes could occur in the micro-environment within the column due to the differences in modulator levels, which could potentially change the overall separation.

Benzethonium chloride was found to have good potential: it was highly soluble, strongly retained, and easily detected by UV (because of the benzyl rings). Other candidates tested were 5-nonanone, 4-heptanone, 3,4 dimethoxybenzylalcohol, n-benzyl benzamide, diethyl dodecamide, heptoxybenzylchloride, and TMEEA. Amongst these

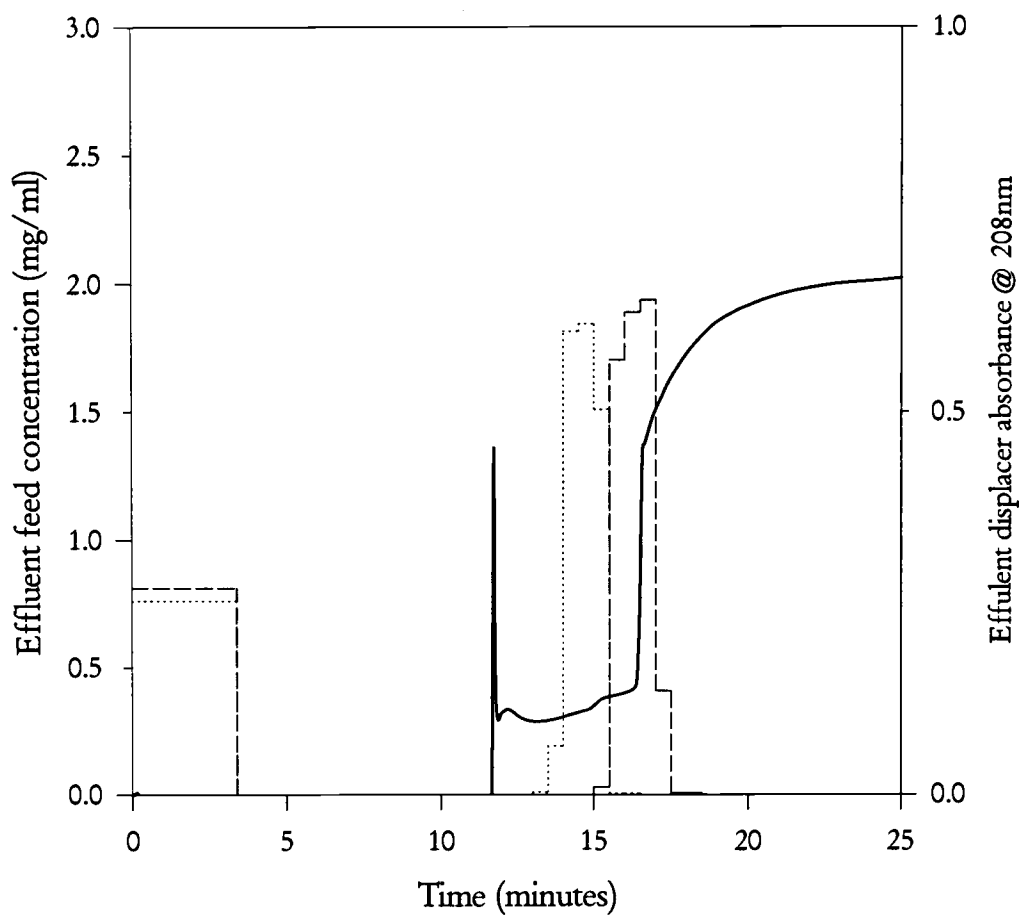


Figure 2.10a. Displacement chromatogram using 3 mg/ml TMEEA as displacer in 15% ACN. Feed: 0.76 mg/ml P (shown by dots) and 0.81 mg/ml T (shown by dashes) in 3.4 ml feed volume in 10% ACN. All other conditions as in Figure 2.8.

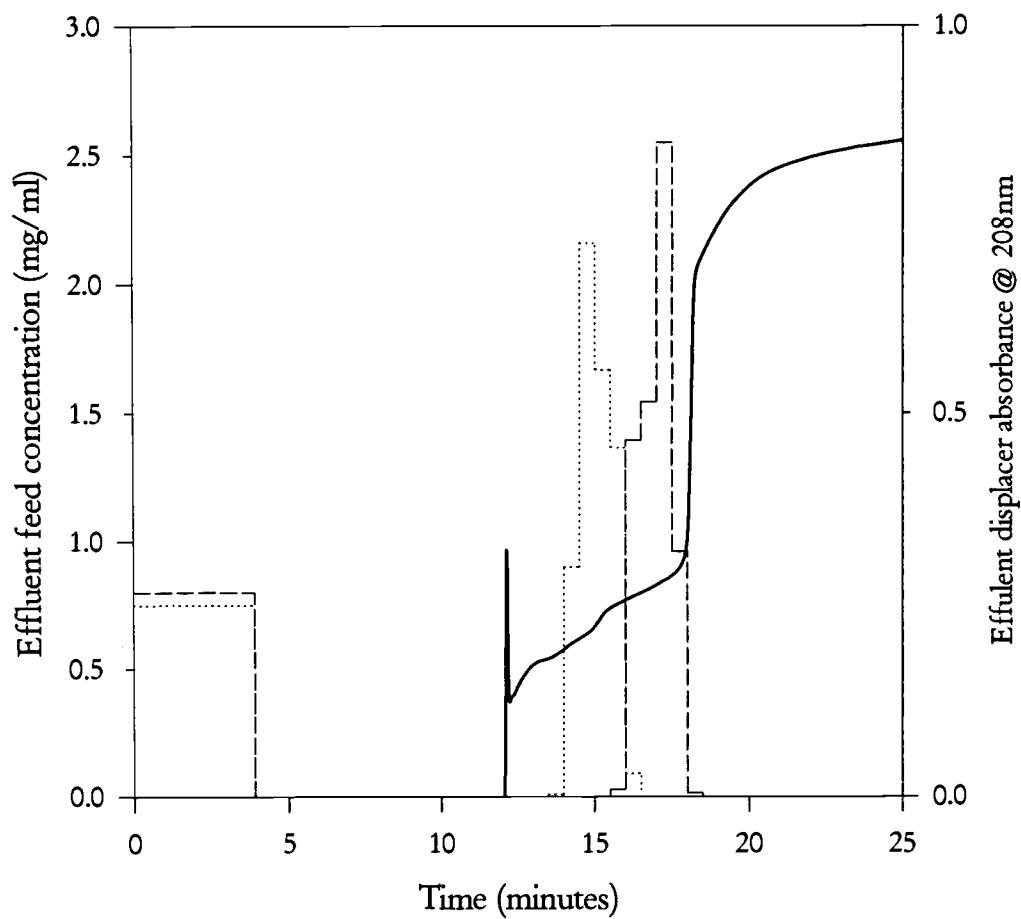


Figure 2.10b. Displacement chromatogram using 3 mg/ml TMEEA as displacer in 15% ACN. Feed: 0.75 mg/ml P and 0.80 mg/ml T in 3.9 ml feed volume. All other conditions as in Figure 2.8.



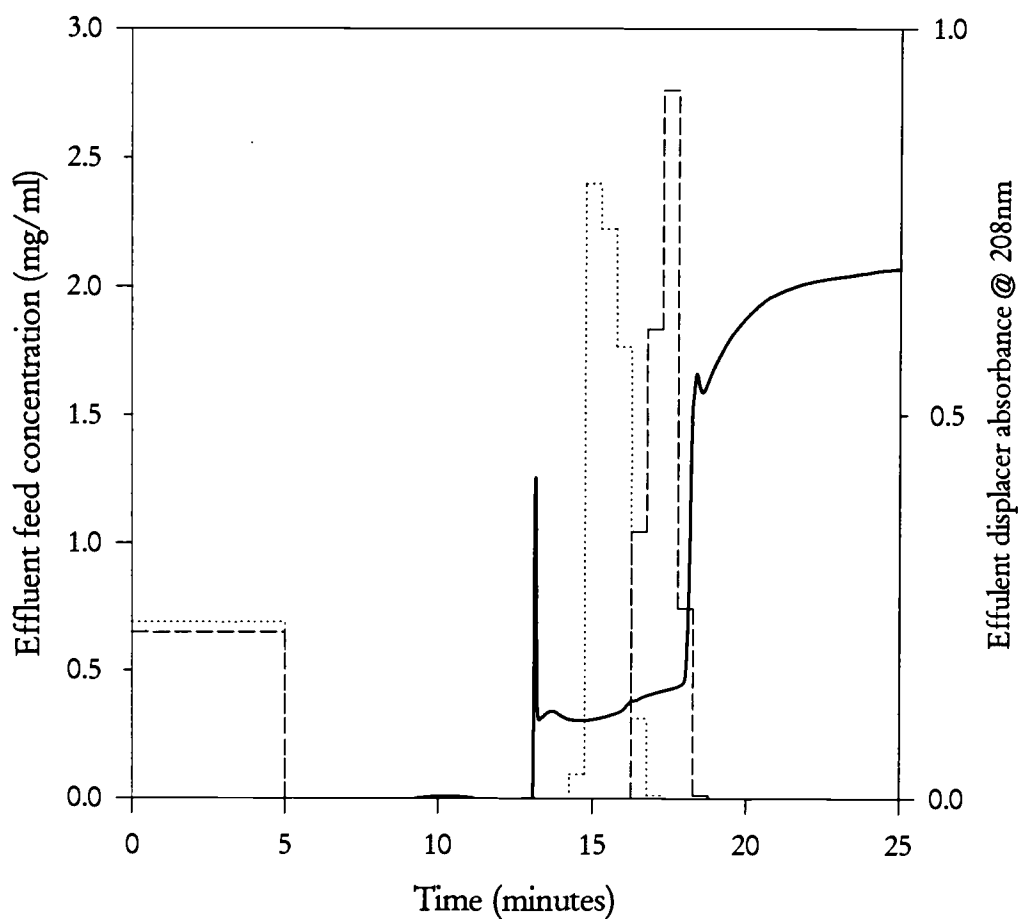


Figure 2.10c. Displacement chromatogram using 3 mg/ml TMEEA as displacer in 15% ACN. Feed: 0.69 mg/ml P and 0.65 mg/ml T in 5.0 ml feed volume. All other conditions as in Figure 2.8.

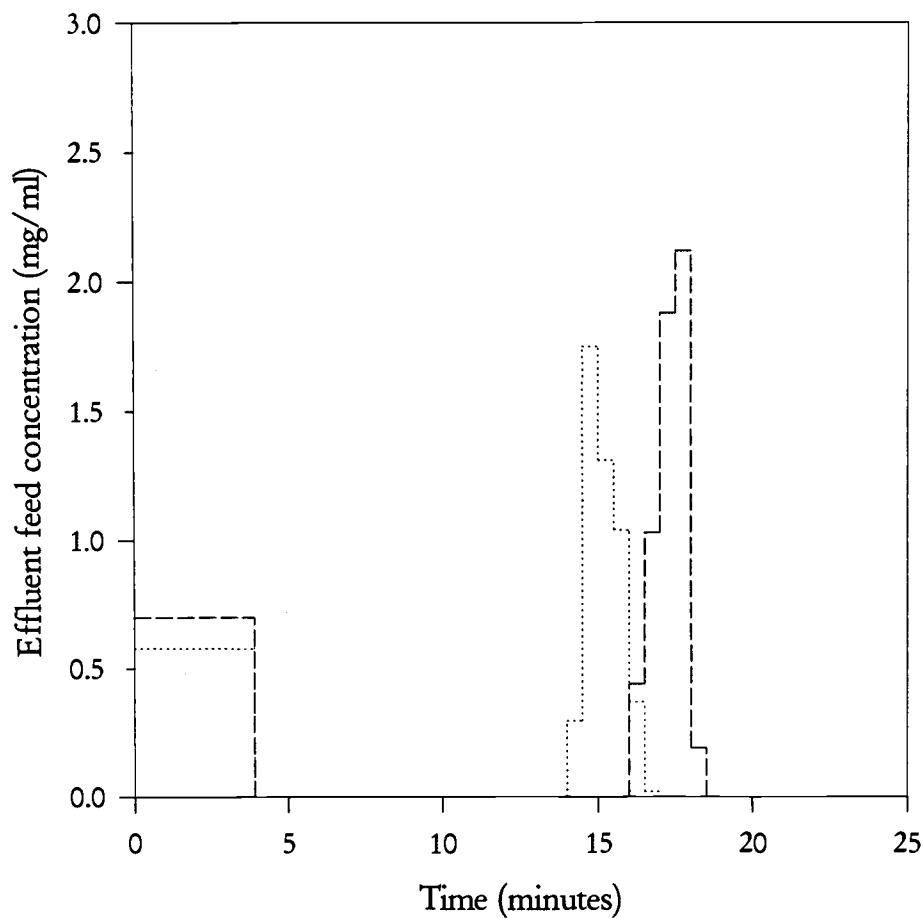


Figure 2.11. Displacement chromatogram using 3 mg/ml "newer stock" TMEEA as displacer in 15% ACN. Feed: 0.58 mg/ml P (shown by dots) and 0.70 mg/ml T (shown by dashes) in 3.9 ml feed volume in 10% ACN. All other conditions as in Figure 2.8.

compounds only TMEEA, a tertiary amine, was found to satisfy both solubility and retention requirements

*Choice of modulator level:* The retention of many molecules decreases exponentially with increasing organic modulator level (Snyder, 1980). Hence, minor differences in modulator levels both in the initial feed state and in the displacer could cause significant differences in retention. High initial modulator levels could potentially help in reducing the separation time; however, they may also result in less efficient separation because of decreased selectivity. On the other hand, when low initial modulator levels are used, the molecules bind strongly to the stationary phase, thereby increasing the loading capacity, but increasing the separation time. Hence, the highest productivity, reflecting the balance between loading and selectivity on the one hand and the separation time on the other, will be obtained by a judicious choice of modulator levels in the feed state and in the displacer (these need not be equal).

Separations using TMEEA as displacer suspended in 15% ACN for a 3.4 ml feed volume where the initial state of column was also at 15% ACN is shown in Figure 2.12 (hereafter, 15 initial/15 displacement). By comparing this to the 3.4 ml run (Figure 2.10a) when the displacer solution was fed at 15% ACN level keeping both the initial state of column and the feed state at 10% ACN (10 initial/15 displacement), it can be observed that the latter resulted in complete separation, but had a greater separation time. The 10 initial/15 displacement resulted in concentrated and roughly rectangular bands. In the 15 initial/15 displacement run, the feed components moved too fast for the displacer to affect them appreciably, resulting in lower productivities. A third choice, where both displacer

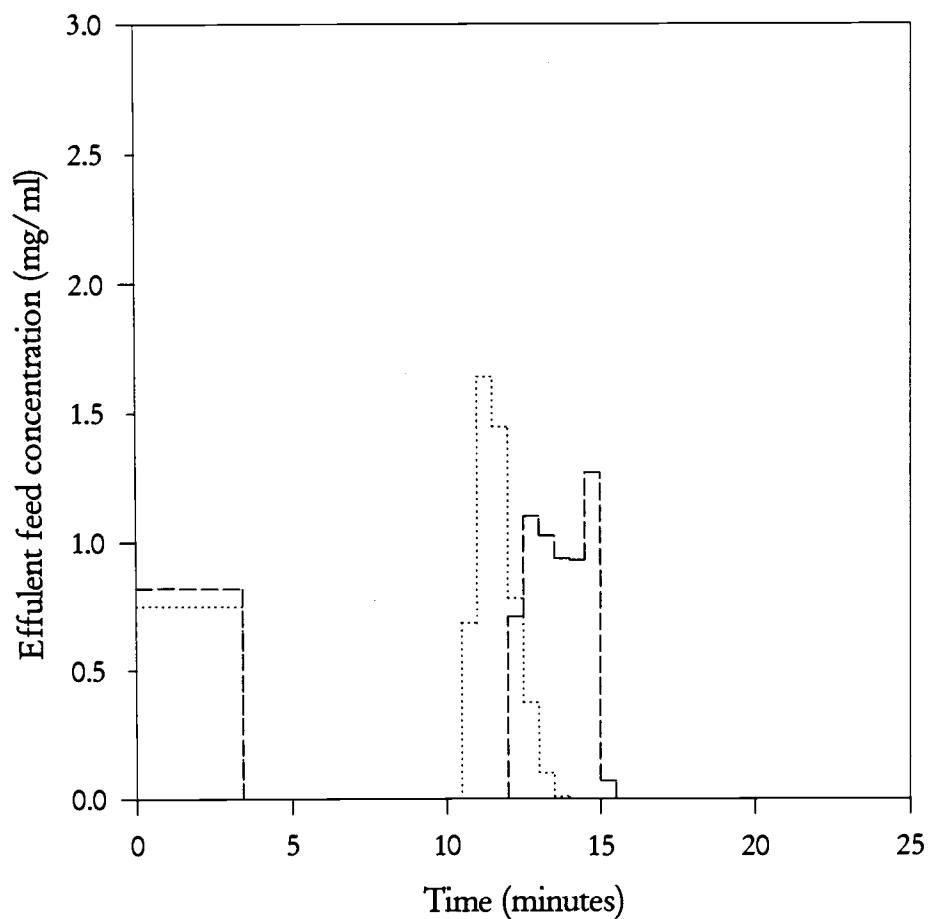


Figure 2.12. Displacement chromatogram using 3 mg/ml TMEEA as displacer in 15% ACN. Feed: 0.75 mg/ml P (shown by dots) and 0.82 mg/ml T (shown by dashes) in 3.4 ml. The initial state of column was 15% ACN. All other conditions as in Figure 2.8

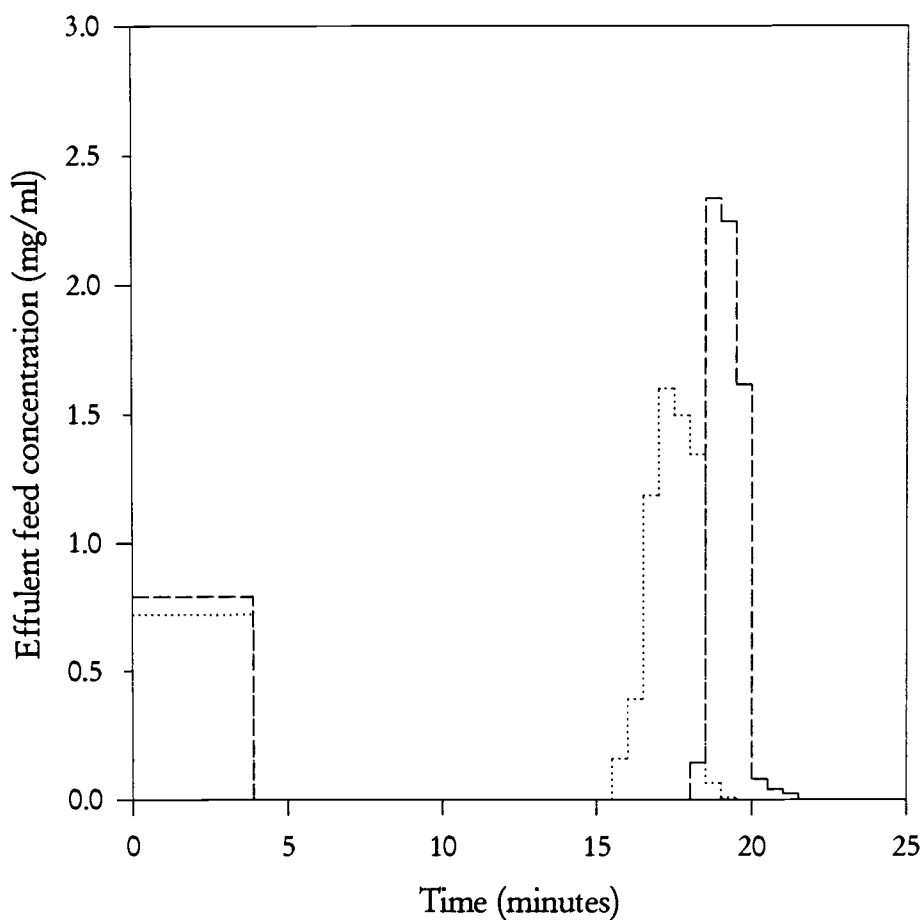


Figure 2.13. Displacement chromatogram using 3 mg/ml TMEEA as displacer in 15% ACN. Feed: 0.72 mg/ml P (shown by dots) and 0.79 mg/ml T (shown by dashes) in 3.9 ml. The initial state of column was 10% ACN. All other conditions as in Figure 2.8.

and initial state of the column was at 10% ACN (10 initial/10 displacement) is shown in Figure 2.13. While the separation was good, the substantially longer separation time resulted in lower productivities. It can be concluded from this series of experiments that the best results are achieved when the feed is loaded at low modulator levels (thereby exploiting the discrimination of stationary phase for the feed components) and displacements carried out with higher modulator levels in the displacer solution producing rapid separations. The potential difficulty with high modulator level in the displacer is that the feeds may elute in the enriched modulator band formed ahead of the displacer. However, that does not occur in the TMEEA runs.

*Choice of flow-rate:* It has been found that low flow-rates can result in improved displacement separations (Horvath et al., 1981). In order to test the effect of flow-rate on separation of P and T, displacements at low flow-rates (0.1 ml/min for benzethonium chloride and 0.1 ml/min and 0.75 ml/min for TMEEA) were attempted for both displacers. The low flow-rate run for benzethonium chloride could not be carried out because P crystallized immediately after elution from the column. Experiments carried out with TMEEA at 3 mg/ml for 3.4 ml feed volume at 0.1 ml/min and 0.75 ml/min is shown in Figure 2.14. In comparison with the run at 1 ml/min (Figure 2.12), the separations, including the extent of mixing, were quite similar. Thus, the flow-rate is not a limiting factor in these runs.

*Choice of displacer concentration:* The single-component isotherms of feed components and displacer serve as the starting point for selecting the displacer concentration. The classical final-pattern concentrations for the feed components are found by drawing a chord between the origin and a given displacer concentration

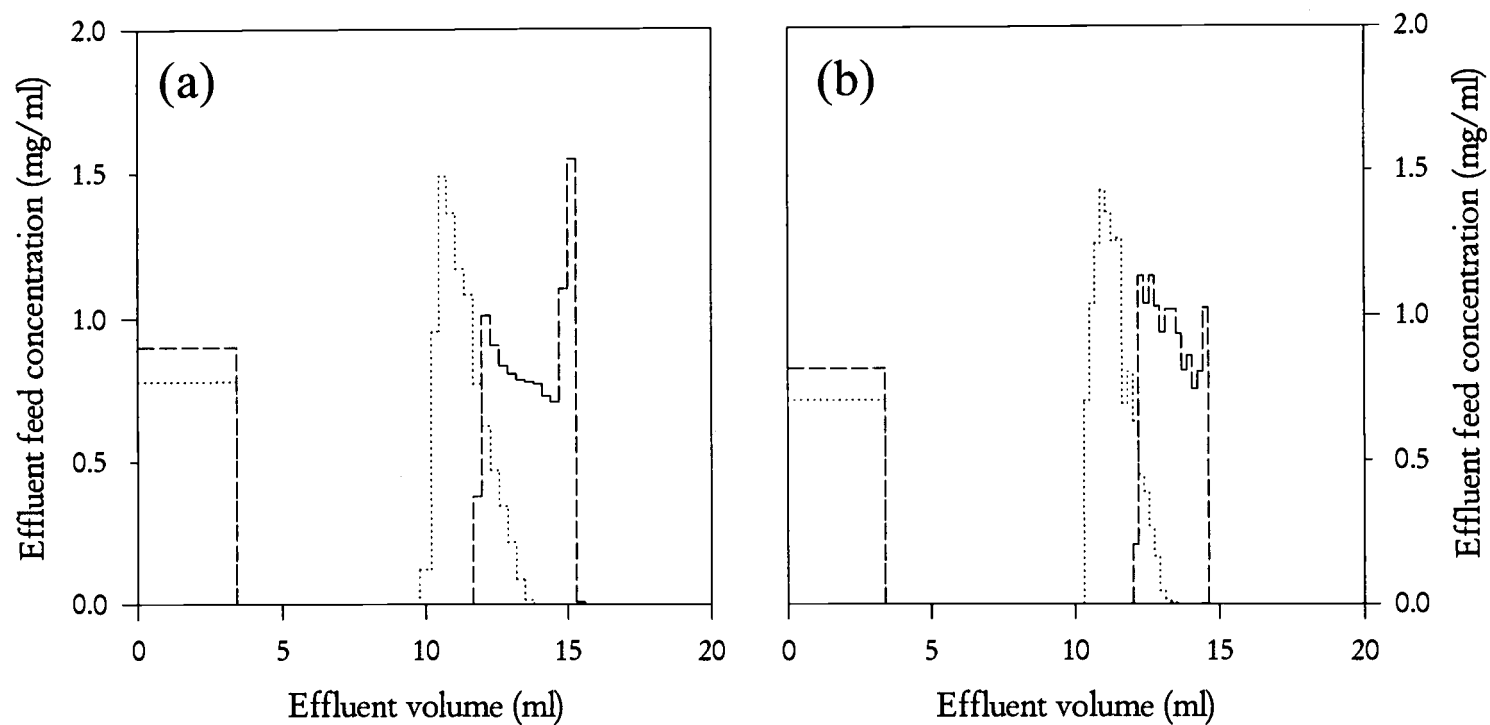


Figure 2.14. Displacement chromatogram using 3 mg/ml TMEEA as displacer in 15% ACN. Feed: 0.78 mg/ml P (shown by dots) and 0.90 mg/ml T (shown by dashes) in 3.4 ml. (Panel a) Flow-rate was 0.1 ml/min, (Panel b) 0.75 ml/min. All other conditions as in Figure 2.8.

(operating line), and identifying the points of intersections of the feed single-component isotherm's with this operating line (see Figures 2.6 and 2.9). For systems where feed solubility is a constraint, lower displacer concentrations are often useful. However, lower concentration fronts move slower than higher concentration fronts, thereby increasing separation time. The frontal run for 7.5 mg/ml benzethonium chloride resulted in a displacement front at 19 min. From the gradient runs at 15% ACN [29] for a 2.4 ml feed volume, both P and T eluted by 20 min. Also, results from the corresponding isocratic runs (Kim and Velayudhan, 1998) suggested that P would elute when using 7.5 mg/ml benzethonium chloride. The elution of P is desired because of its limited solubility.

The single-component isotherm for TMEEA closely overlay the single-component isotherm of feed components in the region of operation at 3 mg/ml, where a frontal run at for the same concentration resulted in a shock at 12.5 min. This is another useful approach when feed solubility is limited, because the feeds are unlikely to be highly concentrated in the final pattern. Hence, 7.5 mg/ml of benzethonium chloride and 3 mg/ml of TMEEA were chosen as starting points for displacement experiments, and were observed to give good productivities without unduly concentrating the P band. 10 mg/ml benzethonium chloride was also tried, and the results were discussed in the previous section. In general, changing the displacer concentration as well as the modulator levels in both feed and displacer are needed to maximize productivity.

*Effect of displacer impurity:* The commercial displacers used in this study contained impurities. benzethonium chloride is 97% pure, and TMEEA 95% pure. Since the displacer is continuously fed into the column, impurities, although present in small



levels, could potentially affect the overall separation quality. Frontal runs for TMEEA (Figure 2.9a) show a peak well ahead of the main front, which is likely to be a weakly retaining impurity. Previous reports on displacer impurities by Zhu et al. (1991) and Jen and Pinto (1993) show that a weakly retained impurity could form a sharp peak followed by a plateau before the displacer front. This impurity is almost unretained, and is unlikely to affect the separation quality. In the case of benzethonium chloride, as evident from the frontal runs (Figure 2.6), there were several impurities; one impurity that was quantifiable in analytical runs was found to occur at significant levels in the fraction just ahead of the displacer front itself. Hence, it is reasonable to assume that this impurity's adsorption was similar to that of benzethonium chloride. However this impurity degraded rapidly in solution at room temperature, and was often not seen under analytical conditions. By contrast, benzethonium chloride was quite stable under the same conditions.

*Comparison of efficacy of operational modes:* Productivity for P and T for various operational modes were calculated using equation 2.1, and given in Table 2.1 and Figure 2.15, for the various runs. The cycle time for all runs was 20 minutes (10 minutes for regeneration and 10 minutes for equilibration).

From Figure 2.15 it is evident that no one mode is superior for P. However, displacement by TMEEA and BC (10 mg/ml) was somewhat better for T. The cost of separating the displacer from purified T was not accounted for. TMEEA gave a maximum productivity of 9.0 mg/ml-hr for P and 7.2 mg/ml-hr for T. Enrichments of P and T increased with increasing feed volumes. Solubility constraints on P prevented further increase in feed volume, which otherwise could have increased the productivity of P. However, productivity of T went through a maximum, and hence further increases in

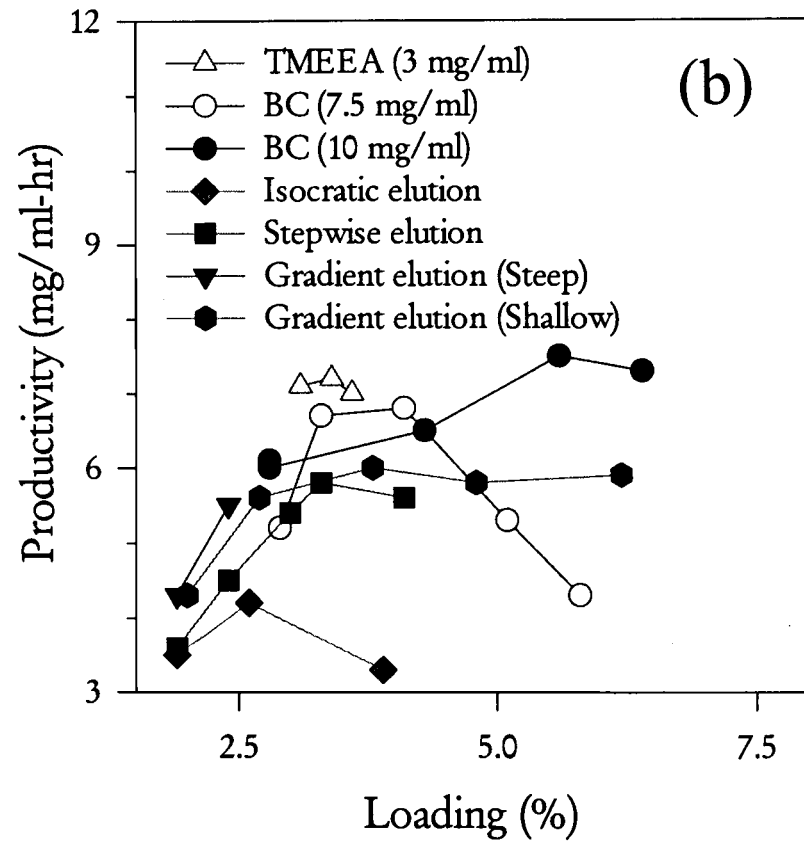
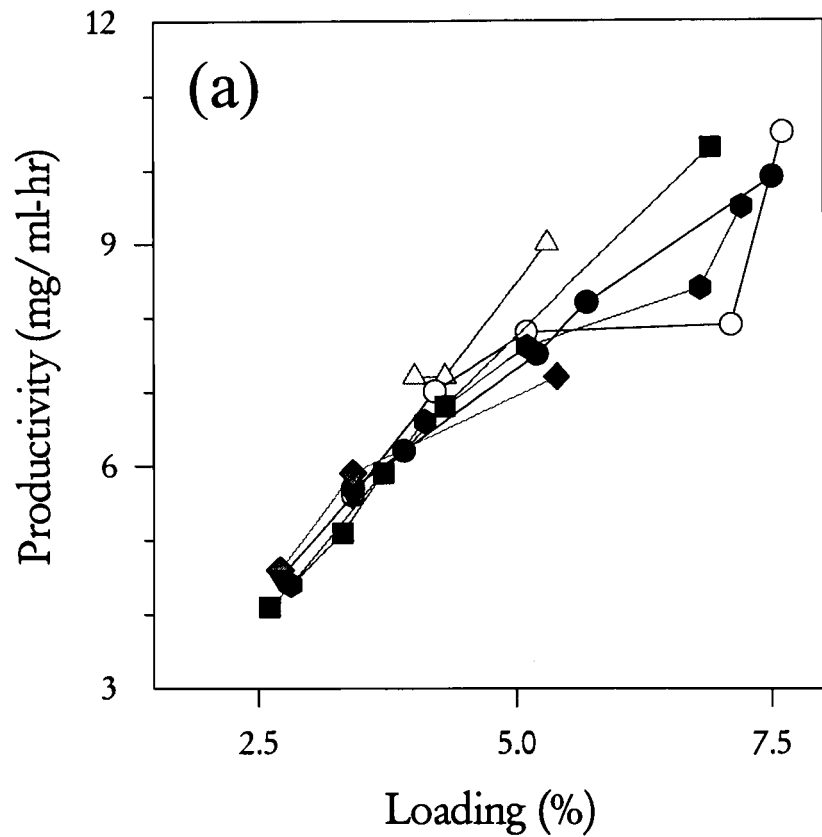


Figure 2.15. Productivity plots for P (panel a) and T (panel b).

n-formyl-Met-Phe				n-formyl-Met-Trp		
Feed (ml)	Loading (%)	Productivity (mg/ml-hr)	Yield (%)	Loading (%)	Productivity (mg/ml-hr)	Yield (%)
Isocratic runs (15% ACN)						
2.4	2.7	4.6	97	1.9	3.5	93
3.0	3.4	5.9	94	2.6	4.2	78
5.0	5.4	7.2	69	3.9	3.3	47
Stepwise elution runs (10 to 15 in 0.5 min)						
3.4	3.7	5.9	98	3.0	5.4	100
3.9	4.3	6.8	98	3.3	5.8	99
5.0	6.9	10.3	93	4.1	5.6	79
Gradient elution (10 to 40 in 30 min)						
5.0	5.1	7.6	95	3.8	6.0	78
6.5	7.2	9.6	84	4.8	5.9	61
7.4	6.8	8.4	80	6.2	5.9	49
TMEEA: 3 mg/ml						
3.4	4.0	7.2	100	3.1	7.1	100
3.9	4.3	7.2	99	3.4	7.2	98
5.0	5.3	9.0	95	3.6	7.0	83
Benzethonium chloride: 7.5 mg/ml						
3.4	3.4	5.6	98	2.9	5.2	89
3.9	4.2	7.0	98	3.3	6.7	99
5.0	5.1	7.8	94	4.1	6.8	85
6.5	7.6	10.5	84	5.1	5.3	54
7.4	7.1	7.9	68	5.8	4.3	40
Benzethonium chloride: 10 mg/ml						
3.4	3.4	5.7	98	2.8	6.1	100
3.9	3.9	6.2	98	2.8	6.0	100
5.0	5.2	7.5	88	4.3	6.5	73
6.5	5.7	8.2	87	5.6	7.5	68
7.9	7.5	9.9	82	6.4	7.3	59

Table 2.1. Productivity table for P+T separation under various modes.

feed volume were not useful. Productivity for displacement runs using benzethonium chloride at 7.5 mg/ml went through a maximum for both P and T. Enrichments for P increased with increasing loading, while enrichment for T went through a maximum. Benzethonium chloride at 10 mg/ml gave maximum productivities of 9.9 mg/ml-hr and 7.5 mg/ml-hr. Solubility constraints on P limited further increase in loading. However, the productivity of T went through a maximum, making further increases in loading pointless. In all cases the yield of T dropped faster than for P since high concentrations of T, at the front of the T band, become mixed with low concentrations of P, at the rear of the P band.

Results from other modes gave comparable productivities for the P+T separations in isocratic, stepwise and gradient elution. For example, productivities for a 5 ml feed under isocratic conditions at 15% ACN were 7.2 mg/ml-hr for P and 3.3 mg/ml-hr for T. Similar results for stepwise elution from 10-15% ACN in 0.5 min gave 10.3 mg/ml-hr for P and 5.6 mg/ml-hr for T, and a gradient run from 10-40% in 30 min gave 7.6 mg/ml-hr for P and 6.0 mg/ml-hr for T.

Although no one method was clearly superior, separations under displacement mode gave somewhat higher productivity coupled with sample enrichment.

In order to assess the efficacy of these displacement separations, productivities were estimated from the literature for displacements of other biomolecules on reversed-phase adsorbents. The regeneration time for all separations was assumed to be same as that of P+T system (20 min). Additionally, the total porosity (interstitial + intraparticulate) for all the columns was assumed to be 0.7. Productivity estimates for the displacement separations of melanotropins (Viscomi et al., 1988) on a home-made

octadecylsilica column using ACN-water as mobile phase and benzyldimethyldodecyl ammonium bromide as displacer (hereafter the details are listed as: column; mobile phase; displacer) was 5.1 mg/ml-hr. Separation of oligomycins (Valko et al., 1987; LiChrosorb RP-18; methanol-water; palmitic acid) resulted in 5.2 mg/ml-hr. Viscomi et al. (1991b) report scaling-up displacement separations without significant loss in productivity for a peptide fragment of human interleukin- $\beta$  (LiChrosorb RP-18; water-TFA; benzyltributyl ammonium chloride). The productivity obtained on a  $250 \times 4$  mm column gave 12.6 mg/ml-hr; scaling up by a factor of 20 gave productivity of 10.6 mg/ml-hr. A detailed account of productivity under elution modes is given in Kim (1997). The productivities obtained for P+T were comparable to, and some cases substantially higher than, the estimates for other biomolecules in spite of stringent solubility limitations.

## CONCLUSIONS

Optimization in nonlinear chromatography was carried out for the first time for a biomolecule mixture. Good productivities were obtained despite the limited solubility of the feed components. Maximum productivities were achieved in displacement mode under non-isotachic conditions. Flow rates similar to those typical of elution modes were used effectively for the displacement runs. Displacer impurities did not affect separation quality in these runs. For this system, feed loading should be at low modulator levels followed by displacements at higher modulator concentrations. For feeds with limited solubility, displacers with single-component isotherms that are only slightly higher than those of the feeds or shallow gradients are preferable.

## Chapter 3

### Isolation and Preparative Chromatography of Microcystin Variants

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**Keywords:** preparative chromatography, microcystins, toxins, peptides.

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## ABSTRACT

Preparative reversed-phase liquid chromatography was successfully used to purify two microcystins (microcystin LR and microcystin LA) from a cyanobacterial process waste. The separation protocol involved extraction of lyophilized cells by methanol, isolation and concentration by solid-phase extraction, and purification by reversed-phase HPLC. Milligram-level loading of microcystins was obtained on a solid-phase extraction cartridge packed with 0.5 g of C<sub>18</sub> stationary phase. The separations were first carried out on an analytical column and then scaled-up to a preparative column. The microcystins were quantified by HPLC and ELISA. A method to remove microcystins rapidly and economically from the cyanobacterial process waste is also described.

## INTRODUCTION

*Microcystis*, a cyanobacterial species, is known to produce a class of cyclic heptapeptides called microcystins (Huges et al., 1958; Watanabe et al., 1996; Carmichael and Bent, 1981). Microcystins have been shown to inhibit protein phosphatases (Runngear et al., 1995) and promote tumors (Codd et al., 1994) and have been reported to be the cause of numerous animal fatalities [Carmichael, 1994; Billings, 1981; Turner et al., 1990). There has been an increasing need for highly pure microcystin standards in recent years (Codd et al., 1994) to serve the demands of chemists and toxicologists for structural and toxicological studies and enzyme-based assay development. It is therefore of continuing importance to develop preparative separation methods for these compounds.

Microcystins are a family of mono-cyclic heptapeptides, consisting of D-alanine,  $\beta$ -linked erythro- $\beta$ -methylaspartic acid,  $\gamma$ -linked glutamic acid, the two unusual amino acids N-methyldehydroalanine (Mdha) and 3-amino-9-methoxy-10-phenyl-2,6,7,-trimethyldeca-4, 6-dienoic acid (Adda), and a pair of variable L-amino acids. The multitude of variable L-amino acids coupled with slight variations in the side chains of the other amino acids has given rise to about 50 known microcystins so far (Watanabe et al., 1996) Hence, a desired microcystin must be separated not only from other classes of compounds such as nodularins (Lawton et al., 1994) that also have the hydrophobic Adda moiety (and hence may exhibit similar chromatographic properties), but also from closely retaining microcystins.

The focus of many articles on microcystins thus far has been the discovery of a new microcystin and its structural characterization (Reinhart., 1994; Watanabe et al., 1996). There have been fewer reports on efficient methods for the preparative purification of microcystins (Edwards et al., 1996a and 1996b). In this paper, we report a method to isolate both microcystin LR (MC-LR) and microcystin (MC-LA) from a cyanobacterial process waste stream. The isolated microcystins were identified by electro-spray mass spectrometry, and quantified by analytical HPLC and ELISA. Finally a simple method for the economical removal of microcystins is described.

## **MATERIALS AND METHODS**

### *Materials*

HPLC-grade acetonitrile was obtained from EM Science (Gibson, NJ, USA) and sequanal-grade trifluoroacetic acid (TFA) from Pierce (Rockford, IL, USA). Deionized-



distilled water was obtained using Milli-Q ultra-pure water system (Millipore Co., Bedford, MA, USA). Sep-Pak C<sub>18</sub> (0.5 g) cartridges were obtained from Waters (Milford, MA, USA). Activated carbon was purchased from Westvaco (Covington, VA, USA). Microcystin standards were purchased from Calbiochem-Novabiochem Corporation (La Jolla, CA, USA). The enzyme immunoassay kit for microcystin-LR was purchased from Strategic Diagnostics, Inc. (Newark, DE, USA).

### *Apparatus*

The HPLC system consisting of a quaternary pump (Model 600), UV detector (Model 486), and an autosampler (Model 717 plus), was controlled by a DEC (Nashua, NH, USA) personal computer using Waters Millennium software. A Rheodyne Model 7125i injector (Cotati, CA, USA) was used to inject preparative samples. Preparative column (25 cm × 2 cm I.D.) and a guard column (5 cm × 2 cm I.D.) was from Kromasil (Bohus, Sweden). Analyses were made using both Kromasil and Alltech (Deerfield, IL, USA) Alltima C<sub>18</sub> column (25 cm × 0.46 cm I.D.). A Buchi RE 111 rotary evaporator (Flawil, Switzerland) was used to concentrate the liquid samples. The molecular mass of the samples was determined using a Perkin-Elmer Sciex API III mass spectrophotometer (Norwalk, CT, USA).

### *Procedures*

*Extraction:* A.F.A., Inc. (Klamath Falls, OR, USA) supplied us with various batches of wet (slurry) and dry (lyophilized) process waste streams containing microcystins. The dry cells (20 g) were extracted with 600 ml of MeOH-water (75:25) by

continuous stirring using an orbital shaker set at 400 rpm for 1 hour at room temperature. After one hour, the sample was centrifuged at  $9650 \times g$  for 15 minutes. The extraction procedure was repeated once with 400 ml of the same solvent. The supernatants resulting from both the steps were pooled and reduced to approximately a tenth of its initial volume by rotary evaporation at  $35\text{ }^{\circ}\text{C}$ . The liquid concentrate was stored at  $4\text{ }^{\circ}\text{C}$ . The effect of sonication on microcystin extraction was studied on slurry samples using a Branson sonifier model 250 set at 50% cycle time and an intensity of 5, for 10 minutes.

*Isolation:* The concentrate from the extraction step was brought to room temperature and centrifuged at  $11,300 \times g$  for 10 minutes. A 25 ml aliquot of the microcystin-rich concentrate was applied to a preconditioned Sep-Pak  $\text{C}_{18}$  cartridge (0.5 g) at 1 ml/min. The preconditioning step included washing with 20 ml each of neat methanol and by 20% aqueous methanol. The loaded cartridge was washed with 20% aqueous methanol, after which the microcystins were eluted using 5 ml of 80% methanol at 1 ml/min. The cartridges were regenerated by washing with 20 ml each of methanol, chloroform and hexane. Liquid-liquid extraction (LLE) was also studied as an isolation step. The microcystin extract was contacted with an organic solvent (hexane, n-butanol or methyl-ethyl ketone; 1:1), and shaken vigorously for 2 minutes using a vortexer. Centrifugation of the liquid mixture at  $805 \times g$  for 15 minutes separated the organic layer from the aqueous layer. Samples from both layers were analyzed for microcystin content by HPLC.

*Chromatography:* The concentrated microcystin extract from the SPE was injected into either the Alltech or the Kromasil column. The chromatographic profile and the retention times for the microcystins and their impurities were very similar on these

two analytical columns, and hence both were used interchangeably for microcystin analysis. The methods developed on the analytical column were scaled-up to a preparative column (Kromasil, 25 cm × 2 cm I.D.). The MC-LR and MC-LA fractions were collected and analyzed as described below.

*Analysis:* The microcystins MC-LR and MC-LA were both analyzed quantitatively by HPLC based on a calibration curve constructed by analyzing commercially available MC-LR standards. The purity of the MC-LR fractions was assessed by quantifying the closely retaining impurities by fitting them to the MC-LR calibration curve. A similar procedure was adopted for assessing MC-LA's purity. In addition to HPLC, the samples were analyzed by an enzyme-based assay kit from Strategic Diagnostics Inc. (Newark, DE, USA). Typically, a dilution factor of 200,000:1 was essential to bring the sample concentration within the calibration curve. Further, the purified microcystins were identified by a Sciex API III+ triple quadrupole ionspray mass spectrometer.

*Removal of Microcystins:* The process waste (1000 ml) was brought to room temperature and contacted with 20 g of activated charcoal. The resulting mixture was stirred, and samples were collected at 30 minute intervals for residual microcystin analysis by HPLC.

## RESULTS AND DISCUSSION

*Extraction:* Refrigerated slurry samples (non-lyophilized) was used to study the effect of sonication on cell rupture, and hence microcystin extraction. No significant improvement in microcystin content of the sonicated sample was found. Further,

independent experiments on the sonication of commercial MC-LR standard solutions showed degradation. Hence, it's possible that sonication yielded higher amounts of MC-LR through cell disruption but also degraded some of the MC-LR. These effects could offset each other, resulting in comparable amounts in both sonicated and un-sonicated samples. Consequently, cell disruption technique was not incorporated in the extraction protocol.

The extraction efficacy plot (Figure 3.1) shows comparable extraction of MC-LR by most solvents, while 75% methanol in water was somewhat more efficient than the others. MC-LA, however, was less efficiently extracted by solvents containing acetic acid. We also found that no one solvent was superior in reducing the co-extraction of impurities. While Harada et al. (1988) reported near-complete extraction of MC-LR using 5% acetic acid, more hydrophobic microcystins could not be extracted using this method. A more non-polar solvent mixture (butanol-methanol-water, 5:20:75) used by Krishnamurthy et al. (1986) and Brooks and Codd (1986) resulted in a better extraction of several microcystins. However, Lawton et al. (1995) found methanol to be superior to both acetic-acid and butanol-containing solvent mixtures for extracting most microcystin variants. We found 75% methanol to be modestly superior to both acetic-acid and butanol-methanol-water (5:20:75) for MC-LR and MC-LA extraction, which was in agreement with previous reports. This choice (75% methanol) had the added advantage of lower time consumption during the rotary evaporation step, because of higher organic content.

*Isolation:* SPE cartridge capacity was estimated by increasing feed loading, as shown in Table 3.1. The analysis of eluant from the various steps, for increasing loading,

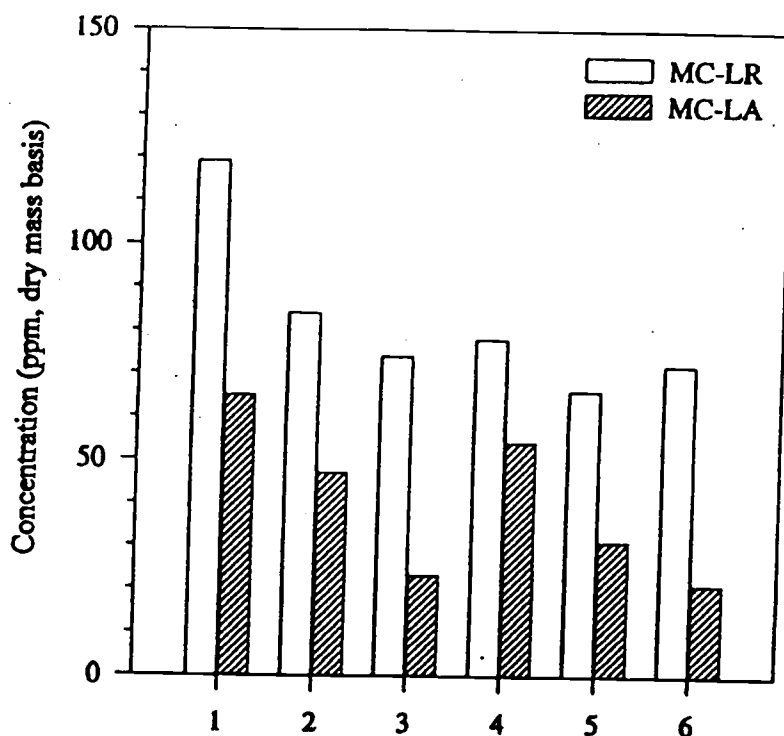


Figure 3.1. Efficacy of extraction of microcystins (MC-LR, lines; MC-LA, no lines) by various solvent systems. The x-axis corresponds to the solvent compositions used in the extraction, and are (1) MeOH-water (75:25); (2) Butanol-MeOH-water (5:20:75); (3) Butanol-MeOH-acetic acid-water (5:20:1:74); (4) MeOH-acetic acid-water (25:1:74); (5) MeOH-acetic acid-water (25:1:74); (6) Acetic acid-water (5:95). The samples were quantified by HPLC as per conditions given in Figure 3.2.

showed the presence of microcystins (both MC-LR and MC-LA) primarily (> 98%) in the MeOH-water (80:20) step. The 85% methanol step contained insignificant amounts of MC-LR and MC-LA (< 2 % of the total amount extracted). The purity of the microcystins in the SPE effluent was found to decrease slightly (from 85% to 80%) when the sample was enriched from 2 to 5 times. However, we were able to load up to ca. 1 mg each of MC-LR and MC-LA on this cartridge (Sep-Pak C<sub>18</sub>, 0.5 g). Our loading capacity for MC-LR (2 mg/ml, empty cartridge volume) was comparable to our estimate for Edward et al.[11] (1.7 mg/ml, empty column volume), where they used a flash-column.

Since our objective in preparative chromatography was to maximize the combined productivity, obtaining MC-LR and MC-LA in a single SPE fraction proved to be of significant advantage. While many reports (Moolan et al., 1996; Harada et al., 1991; Gathercole and Thiel., 1987) suggest 100% methanol for microcystins elution, we found that 80% methanol to be sufficient for eluting both MC-LR and MC-LA (Table 3.1) in a single step. Other stepwise elution sequences (e.g., loading step at 20% methanol, followed by elution using 35%, 45%, 55% methanol respectively) were tried, which not only had the disadvantage of not recovering all the MC-LR and MC-LA in a single step, but also did not remove additional impurities.

LLE experiments with solvents that are partially miscible with water (n-butanol and methyl-ethyl ketone) resulted in the formation of an intermediate layer. The microcystins were often present in more than one phase, thereby decreasing the efficiency of extraction. For example, in the butanol-water experiment, the MC-LR distributed as follows: 7% in the butanol phase, 43% in the intermediate layer, and 50% in the water phase. Similar intermediate layer formation was reported by Birk et al. (1989) when they

Sample	Load vol. (ml)	Elu.vol. (ml)	MC-LR ( $\mu\text{g}$ )	MC-LA ( $\mu\text{g}$ )
Feed	10	n/a	535 ( $\pm$ 22)	415 ( $\pm$ 15)
Loading step	n/a	10	0	0
20% MeOH	n/a	5	0	0
80% MeOH	n/a	5	558 ( $\pm$ 25)	389 ( $\pm$ 19)
85% MeOH	n/a	5	0	0
Feed	25	n/a	1338 ( $\pm$ 54)	1038 ( $\pm$ 38)
Loading step	n/a	25	0	0
20% MeOH	n/a	5	0	0
80% MeOH	n/a	5	1448 ( $\pm$ 51)	1157 ( $\pm$ 53)
85% MeOH	n/a	5	5.0 ( $\pm$ 0.2)	3.0 ( $\pm$ 0.5)

The feed represented the aqueous methanol extract of the cyanobacterial sample concentrated by ca. 10 times (full details on the feed and the extraction in the text). The effluent from the SPE cartridge was sampled during the feed loading step as well as the subsequent steps of 25, 80 and 85% MeOH. The microcystin concentration in each step was obtained from HPLC, using the conditions given in Figure 3.2.

Table 3.1. The loading capacity for microcystins on the SPE cartridge.

used diethyl ether as the extraction solvent. We found hexane to be the most efficient in terms of easy phase separation and removal of highly non-polar impurities. No intermediate layer was formed in this case. However, the microcystins were not concentrated (unlike SPE) because they remained in the aqueous phase, whose volume does not change appreciably in LLE.

The efficacy of SPE and LLE was compared by quantifying the neighboring impurities (peaks between 7 minutes and 13 minutes for MC-LR, and between 23 minutes and 30 minutes for MC-LA). A 5 ml feed volume was used in both SPE and LLE runs. Both methods resulted in comparable purity levels for MC-LR ( $77\pm 1\%$  by SPE and  $71\pm 0.2\%$  by LLE) and MC-LA ( $83\pm 2\%$  by SPE and  $82\pm 4\%$  by LLE). Albeit the processing times for both SPE and LLE were comparable, SPE was preferred as the

isolation step since it not only resulted in the removal of strongly hydrophobic impurities (achieved by LLE), but was also able to concentrate the microcystins.

*Purification:* Figure 3.2 shows the separation of MC-LR and MC-LA from their respective impurities on an analytical column. For preparative work (Figure 3.3a), this modulator schedule was slightly altered: the gradient was made less steep in order to prevent the potential contamination of MC-LA. This method resulted in milligram quantities of purified MC-LR and MC-LA. Microcystin fractions collected from the preparative run were quantified by analytical HPLC, and the reconstructed chromatograms are shown in Figures 3.3b and c. Each microcystin had several closely retaining impurities. The chromatographic profile for MC-LR (Figure 3b) shows a non-Gaussian peak with a dip in the middle. The tailing effect is attributed to nonlinear adsorption, while the increase in concentration after the dip is due to its desorption induced by the adjacent higher-retaining impurity. Figure 3.3c shows enrichment of MC-LA, which is attributed to the focussing effect of the gradient (Snyder 1980; Yamamoto 1988).

A convenient measure of the efficacy of a preparative run is productivity, which is

defined as

$$\text{Productivity} = \frac{\text{Product at specified purity}}{\text{Overall run time} \times \text{Empty column volume}} \quad (\text{mg/ml} \cdot \text{hr})$$

where the overall run time is the sum of the time required for the elution of desired peak and the time required to regenerate the column.

Since MC-LR and MC-LA have comparable toxicity and commercial value, our objective was to optimize the conditions that resulted in the maximum combined productivity. We recovered 3 mg of MC-LR at 90% purity and 4 mg of MC-LA at



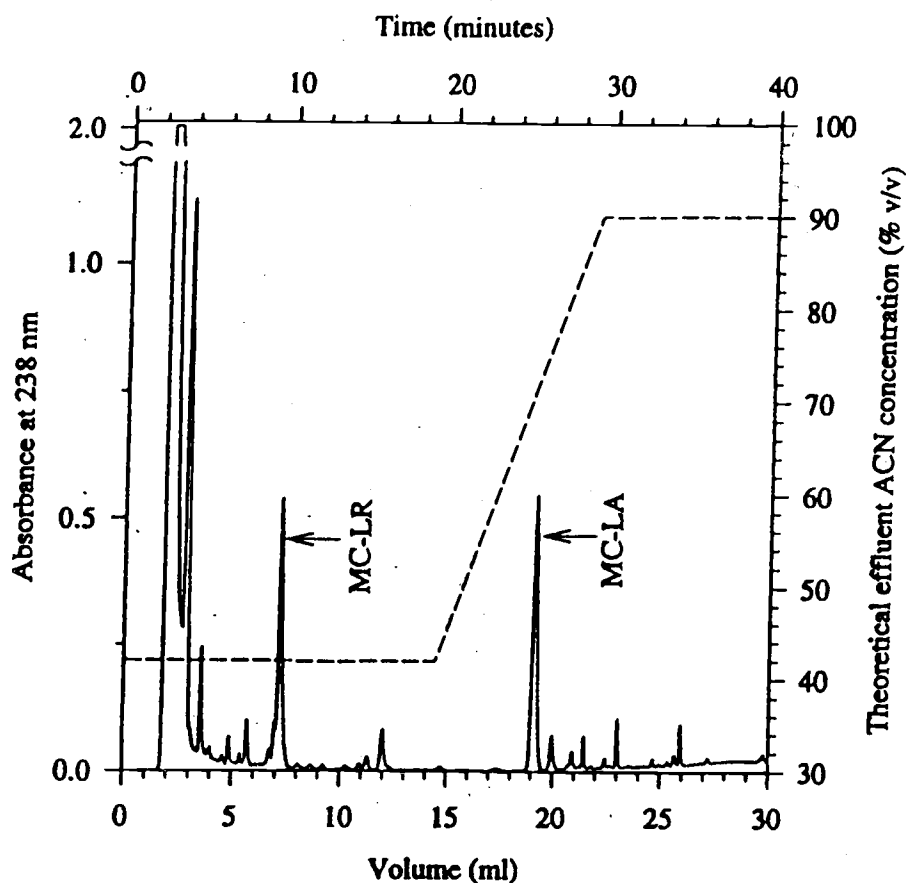


Figure 3.2. Separation of MC-LR and MC-LA on an analytical column (25 cm X 0.46 cm I.D.). The feed volume was 50  $\mu$ l. The chromatographic conditions are as follows: isocratic ACN-water-TFA (42:58:0.05) for 15 minutes followed by a gradient of ACN-water-TFA (42:58:0.05) to ACN-water-TFA (95:5:0.05) in 10 minutes. The flow-rate was 0.75 ml/min. The gradient delay in our system was 5.6 ml. The theoretical effluent ACN accounts for system delay but does not account for ACN retention on the sorbent.

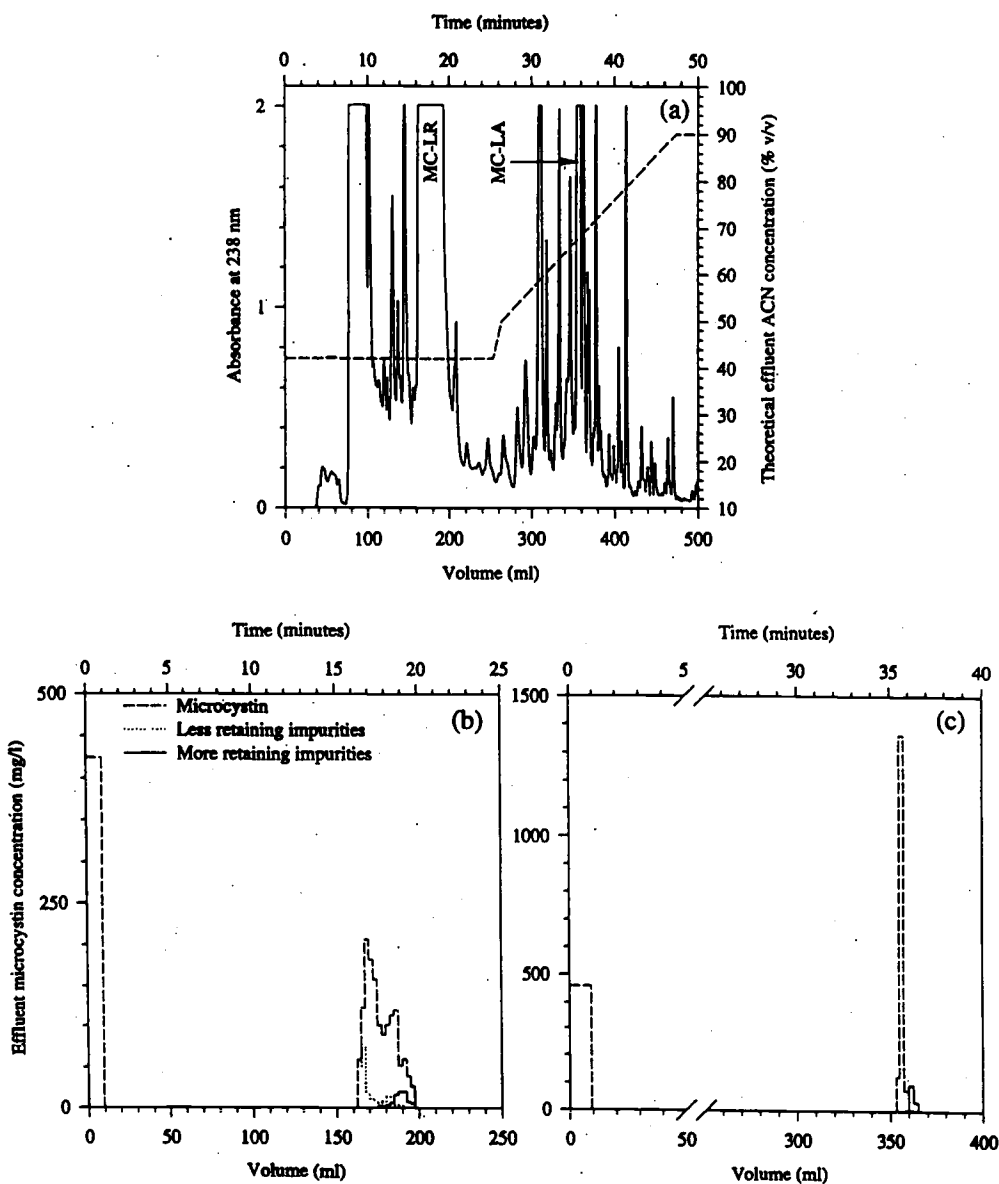


Figure 3.3. Separation of MC-LR and MC-LA on a preparative column (25 cm X 2 cm I.D.). The trace from the preparative run is given in panel (a). The column conditions immediately after sample injection was: isocratic ACN-water-TFA (42:58:0.05) for 25 minutes followed by a step gradient of ACN-water-TFA (50:50:0.05) in 1.0 minutes and then to ACN-water-TFA (90:10:0.05) in 21 minutes. The flow-rate was 10 ml/min. The loading was 4.0 mg of MC-LR and 4.3 mg MC-LA. The panels (b) and (c) are the reconstructed chromatograms of MC-LR and MC-LA respectively in the preparative run, analyzed as per conditions given in Figure 3.2.

greater than 98% purity in a single run, which resulted in a combined productivity of 0.09 mg/ml-hr. If the purity requirement for MC-LR was 95%, the combined productivity dropped to 0.06 mg/ml-hr (0.9 mg of MC-LR and 3.9 mg of MC-LA was recovered). These values were comparable to many found in the literature (see Table 2 for comparison). Experiments carried out with a lower loading (1 mg each of MC-LR and MC-LA) run under the same conditions resulted in lower productivity (0.03 mg/ml-hr). Other gradient schedule at a loading of 2 mg each of MC-LR and MC-LA also resulted in a lower productivity (0.04 mg/ml-hr). Although the runs with lower loading resulted in higher yields, their productivity values were lower, while significantly overloaded run gave higher productivity.

The comparison of our post-SPE (80% methanol fraction) chromatogram with the analysis of the post-flash MC-LR-rich fraction of Edwards et al. (1996) shows fewer peaks for the latter. Since methanol was used as the extraction solvent in both cases it appears that our starting sample had significantly more components than that of Edwards et al. However, the microcystin content in our dry samples was estimated to be lower by a factor of 10 than many reported values (see Table 3.2). Hence a more equitable comparison of the effectiveness of microcystin purification method is obtained by taking the ratio of obtained productivity (P) to the initial level (I.L., concentration on dry weight basis). Despite the lower microcystin levels in the starting material and a complex feed, we achieved P/I.L. values better than or comparable to many results, as seen in Table 3.2. Hence, it is quite possible that our method could potentially yield much higher productivities when used on samples with higher microcystin content.

*Identification and Analysis of Microcystins:* Figures 3.4a and b are the mass spectra of purified MC-LR and MC-LA fractions. The published m/z values for MC-LR and MC-LA are 995 and 910 respectively (Watanabe 1996). In Figure 3.4a, besides the m/z peak at 995 we observed an additional m/z peak of 498.4. This is due to the association of two protons with MC-LR. For MC-LA we noticed two peaks, one at 910 and the other at 932. While the former is the signature of MC-LA, the latter could be due to the presence of trace amounts of sodium salt of MC-LA, possibly formed from the sodium hydroxide added to adjust the pH of the HPLC mobile phase. The association of alkali metals with microcystins has been previously reported by Dale et al. (1994) and by Yuan et al. (1999). The chromatograms of purified MC-LR and MC-LA fractions are given in Figure 3.5a and b. The increase in baseline after MC-LA elution is due to the UV absorption of ACN.

ELISA analysis of purified MC-LR fractions resulted in a good correlation with HPLC analysis only when the samples were free of HPLC solvents (ACN and TFA) and limited dilution. Significant gain in correlation was achieved only when the required dilutions were lower.

*Removal of Microcystins:* The use of activated carbon to remove microcystins was studied. The liquid filter-waste sample with microcystin concentration of  $18 (\pm 3)$  ppm (MC-LR and MC-LA combined) was reduced to undetectable levels within an hour. Rate experiments reported by Donati et al. (1994) with starting MC-LR concentrations of 2.2 ppm showed presence of MC-LR even after 72 hours. This was due to lower amounts of activated carbon used in comparison to that of ours [10-30 mg/L (water) versus 20 g/L (slurry process stream)]. We used large amounts of activated carbon in order to reduce

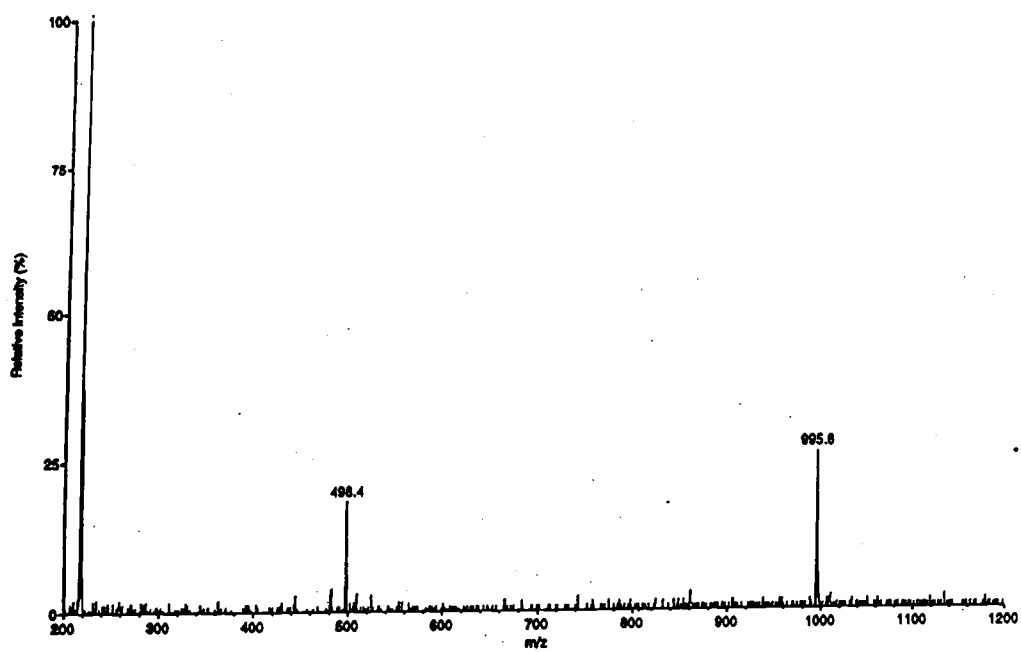


Figure 3.4. Electrospray mass-spectrophotometry of microcytin-LR from chromatography.

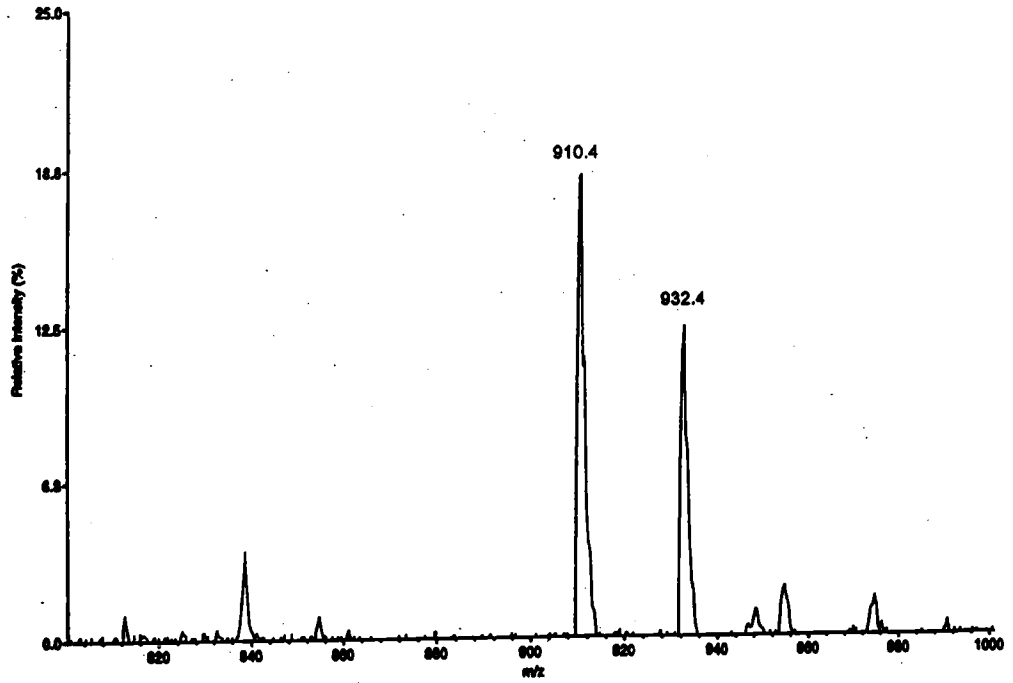


Figure 3.5. Electrospray mass-spectrometry analysis of microcystin-LA from chromatography.

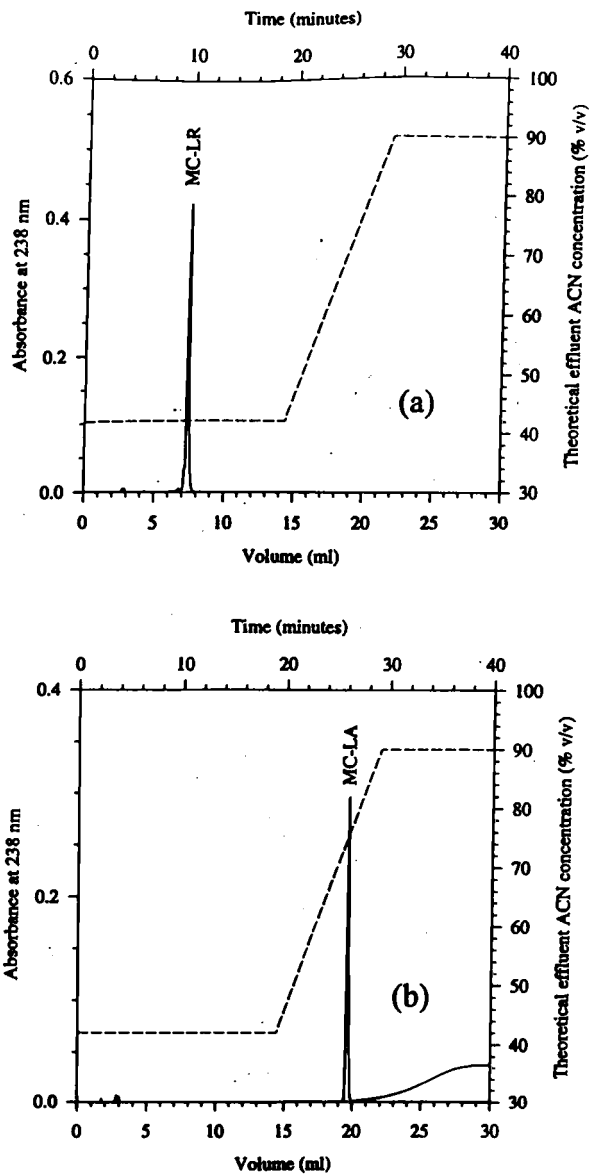


Figure 3.6. HPLC analysis of the purified sample. (a) MC-LR (b) MC-LA. All experimental conditions are same as in Figure 3.2.

Table 3.2. Productivity of microcystins.

Component	I.L. <sup>a</sup> (ppm)	Column L (cm)×I.D. (cm)	Recovery (mg)	<i>P</i> (mg/ml h)	<i>P</i> /I.L. <sup>b</sup> ×10 <sup>-4</sup>	Refs.
MC-LR	2147	Shandon HS BDS C <sub>18</sub> 15×7.5	260	0.67	3.12	[11]
MC-LR	4841	Hyperprep HS C <sub>18</sub> 15×7.5	416	1.08	2.20	[12]
MC-LR	7030	Novapak C <sub>18</sub> 10×2.5	70.3	0.38	0.54	[16]
5 Methyl ester derivatives of MC-LR	700	μ-Bondapak 15×1.9	0.35	0.01	0.20	[27]
MC-LR	240	μ-Bondapak 15×1.9, 30×1.9 Alltech ODS (25×0.46)×2	1.20	0.004	0.18	[26]
Mixture of 7 MC-LRs	3054	μ-Bondapak 15×1.9	15.3	0.06	0.18	[29]
3-Demethyl MC-LR	230	Chromatex ODS 94×1.1	2.80	0.05	2.34	[18]
7-Demethyl MC-LR	3333	Chromatex ODS 91×1.1 Toyopearl HW-40 91×1.2	14.3	0.09	0.27	[28]
3,7-Didesmethyl MC-LR	350	Cosmosil 5C <sub>18</sub> -P 25×1	1.50	0.02	0.49	[28]
MC-LR	1000–4000	Altex C <sub>18</sub> 25×0.94	0.75–3.00	0.07–0.30	0.70–0.75	[14]
MC-LR and MC-LA	210	Kromasil C <sub>18</sub> 25×2	4.80	0.06	1.60	Present work

<sup>a</sup> μg/g of dry biomass.<sup>b</sup> g of dry biomass/ml h. I.L., initial level; *P*, productivity.



the competition between microcystins and other biomolecules for the binding sites. Since activated carbon is inexpensive, large quantities of liquid sample can be stripped of microcystins economically. The agreement of our results with those in the literature for different feed stocks indicates that this is likely to be an economical large-scale method for the removal of microcystins.

## **CONCLUSIONS**

Microcystins LR and LA were purified from processed natural samples by preparative reversed phase chromatography. Amongst the many organic solvents tried, methanol was found to be the best extracting agent, which is in agreement with several previous reports. Very high loading of microcystins on the solid-phase extraction cartridge was achieved. The analytical HPLC method was scaled-up to a preparative column, with substantial overloading, where milligram quantities of MC-LR and MC-LA at higher than 95% purity was obtained in a single step.

## **ACKNOWLEDGMENTS**

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## Chapter 4

### Isolation of Phycobiliproteins: Large scale Production and Process Economics

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In review: *Bioresource. Tech.*

## **ABSTRACT**

Phycobiliproteins are naturally occurring intensely colored pigments of immense commercial potential. Here, we report a simple and economical process to purify phycocyanins and allophycocyanins from a cyanobacterial process waste stream for two applications: as an inexpensive blue food colorant and as a biomedical marker. The process for the food-grade product involved size-exclusion chromatography with Sephadex G-25 (MW cutoff, 5 kDa) to eliminate the microcystins that could potentially be present in the wild-type samples. Consequently, the reversed-phase HPLC of purified product showed no microcystins. The purity of the product, measured as the ratio of absorbances at 620 nm and 280 nm, was four times greater than the purity of commercially available product. The process for biomedical-grade product included salt precipitation followed by anion-exchange chromatography. The purity of the product was measured by SDS-PAGE, absorption spectra and fluorescence spectra. A detailed design for the large-scale production of biliproteins for both applications is also presented. Economic evaluation of the process resulted in comparable costs with the current market price for the food-grade product and substantially lower cost for the biomedical grade product.

**KEYWORDS:** Phycobiliproteins, phycocyanin, allophycocyanin, cyanobacteria, ion-exchange, economics.

## INTRODUCTION

Phycobiliproteins belong to a family of light-harvesting photosynthetic pigments, and are present in the chloroplasts of intact cyanobacterial cells, red algae and cryptomonads (MacColl and Guard-Friar, 1987; Packer and Glazer, 1988). Biliproteins are the constituents of a complex assembly, the phycobilisome (Packer and Glazer, 1988), and are broadly classified according to their structure and spectroscopic properties: phycocyanins (PC, absorption maximum, 610-620 nm), allophycocyanins (APC, absorption maximum, 650-655 nm) and phycoerythrins (PE, absorption maximum, 545-565 nm). Biliproteins are brightly colored due to the presence of a linear tetrapyrrole chromophore (bilin) to which the apoproteins (polypeptide chains,  $\alpha$  and  $\beta$ ) are covalently linked through specific cysteine residues. The physical and chemical properties of biliproteins are extremely complex (Packer and Glazer, 1988). They are highly charged, extremely soluble in water, and are known to exist in several aggregation states (monomer-dimer, monomer-trimer and monomer-hexamer) depending on the pH, ionic strength, concentration, temperature and buffer composition.

Biliproteins have many uses. They are used as a natural dye in the food industry (Hendry and Houghton, 1996; CQVB, 1988), as a fluorescent tag in immunoassays (Kronick, 1986), as a standard in iso-electric focusing, and in the cosmetic industry. A detailed discussion on the applications of biliproteins is given in Glazer (1994). At present there are no naturally derived, food-grade blue colorants available in the United States (Pszczola, 1998).

There are several structural proteins present in the phycobilisome (Packer and Glazer, 1988) that can interfere during the purification process. The chemical complexity

of the impurities coupled with the increasing demand for biliproteins makes their large-scale purification a challenging and relevant problem.

Although there are several reports that describe the purification of biliproteins (Cohen-Bazire et al., 1977; MacColl et al., 1971; Boussiba and Richmond, 1979), the raw material for their processes was a homogenous cyanobacterial culture that requires substantial effort to maintain and grow at large-scale. In contrast, we describe a simple and easily scalable process to isolate biliproteins from an industrial process waste, thereby resulting in a truly value-added product of significant commercial value. A detailed design and economic evaluation for the large-scale production of biliproteins for two kinds of applications (food-grade and biomedical grade marker) is also provided.

Every summer, a natural cyanobacterial bloom is formed on Klamath Lake in Oregon. More than 90% of this bloom consists of *Aphanizomenon flos-aquae* (Kann, 1998). The residue is a mixture of various species of cyanobacteria, e.g., *Microcystis Sp.* and *Anabaena Sp.* *Aphanizomenon flos-aquae*, sold as a nutrient supplement, was separated from *Microcystis Sp.* and *Anabaena Sp.* by a filtration process. The filtrate is the process waste that served as the starting material for the process reported here.

## **Methods**

### *Materials*

Q-Sepharose Fast Flow ion exchange and Sephadex G-25 size exclusion sorbents were obtained from Pharmacia (Piscataway, NJ, USA). Kimble-Kontes Glass columns (2.5 cm I.D. × 60 cm L) were purchased from Fischer Scientific (Pittsburg, PA, USA). Tris-(hydroxymethyl) aminomethane, sodium chloride, blue dextran, and protein

standards for PAGE were purchased from Sigma Chemicals (St. Louis, MO, USA). The reagents (sodium dodecyl sulfate, glycerol, 2-mercaptoethanol, bromophenol blue, glycine, and ready made 12% polyacrylamide gels) were from Biorad (Hercules, CA, USA). The peristaltic pump (Catalog No. 54856-075) was purchased from VWR Scientific (South Plainfield, NJ, USA). The raw material, a heterogeneous mixture of natural cyanobacterial blooms, was provided by A.F.A., Inc. (Klamath Falls, OR, USA).

### *Apparatus*

Waters (Milford, MA, USA) HPLC system, which consisted of a quaternary pump (Model 600), UV detector (Model 486), and an autosampler (Model 717 plus), was used for analyzing phycocyanin samples. All the instruments were controlled by a DEC (Nashua, NH, USA) personal computer using Waters Millennium software. The protein samples were analyzed using an Alltima C<sub>18</sub> column (Alltech, Inc., Deerfield, IL, USA; 0.46 cm I.D. × 25 cm L). A Beckman Model J2-MI (Fullerton, CA, USA) centrifuge was used in the isolation sequence to remove cell debris from the supernatant containing phycobiliproteins. A Savant speed-vac (Holbrook, NY, USA) was used to concentrate the liquid samples. SDS-PAGE runs were carried out on a Mini-Protean II (Biorad, Hercules, CA, USA) system. Absorption spectra were measured with a Beckman Model 605 (Fullerton, CA, USA) spectrophotometer. Fluorescence spectra of the purified samples were obtained using a Perkin-Elmer Model CL-50B (Norwalk, CT, USA) chemiluminescence spectrophotometer.

## *Procedures*

The cyanobacterial process waste stream (300 g) stored at  $-20\text{ }^{\circ}\text{C}$  was chipped into smaller pieces, thawed and centrifuged at  $11,300 \times g$  for 30 minutes at  $18\text{ }^{\circ}\text{C}$ . Freezing causes the cell walls to rupture. The supernatant (200 ml) containing the phycobiliproteins was saved for further purification, and the green pellet was discarded. The pellet was re-dissolved in water, and was found to contain no biliproteins. However, sonication of the cells yielded an additional 30% of proteins. Since the biomass did not constitute for any expenses and was available in plenty, sonication of unbroken cells was not carried out during the purification process. Figures 4.1 and 4.2 show the sequence of operation for the food-grade and biomedical-grade biliprotein production, respectively.

*Precipitation:* The supernatant from the centrifugation step was brought to 40% of saturation by the addition of solid ammonium sulfate. The protein extract was allowed to stand in the refrigerator at  $4\text{ }^{\circ}\text{C}$  for one hour. The blue biliproteins precipitated leaving behind a dark yellow liquid. The protein precipitate was further separated from the bulk liquid by centrifugation at  $24,010 \times g$  for 20 minutes. The centrifugation process was carried out at  $4\text{ }^{\circ}\text{C}$  to avoid the dissolution of protein back in to the bulk liquid.

*Size Exclusion Chromatography (SEC):* A Kimble-Kontes glass column (2.5 cm I.D  $\times$  25 cm L) was packed with Sephadex G-25 material that was pre-swollen and equilibrated with Tris-HCl buffer (20 mM, pH 7.0). The feed was concentrated five-fold using a rotary evaporator at  $30\text{ }^{\circ}\text{C}$ , and a 20-ml aliquot was applied at the top of the bed. The biliproteins were eluted using distilled water. Immediately after the elution of blue band, which occurred at the empty column volume, the column was washed with 300 ml of 20% aqueous ethanol. The column was then stored in the 20% aqueous ethanol to

avoid pyrogen growth. Washing with three empty column volumes ( $123 \text{ ml} \times 3$ ) of the eluant prepared the column for the subsequent run.

*Ion-Exchange Chromatography:* The Q-Sepharose Fast Flow anion-exchange material was packed in a glass column ( $2.5 \text{ cm I.D.} \times 25 \text{ cm L}$ ). The column was equilibrated using three empty column volumes ( $123 \text{ ml} \times 3$ ) of the starting buffer. The equilibration step was followed by the feed loading step and the step-wise elution of proteins by modulator. Tris-HCl ( $20 \text{ mM}$ ,  $\text{pH } 7.0$ ) always served as the equilibration buffer, while sodium chloride dissolved in equilibration buffer was the modulator. The phycocyanin-rich fraction was first isolated from an allophycocyanin-rich fraction. The starting condition for this step was  $75 \text{ mM}$  modulator. The protein mixture previously dissolved in the equilibration buffer was introduced at the top of the column using a peristaltic pump at  $1 \text{ ml/min}$ . The PC and APC rich fractions were eluted using  $140 \text{ mM}$  and  $235 \text{ mM}$  modulator respectively. All the PC-rich fractions with  $A_{620}/A_{280}$  value of  $3.2$  or greater, and all APC fractions with  $A_{650}/A_{280}$  value of  $3.5$  or greater, were pooled and rechromatographed separately. The ratio of absorbances measured at  $620 \text{ nm}:280 \text{ nm}$  and  $650 \text{ nm}:280 \text{ nm}$  represent a measure of purity. Typically, if the ratios were greater than  $4.0$ , then the sample was considered to be high/moderate purity (Packer and Galzer, 1988).

*Rechromatography of biliproteins:* The PC fractions with  $A_{620}/A_{280}$  greater than  $3.2$  (marked I in Figure 4.4a) were pooled, desalted and loaded at  $1 \text{ ml/min}$  on to an ion-exchange column that was equilibrated at  $75 \text{ mM NaCl}$ . After the feed loading step, the modulator level was switched to  $100 \text{ mM NaCl}$  at  $2 \text{ ml/min}$ . A red impurity eluted while phycocyanin band spread to almost half the total column length during this step. The



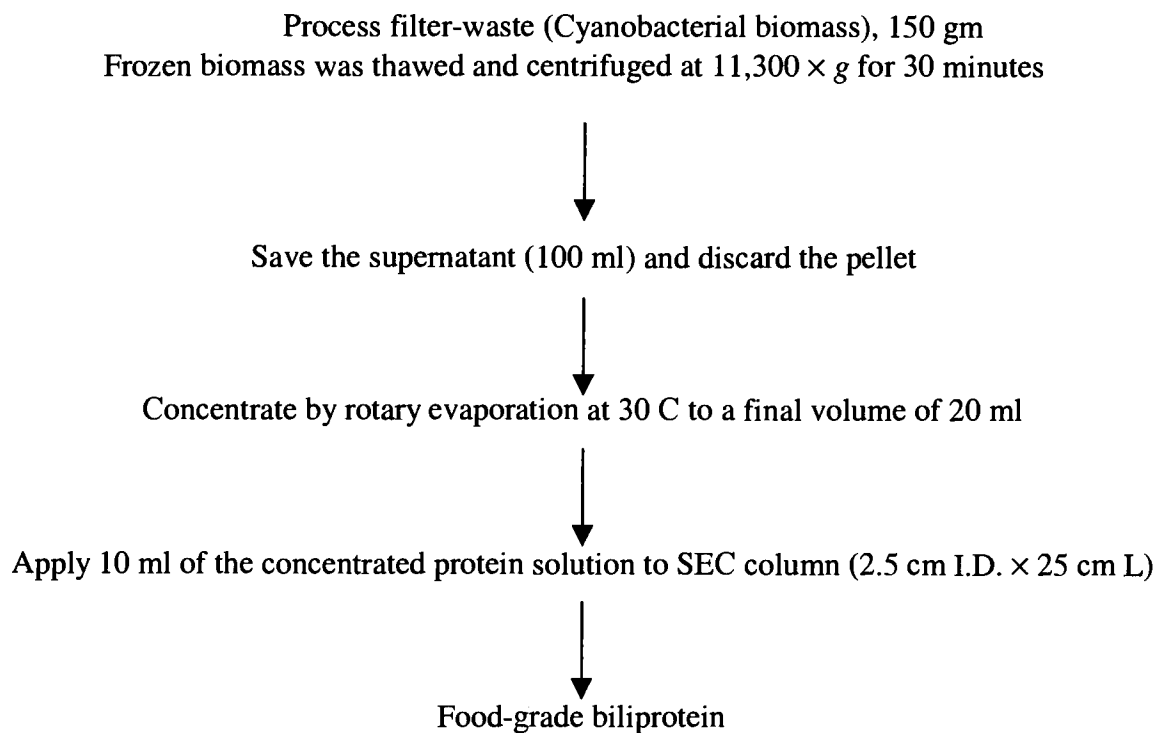


Figure 4.1. Process flow-sheet for the lab-scale production of food-grade phycobiliprotein.

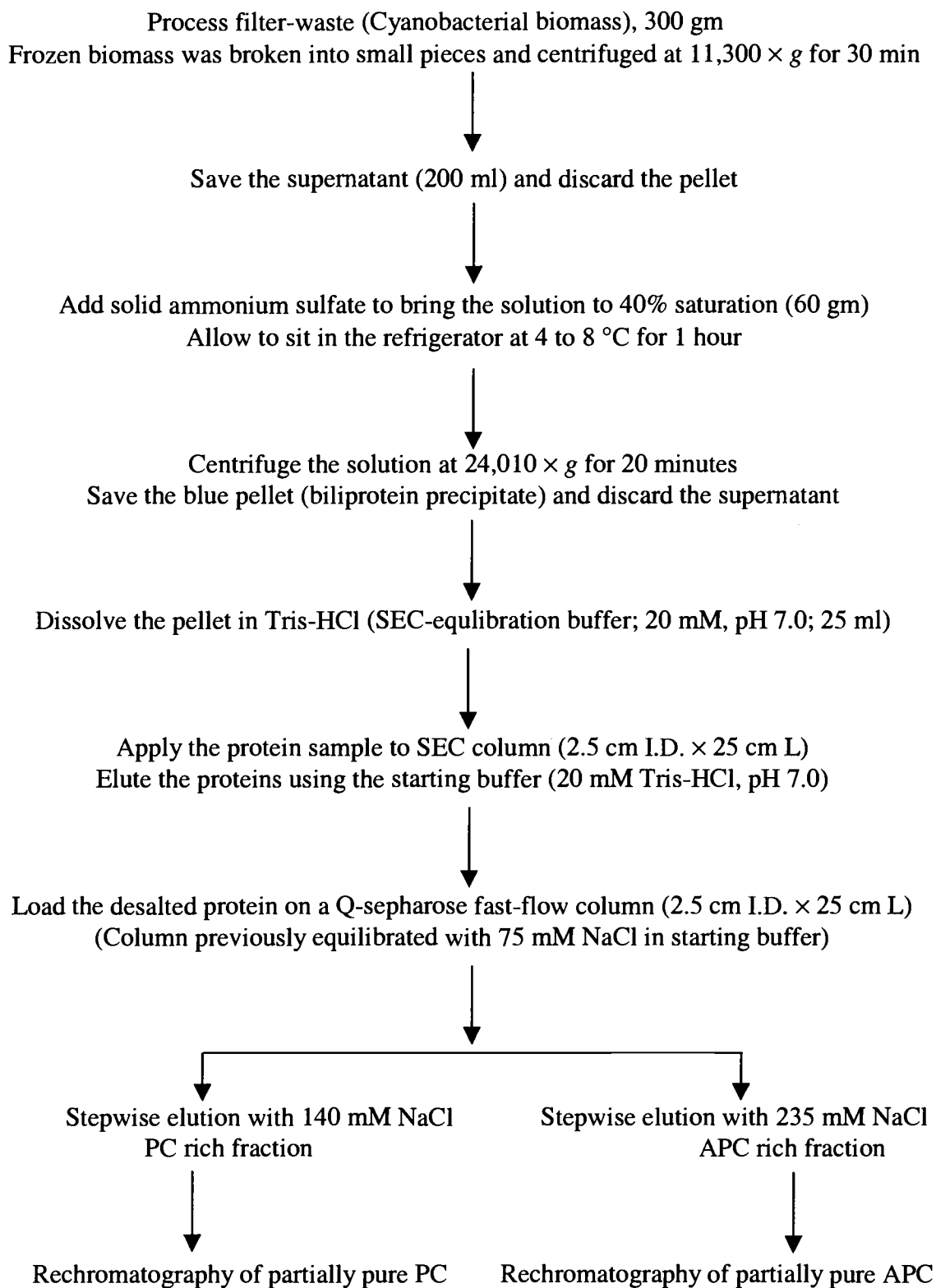


Figure 4.2. Process flow-sheet for the production of biomedical-grade biliprotein.

phycocyanin band was eluted by 125 mM NaCl at 2 ml/min, and 15-ml fractions were collected. The tail portion of the phycocyanin fraction was eluted by 140 mM NaCl. The column was washed with 160 mM salt after the elution of the PC fraction.

Allophycocyanin fractions marked II in Figure 4.4a were pooled and desalted for further purification. The desalted protein was loaded on to a column previously equilibrated with 140 mM NaCl. The feed loading step was carried out at 1 ml/min while the rest of the run was carried out at 4 ml/min. During the feed loading step, a pink band eluted at the empty column volume. The column was washed with one column volume (123 ml) of 160 mM NaCl to ensure the complete removal of this pink band. The modulator level was switched to 175 mM NaCl that resulted in the elution of APC; 20-ml fractions were collected. The column was washed with 235 mM salt after the elution of APC band to free the column of adsorbed proteins.

### *Analytical methods*

*Absorption spectra:* The purity of the protein fractions from chromatography was measured by UV-visible spectroscopy. The samples were either diluted in Tris-HCl buffer or concentrated to allow the raw readings to be between 0.1 and 1.0 AU, and the absorption was measured at 280, 620 and 650 nm. The protein standards from a commercial source showed a linear relationship between the concentration and the absorbance in this range, and hence this range was adopted for our samples.

*Fluorescence spectra:* The fluorescence spectra of the purified samples were measured by diluting the sample in Tris-HCl buffer. The PC and APC samples were excited at 600 and 580 nm respectively. The emission spectra for PC fractions were

recorded between 610 and 800 nm and that for APC fractions between 590 and 800 nm.

*SDS-PAGE*: Electrophoresis under denaturing conditions was carried out according to Laemmli (1970) on a 12% polyacrylamide gel at pH 8.3. The denatured protein samples (20  $\mu$ l) were loaded on the gel, and the reservoir was filled with the running buffer. The edges of the plate were sealed using agarose gel to prevent the leakage of running buffer from the dam.

*HPLC*: The biliprotein fractions eluting from the size exclusion step were processed through a column packed with Q-sepharose fast-flow material (2.5 cm I.D  $\times$  5 cm L) to remove the highly adsorbing molecules prior to biliprotein analysis by HPLC. The samples were then injected into an Alltima column (0.46 cm I.D.  $\times$  25 cm L) previously equilibrated with MeOH:water:TFA (60:40:0.05). Immediately following the sample injection, the methanol level in the mobile phase was increased from MeOH:water:TFA (60:40:0.05) to MeOH:water:TFA (90:40:0.05) in 40 minutes duration. The flow rate was maintained at 0.75 ml/min.

## RESULTS AND DISCUSSION

### *Characterization of feed*

The typical concentrations of biliprotein in cyanobacteria may be up to 24% of the dry weight of the cells, and well over half of the total soluble protein (Myers, 1955; Bennett, 1973). The liquid concentration of PC and APC in the process waste stream, after the initial centrifugation step, were 1.72 ( $\pm$  0.30) mg/ml and 1.32 ( $\pm$  0.30) mg/ml respectively. Analysis of two process waste streams supplied to us (corresponding to algae samples collected from Klamath Lake at different times) revealed no significant

variation in biliprotein concentration (Batch # 2: PC,  $1.87 \pm 0.05$  mg/ml; APC  $1.02 \pm 0.03$  mg/ml). The overall protein content measured as the absorbance at 280 nm was also consistent across samples (< 5%). The primary impurities in PC and APC purification are phycobilisome constituents, e.g., phycoerythrins (red proteins) and linker polypeptides (colorless).

#### *Purification for food colorant application*

The process for food-grade biliprotein involved the isolation of small molecules from the biliproteins by size-exclusion chromatography (SEC). The blue protein band was eluted by distilled water under gravity (linear velocity, 1.2 cm/min). The isolated protein fractions were lyophilized and stored.

Although the biliprotein fraction contained other proteins, the purity, as measured by absorbance ratio  $A_{620}/A_{280}$ , was typically 2. The purity for the product according to this report was higher than several previous reports: 0.54 for commercial food-colorant from Dainippon Ink & Chemicals, Inc.; 0.94 according to Hohlberg et al. (1989); and 1.0 reported by Reis et al. (1998). This is a significant improvement, given that our starting biomass was an industrial waste stream. The economic evaluation of our process and a comparison to the work of Reis et al. (1998) is described in a later section.

The cyanobacterial process waste stream is known to contain microcystins, a class of cyclic heptapeptides, with molecular mass of ca. 1 kDa (Watanabe et al., 1996; Ramanan et al., 2000). Microcystins are potentially harmful (Carmichael, 1994) and hence should be removed from food-grade product. This was achieved by utilizing a SEC with molecular mass cut-off of 5 kDa. Thus the SEC step simultaneously removes

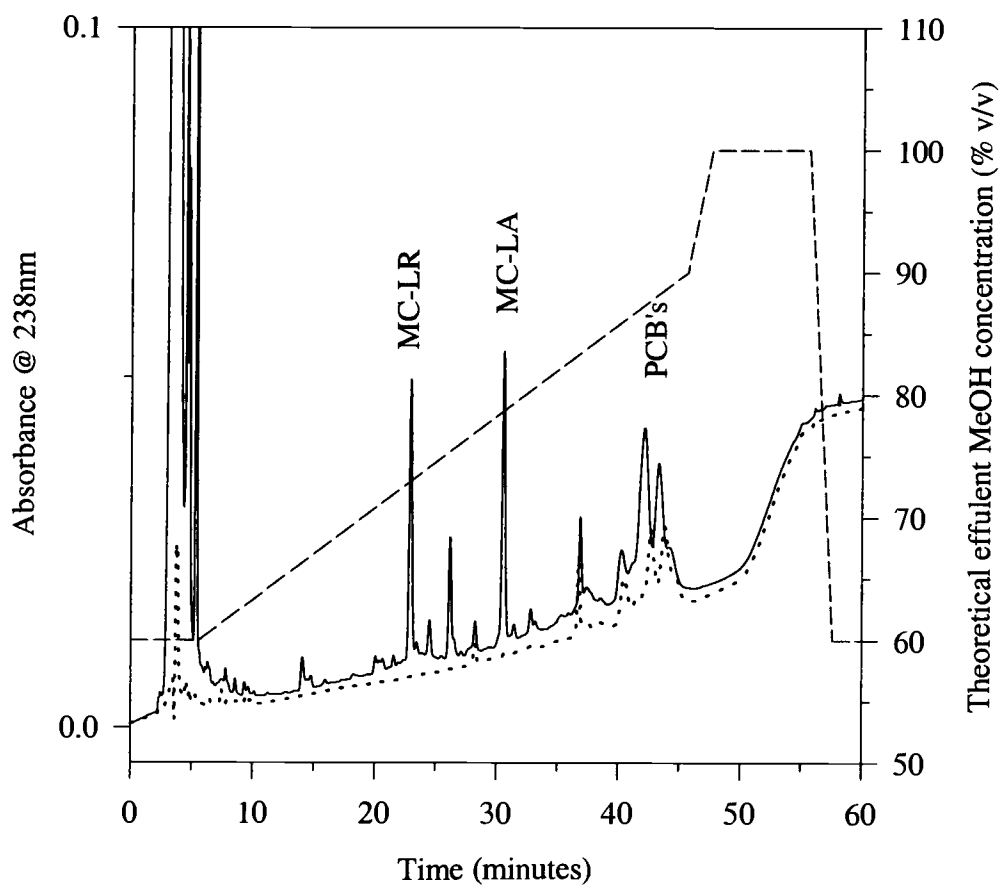


Figure 4.3. Reversed-phase HPLC analysis of food-grade biliprotein mixture prior to SEC (solid line) and after the SEC step (dots), showing the removal of microcystin. Experimental conditions: linear gradient of MeOH:water:TFA (60:40:0.05) to MeOH:water:TFA (90:40:0.05) in 40 minutes. The flow rate was maintained at 0.75 ml/min. The gradient delay in our system was 5.6 min. The gradient is shown in broken lines.

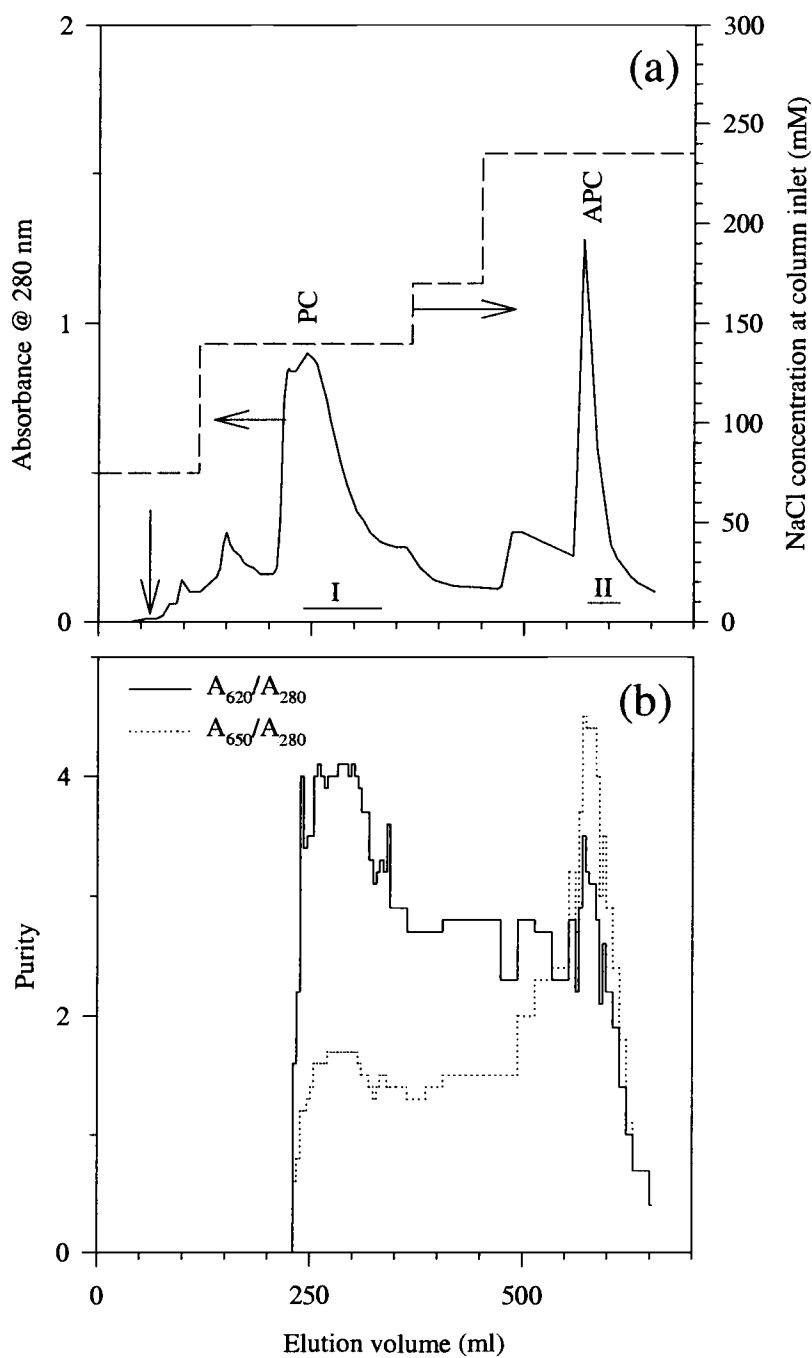


Figure 4.4. Chromatographic profile of phycocyanins and allophycocyanins on a Q-Sepharose Fast-Flow column (2.5 cm I.D  $\times$  20 cm L) is shown in panel a. The secondary y-axis shows the modulator concentration (broken lines). The purity as the ratio of absorbance measured at 620/280 nm for phycocyanin and 650/280 nm for allophycocyanin fractions is shown in panel b.

microcystins and other small molecules that would otherwise reduce the  $A_{620}/A_{280}$  ratio. Figure 4.3 shows the HPLC analysis of phycocyanin fraction prior to and after the post size-exclusion step. The microcystins LR and LA, where LA and LR stands for the two variable amino acids in the back bone, (Figure 4.3, solid line) elute at 23 and 31 minutes respectively, while the biliproteins elute between 41 and 45 minutes. The purified fraction (Fig. 4.3, dotted line) showed no microcystins peaks. An unidentified peak eluting between MC-LR and MC-LA at 28 min was found in the purified fraction. However, a sensitive enzyme based assay for microcystins (data not shown) carried out on post-SEC phycocyanin fractions also showed no microcystins, indicating that the size-exclusion step was sufficient to remove the microcystins.

#### *Purification for biomedical application*

*Isolation of PC and APC from impurities:* The concentrated biliprotein mixture from the precipitation step was desalted and purified further on an anion-exchange column. Figure 4.4a shows the elution profile from a Q-Sepharose Fast Flow column. Fractions were collected every 4 ml, and their purity assessed by absorption spectroscopy is given in Figure 4.4b.

The loading step was carried out at 75 mM NaCl in 20 mM Tris-HCl (pH 7.0), at 1 ml/min (the arrow in Figure 4.4a represents the end of the loading process).

Preliminary anion-exchange runs showed that a moderate salt concentration was essential for easy removal of less retaining impurities while imparting retention of PC and APC to the column. During the loading step, an unretained yellow band eluted, and was discarded. Immediately after the elution of the yellow band, the salt concentration was



increased to 140 mM NaCl. The flow-rate was increased simultaneously to 2 ml/min. A pink band (peak between 140 and 180 ml in Figure 4.4a), which followed the yellow band, was also discarded. Phycocyanin fractions followed the pink colored component and 4 ml fractions were collected. These fractions were saved for spectrophotometric analysis and rechromatography. The absorption measurements showed  $A_{620}/A_{280} > A_{650}/A_{280}$ . Since PC's typically have an absorption maximum between 615-620 nm (Glazer and Packer, 1988), this peak was identified as phycocyanin and labeled as PC in Figure 4.4a. After the elution of the PC band, the bottom one-third of the column was bluish-red in color, while the allophycocyanin band had spread to almost half the column length. The bluish-red impurity was eluted using 170 mM NaCl at 4 ml/min. Immediately following the breakthrough of the bluish-red front, the modulator level was increased to 235 mM NaCl at the column inlet. Allophycocyanin fractions (4 ml each) were collected and stored for analysis and further purification. The absorption measurements of the fractions showed  $A_{650}/A_{280} > A_{620}/A_{280}$ . Since APC's typically have an absorbance maximum at 650-652 nm (Glazer and Packer, 1988), this peak was identified as allophycocyanin and labeled as APC in Figure 4.4a.

The process parameters such as flow-rate and salt level for each step were chosen based on several preliminary runs. To minimize band spreading, flow rate of 1 ml/min was chosen for the loading step. A flow rate of 2 ml/min for the phycocyanin elution step was found to give the best compromise between minimizing separation time and maximizing resolution. Allophycocyanins were efficiently recovered at 4 ml/min.

*Rechromatography of biliproteins:* The spectrophotometric analysis of all the phycocyanin fractions collected from the second chromatography runs showed an

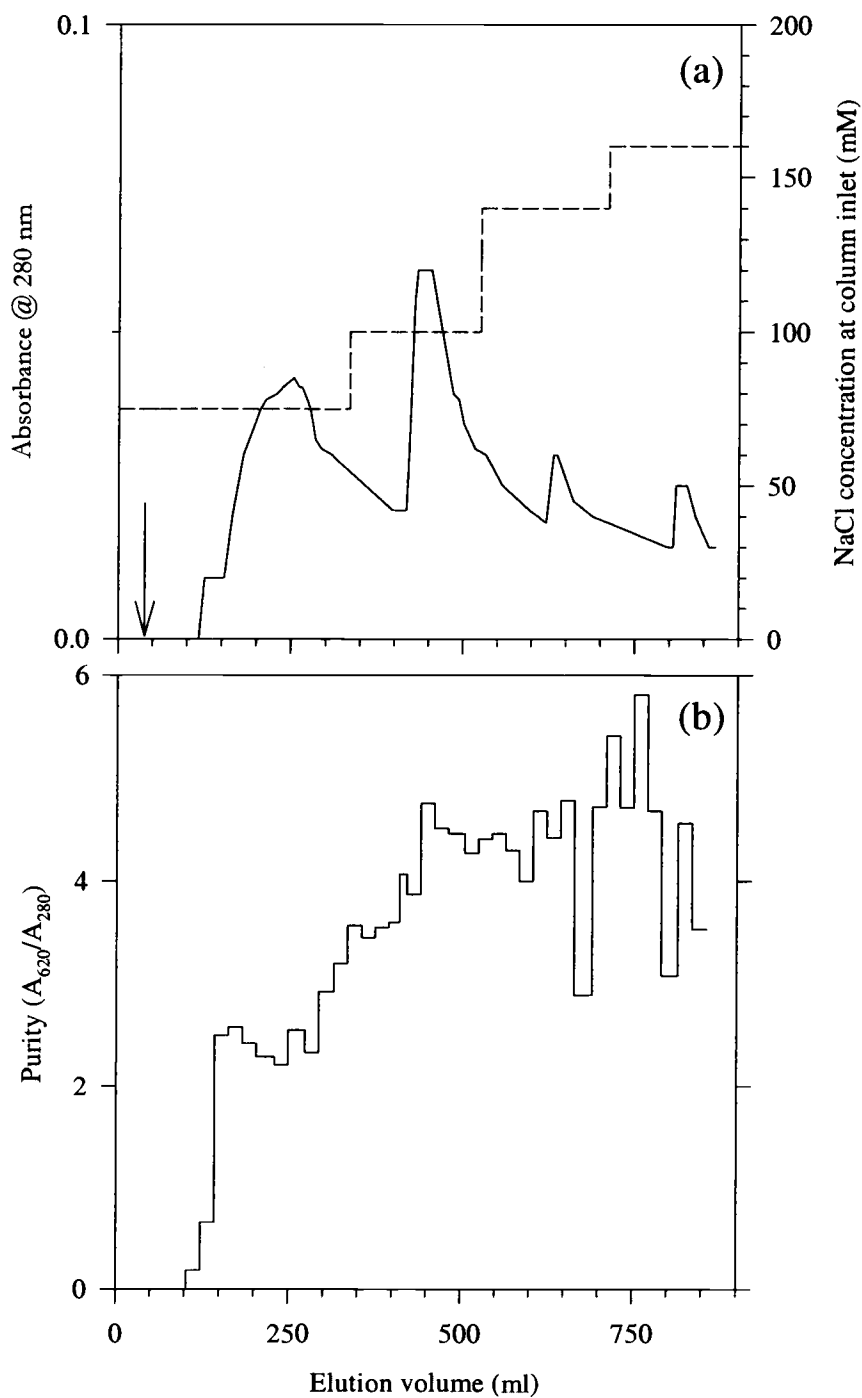


Figure 4.5. Rechromatography of phycocyanin fractions (zone marked I in Figure 4a) pooled from ion-exchange run shown in Figure 4a. The secondary y-axis shows the modulator concentration (broken lines). The purity as the ratio of absorbance measured at 620/280 nm is shown in panel b.

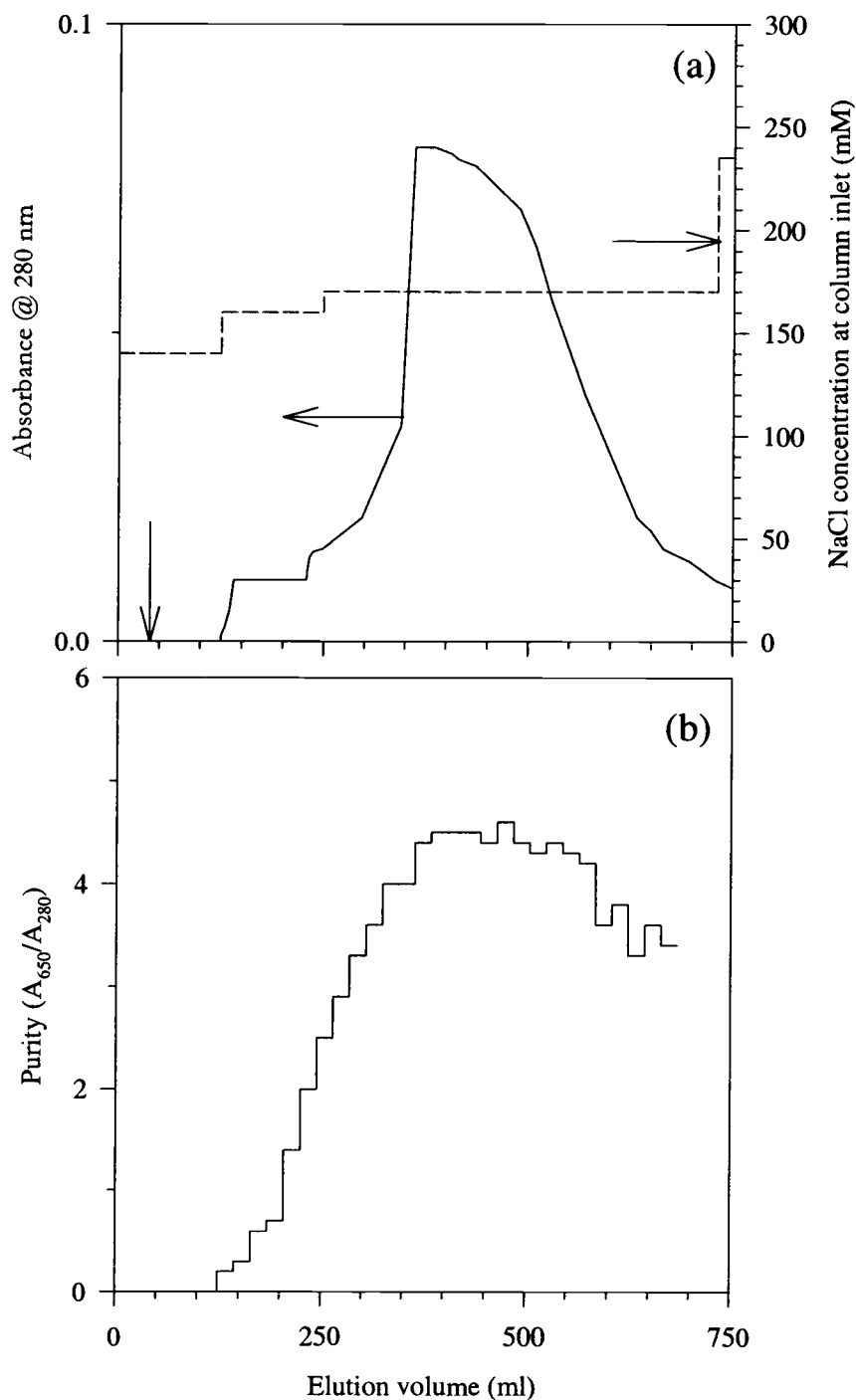


Figure 4.6. Rechromatography of allophycocyanin fractions (zone marked II in Figure 4a) pooled from ion-exchange run shown in Figure 4a. The secondary y-axis shows the modulator concentration (broken lines). The purity as the ratio of absorbance measured at 650/280 nm is shown in panel b.

increase in purity for a significant number of fractions, and is shown in Figure 4.5b. The impurities were primarily present in the 100 mM and 160 mM fractions. All the fractions collected during the 125 mM NaCl had purity value above 4.0, with a few fractions above 4.5. The rechromatography of allophycocyanins also revealed a similar result (significant increase in the  $A_{650}/A_{280}$  ratio; Figure 4.6b).

The entire procedure was repeated three times and was found to be reproducible. The phycocyanin and allophycocyanin production after their respective rechromatography steps were  $24 (\pm 3)$  mg and  $42 (\pm 3)$  mg. Since allophycocyanins are currently used as the biomedical marker in the market, primarily due to its very high Stokes shift (Glazer and Stryer, 1984), we scaled-up the APC process for large-scale production of biomedical-grade biliprotein.

The absorption spectra and the spectroscopic characterization of the biliproteins after each purification step are given in Figure 4.7 and Table 4.1 respectively. Figure 4.7 shows that the absorption at 280 nm decreases after each step implying the removal of proteins. The absorption spectra reported here are similar to previous reports (Boussiba and Richmond, 1979; Glazer, 1988), in which the phycocyanins were obtained from a homogenous culture. This corroboration suggests the product obtained according to the method described here is likely to be comparable in quality to those described in previous reports. Figure 4.8 shows the fluorescence emission spectra of representative PC and APC fractions from their respective rechromatography steps. The emission maximum for PC was 643 nm and that for APC was 657 nm. Despite our starting sample being an industrial waste stream, the purified biliproteins had comparable fluorescence spectra

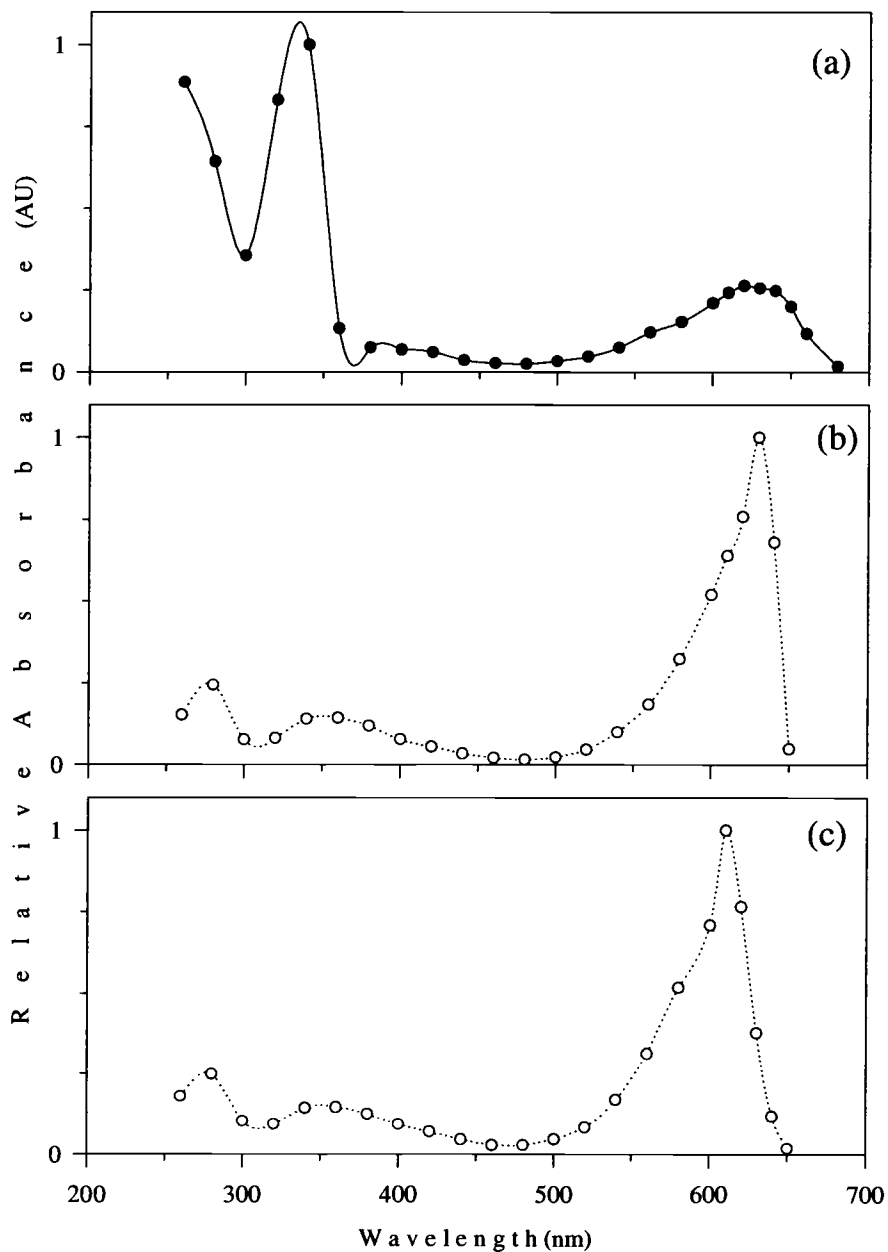


Figure 4.7. Absorption spectra of the biliprotein extract from the purification steps. (Panel a) Extract from the centrifugation step of frozen biomass. (Panel b) Precipitated biliprotein mixture resuspended in Tris-HCl buffer (20 mM, pH 7.0) prior to the first ion-exchange step. (Panel c) Biliprotein mixture after the size exclusion step.

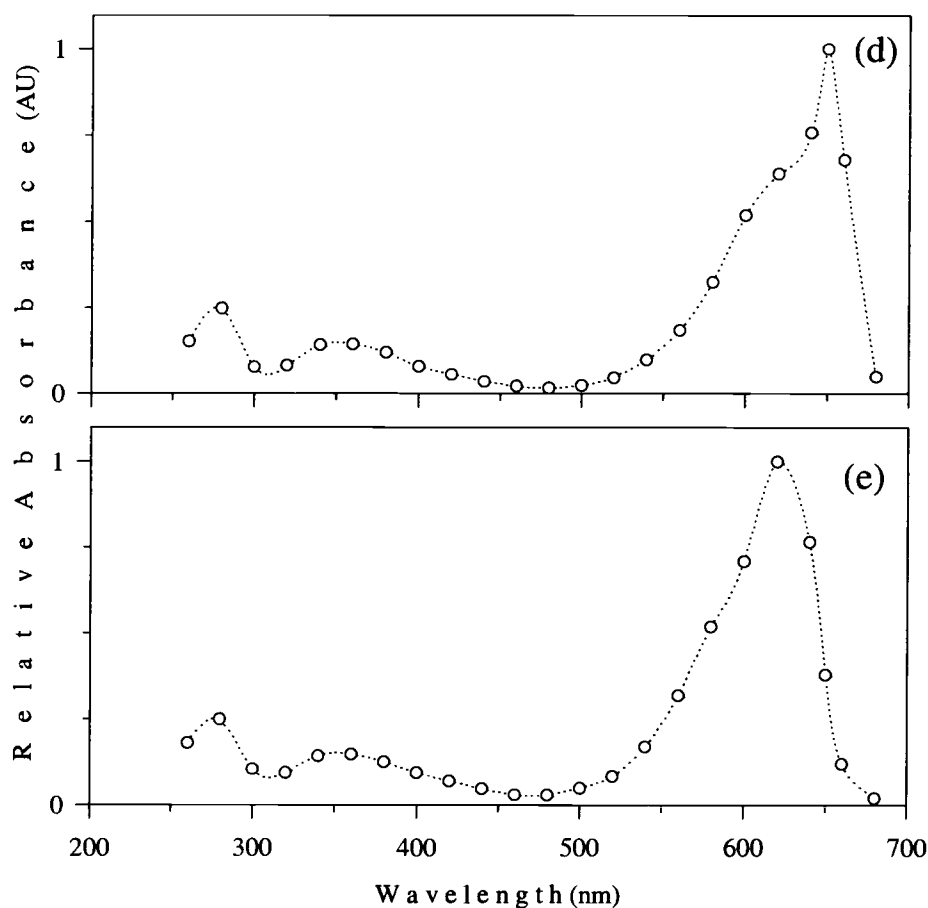


Figure 4.7 (contd) Absorption spectra of the biliprotein extract from the purification steps. (Panel d) Purified Allophycocyanin fraction from the zone marked ion-exchange rechromatography step. (Panel e) Purified phycocyanin fraction from the ion-exchange rechromatography step. The relative absorbance was calculated by taking the ratio of data points to the maximum value. The absorbance maximum for purified allophycocyanin is 650 nm while the corresponding value for purified phycocyanin is 620 nm.

Process	PC	APC
	$A_{620}/A_{280}$	$A_{650}/A_{280}$
Initial extract	0.5	0.3
Precipitation	1.9	1.3
Q-Sepharose chromatography	3.2	3.5
Rechromatography	4.0 to 4.6	4.0 to 4.8

Table 4.1. Spectroscopic characterization of phycobiliproteins after each purification step.

with several previous reports (Glazer and Stryer, 1984; Kronick, 1986; Downes and Hall, 1998), where the biliproteins were purified from a homogenous culture. In addition, our process involved only one mode of chromatography (electrostatic interaction) while many reports use multi-modal, multi-step separations to reach purity by absorption spectroscopy above 4.0, e.g., anion-exchange followed by hydroxylapatite (Brejc et. al., 1995), hydroxylapatite followed by anion-exchange (Boussiba and Richmond, 1979).

To further characterize the final product, the biliprotein fractions were subjected to electrophoresis under denaturing conditions. The result is shown in Figure 4.9. PC fractions with spectroscopic purity above 4.0 showed only two bands (lanes 9 and 10). The molecular masses of these two bands correspond to those of the  $\alpha$  and  $\beta$  chain of the phycocyanin (Parker and Glazer, 1988; MacColl and Guard-Friar, 1987), and was estimated as 17,500 Da and 18,700 Da respectively. Early eluting PC fraction with purity below 4.0 showed few fractions ranging from 15,500 Da to 36,600 Da. However, based on the bandwidth and intensity we concluded that the impurities were present at lower levels in comparison to PC. The purity of PC increased with the subsequent fractions,

supported by the  $A_{620}/A_{280}$  ratio and the presence of only two bands in SDS-PAGE.

Based on the elution profile, absorption spectroscopy and electrophoresis, we deduced that the primary contaminant in the rechromatography step was a lesser retaining peak, eluting between 110 and 350 ml.

SDS-PAGE of the representative APC fractions from the rechromatography step is shown in Figure 4.9 (lanes 2 to 5). The samples in lanes 2 and 3 had spectroscopic purity above 4.0, while the ones in lanes 4 and 5 were lower than 4.0. The molecular masses of the two bands were estimated as 16,700 and 18,300, respectively. The later retaining fraction (in comparison to APC) had a 41,300 Da impurity. Based on the elution profile, absorption spectroscopy and electrophoresis, we deduced that APC was engulfed between a lesser retaining impurity (pink band eluting between 120 ml and 250 ml), and a later retaining impurity in the rechromatography step.

HPLC runs on an ion-exchange column for the highly pure APC fractions (figures not shown) showed one peak with several shoulders, even though the spectroscopic purity was above 4.0. This result was consistent with a previous report by Glazer (1988), where it was suggested that the spectroscopy data alone may not be sufficient to assess the purity of the sample. However, SDS-PAGE of the purified sample showed only two bands (similar to several previous reports for  $\alpha$  and  $\beta$  subunits of APC), and the fluorescence spectra were comparable to previous reports, we believe that our purified samples are a mixture of APC's.

Previous reports on fluorescent applications of biliproteins (Hock et al., 1998; Bueso-Ramos et al., 1988; Oi et al., 1982) were analyzed to determine the feasibility of using the biliproteins obtained according to the method reported here as a biomedical



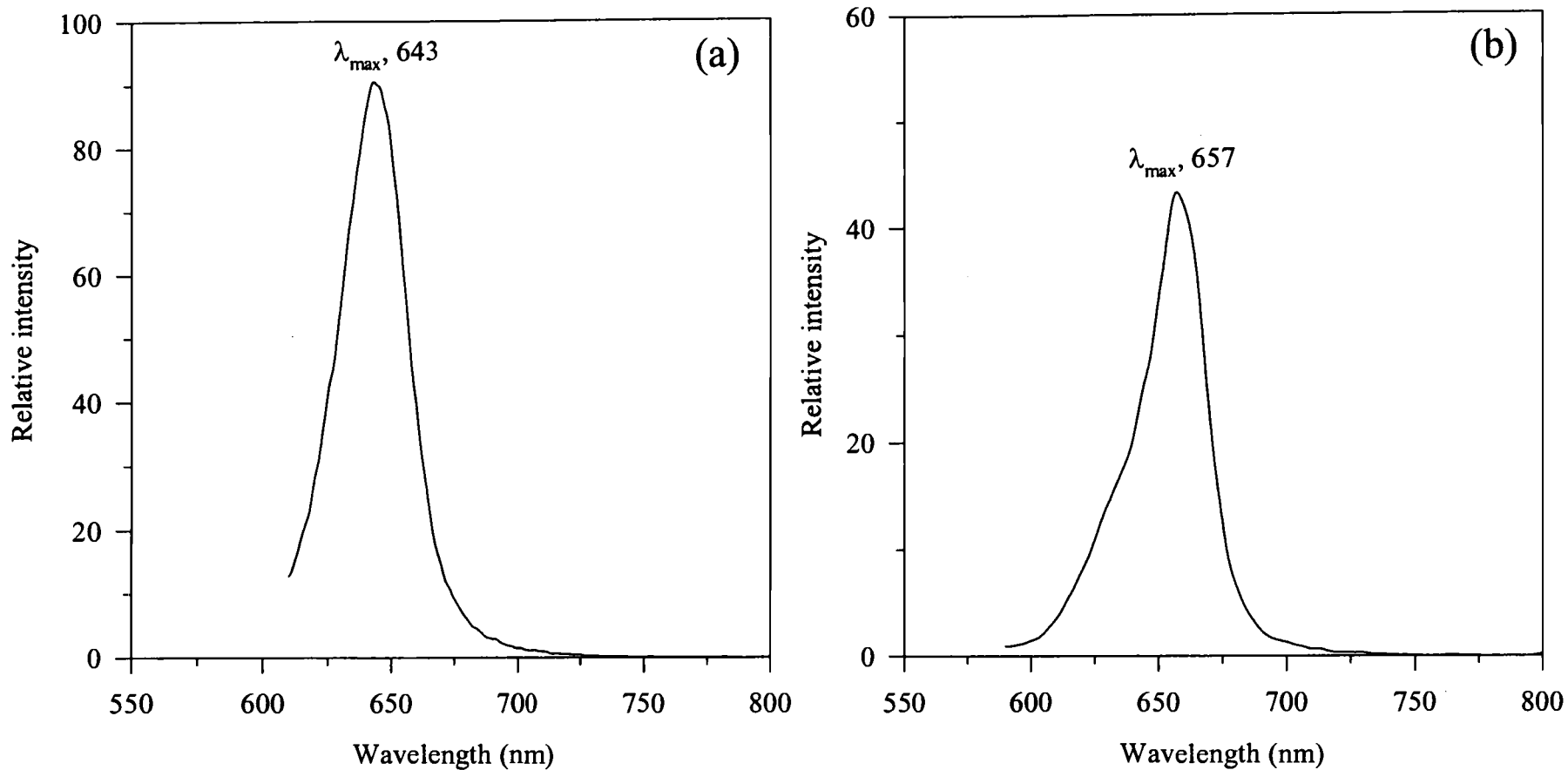


Figure 4.8. Fluorescence emission spectra of phycocyanin (Panel a) and allophycocyanin (panel b) fraction from their corresponding rechromatography runs. The samples were suspended in Tris-HCl (20 mM, pH 7.0).

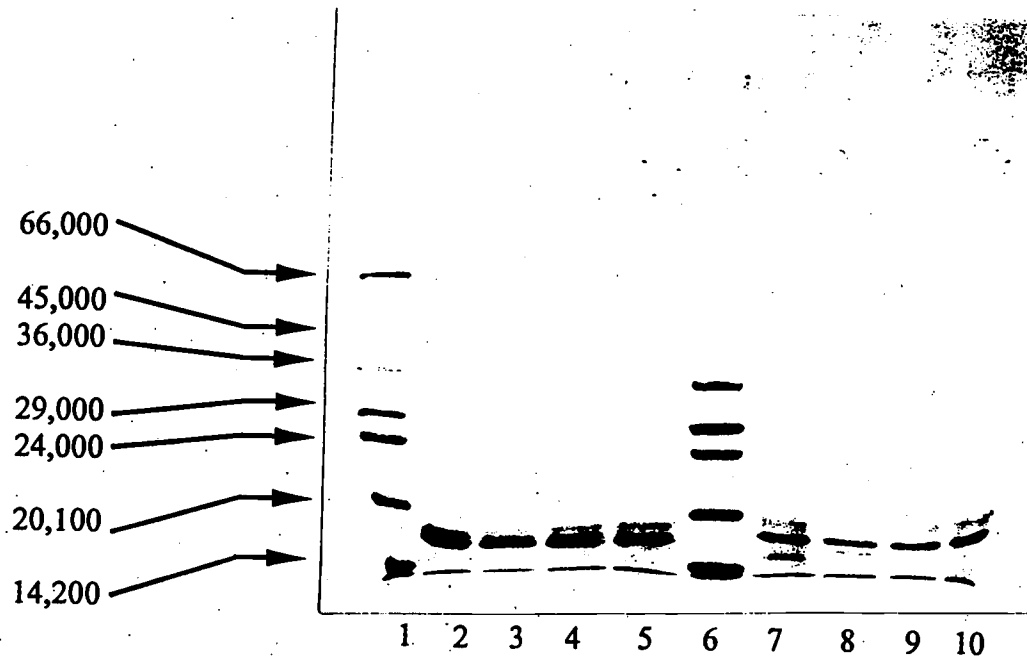


Figure 4.9. SDS-PAGE run of purified phycocyanin and allophycocyanin fractions from their respective rechromatography runs. The sample description in each lane is as follows (lane 1) molecular mass standards (from top: 66.0; 45.0; 36.0; 29.0; 24.0; 20.1; 14.2 kDa); (lanes 2 and 3) APC fraction with spectroscopic purity above 4.0; (lane 4) APC fraction with purity less than 4.0 eluting earlier than APC; (lane 5) APC fraction with purity less than 4.0 eluting later than APC; (lane 6) molecular mass standards, 5 proteins only; (lanes 7 and 8) PC fractions with purity less than 4.0; (lanes 9 and 10) PC fractions with purity greater than 4.0.

grade marker. In all these reports, an antibody was treated with an excess biliprotein solution to facilitate protein-antibody binding. The unbound protein was separated from the bound antibody by either size-exclusion or ion-exchange chromatography. While it is possible that our product, a heterogeneous mixture of PC's or APC's, could bind to the conjugate differently, their characteristic emission makes them easy to differentiate from other probe molecules. From Figure 4.8, it is clear that the heterogeneous protein mixture has emission spectrum similar to those previously reported for homogeneous samples (Glazer, 1994). Therefore it is possible that the biliproteins obtained here despite their heterogeneity, could be used effectively in molecular biological applications.

### *Scale-up and cost estimation for food-grade biliprotein production*

#### *Design basis*

The plant was designed to yield 2000 kg of lyophilized protein on a yearly basis. The operation was carried out in 200 working days, with eight-hours of operation each day. Therefore, the daily basis for operation was 100 kg of protein. A 2000 kg/year basis was chosen to compare the production cost of the current process with that reported by Reis et al. (1998). Figures 4.10a and 4.10b shows process flow-sheets for large-scale food-grade biliprotein production by two methods. The Production cost, which includes fixed-capital costs and operational costs, is itemized in Table 4.4 and Table 4.5. The following section describes process design and economics.

#### *Process equipment and their design*

The list of essential process units utilized in food-grade biliprotein production are:

- (a) Centrifuge to separate solids from supernatants continuously at 202 L/hr.

- (b) Feed and product stream concentration by evaporation.
- (c) Feed and product stream concentration by ultrafiltration.
- (d) Large-scale size exclusion chromatography.

(a) Centrifuge for cell separation: To separate the biliprotein-rich broth from the cells, a disc-bowl centrifuge with nozzle for continual ejection of solids was designed according to West, 1985. Disk bowl centrifuge with nozzles for solids ejection was selected to enable continuous removal of solids (Perry et al., 1984; West, 1985). The following assumptions were made during the centrifuge design: diameter ( $d_p$ ) of the smallest cell particle to be separated was  $1 \mu\text{m}$  (the average size of *Microcystis. Sp* is  $4 \mu\text{m}$ ; Watanabe et al., 1996); density ( $\rho_s$ ) of the smallest particle was  $1.02 \text{ g/cm}^3$ ; the outer and inner diameters of the disc were  $15.7 \text{ cm}$  and  $6 \text{ cm}$  respectively (West, 1985); the disc angle ( $\theta$ ) was  $40^\circ$ ; bowl rotation speed ( $\omega$ ) was  $5000 \text{ rpm}$ .

The centrifuge design involved estimating the terminal settling velocity and number of discs. The terminal settling velocity ( $v_g$ ) was calculated first according to equation 4.1. This value for  $v_g$  was used to estimate the number of discs required to process the desired flow-rate of slurry according to equation 2 (West, 1985).

$$v_g = \frac{d_p^2 (\rho_s - \rho) g}{18 \mu} \quad (4.1)$$

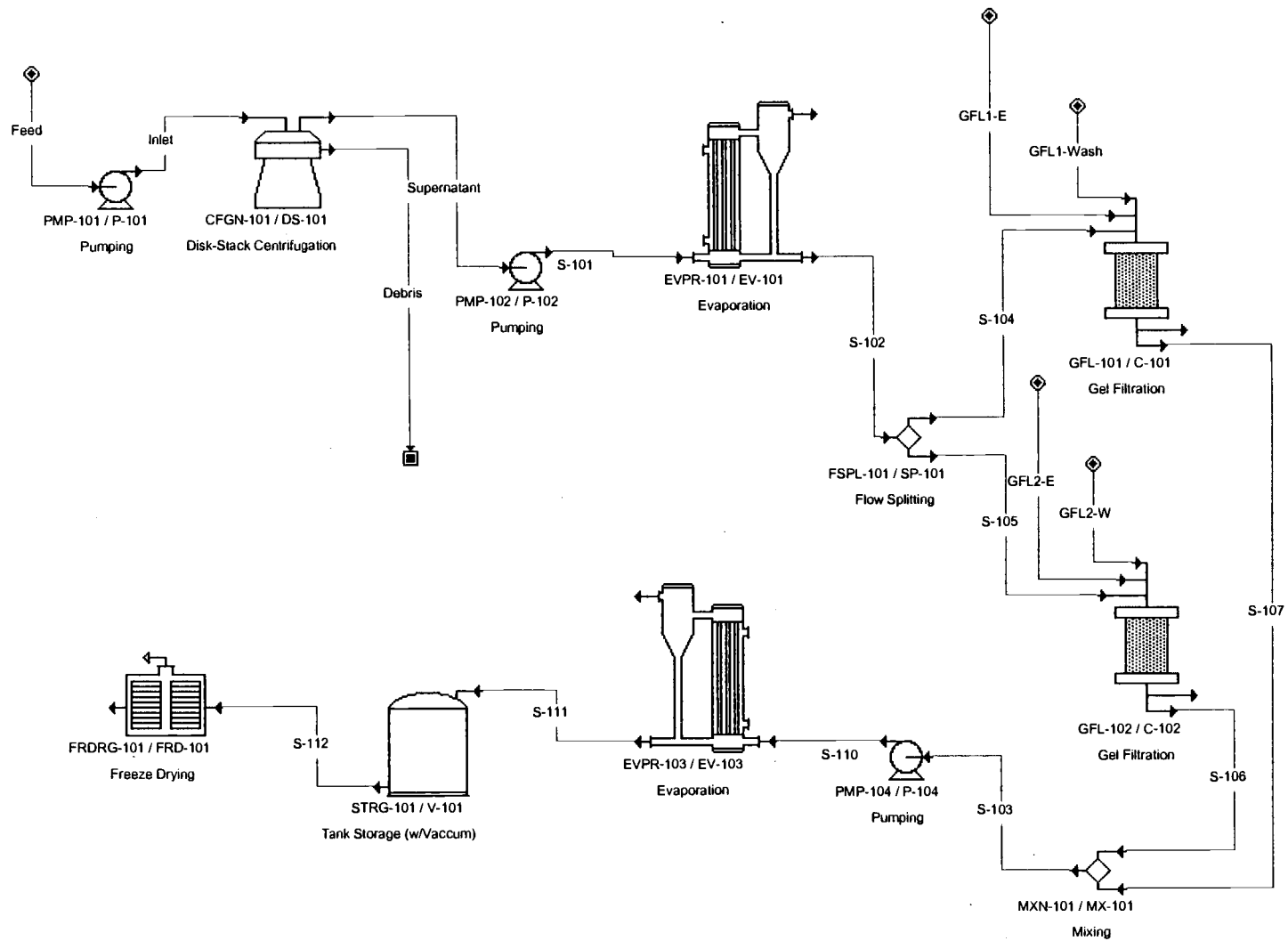


Figure 10. Process flow-sheet for large scale production of food-grade biliproteins.

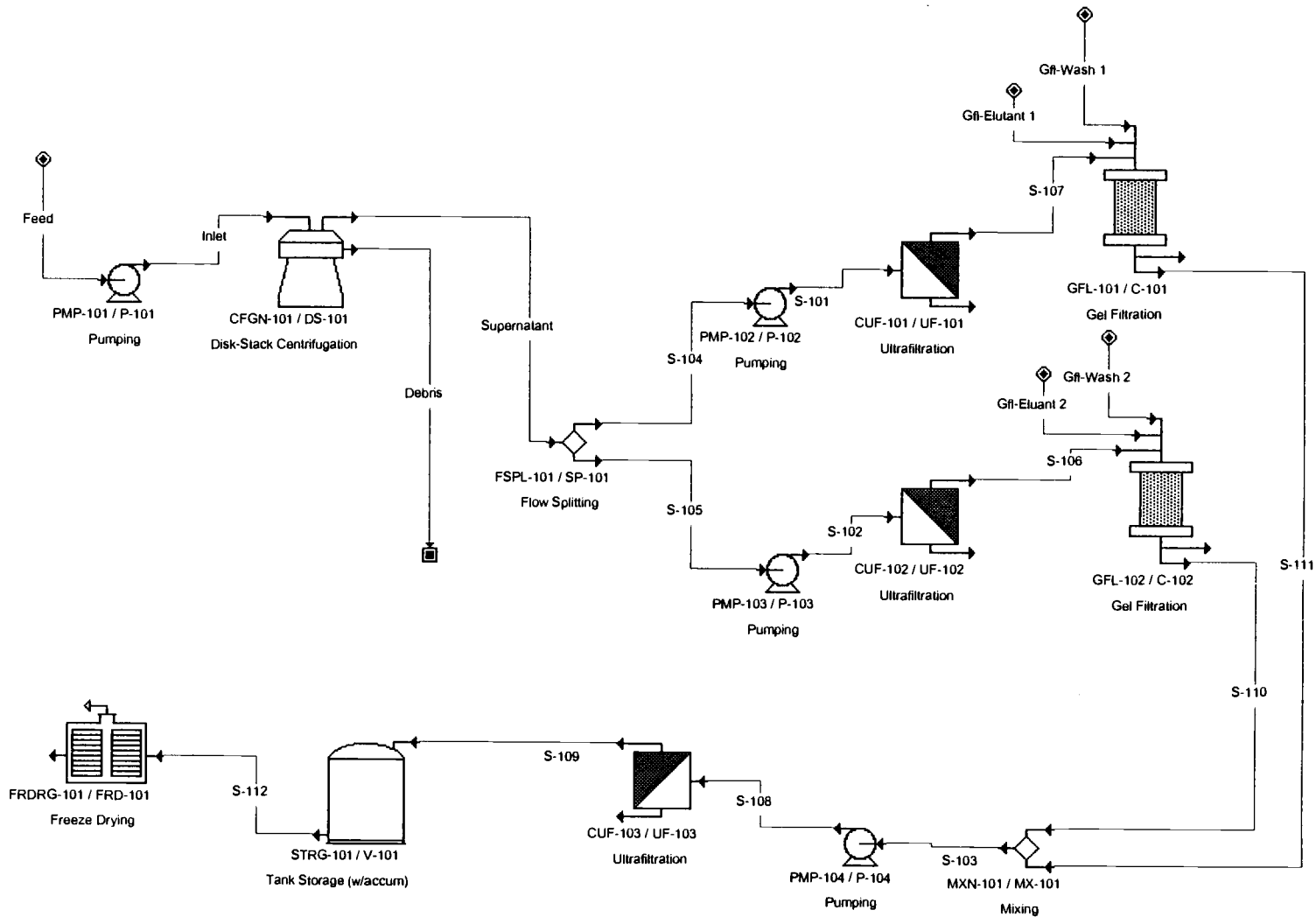


Figure 4.10. (contd.) Process flow sheet for the purification of food-grade biliprotein production in large scale using ultrafiltration as the concentration step.

$$Q = v_g \Sigma, \text{ where } \Sigma = \frac{2 \pi (n-1) (r_2^3 - r_1^3) \omega^2}{3 g \tan \theta} \quad (4.2)$$

The terminal settling velocity was  $9.9 \times 10^{-7}$  cm/sec. To obtain 100 kg of product in an eight-hour operation, a centrifuge that can process approximately 1600 L of process waste was needed. According to equation 4.2, a centrifuge with 24 discs resulted in settling area  $5870 \text{ m}^2$  adequate to process 209 L/h (1672 L/8-hour). However, the actual performance may vary considerably from that predicted by equation 2 (West, 1985) due to factors such as particle size distribution, shape, cross-flow mixing, non uniform flow distribution or hindered settling within the centrifuge. Therefore, a safety factor of 2 was incorporated into the design. Hence, the number of discs were increased to 48, giving a settling area of  $11990 \text{ m}^2$ .

- (b) Concentration of liquid by evaporation: Several unit operations were considered for the feed enrichment step. Feed enrichment by precipitation was found to be prohibitively expensive. The falling-film evaporators have been successfully used in the food industry to concentrate heat-sensitive materials (Moore and Hesler, 1963; Carter and Kraybill, 1966; Wiegand, 1971). In addition, lab-scale concentration of biliproteins on a rotary evaporator showed no degradation when operated at  $35 \text{ }^\circ\text{C}$ . Hence a falling film evaporator was used to concentrate the biliproteins at large-scale at  $30 \text{ }^\circ\text{C}$ .

The following assumptions were made for the evaporator design: evaporation process was carried out at 0.042 bar ( $30 \text{ }^\circ\text{C}$ ) to enhance the evaporation rate while minimizing potential thermal degradation; steam at  $120 \text{ }^\circ\text{C}$  (2 bar) was generated in-house using a natural-gas powered boiler; since the feed stream was dilute, the boiling

point elevation was insignificant; since the evaporation process takes place at 30 °C, the feed was assumed to be available at 30 °C to enable the efficient use of evaporator space.

In general, the protein concentration in feed during the gel filtration process should not exceed 40 g/L (Janson and Pettersson, 1992). Therefore, we designed the evaporator to enrich the feed stream to 30 g/L. The evaporator design involved estimating the steam requirement and the heating surface area. The steam required to concentrate the feed (202 kg/h with 0.62% solids) by five-fold to 3% solids was calculated by carrying out an enthalpy balance across the evaporator according to Singh, 1993, using steam tables data from Smith et al., 1996, and given as follows:

$$m_f H_f + m_s H_s = m_v H_v + m_p H_p + m_c H_c \quad (4.3)$$

where  $m_f$ ,  $m_s$ ,  $m_v$ ,  $m_p$  are the mass flow rates of feed, steam, vapor from the evaporation process, and concentrated liquid stream respectively. The symbols  $H_f$ ,  $H_s$ ,  $H_v$ ,  $H_p$ , and  $H_c$  stand for the enthalpy of feed stream, saturated steam, saturated vapor from the evaporation process, product stream and steam-condensate stream respectively. The enthalpy for the feed and the product streams were estimated according to the following equations:

$$H_f = C_{pf} (T_f - 0^\circ\text{C}) \quad (4.4a)$$

$$H_p = C_{pp} (T_f - 0^\circ\text{C}) \quad (4.4b)$$

where  $C_p$  is the specific heat at constant pressure, and estimated according to the following equation (Singh, 1993):

$$C_p = 1.424 \Phi_c + 1.549 \Phi_p + 1.675 \Phi_f + 0.837 \Phi_a + 4.187 \Phi_m \quad (4.5)$$



where,  $\Phi$  is the mass fraction and the subscripts c, p, f, a and m stand for carbohydrates, protein, fat, ash and moisture, respectively. Since the feed stream was dilute, only the protein concentration and water content were used in equation 5. All other terms were set to zero. The data given in Table 4.2 were used in equations 4.3 and 4.5, that resulted in a steam demand of 176.5 kg/h to enrich 202 kg/h of feed from 0.62% to 3% protein content.

The heating surface area was calculated based on the expression for heat transfer rate given below;

$$q = m_s (H_s - H_c) = U A \Delta T \quad (4.6)$$

where U is the overall heat transfer coefficient of stainless steel; A is the heating

Process parameters	Symbol	Data
Mass flow rate of feed	$m_f$	0.0560 kg/s
Mass flow rate of condensate	$m_c$	0.0116 kg/s
Mass flow rate of vapor	$m_v$	0.0444 kg/s
Enthalpy of feed	$H_f$	125.1 kJ/kg
Enthalpy of steam	$H_s$	2706 kJ/kg
Enthalpy of condensate	$H_c$	503.7 kJ/kg
Enthalpy of vapor	$H_v$	2556 kJ/kg
Steam temperature	$T_s$	393 K
Feed temperature	$T_f$	303 K

Table 4.2. Data for evaporator design.

Process parameters	Symbol	Data
<b>FEED CONCENTRATION STEP</b>		
Active surface area	A	7.64 m <sup>2</sup> /element
Average flux (feed concentration)	j	28 L/m <sup>2</sup> -h
Feed volume	V <sub>o</sub>	1613 L
Product volume	V	333 L
Universal gas constant	R	0.082 L-atm/mol-K
Temperature	T	298 K
Pressure	ΔP	4.1 atm
<b>PRODUCT CONCENTRATION STEP</b>		
Active surface area	A	7.64 m <sup>2</sup> /element
Average flux (feed concentration)	j	15 L/m <sup>2</sup> -h
Feed volume	V <sub>o</sub>	435 L
Product volume	V	100 L

Table 4.3. Data for ultrafiltration design.

surface area and  $\Delta T$ , the temperature differential between the steam and the feed temperature. The typical overall heat transfer co-efficient listed in McCabe and Smith (1993) for natural circulation long-tube vertical evaporators was 1000 - 2500 W/m<sup>2</sup>-K. We assumed the overall heat transfer co-efficient for the evaporator to be 1000 W/m<sup>2</sup>-K (3600 kJ/m<sup>2</sup>-K). The surface area estimated according to equation 4.6 was 1.2 m<sup>2</sup>. Based on the typical average tube internal diameter for falling film evaporator (Earle, 1966) the tube side dimensions were specified as 7.6 cm (3") I.D × 5 m L.

The product stream eluting from the size-exclusion chromatography was concentrated using a second unit with same configuration. Although the product

volume to be concentrated was smaller in comparison with the feed concentration step, the time involved was lower (2.5 hours for product concentration versus 8 hours for feed concentration), thereby resulting in lower operating costs. Hence, the design from the previous step was replicated.

- (c) Feed and product stream concentration by ultrafiltration: It is widely known that an evaporator utilizes large amounts of energy and may be deleterious to many biological products (Jackson, 1991). Although lab-scale rotary evaporation of biliprotein did not show any degradation, we designed an ultrafiltration unit to concentrate the feed to compare its competitiveness against evaporation.

The following assumptions were made during the design: a spiral-wound ultrafiltration membrane of type PW3840C typically used in food related processes (Osmonics, Inc., MN, USA) was selected to concentrate the feed stream; concentration polarization was assumed to be negligible; membranes were changed after processing 80,000 L of feed; the initial flux for the feed and the product streams were  $28 \text{ L/m}^2\text{-h}$  and  $15 \text{ L/m}^2\text{-h}$  respectively at 60 psi for 4 elements connected in series.

The ultrafiltration design involved estimating the time required to enrich approximately 1600 L of feed from 0.62% to 3% protein content using one unit containing 4 membrane elements ( $4 \times 7.4 \text{ m}^2$ ). Then, the number of units to process the entire feed in an eight-hour operation was estimated. This was achieved by solving a mass balance equation on the solvent in conjunction with the equation for flux, according to Belter (1988), and given below:

$$\frac{dv}{dt} = -A j \quad (4.7)$$

where A is the surface area of the membrane. The flux across the membrane, j, is given by

$$j = L_p (\Delta P - \sigma \Delta \Pi) \quad (4.8)$$

where  $L_p$  is the permeability,  $\Delta P$  is the applied pressure,  $\sigma$  is the reflection coefficient, and  $\Delta \Pi$  is the osmotic pressure. Equations 4.7 and 4.8 were combined, and integrated using the initial condition at  $t = 0$ ,  $V = 1613$  L, which resulted in the following expression:

$$t = \left[ \frac{1}{A j} \right] \left\{ (V_o - V) + \left( \frac{RTn_1}{\Delta P} \right) \ln \left( \frac{V_o - \frac{RTn_1}{\Delta P}}{V - \frac{RTn_1}{\Delta P}} \right) \right\} \quad (4.9)$$

where A, total active surface area of the membrane; j, permeate flux across the membrane;  $V_o$ , feed volume; V, product volume; R, universal gas constant; T, operation temperature;  $\Delta P$ , pressure drop across the membrane;  $n_1$ , total amount of solids. The analysis of several charts for flux versus protein concentration given in Cheryan, 1986 showed feed streams with protein concentration < 3% had initial flux greater than 30 L/m<sup>2</sup>-K. Using the data given in Table 4.3 in equation 4.9 resulted in an operation time of 15.4 h. Therefore, two units with 4 elements each were operated simultaneously.

The product eluting from the size-exclusion step was typically 2.3% proteins, which needed to be concentrated to 10% proteins prior to the lyophilization step. According to the data sheet for this membrane (type PW3840C) the typical flux for whey proteins was 17 L/m<sup>2</sup>-h when the initial solid content was 13%. Therefore, a

conservative assumption for the average flux was made as 15 L/m<sup>2</sup>-h. This resulted in 7.5 hours of operation to concentrate the liquid stream eluting from size-exclusion column. The effectiveness of evaporation and ultrafiltration as concentration steps is assessed later.

- (d) Size-exclusion chromatography: Since there is no adsorption process involved in the size-exclusion column, pore diffusion was considered as the limiting step. The column dimensions were scaled-up according the standard method (e.g., Ladisch and Velayudhan, 1995). First, the column volume was specified according to the following equation:

$$\left( \frac{V_{\text{column,l}}}{V_{\text{column,b}}} \right) = \left( \frac{V_{\text{sample,l}}}{V_{\text{sample,b}}} \right) \quad (4.10)$$

where V stands for the volume, while the suffixes l and b denote large and bench-scale respectively. Since the largest available glass cylinder had an internal diameter (80 cm; Ace Glass, Inc., NJ), the column length was specified based on the constraint that the ratio of sample volume to the column volume be constant at the lab-scale and at the production scale. This resulted in the dimensions for production column as 80 cm I.D. × 100 cm L. The resolution in linear chromatography is proportional to the square root of column length (Snyder and Kirkland, 1979). Therefore, an increase in column length by a factor of 4 for the production scale will result in a factor of 2 increase in resolution between the protein fraction and the small molecules.

However, to ensure the complete elimination of components lower than 5 kDa in the product, a safety factor of 2 in sample volume was introduced. This resulted in 40.3 L

of feed for each cycle. The linear velocity obtained in the lab-scale run was reproduced in large-scale by utilizing a sterile, food-grade positive displacement pump (Jabsco Inc., CA, USA). Sephadex G-25 media is very stable, and can withstand the higher pressures (upto 5 psi) resulting from the increased flow-rate (Amersham-Pharmacia, 1999). Each run under scaled-up conditions was carried out in 4 hours (2 hours for separation and 2 hours for regeneration). Previous report on the large-scale protein separation using Sephadex G-25 (column volume 75 L) was able to function well for six years (70,000 L of plasma) before being discarded (Marrs, 1993). Since they did not specify the number of cycles, we made an estimate assuming the maximum possible loading of 25% of the column volume (Janson and Pettersson, 1992); this resulted in 3733 cycles. Friedli and Kistler (1972) reported using a production scale Sephadex G-25 column (75 L) for greater than 7000 cycles during the deethanolization process of human serum albumin. Therefore, a conservative estimate of 2000 cycles (5 years) was assumed for our process.

#### *Other capital costs*

The fixed capital investment included the cost for walk-in cooler, product quality control, and other fixed costs (installation, instrumentation, insulation, piping, electrical auxiliaries, engineering and contingency). The footnotes for Table 4.4a describe in detail the method to estimate the other capital costs.

Processing equipment and supplies	Investment (US\$)	Amortization (years)	Cost <sup>a</sup> (US\$)
<b>Fixed-capital cost (purchased equipment)</b>			
Evaporator (2 units)			
Falling-film evaporator <sup>b</sup>	20,000	10	3,300
Vacuum pump	4,000	10	700
Gas powered boiler	20,500	10	3,300
Feed pump	2,500	10	400
Centrifuge <sup>c</sup>	85,000	10	13,800
Pumps <sup>d</sup>	9,600	10	1,500
Columns <sup>e</sup>	12,000	10	2,000
Column accessories	8,000	10	1,300
Pump	4,000	10	600
Megapure water <sup>f</sup>	6,730	10	1,100
HPLC unit, columns, accessories	31,000	10	5,000
Walk-in cooler <sup>g</sup>	27,860	10	4,500
<b>Other fixed-capital investment (see note below)</b>			
Installation	38,450	10	6,200
Instrumentation and control	8,460	10	1,400
Insulation	3,760	10	600
Piping	43,120	10	7,000
Electrical auxiliaries	14,620	10	2,400
Engineering	14,620	10	2,400
Contingency	34,480	10	5,600
<b>Total fixed costs</b>			<b>62,200</b>

- (a) In order to compare the costs with Reis et al. (1998), the same equations (Mohr and Contreas, 1991) were used to estimate the depreciation cost for the equipment. The interest rate, the maintenance cost and the cost of electric power were also the same as that of Reis et al. (1998), and were 8.5% per year, 2% of the capital cost, and US\$ 0.125 per Kwh respectively.
- (b) The cost estimate for falling film evaporator was obtained from Doyle & Roth Mfg. Co., Inc., NY, USA; the cost estimate for the accessories were obtained from the following companies: natural gas powered Fulton brand boiler (Mechanical Sales, Inc., WA, USA); vacuum pump (KNF Neuberger, Inc., NJ, USA). The capital costs which includes costs for purchased equipment, installation, instrumentation, insulation, electrical auxiliaries and engineering for the evaporator unit was estimated according to Renshaw et al. (1982). The cost of natural gas was US\$ 0.60 per 29.4 Kw (Northwest Natural, OR, USA).
- (c) The cost estimate for an industrial scale centrifuge was obtained from Alfalaval, Inc., USA. The unit was pre assembled in the factory, and hence no installation cost was incorporated.
- (d) The food-grade positive displacement pumps (for centrifugation and evaporation) were from Jabsco, Inc., USA.
- (e) The cost estimate for large-scale glass columns was from Ace Glass Inc., NJ, USA.
- (f) The mega-pure water needed for the eluting the proteins was obtained by utilizing a reverse-osmosis unit. The amortization years for the equipment was assumed to be 10 years, while the membranes were replaced each year. The cost of the membrane was US\$ 1900 (Osmonics, Inc., MN, USA)

### Table 4.4a (Continued)

(g) The cost estimate for a walk-in cooler (40' X 40' X 10') was obtained from Stanley Marsh & Sons, Inc., USA.

#### NOTE:

The installation cost (pumps, 40% PE; size-exclusion columns and accessories, 20% PE, reverse-osmosis unit for water purification, 20% PE, walk-in cooler, 20% PE) was estimated from an average value given in Table 6 (p.169), Peters and Timmerhaus (1980). Since the centrifuge and HPLC units were pre assembled in the factory, no installation cost was assumed. The installation cost for evaporator unit was calculated according to Renshaw et al. (1982), 47% of PE.

The instrumentation and insulation costs for the evaporator and its accessories was 18% of PE and 8% of PE respectively (Renshaw et al., 1982). All other units did not have both instrumentation and insulation costs.

The cost of piping for the evaporator was 60% of PE, while piping for pump leading to the centrifugation unit, size-exclusion columns and water purification unit were 30% of PE. The walk-in cooler, centrifuge and HPLC unit did not have any piping cost associated with the unit.

The electrical accessories constituted 18% of PE for the evaporator. The electrical accessories for the other units (pumps, water purification unit and HPLC) were 12% of PE. The walk-in cooler had electrical parts pre installed.

The engineering and contingency of 10% of PE and 15% of PE was assigned for all units.

Table 4.4a. Fixed costs for the food grade biliprotein production. The purification sequence involves falling-film evaporator to concentrate the feed and product. Basis: 2000 kg/year production.



Item	Investment (US\$)	Amortization (years)	Cost (US\$)
Biomass	0		0
Accessories for mega pure water	3,400		3,400
Size exclusion gel <sup>a</sup>	397,452	5	104,300
Reagents	1,000		1,000
Lyophilization <sup>b</sup>	88,100		88,100
Solvent for HPLC	900		900
Labor	160,000		160,000
Transportation	1,700		1,700
Energy	19,100		19,100
<b>Total operating costs</b>			<b>378,500</b>

(a) The pricing for the Sephadex G-25 size-exclusion gel was obtained from the bulk media division of Amersham-Pharmacia Biotech, Inc., NJ, USA.

(b) The cost estimate for lyophilization process was obtained from Oregon Freeze Dry, Inc., OR, USA.

Table 4.4b. Operating costs for the food-grade biliprotein production. The purification sequence involves falling-film evaporator to concentrate the feed and product. Basis: 2000 kg/year production.

Processing equipment and supplies	Investment (US\$)	Amortization (years)	Cost (US\$)
<b>Fixed-capital cost (purchased equipment)</b>			
Ultrafiltration (3 units)			
Membrane housing	2,820	10	500
Pump (large)	7,500	10	1,200
Pump (small)	3,750	10	600
Pressure controller	1,800	10	300
Centrifuge	85,000	10	13,800
Pump	3,200	10	500
Column	12,000	10	2,000
Pump	4,000	10	700
Column accessories	8,000	10	1,300
Megapure water	6,730	10	1,100
HPLC unit, columns, accessories	31,000	10	5,000
Walk-in cooler	27,860	10	4,500
<b>Other fixed-capital investment (see note below)</b>			
Installation	19,350	10	3,100
Instrumentation and control	3,430	10	600
Piping	14,400	10	2,300
Electrical auxiliaries	10,640	10	1,700
Engineering	10,870	10	1,800
Contingency	29,050	10	4,700
<b>Total (US\$ per 2000 kg)</b>			<b>45,700</b>

## NOTE:

The other capital costs for all units, except the installation cost for the ultrafiltration unit, were the same as Table 4.4a. The installation cost for the ultrafiltration unit was 40% of PE.

Table 4.5a. Fixed costs for the food grade biliprotein production. The purification sequence involves ultrafiltration to concentrate the feed and product. Basis: 2000 kg/year production.

Item	Investment (US\$)	Amortization (years)	Cost (US\$)
Biomass	0		0
Accessories for mega pure water	3,400		3,400
Membranes	39,700		39,700
Size exclusion gel	397,452	3	104,300
Reagents	4,000		4,000
Lyophilization	88,100		88,100
Solvent for HPLC	900		900
Labor	160,000		160,000
Transportation	1,700		1,700
Energy	10,600		10,600
<b>Total (US\$ per 2000 kg)</b>			<b>412,700</b>

Table 4.5b. Operating cost for the food grade biliprotein production. The purification sequence involves ultrafiltration to concentrate the feed and product. Basis: 2000 kg/year production.

### *Operating costs*

The operating cost included costs for size-exclusion media, reagents, transportation, lyophilization and labor. The cost estimate for the size-exclusion media was obtained from Amersham-Pharmacia Inc. (NJ, USA). The concentrated product was stored in the cooler, and transported once every four weeks to Oregon Freeze Dry Inc. (OR, USA) for lyophilization. Transportation costs, including the rent for a refrigerated truck (\$115/day), mileage (\$0.25 each mile for 500 miles) and refrigeration (\$1.25/hr for 24 hours), were obtained from PV Rentals, TX, USA.

The production of 2000 kg of food-grade product was completed in 200 days. Two process options were available for the food-grade product production: feed and product enrichment by evaporation (option 1) and feed and product enrichment by

ultrafiltration (option 2). The duties involved for both these options were divided among four full-time technicians. The scheduling for process option 1 is as follows:

Technician 1: moving biomass from the cooler to the centrifuge (0.5 h/day);  
evaporation unit for the feed stream (startup and period monitoring, 4 h/day);  
evaporation of product stream (startup and period monitoring, 2 h/day).

Technician 2: cell separation by centrifugation (4 h/day) and transportation of the product to the lyophilization unit once every 4 weeks (12 h/4 weeks).

Technician 3 and 4: size-exclusion chromatography and product storage in the cooler (7.5 h/day).

Contingency: 20 hours per week was incorporated for maintenance, troubleshooting, emergencies, etc.

The technicians were paid at \$20/h, resulting in an annual labor cost of \$40,000 per person.

The scheduling for process option 2:

Technician 1: moving biomass from the cooler to the centrifuge (0.5 h/day);  
ultrafiltration units for the feed stream (startup and period monitoring, 4 h/day);  
ultrafiltration unit for the product stream (startup and period monitoring, 2 h/day).

Technicians 2, 3 and 4: same as option 1.

The production costs according to Tables 4.4(a and b) and 4.5(a and b) were US\$ 0.22 and US\$ 0.23 per g biliprotein, respectively. Although the energy costs for the process with evaporation (Table 4.4b) was higher, as expected, than the process with ultrafiltration (Table 4.5b), the membranes have to be replaced periodically, resulting in

comparable operating costs. These cost estimates are only slightly higher than that reported by Reis et al. (1998) despite their report neglecting several fixed-costs (installation, instrumentation, piping, electrical auxiliaries, engineering and contingency) and operation costs (lyophilization, transportation and labor). A substantial portion of their cost is due to cell culture.

The cost of food-grade biliprotein according to Cyanotech Corp. (HI, USA) was in the range of 20-130 US \$ per g biliprotein, while Dainippon Ink & Chemicals, Inc. (Japan) has a current market price of US\$ 0.29-0.79 per g of biliprotein. Our production cost compares favorably with these numbers, indicating that this process may be economically viable. We emphasize that our goal is only to provide a detailed engineering design of the process. We do not account for market research and other costs that are involved in actually bringing a product to market. We also note that while biliproteins are available as food-grade colorants in several countries (e.g., Japan, Taiwan), they have not yet been approved by the US FDA, and are not yet commercially available in the US (Francis, 2000).

#### *Scale-up and cost estimation for biomedical grade biliprotein production*

The process for biomedical-grade biliprotein was scaled-up to make 500 g each year. This basis was chosen to enable direct comparison of production costs against those of Reis et al. (1998). Figure 4.11 shows the flow-sheet for large-scale production of biomedical grade biliprotein. The production cost estimate, which includes the fixed-capital cost and operation cost is itemized in Table 4.6.

### *Process equipment requirements and their design*

The process units required for the large-scale biomedical grade biliprotein production are: a centrifuge to separate solids from supernatants continuously at 42 L/hr, and large columns for ion-exchange and size exclusion chromatography.

Given the processing volume for this product (42 L of feed/day), a Carr bowl centrifuge (CE pilot powerfuge) with a processing capacity of 60 L/h and capability for automatic solids removal was used for cell separation.

Precipitation of proteins was found to be more economical for feed enrichment than evaporation and reverse osmosis (less expensive by 3 times and 2 times, respectively), and hence ammonium sulfate precipitation was used. The precipitated protein was decanted and re-dissolved in the buffer prior to the large-scale size exclusion step.

The biliprotein was desalted in a large column (60 cm I.D. × 50 cm L) packed with Sephadex G-25 gel. The scale-up was carried out according to the method given in the previous section. The linear velocity obtained in the lab-scale run (1.2 cm/min) was duplicated in large-scale run by utilizing a sterile positive displacement pump. Each run under scaled-up conditions consumed two hours (one hour each for separation and regeneration).

The effluent resulting from the size-exclusion step was processed using an ion-exchange column (Q-Sepharose Fast Flow sorbent; column dimensions 53 cm I.D. × 25 cm L). The column was scaled-up in diameter while keeping the length constant. This ensures the reproducibility of resolution achieved in the bench-scale. Allophycocyanin

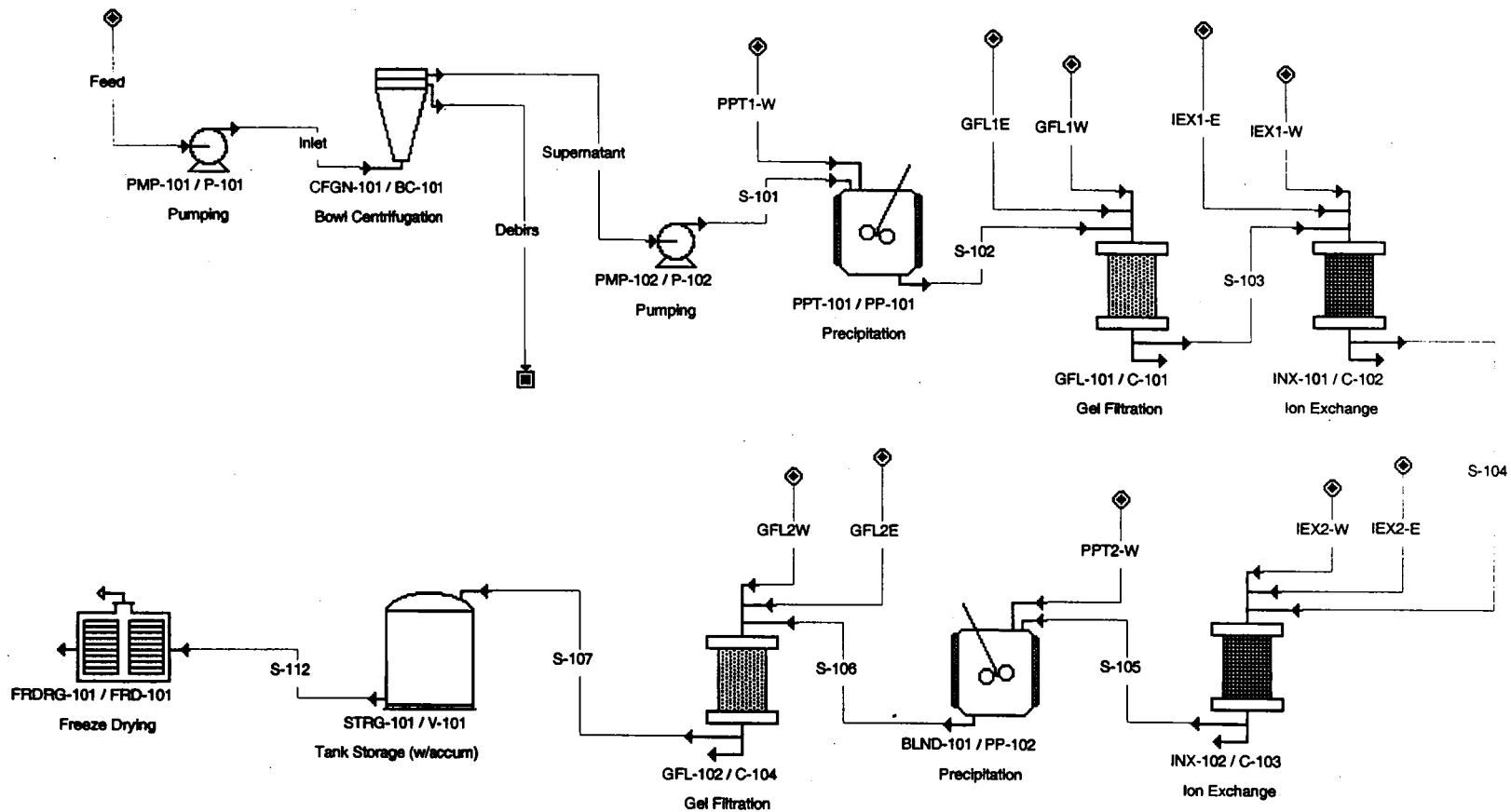


Figure 4.11. Process flow sheet for the purification of biomedical-grade biliprotein production in large scale.

Processing equipment and supplies	Investment (US\$)	Amortization (years)	Cost (US\$)
<b>Capital costs</b>			
Pump (centrifugation)	3,200	10	500
Centrifuge (precipitation step)	50,000	10	8,100
Mixers	2,000	10	300
Mega-pure water system <sup>b</sup>	6,500	10	1,100
Ion exchange column	4,500	10	700
Size-exclusion column	3,000	10	500
Pump (chromatography)	1,000	10	200
Walk-in cooler	4,530	10	700
HPLC unit, column, accessories	31,000	10	5,000
<b>Other fixed-capital investment (see note below)</b>			
Installation	6,180	10	1,000
Instrumentation and control	810	10	100
Piping	5,460	10	900
Electrical auxiliaries	6,330	10	1,000
Engineering	9,250	10	1,500
Contingency	13,880	10	2,300
<b>Total fixed cost</b>			<b>23,900</b>

(a) All definitions and cost estimates for the processing equipment were the same as Table 4.4 unless mentioned otherwise.

(b) The cost of laboratory scale mega-pure water system was obtained from Millipore Corp., MA, USA.

NOTE:

The other capital costs for all units, except the installation cost for the ion exchange step, were the same as Table 4.4a. The installation cost for the ion exchange column and accessories was 40% of PE.

Table 4.6a. Fixed costs for the biomedical grade phycobiliprotein production<sup>a</sup>. Basis: 500 g/year production.



Item	Investment (US\$)	Amortization (years)	Cost (US\$)
Biomass	0		0
Ammonium sulfate	2,650		2,650
Ion exchange gel	36,000	a	7,600
Reagents	1,000		1,000
Size exclusion step <sup>b</sup>	29,110	10	4,700
Energy	160		160
Labor	28,800		28,800
Lyophilization	100		100
<b>Total operating costs</b>			<b>45,010</b>

- (a) The life of first ion exchanger was 5 years (300 cycles) while the second one lasted 10 years (600 cycles).
- (b) The cost of size-exclusion process was calculated similar to Table 4.4a. However, only the cost of column, accessories and the gel was accounted for. A column with 60 cm I.D × 50 cm L) was found used for feed-desalting step, while another column of 12.5 cm I.D × 25 cm L was used for the product-desalting step.

Table 4.6b. Operating cost for biomedical grade phycobiliprotein<sup>a</sup> production.

fractions eluting at 235 mM NaCl was diluted to 75 mM NaCl using mega-pure water and rechromatographed in a separate column with the same dimensions.

The "other fixed capital investment" listed in Table 4.6 was estimated according to Peters and Timmerhaus, 1980.

### *Operating costs*

The operating cost included costs for the size-exclusion media, ion-exchange media, reagents, transporting the purified product to the lyophilization plant, lyophilization and labor. The lyophilization of the product was contracted to Oregon Freeze Dry Inc. (OR, USA), and the rental cost for transporting the purified product was

also included. The entire process was carried out in 60 days using 3 full-time technicians who were paid at \$ 20/hour. A contingency of 15 hours of labor was included in the production cost to offset any emergencies. The scheduling for process is as follows:

Technician 1: cell separation (1 h/day); feed precipitation step (2 h/day); size-exclusion of feed and product streams (3 h/day); storage (1 h/day).

Technician 2 and 3: ion-exchange columns and product precipitation (7 h/day)

The costs for size-exclusion gel was prorated for 10 years since the number of cycles each year was only 120. The Sepharose ion-exchange gels have been reported to last between 600 (Tayot et al., 1986) and 1000 cycles (Sofer and Nystrom, 1989) during plasma proteins purification and albumin purification. Hence, a conservative estimate of 300 cycles for the first-ionexchanger and 600 cycles for the second ion-exchanger was used before replacing the media.

The production cost estimate for biomedical grade biliprotein according to process was US\$ 137 per g (Tables 4.6a and 4.6b). The production cost reported here was slightly higher than reported by Reis et al. (1998). The difference is primarily due to Reis et al. (1998) neglecting several costs (other fixed-cost, product quality testing and labor). However, both these values were substantially lower than current market price of US\$ 15 per mg (Cyanotech. Corp, HI, USA; CQVB, 1988; Prozyme, Inc., CA, USA, 2000).

#### *Robustness of design:*

The phycocyanin concentration in the supernatant after the initial centrifugation step was 1.72 ( $\pm$  0.30) mg/ml while the corresponding values for allophycocyanin was

1.32 ( $\pm$  0.30) mg/ml. Analysis of two different batches collected at two different times revealed no significant variation in biliprotein concentration (deviation of PC by 4% and APC by 13%). The total protein concentration was 6 mg/ml. Only the above mentioned two batches of samples were available to us. However, if the protein concentration were lower in a different batch, the feed could be concentrated without significant additional cost using either an evaporator or an ultra-filtration unit as described in this article.

According to the current design only 8 hours/day for operation was needed to concentrate approximately 1600 L of feed. However, this can be easily expanded with additional employees and energy costs. No additional equipment may be needed. For example, if the protein concentration were 50% of current value (3 g/L of slurry), we would need an additional employee and energy costs associated with evaporator operation.

Incorporating these costs into Table 4.4, we obtained the production cost of US\$ 0.24/g (a 2 cent increase per gram of product). Therefore, the production cost is likely not to increase significantly. Further, various unit operations had several safety factors incorporated into their designs (e.g., the factor of 2 in the SEC column) which also provide a measure of security against large variations in the feed.

## CONCLUSIONS

A simple and scalable process to purify phycobiliproteins from a cyanobacterial process waste was developed for two applications: food-grade colorant and biomedical-grade fluorescent marker. A size-exclusion column packed with Sephadex G-25 material successfully eliminated microcystins from the food-grade product. The process for purifying fluorescent-grade biliprotein required two passes through an anion-exchange

column. The purified biliproteins were a heterogeneous mixture of PC's and APC's. However, their absorption and fluorescence spectra matched with several previous reports, when the protein was purified as a homogeneous sample. Process designs for large-scale production of food-grade (2000 kg/year) and biomedical grade (500 g/year) biliprotein production were provided. The production cost estimate according to the two processes (evaporation followed by size-exclusion chromatography vs. ultrafiltration followed by size-exclusion chromatography) reported here for the food-grade biliprotein production resulted in comparable costs. The cost estimate for food-grade biliprotein resulted in values comparable to the current market price. While the cost estimate for large-scale biomedical grade biliprotein resulted in lower values than the current market price. The process designs provided in this report is likely to serve as a good reference for commercial production of biliproteins, should biliproteins be permitted as food-colorants in the US.

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## Chapter 5

### Conclusions

Systematic optimization of separation for a biomolecule mixture was carried out for the first time under various modes of chromatography: nonlinear isocratic elution, gradient elution and displacement chromatography. Loadings far higher than classical approach was obtained resulting in efficient use of resources. This approach -- over loaded chromatography -- was applied to two realistic problems and good results were obtained.

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