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Hydrodynamic Evaluation and Characterization of Capillary Channeled Polymer (C-CP) Fibers as a Stationary Phase in HPLC of Macromolecules

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HYDRODYNAMIC EVALUATION AND CHARACTERIZATION OF CAPILLARY
CHANNELED POLYMER (C-CP) FIBERS AS A STATIONARY PHASE
IN HPLC OF MACROMOLECULES

A Dissertation
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
Chemistry

by
Kelum M. Randunu
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Accepted by:
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ABSTRACT

High Performance Liquid Chromatography (HPLC) is the most used analytical technique for the separation of samples in solution. HPLC has progressed with successive use of new supports such as silica, polysaccharides, monoliths, and organic polymers. Although porous silica phases are very effective in small molecule separations, they suffer from certain drawbacks including pH stability, chemical robustness, high backpressure, and slow mass transfer for macromolecule separations. Thus, the most protein separations are carried out using nonporous, partially porous, and superficially porous phases to overcome mass transfer limitations. As a competing methodology, fiber based polymer support/stationary phases are also developed for protein separations.

Capillary-channeled polymer (C-CP) fibers are being developed and characterized in the Marcus laboratory as a platform for variety of separations, predominantly for HPLC separation of macromolecules. C-CP fibers interdigitate to form a network-like structure when they are packed in a column that enables high fluid transport efficiency with low backpressure characteristics. This feature was successfully utilized for macromolecule separations with increased mass transfer characteristics at high linear velocities.

In this study polypropylene (PP) C-CP fibers were utilized to evaluate the zone-broadening process of C-CP fiber columns, leading to the optimization of fiber diameter, column dimensions, and packing density (interstitial fraction) for analytical chromatographic separations. The optimized results were applied

successfully for the separation of three-protein suite under reversed phase (RP) gradient conditions. Microbore PP C-CP columns were employed for rapid RP HPLC of proteins, evaluating the roles of column length, linear velocity, and radial compression on the separation performance. Nylon-6 C-CP fiber packed columns were characterized as substrates for the downstream processing of biomacromolecules. An evaluation of adsorption and desorption characteristics of lysozyme on nylon-6 fibers have been investigated with the aim of determining frontal throughput and % yield. The results presented here show lot of promise in developing C-CP fibers as a combined support/stationary phase for biomacromolecule separations.

DEDICATION

This Dissertation is dedicated to all those who believed in me with their love, support and encouragement.

To my loving wife Namal, my mother, father, sister and two brothers, and all other family members for your endless love and constant encouragement you provided me throughout.

To all my teachers who taught me to this success.

To all my friends and colleagues at Clemson University for your support and friendship.

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CHAPTER ONE

INTRODUCTION

Introduction to Liquid Chromatography

Liquid chromatography (LC) is an analytical chromatographic technique that is used to separate ions or molecules by their affinity for a solid support (stationary phase) while in the presence of a moving stream liquid (mobile phase). Since the origin of what is now called high performance liquid chromatography (HPLC) about 39 years ago, it has become an indispensable technique for the analysis of samples, isolation and purification components from complex mixtures [1]. HPLC is the “high pressure” form of LC, which uses the same fundamental mechanism of LC to achieve better separation efficiency and throughput. There are three major components that can be manipulated in terms tuning separation performance of a HPLC system: the mobile phase, stationary phase and instrumentation. The mobile phase modifications involve choice of isocratic or gradient composition, mobile phase velocity, pH and additives to affect separation. Over the years there has been a continuous development of stationary phases for columns that provide wide variety of separations. The development of support/stationary phases focused on various packing materials, bonded stationary phases with a range of functionalities, packing density, porosity, and particle size. The cost of a chromatographic system is usually determined by the number of pumps and the number of pistons per pump.

Theory of Chromatography

The basic goal of chromatography for analytical purposes is to develop a fast HPLC with a sufficiently high efficiency [2]. When a mixture of two components is injected to the top of a column, they migrate along the column forming bands depending on their affinities to the column. To achieve high resolution of the two elution peaks the distance between the two peaks should be wide as possible and the width of them as narrow as possible. The position of elution peaks depends on retention equilibrium thermodynamics and the degree of band broadening depends on both retention equilibrium and mass transfer kinetics [3].

A chromatographic band is considered to be a statistical distribution of molecules. The peak width of the elution band is proportional to the square root of the length (L) that it has travelled [1]. The first theory of band dispersion in chromatography was the plate theory of Martin and Synge (1941), who defined the height equivalent to a theoretical plate ($HETP = H$) [4]. The slope of a plot of variance of peak width (σ^2) vs. length is defined as plate height H. It has been the traditional way of understanding the hydrodynamics of chromatography by analyzing the dependence of HETP on the mobile-phase linear velocity (Fig 1.1). The van Deemter equation (1959) is the simplest form of the equations that describes the relationship between HETP and linear velocity (U) (Eq. 1.1).

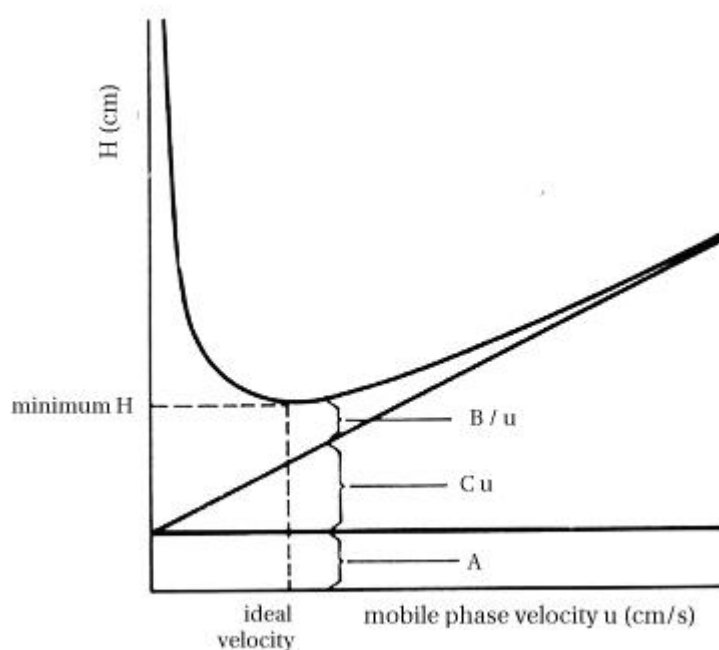


Figure 1.1. The van Deemter plot illustrating the contribution from individual term to band broadening

The generalized van Deemter equation describes the major contributions to band dispersion in chromatography: axial dispersion, longitudinal diffusion, and mass transfer to/with the stationary phase.

$$H = A + \frac{B}{U} + CU \quad (1.1)$$

The A-term represents the eddy diffusion arise from flow inhomogeneities, the B-term represents the molecular diffusion in the axial direction which is inversely proportional to linear velocity, and the C-term represents all sources of resistance to mass transfer. Since these contributions are independent of each other they add up to form a curved profile as depicted in Fig 1.1. Later, a number

of other equations (Giddings and Knox equations) were derived based on the van Deemter equation on the agreement with experimental data [5-7].

Stationary Phases for Protein Separations

A proteome sample may contain 10,000-50, 000 proteins and 100,000-500,000 peptides digested from proteins for the analysis [8,9]. Because of its ability to resolve complex mixtures, HPLC is expected to play a prominent role in the field of proteomics. The characterization and development of new supports and stationary phases for protein chromatography has expanded over the years such as polysaccharides [10], silica [11-13], organic polymers [14], monoliths [15], and fibers [16-19] . Different types of liquid chromatographic approaches are employed in protein chromatograph: reversed phase (RP), ion exchange (IEC), hydrophobic interaction (HIC) and size exclusion (SEC). Among them the great majority of separations are carried out using reversed phase (RP) conditions. RP-HPLC utilizes a less polar stationary phase and polar, water bases mobile phases [20]. IEC has been widely used in downstream processing of therapeutic proteins and isolation of bacterial enzymes [21]. IEC conditions provides a gentle environment for biomolecules near physiological conditions allowing greater flexibility for optimizing separation conditions in method development stage [21].

Although porous phases are very effective in small molecule separations, they suffer from slow mass transfer characteristics (due to intra-stationary phase diffusion) for protein separations. The majority of protein separations are carried out with either non-porous or limited porosity (Porous shell) phases to overcome

mass transfer limitations [22-24]. Fibrous support/stationary phases have been developed to affect protein separation for almost 20 years [16,19,25-28]. The ability to employ high mobile phase velocities with minimal backpressures, ease of packing, and low material cost are the major advantages of the fiber based phases in comparison to other phases.

This focus of this research is based on the development of capillary channeled polymer (C-CP) fibers as a combined support/stationary phase for macromolecule separations. In fact C-CP fibers are being developed in Marcus laboratory as a platform for macromolecule separation since 2003 [19]. C-CP fibers are manufactured by spin melt process in the Clemson University School of Materials Science and Engineering (Clemson, SC, USA) [29]. C-CP fibers have been employed for the separations of hydrocarbons, inorganic compounds, amino acids [30], polymers [31], proteins [19,27,28,32-34] and for the extraction of metals [35]. These C-CP fibers come in a variety of base polymers: polypropylene (PP), nylon-6, polyester (PET). They have a unique structure with eight capillary channels running axially along the entire length of the fiber (fig. 1.2), which act in concert as an efficient transport medium for pressurized flow, yielding relatively low back pressures at high flow velocities. Utilizing the functionality of C-CP base polymer, a variety of solute-surface interactions (hydrophobic, ionic, hydrogen bonding, π - π interactions) can affect protein separations.

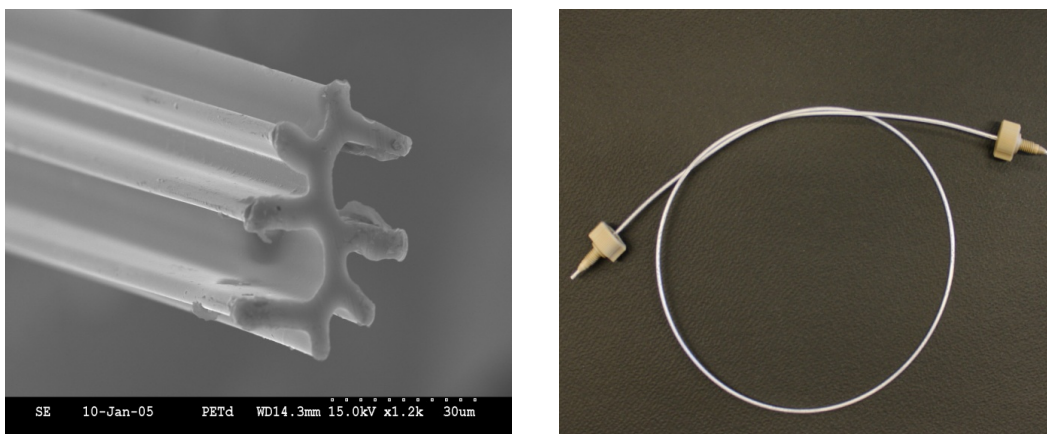


Figure 1.2. a) SEM image of a PET C-CP fiber [36] , b) Image of a microbore column packed with C-CP fibers

The use of microbore column format (Fig 1.2b) packed with C-CP fibers have shown to be the most effective means of achieving high separation efficiencies for rapid analytical separations.

Summary

The focus of the work presented in this dissertation is based on the evaluation of fluid dynamics and characterization of C-CP fiber stationary phases for macromolecule separations. Chapter two describes the use PP C-CP fibers to examine the column hydrodynamics of C-CP fiber columns. The hydrodynamic studies were focused on optimizing the size of fiber, packing densities (interstitial fraction), and column dimensions to achieve high separation efficiency for the separation of two small molecules (uracil and butylparaben). Optimized hydrodynamic characteristics for small molecules were successfully applied for the separation of a three-protein suite using PR-HPLC to achieve high separation efficiency.

Chapter three continues to demonstrate the use of PP C-CP fibers in microbore column format for rapid RP-HPLC separation of proteins with enhanced separation performance. More specifically, the role of column length (40 -110 cm) on peak width, resolution, and peak capacity was evaluated. Also the radial-compression is used to increase the packing uniformity of microbore C-CP fiber columns and thereby more homogeneous mobile phase dispersion to affect improved separation. The ability to achieve high linear velocities (up to 100 mm s⁻¹) at low mobile phase flow rates was driving force of enhanced performance of microbore C-CP columns.

Chapter four presents the initial evaluation of nylon-6 C-CP fibers as a stationary phase for downstream processing of bio-macromolecules. Ionizable amine and carboxylic acid groups naturally present in nylon-6 C-CP fibers allow for ion exchange chromatography (IEC) on the native nylon surface. Adsorption and desorption characteristics of lysozyme on nylon-6 C-Cp fibers were evaluated using frontal development to calculate the frontal throughput and yield. The effect of elution salt strength, protein feed concentration and linear velocity on throughput and yield were determined. The low cost and ability to operate at high linear velocities and low back pressures are the most important advantages of C-CP fibers for preparative-scale macromolecule separations.

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CHAPTER TWO
DYNAMIC EVALUATION OF POLYPROPYLENE CAPILLARY-CHANNELED
FIBERS AS A STATIONARY PHASE IN HIGH PERFORMANCE
LIQUID CHROMATOGRAPHY

Introduction

The use of polymer stationary phases is receiving increased attention in liquid chromatography applications, primarily because of their chemical stability (pH stability and absence of silanol groups), versatile surface chemistries and good performance in macromolecule separations. Thanks to these advantages, polymer stationary phases are commonly used as supports for size exclusion, ion-exchange, and hydrophobic interaction chromatography techniques [1-4]. However, polymer beads are endowed with low specific permeability, limited mechanical stability, swelling in certain mobile phase environments and hindered mass transfer within the polymer network [5,6].

A number of fiber-form stationary phases have been developed for HPLC as an alternative approach to polymer beads [7-12]. A pair of recent review articles by Marcus described the physical and chemical attributes for the use of fibrous stationary phases in HPLC separations [13,14]. Fibrous materials can serve as a combined support and stationary phase, where their native surface chemistry can be easily modified accordingly to the chromatographic separation mode of choice. The primary advantages of fiber phases are broad range of potential surface chemistries, low cost materials, ease of column packing, and

reduced back pressures. Polymeric fiber stationary phases have been utilized in size-exclusion, reversed-phase, ion exchange, and capillary electrochromatography mode separations for the analysis of chemical and biological species ranging from inorganic ions to proteins [7-11].

Marcus and co-workers have specifically demonstrated the use of capillary-channeled polymer (C-CP) fibers as a combined support/stationary phase for chromatographic separations [2,15-22]. These fibers are made in a melt-spin process in which the fiber geometry is created by extrusion through an orifice with the desired shape. These C-CP fibers are unique in that they have eight capillary channels on the periphery that run the entire length of each fiber, which act in concert in a column format as an efficient transport medium for pressurized flow, yielding relatively low back pressures as well as efficient mass transfer characteristics [2,13-22]. C-CP stationary phases exhibit relatively poor performance for the separation of *small molecules*, predominately attributable to their relatively low specific surface areas, implying low binding capacity [20,21]. On the other hand, they have been effectively utilized in macromolecule separations, where adsorption/desorption processes in the absence of significant mass transfer limitations yield very high recoveries and efficiencies at high linear velocities and gradient rates [2,16-19,22]. In particular, mass transport of the solutes from the bulk liquid phase to the surface of the C-CP fibers is not hindered by slow molecular diffusion inside the dead end pores as in the case of traditional porous polymeric beads [23]. Boundary layer mass transfer in packed

beds is much slower than the convective transport through the channels in the C-CP columns [23]. In addition, the large solutes tend to bind only on the external surface of porous chromatographic beads reducing the overall ligand efficiency, while the open structure of C-CP fibers allows the whole surface area available for adsorption to be accessed and thus the capacity is practically maximized [24]. Analogous to previous studies carried out on alternative stationary phases such as membranes [25] and monoliths [26], the mass loading capacity for C-CP fibers increases with the size of the target molecule as the larger size results in more mass bound per unit of surface area.

Two basic parameters that can be manipulated to optimize liquid chromatography (LC) separations are the physical and the chemical characteristics of the stationary phase used [6,27,28]. A basic understanding of the hydrodynamic and kinetic properties of a stationary phase is essential in predicting and tailoring column performance. In addition, the investigation of the underlying phenomena of zone formation and band broadening offers insights for improved column design to enhance column performance. The standard for characterization and optimization of stationary phases for HPLC are isocratic separations, as the mobile phase composition is held constant so that column bed variances can be readily evaluated [6,29-34]. The hydrodynamic aspects of the C-CP columns have been studied by varying fiber packing density and column inner diameter, using uracil (an unretained marker compound) to probe A-term contributions to band broadening [21]. Those studies conducted with

polypropylene (PP) and polyester (PET) C-CP fibers showed that optimal peak width and peak asymmetry characteristics were found at lower packing densities ($< 0.5 \text{ g/cm}^3$ and $\epsilon_i < 0.65$) and columns of smaller inner diameters (3.2 mm i.d. vs. 4.6 and 7.7 mm i.d).

The evaluation of the roles of fiber diameter, packing density (interstitial fraction), and column inner diameter on the elution characteristics of unretained and retained species using PP C-CP fibers is extended in the present study. Polypropylene is chosen in this demonstration due to its simple chemical structure, ensuring that only hydrophobic interactions control analyte retention. The contributions to band broadening are evaluated using uracil and butylparaben as unretained and retained model compounds, respectively. In order to identify the best performing packing characteristics, elution experiments were performed under isocratic conditions using the small molecule tracers. The optimized PP C-CP fiber column was then challenged for macromolecule separations under gradient conditions using a three-protein suite as a test model. This study provides further understanding of the zone broadening process of PP C-CP fiber columns, leading to the optimization of fiber diameter, column dimensions, and interstitial fraction characteristics for analytical chromatographic separations which are envisioned predominately for macromolecule systems.

Experimental

Column Construction

The polypropylene C-CP fibers were manufactured in the Clemson University School of Materials Science and Engineering (Clemson, SC, USA) [35]. The polypropylene fibers (designated as PP1, PP2, PP3, and PP4) were produced with maximum cross sectional diameters of 65, 40, 30, and 40 μm , respectively. The physical characteristics of the different fibers are summarized in Table 2.1, while magnified images of the fibers are presented in the electron micrographs of Fig. 2.1. The last column of the table reports the total fiber surface (SA_T) area per column, based on the assumption that the fibers are totally non-porous. This value is obtained by multiplying the fiber perimeter (p) by the column length (L) and the number of fibers per column. Fibers PP1-3 have essentially the same shape, differing principally in their overall size. PP4 has more uniform channel dimensions, as a different extrusion spinneret was employed. To study the role of the different fibers on separation efficiency, 250 mm x 2.1 mm. i.d. columns were packed [16,20] to an interstitial fractions of $\varepsilon_i \approx 0.63$ [21] (Table 2.1) and tested for the resolution of uracil and butylparaben. Additional experiments were carried out using columns prepared at different degrees of porosity, ranging from $\varepsilon_i = 0.39 - 0.69$ and inner diameters of 1.5, 2.1 and 4.6 cm. Stainless steel HPLC columns (1.5, 2.1, and 4.6 mm i.d. x 250 mm long), 10 μm porosity frits (polyethylene), and end-fittings were purchased from Valco Instruments (Houston, TX, USA). The separation of the three-protein suite was carried out

using a 250 mm long x 2.1 mm. i.d. PEEK-lined stainless steel column (Valco Instruments, Houston, TX).

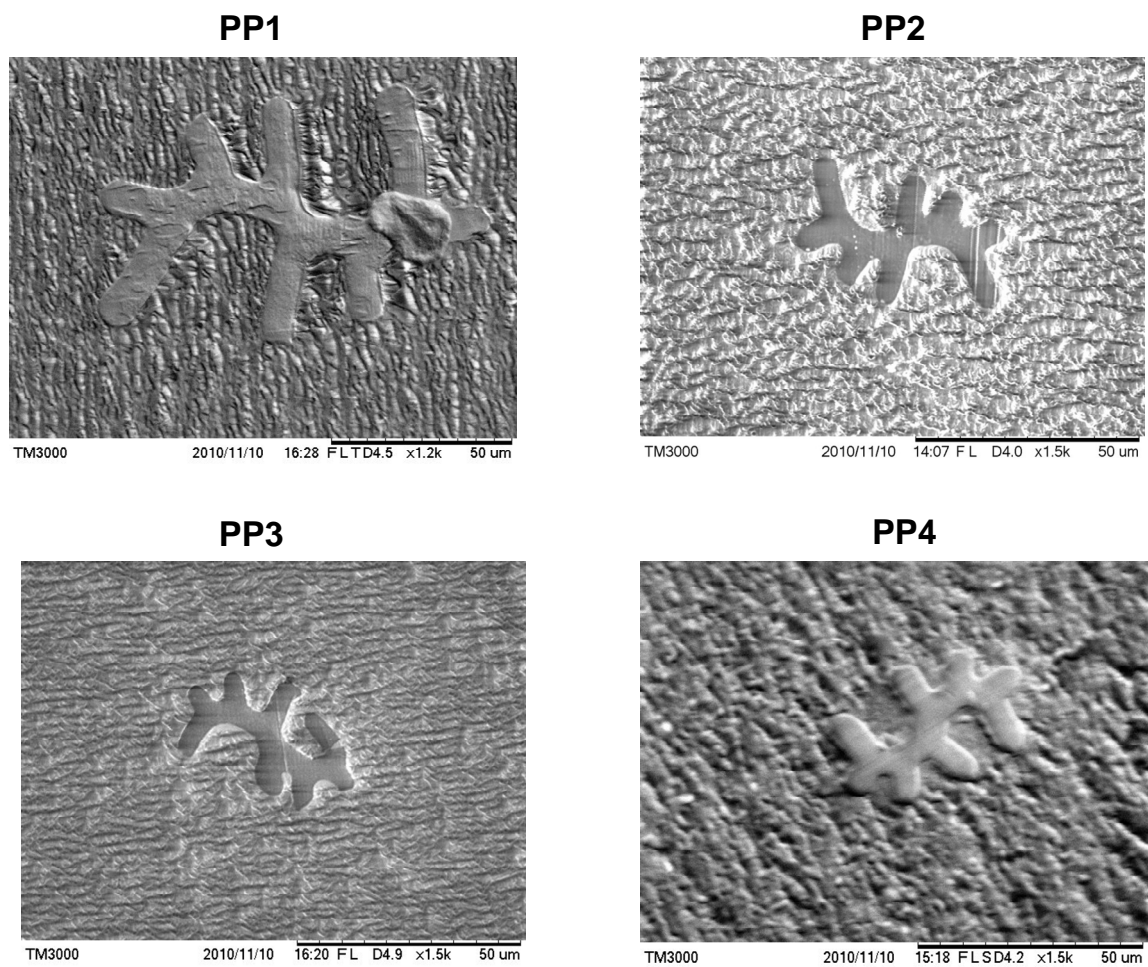


Figure 2.1. Scanning electron micrographs of C-CP fibers a) PP1, b) PP2, c) PP3, and d) PP4. A 50 μm scale is included on each of the micrographs.

Table 2.1. Polypropylene C-CP fiber diameters, perimeters, number density, and surface areas per column area for columns (2.1 x 250 mm) packed to interstitial fractions $\epsilon_i \cong 0.63$.

Fiber (maximum cross sectional diameter)	Perimeter (p, μm)	dpf*	# fibers per column	Surface area per column (SA_T, m^2)	Measured ϵ_i
PP1 (65 μm)	476	21	608	0.0723	0.640
PP2 (40 μm)	252	7.6	1760	0.111	0.635
PP3 (30 μm)	168	3.3	3760	0.158	0.635
PP4 (40 μm)	199	3.0	4080	0.203	0.625

* Denier per filament = g fiber / 9000 m length

Sample Preparation and Solution Delivery

Stock solutions of reagent-grade uracil (2,4-pyrimidinethiol) ($30 \mu\text{g mL}^{-1}$) and butylparaben (butyl 4-hydroxybenzoate) ($90 \mu\text{g mL}^{-1}$), both from Acros Organics (Morris Plains, NJ, USA) were prepared in 75:25 water:methanol ($\text{H}_2\text{O}:\text{MeOH}$). The mobile phase components were ACS grade methanol (VWR International, West Chester, PA, USA) and MilliQ water (conductivity $18.2 \text{ M}\Omega \text{ cm}^{-1}$). The chromatographic system consisted of a Waters HPLC pump Model 600 (Milford, MA, USA) connected to a Rheodyne 7725 six port injector valve (Rohnert Park, CA, USA) fit with an injection loop. In order to prevent volume and mass overload bias, the injection loop volume was proportionally adjusted to the column volume using 5, 10, and 50 μL loops for the 1.5, 2.1, and 4.6 mm i.d. columns, respectively. A Waters 2487 dual wavelength absorbance detector

equipped with a small-volume (2.6 μL) detector cell was used throughout. The chromatograms were recorded using the Waters Millennium 4.0 data acquisition system and processed with Microsoft Excel (Seattle, WA, USA) and XLSTAT 2009 statistical analysis software (New York, NY, USA). All presented data are the means of triplicate injections, with error bars representing an interval equal to one standard deviation. It should be stressed that column-to-column variations in retention characteristics of C-CP columns are typically better than 3% RSD [2,21].

For the separation of the synthetic three-protein suite, a test mixture containing ribonuclease A (bovine pancreas), cytochrome *c* (bovine heart), and transferrin (human) at a concentration of 100 $\mu\text{g mL}^{-1}$ each and spiked with 10 $\mu\text{g mL}^{-1}$ uracil was prepared in MilliQ water with 0.1 % HPLC grade trifluoroacetic acid (TFA). The separation was performed at a flow rate of 2 mL min^{-1} , with a gradient elution program of 80:20 to 50:50 H_2O containing 0.1% TFA (v/v):ACN containing 0.06% TFA (v/v) over 2.5 minutes. The three test proteins and TFA used in the mobile phase were all purchased from Sigma Aldrich (Milwaukee, WI, USA). The HPLC grade ACN used for elution was purchased from Fisher Scientific. Once prepared, the protein test solutions were stored at 6 °C.

The chromatographic system used for the protein separation consisted of a Waters Model 717 autosampler, 600S Controller, 616 HPLC pump and Waters 996 photodiode array detector at 216 nm (Milford, MA, USA). Data was collected

by the Millennium 32 and Empower 2 Chromatography Manager and further processed and managed in Microsoft Excel.

Results and Discussion

Contributions to Band Broadening

In column chromatography, the three major contributions to zone broadening are axial dispersion, longitudinal diffusion and mass transfer to/within the stationary phase [5,6,30]. The contribution of these three factors is explicit in the generalized van Deemter equation, which describes the relationship between the height equivalent per theoretical plate (H or HETP) and linear flow velocity (U) [5,6,30,36].

$$H = A + \frac{B}{U} + CU \quad (2.1)$$

The first term (A-term) represents the dispersive contribution to band broadening arising from flow profile inhomogeneities, non idealities and localized vortices and turbulences, also referred as to eddy diffusion. This factor is minimized with homogeneous packing arrangements and optimized fluid flow within the column [5,6,31,36-38]. The second term (B-term) is associated to molecular diffusion in the axial direction and is inversely proportional to the linear velocity of the mobile phase. The last term (C-term) reflects all contributions related to mass transfer phenomena to/from the stationary phase.

A number of other expressions have been successively derived from the van Deemter equation to improve the agreement with experimental HETP data. The two most popular are the Giddings and the Knox equations, which can be

used to evaluate the plate height of virtually any chromatographic system. The Giddings equation (Eq. 2.2) differs from van Deemter's in the description of the mobile phase dispersion term. In fact, accordingly to Giddings coupling theory, the combined effects of eddy dispersion and transverse diffusion should be harmonically added in order to account for the attenuation of band dispersion [29,31,39].

$$H = \frac{A}{[1 + (\frac{E}{U})]} + \frac{B}{U} + CU \quad (2.2)$$

The Knox equation is the most widely used variant of the van Deemter equation [40] and assigns an empirical 1/3 power dependence for the A term on linear velocity:

$$H = AU^{\frac{1}{3}} + \frac{B}{U} + CU \quad (2.3)$$

These three equations can be used to gain insights on the relative contribution of the relevant mass transport phenomena present in the chromatography column simply from HETP and linear velocity data. The HETP was considered the parameter of choice to evaluate separation efficiency of the C-CP columns. The plate height is derived from the number of theoretical plates, N, present in a column of total length L

$$H = \frac{L}{N} \quad (2.4)$$

The number of theoretical plates is calculated from the elution peak profiles resulting from sample injections

$$N = 16 \left(\frac{t_R}{w} \right)^2 \quad (2.5)$$

where t_R is the retention time and w is the peak width measured at the base line (4σ).

Another relevant parameter that will be considered to evaluate column performance is the peak resolution of two species, R_S , defined as

$$R_S = \frac{2 \Delta t_R}{w_1 + w_2} \quad (2.6)$$

where Δt_R is the retention time difference of the two peaks and w_i are the peak widths of the two species.

Role of PP Fiber Diameter and Shape

The different shape of the fibers strongly affects important characteristics of the column as the specific surface area and the quality of the packing. In order to evaluate the best performing polypropylene (PP) fiber, 250 mm x 2.1 mm i.d. columns were packed with the different polymeric stationary phases considered in the present study. The columns were manufactured at an interstitial fractions (ε_i) of ~0.63, a value found to be optimal in previous C-CP fiber column studies [21]. The actual value ε_i of each column was determined through the retention time of uracil, an unretained compound, using the following simple expression

$$\varepsilon_i = \frac{F \cdot t_0}{V_c} = \frac{V_m}{V_c} \quad (2.7)$$

where V_c is the total column volume, F is the volumetric flow rate, t_0 is the elution time of the unretained tracer and V_m is the volume occupied by the mobile phase equal to the void volumes present in the column. Since there is no chemical

interaction with the stationary phase, unretained compounds remain in the mobile phase and thus elute with the void volume [5,6]. The experimental porosities are summarized in Table 2.1.

In order to determine the best PP fiber geometry/size, the solutes were eluted under isocratic conditions with a 75:25 H₂O:MeOH mobile phase composition. This mobile phase was selected from preliminary experiments carried out at different water-methanol compositions ranging from 0 to 30% MeOH. The best elution buffer was chosen on the basis of minimum peak width and maximum resolution for the separation of uracil and butylparaben.

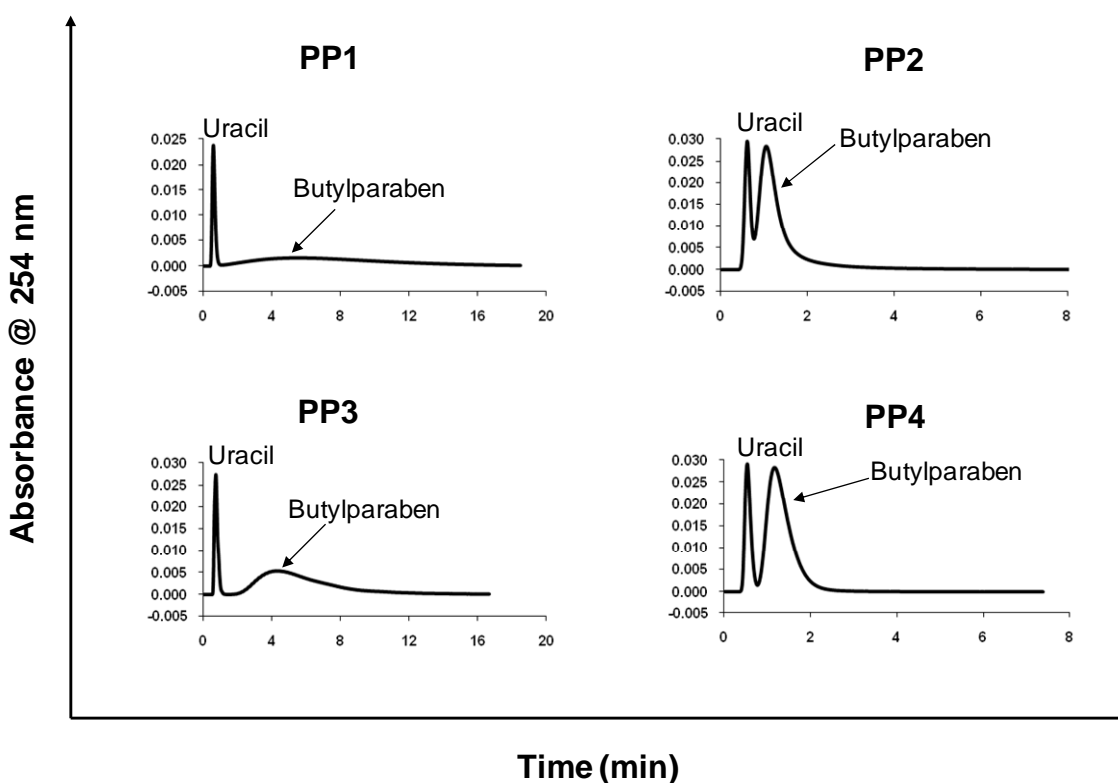


Figure 2.2. Comparison of chromatograms obtained with the PP1 (65 μm), PP2 (40 μm), PP3 (30 μm), and PP4 (40 μm) C-CP fiber columns packed at an interstitial fraction of ~ 0.63 each. Flow rate = 1 mL min^{-1} . Injection volume = 10 μL , concentration = 30 $\mu\text{g mL}^{-1}$ uracil and 90 $\mu\text{g mL}^{-1}$ butylparaben.

In Fig. 2.2 are presented the chromatograms measured from an injection of uracil and butylparaben as tracers for each fiber-type column. Separation efficiency was far superior for columns packed with PP2 and PP4 in comparison to those of PP1 and PP3, where butylparaben is eluted as a very broad peak. Additional information can be obtained plotting the plate height versus mobile phase linear velocity (U) (aka, van Deemter plots) as reported in Fig. 2.3. The large plate heights for the uracil marker associated with the PP1 and PP3 columns, versus those of the PP2 and PP4 packing, is a clear indication of poor performance in terms of band broadening. Interestingly, PP2 and PP4 fiber columns are intermediate in size, suggesting that this dimension allows a better packing arrangement and interdigitation of the fibers.

The plate heights for the retained butylparaben solute are in the range of 13-25 mm for linear velocities ranging from 1 to 37 mm s⁻¹, following the typical convex downward trend with a minimum occurring at ~2.4 mm s⁻¹ followed by a linear increase of HETP with linear velocity. The plate heights for uracil increase from 5 to 10 mm as linear velocity increases from 0.9 to 37 mm s⁻¹. The relative flatness of the uracil curve indicates the A-term dominance on band broadening, through there are C-term contributions. The poor overall performance exhibited here for butylparaben can be attributed to three factors; low surface area, flow path variance, and the porosity of the fiber matrices. The lower surface areas of these PP fibers in comparison to porous silica beads compromise the binding capacity of the fibers, especially when using small organics as tracers.

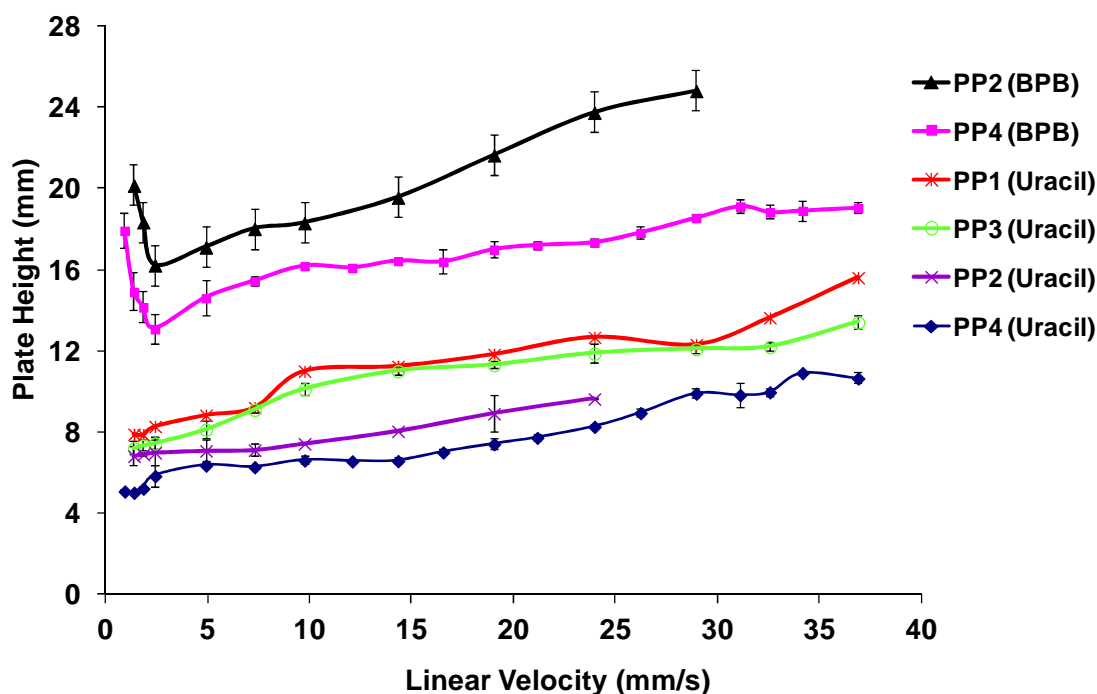


Figure 2.3. Effect of linear velocity on plate height of uracil and butylparaben evaluated on PP C-CP fiber column packed at an interstitial fraction of ~ 0.63 (250 mm x 2.1 mm i.d.). Butylparaben data for PP1 and PP3 are not reported in figure because of their large (out of scale) plate height. Mobile phase composition 25:75 methanol: water, injection volume = 10 μL , concentration = 30 $\mu\text{g mL}^{-1}$ uracil and 90 $\mu\text{g mL}^{-1}$ butylparaben, detection wavelength = 254 nm.

The variance in flow paths is manifest in the characteristics of the van Deemter curves, showing the important contribution of the A term to the HETP. Finally, the increase in plate height for uracil as the linear velocity increases indicates C-term contributions to plate height. This observation is not surprising as C-term contributions are problematic in small molecule separation using polymeric stationary/support phases [6,41,42]. However, it is worth noticing that the slope of the van Deemter curves is qualitatively similar for all the fibers, suggesting a common mass transfer feature. Reasonably, this can be assigned to small molecule accessing the nanopores (<1 nm) present in the polymeric network,

where slow mass transport induces increased contribution to band broadening at higher flow rates. However, this contribution is not a problem in terms of macromolecule separations; in fact, transmission electron microscopy (TEM) imaging of the PP fiber material showed no appreciable level of porosity above the single nm level, demonstrating that the pore size in the network is small enough to exclude large solutes. Therefore mass transfer limitations are not present in the case of large proteins and other bio-polymers as will be discussed subsequently in the text.

The peak widths and calculated plate heights for the retained solute, butylparaben, are tabulated in Table 2.2 for each fiber type over a range of linear velocities from $\sim 2 - 37 \text{ mm s}^{-1}$. This range of linear velocities far exceeds what is common for packed-bed columns ($< 6 \text{ mm s}^{-1}$) [6]. There are only three entries for the peak characteristics of butylparaben on the PP1 column, as there is no distinguished peak above $\sim 10 \text{ mm s}^{-1}$. The smaller C-CP fibers (PP2 and PP3 and PP4) had larger effective ranges of linear velocities over which the two peaks could be resolved. The PP2 fiber was able to resolve the two compounds up to linear velocities of 28 mm s^{-1} (3 mL min^{-1}), with backpressures of $\sim 10 \text{ MPa}$. The PP3 and PP4 fiber packed columns could resolve the two compounds up to 37 mm s^{-1} (4 mL min^{-1}) linear velocity. This clearly indicates poor performance of PP1 associated to inefficient packing and inter-arrangement of the bigger fibers. On the basis of plate height and peak width for butylparaben, PP2 and PP4 fibers perform comparably as stationary phases for HPLC.

Table 2.2. Comparison of peak width (w) of butylparaben in minutes, plate height (H) in mm and resolution of uracil and butylparaben in PP fibers in 2.1 x 250 mm column with an interstitial fraction $\epsilon_i \cong 0.63$.

Linear Velocity (mms ⁻¹)	PP1 (65 μ m)		PP2 (40 μ m)			PP3 (30 μ m)		PP4 (40 μ m)		
	w	H	w	H	Rs*	w	H	w	H	Rs*
2.40	27.28	48.91	4.05	16.23	0.95	14.09	23.67	3.59	13.10	1.09
4.90	16.86	80.01	1.91	17.12	0.93	7.58	27.28	1.83	14.61	1.07
9.73	12.28	92.68	0.77	18.32	0.85	6.22	33.44	0.96	16.20	1.04
19.03			0.48	21.65	0.74	2.77	44.45	0.48	17.00	0.96
28.94			0.29	24.83	0.62	2.50	46.28	0.36	18.56	0.89
36.87						1.79	44.78	0.29	19.06	0.84

$$* \text{Rs} = \frac{\Delta t_R}{0.5(w_{r,1} + w_{r,2})}$$

The actual differences in the values reported in Table 2.2 are attributable to the different perimeters and shapes of PP2 and PP4 fibers (see Fig. 2.1 and Table 2.1). Based on the fact that practically the same interstitial fractions exist, the best performance would be expected from the column/fiber shape that has the most uniform distribution of the spaces between the respective fiber surfaces (channel sizes). These would in turn be dictated by the uniformity of the fiber channel dimensions.

Based on the results depicted in Fig. 2.2 and 2.3 and presented in Table 2.2, the performance of PP4 fiber packed columns is judged superior to that of PP2 fiber packed columns. The better performance of PP4 can be partly attributed to its greater surface area (Table 2.1), which is approximately twice that of PP2 (0.203 m² vs. 0.111 m²). In addition, the lower minimum values in the

van Deemter plots, which are set by the A-term, indicates better hydrodynamic performance for PP4 with respect to PP2. This is born out as well in the van Deemter plots of uracil. As depicted in Fig. 2.1, the PP4 C-CP fiber has more uniform channel sizes with walls that have high breadth-to-length ratios, whereas the PP2 fiber has two large channels (and thinner walls) that have a tendency to bend-open.

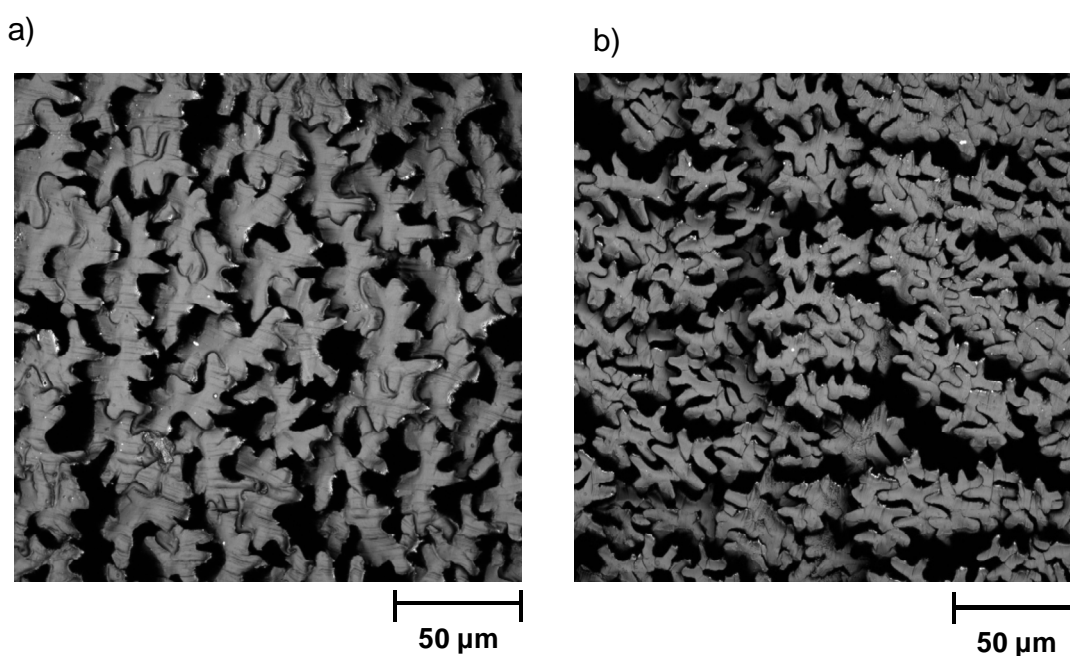


Figure 2.4. Optical micrographs of cross sections of C-CP fiber packed microbore columns a) PP2, and b) PP4.

This subtle yet practically important aspect can be explained in part through optical photographs of the PP2 and PP4 fibers packed into microbore columns as presented in Fig. 2.4. The column cross sections, which reflect the case where the columns are not pressurized with solvent, show a good deal of clustering and inter-digitation. In the case of the PP4 fibers, groups of 4 – 8 fibers commonly

form, whereas for the PP2 fibers there is less extensive interdigitation. Upon pressurization of the columns, it is expected that the interstices will expand to some equilibrium position across the column cross section. Ultimately, it is believed that the greater uniformity among the fiber channels of PP4 (Fig. 2.1) which allows for more homogenous packing.

Another evidence of better performance of PP4 fibers over PP2 can be found in the resolution for the separation of uracil and butylparaben as defined in Eq. 2.6. PP4 showed an average resolution of 0.98, matching the threshold of 1 hence ensuring sufficient peak resolution [32]; on the contrary, PP2 has an average resolution of 0.82 and therefore is not able to sufficiently resolve the elution peaks. In addition, PP2 fibers could not ensure peak resolution at the highest flow rate tested.

One final point of comparison between the PP2 and PP4 fibers can be made. As mentioned previously, the fibers presented here differ in size and shape, as well as drawing conditions. The drawing rate determines the tension under which molten fiber solidifies upon exiting the spinneret and its ultimate crystallinity; in practice, the crystallinity of the drawn fibers will set the inter-chain spacing. In the case of PP4, a higher draw rate was applied, resulting in greater fiber crystallinity that may be reflected in the pronounced lower C-term slope (less mass transfer hindrance) for the butylparaben in the PP4 fiber vs. PP2 (Fig. 2.3). For all of these reasons, further studies were carried out with only the PP4 fibers.

Role of Linear Velocity on Band Broadening

The major contributing factors to chromatographic dispersion of PP4 C-CP fiber packed columns were examined using the plate heights as a function of chromatographic linear velocity for butylparaben. The van Deemter, Giddings and Knox equations (Eqs. 2.1, 2.2, and 2.3) are the three common methods of preference to evaluate the different contributions to band broadening. All three equations were used to fit the plate height data using an XLSTAT 2009 data analysis model. Fitting of the butylparaben plate height data to the three equations is presented in Figure 2.5, with the fitted parameters summarized in Table 2.3.

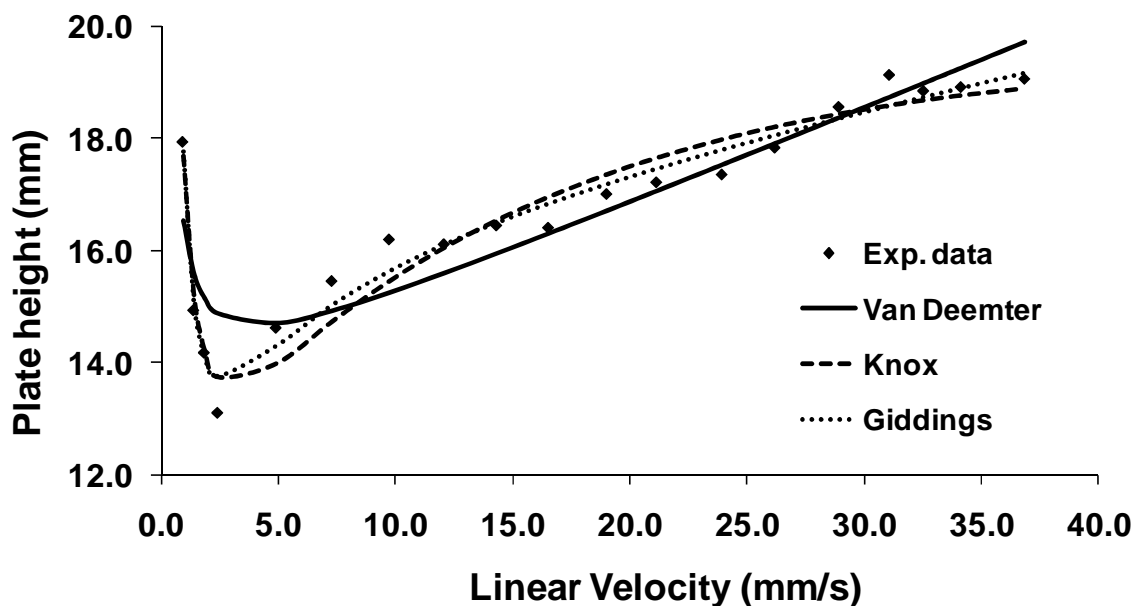


Figure 2.5. Fitting of the van Deemter, Knox, and Giddings equations to the butylparaben plate height data of PP4 C-CP fibers.

Table 2.3. Parameters of the employed plate height equations to determine the band broadening of butylparaben on C-CP fiber stationary phases.

Solute	Equation	Term				R ²
		A	B	C	E	
BPB	van Deemter	13.277	2.845	0.173	-	0.841
	Knox	7.606	9.603	-0.181	-	0.938
	Giddings	16.761	11.772	0.083	2.248	0.966

$$A = \text{mm} \quad B = \text{mm}^2\text{s}^{-1} \quad C = \text{s}$$

The data seem to be well modeled by the Giddings and Knox equations in comparison to the van Deemter equation, as judged by the R² values for the fits of each equation. The greater suitability of the Giddings equation is not surprising due to its additional “E” term which covers the combined effects of eddy diffusion and transverse diffusion across the longitudinal interfaces [39,43]. That said, an equation with more fitting terms will always provide a better fit than an equation with fewer terms [44]. Also greater A-term contributions as observed for butylparaben in the Giddings fit describe the extent of different flow paths within the fiber channels along the column, and those flow paths of butylparaben are influenced by the “pull” of the fiber stationary phase. The diffusional differences evident in the B-term of Table 2.3 are also significant in Giddings fit compared to the other two equations. Butylparaben can diffuse through the stagnant mobile phase within the inter-fiber channels to the fiber surface contributing to B-term broadening. The negative C term observed for Knox equations is surprising.

The minimum plate height of 13 mm was found at a linear velocity of ~ 2.4 mm s⁻¹ for the PP4 fibers. These large plate heights depict the poor performance of C-CP columns in comparison to C₁₈-modified porous silica (0.015 mm at 1.5 mm s⁻¹) and polymeric monolith columns (0.005 mm at 1.3 mm s⁻¹) when separating small molecules [45]. The decrease in column efficiency at increasing flow rates can be related to the slow mass transfer of small molecules within the fiber matrices [6] and possible crimped fiber channels that create voids and stagnant mobile phase zones. This effect was seen in previous studies using columns of different diameter, with and without the use of flow distributors [21]. In this case, high linear velocities shorten the residence time on-column, possibly hindering the achievement of a homogeneous frontal profile.

Role of Interstitial Fraction on Retention Factor, Plate Height, and Resolution

For the C-CP fiber stationary phase, the void fraction is a direct reflection of the available surface area for solute interaction. As fiber packing densities increase, the total surface area that the fibers display increases as well, and that allows more efficient separation. On the other hand, if the packing is too dense the probability of crimped channels is higher and access to part of the surface area is hindered because of excessive interdigitation of the fibers. Therefore, an optimal value of the void fraction must be explored to maximize separation performance. The effect of column porosity was studied on 2.1 mm i.d. PP4 C-CP columns at flow rates ranging from 0.25 to 4 mL min⁻¹ (corresponding to 2 - 37 mm s⁻¹) packed at interstitial fraction values from 0.39 to 0.69. As an

alternative approach to the BET measurements that are tenuous in the single g m⁻² regime, the perimeter of the C-CP fibers was determined from SEM images of multiple individual fibers using the Scale-link (Scalex Corporation, Carlsbad, CA, USA) measurement tool. This method yielded lower fiber specific surface area for these C-CP fibers in comparison to BET measurements done in previous works [15]. This result is expected as any “porosity” of the fibers is not accounted for in this approach. Under the assumptions of negligible fiber stretching, breaking, and fusing during the packing process, the product of fiber perimeter, length and the number of fibers gives a rough estimate of the C-CP fiber surface area in each column as listed in Table 2.4.

Table 2.4. Number of fibers, surface area, and surface area per column area for interstitial fraction study with a 2.1 x 250 mm column. Column i.d. study performed with $\epsilon_i \cong 0.63$.

i.d. (mm)	ϵ_i	# of fibers	SA _T (m ²)	Phase Ratio (β , m ⁻¹)	Hydraulic diameter (μ m)	Apparent fiber cross section (μ m ²)
2.1	0.39	5760	0.287	8.50×10^5	4.7	367
2.1	0.45	5280	0.263	6.75×10^5	6.0	361
2.1	0.55	4800	0.239	5.02×10^5	7.9	325
2.1	0.63	4080	0.203	3.72×10^5	10.9	314
2.1	0.69	3600	0.179	3.00×10^5	13.3	298
1.5	0.61	3120	0.155	5.75×10^5	7.2	221
2.1	0.63	4080	0.203	3.72×10^5	10.9	314
4.6	0.62	21600	1.075	4.17×10^5	9.5	292

The counter effects between accessible surface area and column hydrodynamics are noticeable when one examines the effect of interstitial fraction on retention factors, chromatographic plate height, and resolution. The retention factor (k') is defined as the ratio of the time that an analyte spends in the stationary phase (t_s) to the time it spends in the mobile phase (t_M):

$$k' = \frac{t_s}{t_m} \quad (2.8)$$

Under isocratic conditions, it represents the ratio of the number of molecules in contact with the stationary phase (N_s) to the number of analyte molecules in the mobile phase (N_m). In the case of adsorption chromatography, the retention factor can be described using the following equation [6]:

$$k' = \frac{N_s}{N_m} = \frac{q_s A_s}{c_m V_M} \quad (2.9)$$

where q_s is the concentration of solute molecules per unit surface area, A_s is the surface area of the stationary phase, c_m is the solute concentration in the mobile phase, and V_m is the mobile phase volume. Taken further, the ratio A_s/V_m is designated as the phase ratio (β), given in units of reciprocal length. Given the relative non-porous nature of the C-CP fibers, solute molecules presumably have access to the total fiber surface areas (SA_T) for interactions. Therefore, the phase ratio, as it pertains to adsorption chromatography, can be readily calculated using the product of the column volume (V_C) and the porosity to yield the mobile phase volume, V_M .

$$\beta = \frac{A_s}{V_M} = \frac{SA_T}{V_c \epsilon_i} \quad (2.10)$$

As depicted in Fig. 2.6, higher retention factors are generated with columns packed at low interstitial fraction. As would be expected based on the derivation above, the retention factors do indeed follow the trends of the phase ratios as presented in Table 2.4. For example, the k' -values for the 0.25 ml min^{-1} flow rate increase by a factor of 2.2 as the interstitial fraction decreases from 0.69 to 0.45; a difference in phase ratios of 2.2x. Note that this relationship ceases to exist at the lowest column porosity, $\epsilon_i = 0.39$. It is at this point that the packing density leads to crimping of the interfiber channels and the basic flow patterns in the column become blocked at points. At the same time, the linear velocities of the mobile phase increase to maintain the volume flow rates. Clearly seen in Fig. 2.6 as well is the fact that the solute retention factor values decrease with increasing volume flow rates.

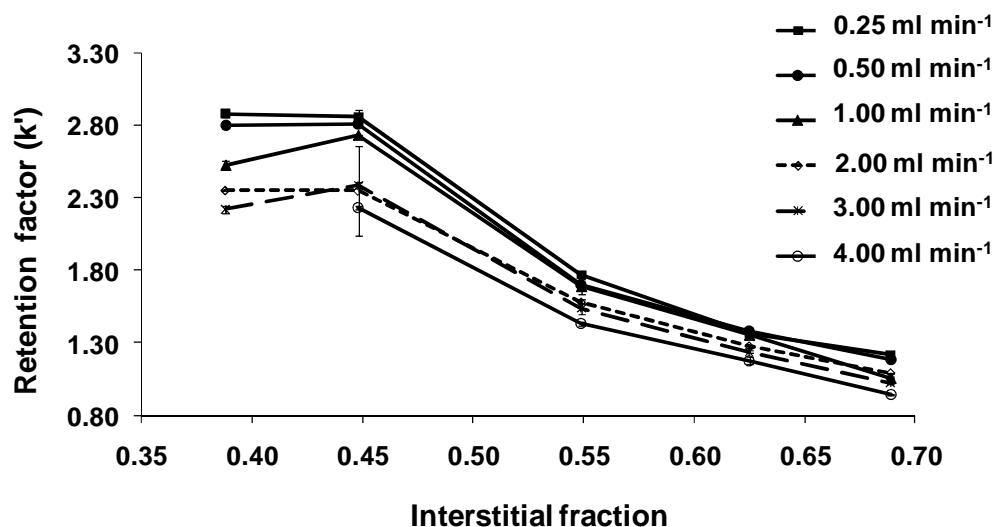


Figure 2.6. Effect of interstitial fraction on retention factor (k') of butylparaben at flow rates ranging from 0.25 to 4 mL min^{-1} . $250 \text{ mm} \times 2.1 \text{ mm}$ i.d. column packed with PP4 C-CP fiber. Mobile phase composition 25:75 methanol: water, injection volume = $10 \mu\text{L}$, detection wavelength = 254 nm .

The retention factor is a reflection of a chemical equilibrium of the solute between the stationary and mobile phases. Thus, as high volume flow rates deplete the mobile phase solute concentration in the near-surface region, the response is more rapid release from the fiber surface to maintain the natural equilibrium, producing lower retention factors. A similar argument thus exists in the case of the lowest interstitial fraction where the mobile phase velocities would be the highest.

Beyond the role of interstitial fraction in the retention factor as predicted by the phase ratio relationship it also affects the column dynamic characteristics. Figure 2.7 highlights the fact that at low interstitial fractions (high packing densities) the columns experience relatively high plate height values, while they improve as the interstitial fraction increases to $\epsilon_i = 0.63$, to yield again poorer separation efficiency at the highest porosity considered. This trend is clearly seen at all flow rates studied here. While low interstitial fractions generally tend to increase the retention factor considerably, the fluid flow path heterogeneity in the inter-fiber channels/gaps becomes more problematic as packing density increases, resulting in greater A-term contributions to broadening and deteriorated column performance. As seen in Fig. 2.7, the plate heights decrease significantly (~ 65%) as the interstitial fraction reaches 0.63. At the same time, a high level of reproducibility in terms of retention times and bandwidths (less than 1% RSD at 1 mL min⁻¹) exists at this point as well. A further increase in interstitial fraction results in an increase in plate height due to

reduced retention times. Overall increases in plate height as a function of flow rate (C-term contributions) is greatest for $\varepsilon_i = 0.69$. In this case, the mass transfer term is amplified as the diffusion (interfiber) distance is the greatest due to the low fiber packing density.

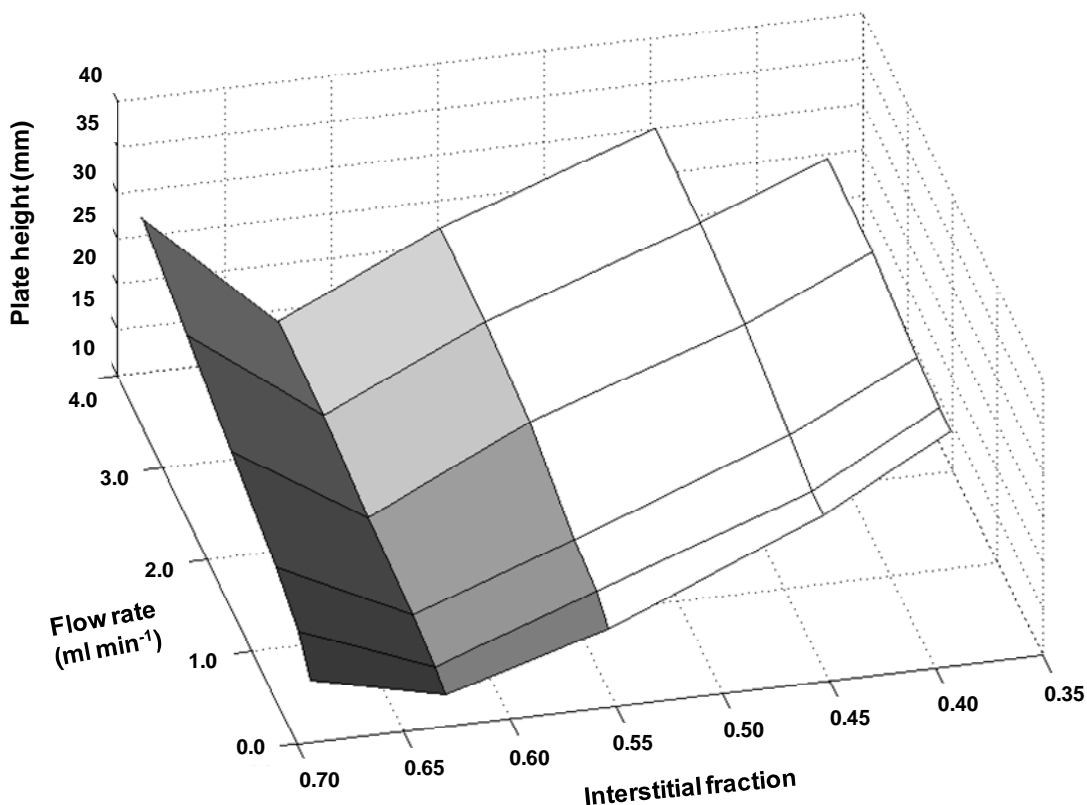


Figure 2.7. Effect of interstitial fraction on plate height (H) of butylparaben at flow rates ranging from 0.25 to 4 mL min⁻¹. 250 mm x 2.1 mm i.d. column packed with PP4 C-CP fiber. Mobile phase composition 25:75 methanol: water, injection volume 10 μ L, detection wavelength = 254 nm.

The resolution of two test compounds (uracil and butylparaben) is a measure of the quality of separation [6] representing the combination of retention factors and band broadening (i.e., k' and H); or more appropriately the balance between the two factors. Figure 2.8 presents the effect of interstitial fraction on

the resolution of uracil and butylparaben. The resolution data follow a similar trend across all flow rates investigated. At low interstitial fractions, high surface areas affect greater retention, but at the price of large amounts of band broadening, yielding low resolution.

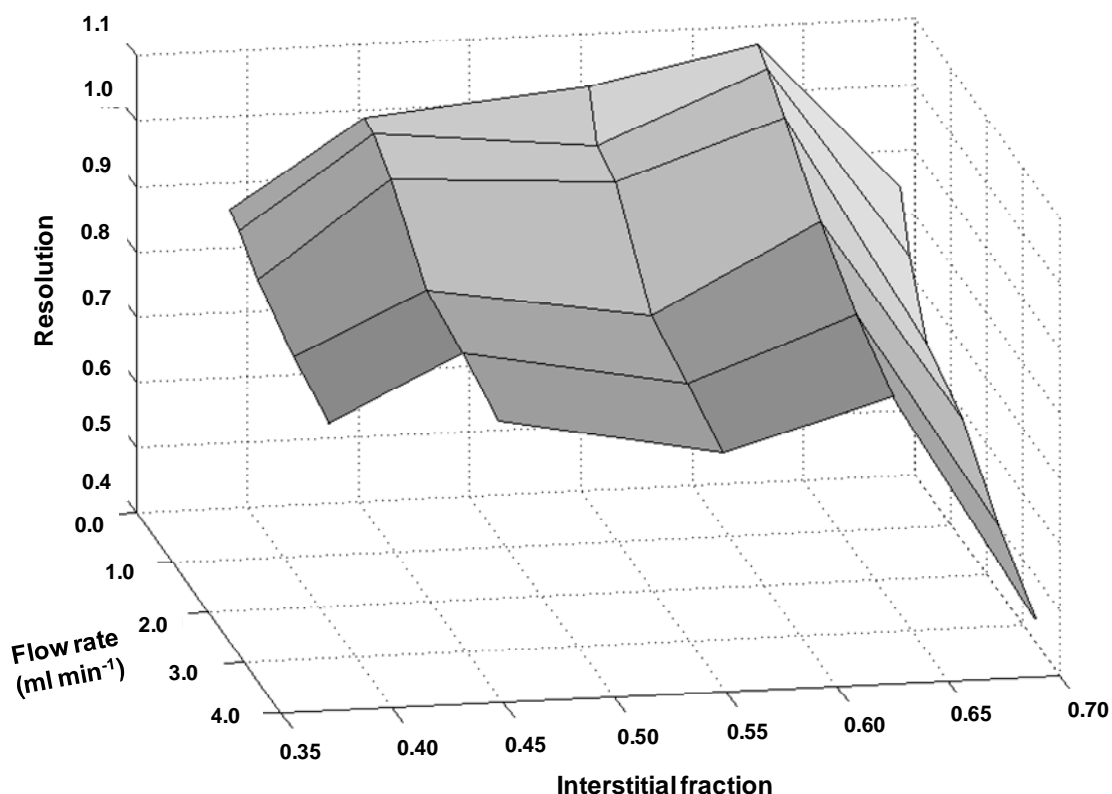


Figure 2.8. Effect of interstitial fraction on resolution of uracil and butylparaben at flow rates ranging from 0.25 to 4 mL min⁻¹. 250 mm x 2.1 mm i.d. column packed with PP4 C-CP fiber. Mobile phase composition 25:75 methanol: water, injection volume 10 μ L, detection wavelength = 254 nm.

This decrease in column performance is due to the increased hydrodynamic heterogeneity at low ϵ_i values, manifest in peak tailing due to possible channeling effects and flow restrictions within closely packed capillary

channels. This is imagined to be partly caused by the frictional heat generated at the interface of fibers and the column wall during the packing process of high density columns [21]. Above $\varepsilon_i = 0.63$, the poor resolution can be related to the lack of solute interactions due to the reduced fiber surface areas and flow inhomogeneities in loosely packed columns. Maximum resolution is found with the three medium porosities (0.45, 0.55, 0.63), with a small maximum observed at $\varepsilon_i = 0.63$. At the optimum ($\varepsilon_i = 0.63$), uracil and butylparaben are resolved ($R_s > 1$) at flow rates lower than 1 mL min^{-1} . This improved resolution is dominated by the peak widths component rather than the retention time differences (Δt_R).

Role of Column Inner Diameter on Plate Height and Resolution

Column performance of micro-particle packed columns is fairly independent of column inner diameter for the columns having diameters ranging from 1 to 10 mm [6]. However, the solvent consumption, sensitivity, and loading capacity are influenced by column inner diameter. Previous studies in this laboratory demonstrated that, in the case of C-CP fiber packed columns, peak width and asymmetry are affected appreciably by the column inner diameter [21]. Unlike the microparticulate columns, multicapillary columns do not possess random flow distributions which act to average out column inhomogeneities [37]. Since the flow paths of C-CP fiber packed columns are longitudinally oriented, uniform radial distribution of solute and solvent across the column inlet and outlet becomes more problematic as the column inner diameter increases. The effect of column inner diameter on plate height and resolution was studied with 4.6, 2.1

and 1.5 mm i.d. columns packed with the PP4 C-CP fibers at the optimal interstitial fraction. The injection volumes were varied in proportion to the column volume in order to prevent volume/mass overload and biasing.

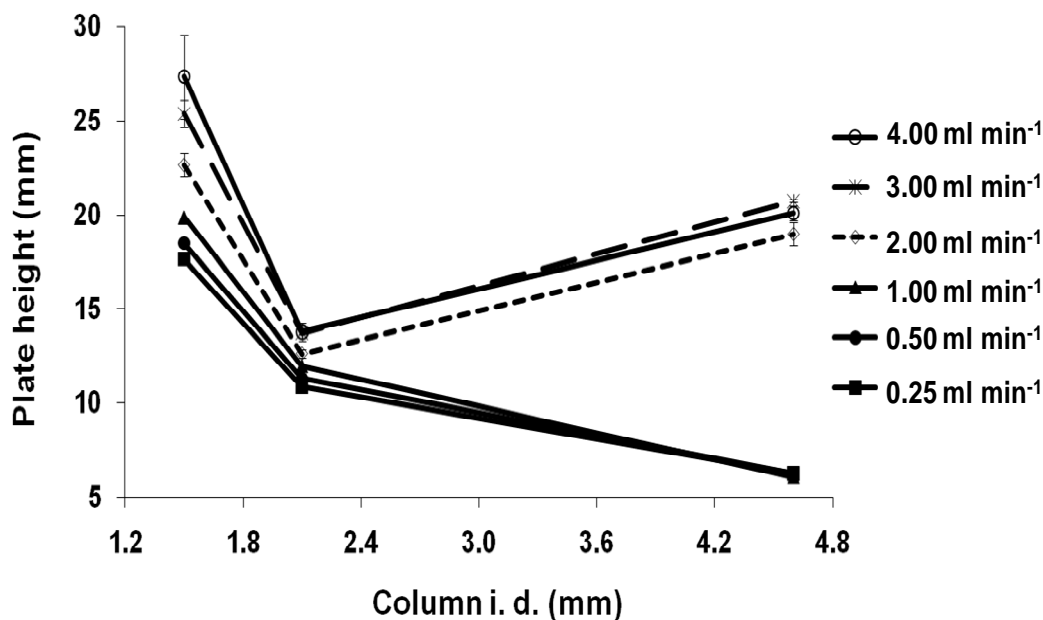


Figure 2.9. Effect of column inner diameter on plate height of butylparaben at flow rates ranging from 0.25 to 4 mL min⁻¹. Columns packed with PP4 C-CP fiber at an interstitial fraction of ~0.63. Mobile phase composition 25:75 methanol: water, injection volumes changed in proportion to the column volume, detection wavelength = 254 nm.

As illustrated in Fig. 2.9, an interesting feature is seen for the larger diameter columns (4.6 mm i.d.); distinct groups of plate height values are seen corresponding to low and high flow velocities reflecting two basic characteristics. As described in above section, this trend can be attributed to the lack of efficient radial mixing across the column profile at high linear velocities resulting in much higher plate heights at high flow velocities [21]. As illustrated in Table 2.4, 4.6

mm i.d. columns possess larger surface area in comparison to 2.1 mm i.d. columns. Unfortunately this high surface area is only accessed while operating at low flow rates. The use of a frit-distributor aids to spread the solution flow radially across the column inlet as in the case of wide-bore packed-bed columns [21].

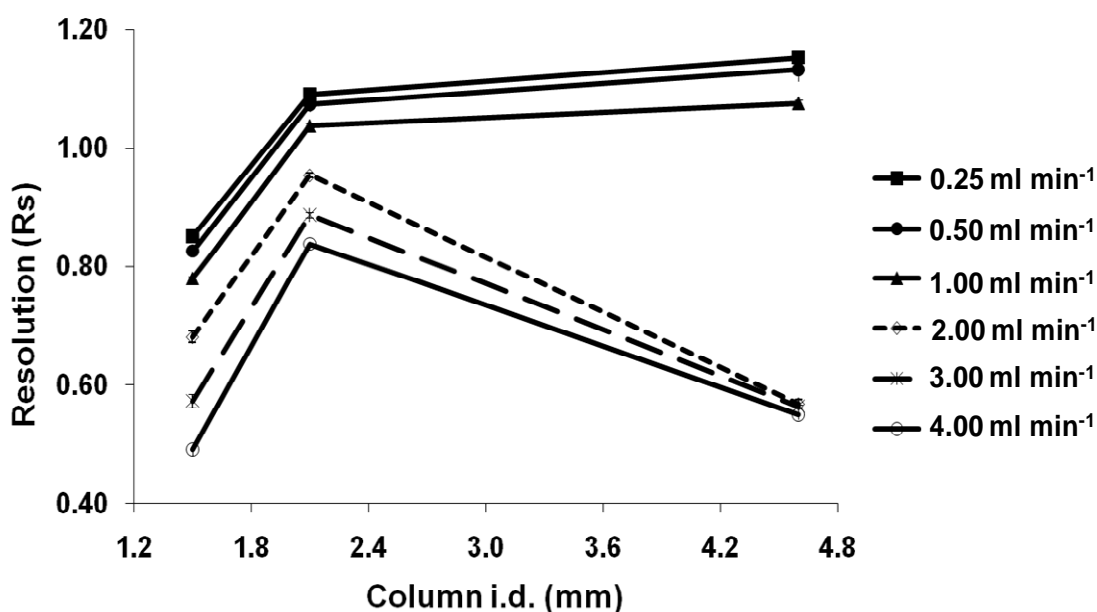


Figure 2.10. Effect of inner diameter on resolution of uracil and butylparaben at flow rates ranging from 0.25 to 4 mL min⁻¹. Columns packed with PP4 C-CP fiber at an interstitial fraction of ~0.63. Mobile phase composition 25:75 methanol: water, injection volumes changed in proportion to the column volume, detection wavelength = 254 nm.

The influence of column inner diameter on the resolution of the uracil/butylparaben pair is reported in Fig. 2.10. A similar trend to that observed in Fig. 2.9 is seen for the larger diameter columns, with an apparent disparity in resolution at low flow rates. Low resolution is due to the poor mobile phase

distribution and collection in the radial direction at high flow rates, while greater retention and less broadening at low flow rates yielded improved resolution. In the case of the 1.5 mm column, the limiting factor becomes the reduced retention of the butylparaben due to reduced overall fiber surface area, even though (as shown in Table 2.4) it has a higher phase ratio. It is believed that there exists a set of trade-offs that may exist in terms of the accessible phase ratios and linear velocities (as suggested with reference to Fig. 2.6) as column diameters are changed. In this case, much higher linear velocities must exist for a fixed volume flow rate at the smallest column diameter. This relationship will be the basis of future works. Based on these results, 2.1 mm would be the optimum column inner diameter (of those tested here) for the separation of small molecules in PP4 C-CP fiber packed columns at the optimized interstitial fraction.

Application of PP4 C-CP Fibers for Bio-macromolecule Separations

C-CP fibers have been developed in this laboratory predominantly as a platform for macromolecule separations [2,15-22]. This is not surprising based on the characteristics presented previously in this report. The most promising separations to date have been observed for proteins, where PP, PET, and nylon C-CP fibers have been utilized as stationary phases in reversed phase, ion exchange, and hydrophobic interaction chromatography [2,16,17]. The ultimate goal here was to evaluate whether the hydrodynamic results obtained for small molecule separations on the C-CP fibers were valid for macromolecules. As presented in Fig. 2.11, baseline separation of three proteins (ribonuclease A,

cytochrome c, and transferrin) is readily achieved by gradient elution with PP4 C-CP fibers at 2 mL min⁻¹ (a linear velocity of ~16 mm sec⁻¹). The gradient method applied here was previously utilized for the reversed phase HPLC separation of proteins on C-CP fiber columns [16,18]. Of particular note is the high level of peak symmetry exhibited by the much higher molecular weight transferrin. The resolution of ribonuclease A and cytochrome c is found to be 1.64 with a variability of ~2% RSD (n=3) at 2 mL min⁻¹.

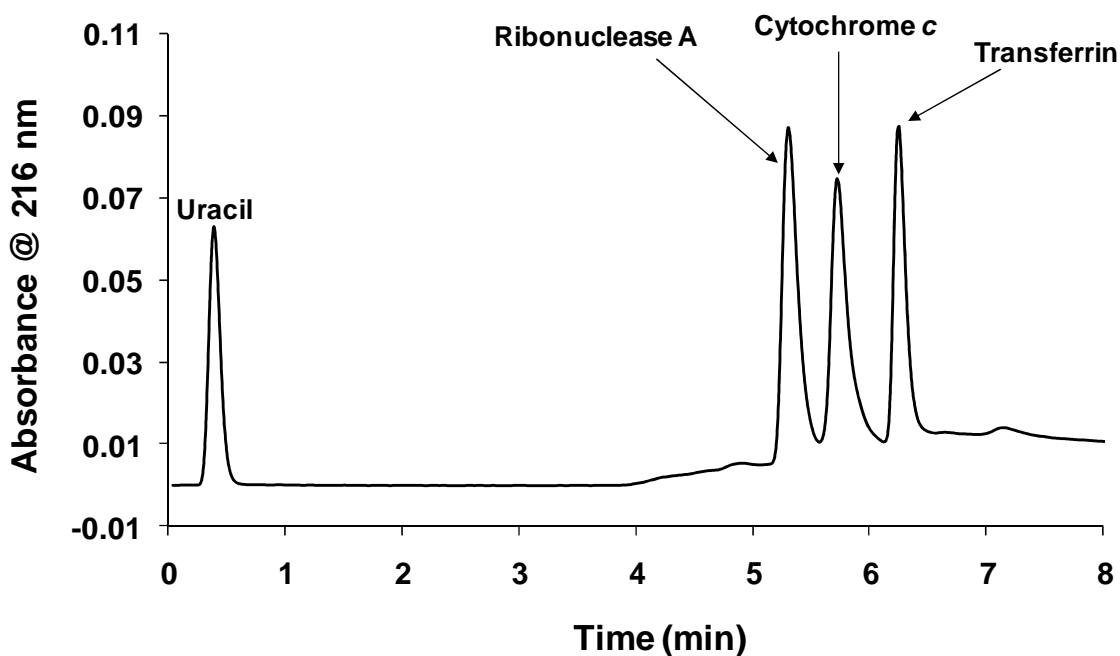


Figure 2.11. Reversed phase separation of uracil and a three protein suite (ribonuclease A, cytochrome c, and transferrin) on a PP4 C-CP fiber packed column (250 mm x 2.1 mm i.d.) at an interstitial fraction of ~0.63. UV detection at 216 nm; flow rate 2 mL min⁻¹; injection volume 10 μ L, gradient elution initiated 2.5 min after injection: 80:20 to 50:50 water containing 0.1% TFA (vol/vol): ACN containing 0.06% TFA (vol/vol) over 2.5 min.

As an evaluation of the ability to extend the lessons of the roles of fiber packing parameters on small molecule separations shown previously, an investigation of the separation characteristics of PP4 C-CP fiber columns with regard to interstitial fraction for the separation of macromolecules was undertaken using the preferred 2.1 mm i.d. column format. Figure 2.12 illustrates the effect of interstitial fraction on the resolution of ribonuclease A/cytochrome c pair for flow rates ranging from 1 to 4 mL min⁻¹ (equivalent to linear velocities of 8 - 31 mm s⁻¹) at the fixed gradient rate. As seen in small molecule separations, the optimum resolution is observed at an interstitial fraction of 0.63.

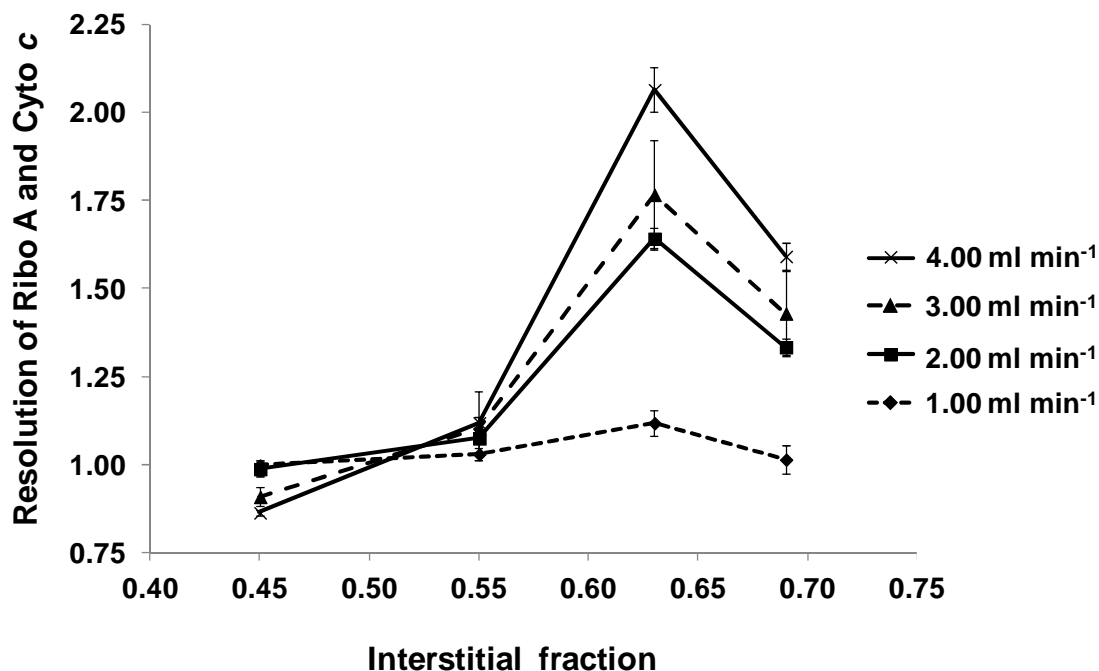


Figure 2.12. Effect of interstitial fraction on resolution of the ribonuclease A/cytochrome c pair at flow rates ranging from 1 to 4 mL min⁻¹. 250 mm x 2.1 mm i.d. column packed with PP4 C-CP fiber. Mobile phase composition: hold time 2.5 min, gradient elution initiated 2.5 min after injection: 80:20 to 50:50 water containing 0.1% TFA (vol/vol): ACN containing 0.06% TFA (vol/vol) over 2.5 min.

The resolution increases as the interstitial fraction increases from 0.45 to 0.63, with a further increase resulting in a considerable decrease in resolution. So it is evident that the optimum packing conditions of C-CP fibers found in small molecule separations can be utilized successfully for the separation of macromolecules with greater separation performance. As seen in the small molecule data of Figs. 2.7 and 2.8, this behavior is more reflective of the factors that affect column efficiency (plate height) than retention factor. This is not surprising as the separation of proteins involves a simple adsorption of proteins on the fiber surfaces (without intra-fiber diffusion or the establishment of dynamic equilibrium) followed by desorption back into the mobile phase at the critical solvent elution strength. Thus, factors affecting the best mobile phase mass transport characteristics are most advantageous.

A more salient, yet practically important, feature seen in Fig. 2.12 is the dramatic improvement in resolution with increased solvent flow rates, which is in distinct contrast to most other HPLC stationary phases (as well as the small molecule separations presented in the previous sections). As seen in the previous van Deemter plots (Fig. 2.3), the limited porosity of the PP C-CP fibers leads C-term broadening effects for uracil and butylparaben at high flow rates (linear velocities). Exclusion of larger solutes from the fiber pore structure negates the potential ill-effects of slow intra-phase diffusion. At the lowest volume flow rate, interstitial fraction has little effect on the obtained resolution for the ribonuclease A/cytochrome C critical pair. On the other hand, as flow rates

are increased to 4 ml min^{-1} the realized resolution increases by $\sim 100\%$ at $\varepsilon_i = 0.63$. As noted in previous works, increases in linear velocity in C-CP fiber protein separations tends to decrease the critical solvent strength for a given protein [2,17]. In this case, the relative elution points among the proteins remain fairly consistent, and thus the Δt_R values remain consistent. On the other hand, as linear velocity increases (up to a value of $U = 31 \text{ mm s}^{-1}$) the respective peak widths decrease as $\sim 1/U^{1/2}$, from average values (for the three proteins) of 0.321 to 0.192 min. To be clear, this realization is only possible due to the absence of C-term effects. By the same token, no appreciable peak asymmetry is introduced at the highest linear velocity. While there are practical advantages in the use of high linear velocities in terms of throughput, this comes at the price of larger elution volumes. In this case, the use of small diameter columns becomes preferable as demonstrated in microbore C-CP fiber column formats [20,46].

Conclusions

The roles of fiber cross sectional diameter and shape, linear velocity, interstitial fraction (packing density), and the inner diameter of columns packed with polypropylene C-CP fibers on separation efficiency and resolution of uracil and butylparaben have been investigated. At constant linear velocity and similar packing density, the $40 \text{ }\mu\text{m}$ polypropylene (PP2 and PP4) fiber-packed columns had improved peak widths and plate counts over smaller and larger diameter fibers. The higher specific surface area of PP4 fibers versus PP2 resulted in

greatly improved column performance in terms of separation efficiency and resolution. Intra-fiber porosity towards both solutes necessitates operation at low linear velocities due to hindered stationary phase mass transport. The observed plate heights for C-CP columns are very large in comparison to the commercial porous phases, predominately due to much lower specific surface areas, flow inhomogeneities, and poor mass transfer characteristics of the polymer fiber phase. The interstitial fraction of C-CP fiber packed columns plays a vital role in column performance due to the combination of accessible surface area and inter-fiber diffusional distances. Based on the chromatographic characteristics, the optimum interstitial fraction was found at ~ 0.63 for PP4 fiber packed columns at each flow rate. Optimum column performance for the separation of small molecules was found to be in a 2.1 mm i.d. Overall, the Giddings equation provided the most accurate fit to experimental data to explain the band broadening of C-CP fiber-packed columns in small molecule separations.

One anticipated outcome of these studies was the extension of the results for isocratic, small molecule separations to the separation of proteins. Under the optimized column packing conditions, baseline separation of a mixture of three proteins was readily achieved using a simple reversed-phase gradient elution program. The optimum interstitial fraction of $\varepsilon_i = 0.63$ was confirmed across all flow rates at a fixed gradient rate. Very different from small molecule separations, the highest level of protein resolution occurred at the highest flow rate of 4.0 mL min^{-1} , equivalent to a linear velocity of $\sim 31 \text{ mm s}^{-1}$. This trend

reflects a lack of porosity of the polymer fibers towards proteins, such that facile mass transfer from the stationary phase to the mobile phase is followed by very rapid transit down the column.

While it is clear that PP C-CP fiber stationary phases are not comparable to commercial, porous bead stationary phases for the separation of small molecules, there are many positive attributes to be capitalized upon for macromolecule separations. Packing characteristics evaluated here under isocratic conditions, point to natural paths forward to achieve enhanced capabilities in rapid protein separations. For example, use of microbore columns of greater length (with implementation of radial compression) would eliminate non-uniform radial velocity distributions. As in the case of packed beds, radial compression would be expected to improve the fiber packing homogeneity.

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CHAPTER THREE
MICROBORE POLYPROPYLENE CAPILLARY CHANNELED POLYMER
(C-CP) FIBER COLUMNS FOR RAPID REVERSED PHASE
HPLC OF PROTEINS

Introduction

The key element in the quality of liquid chromatography separations is the stationary phase, thus the characterization and development of new supports and stationary phases for high performance liquid chromatography (HPLC) is continuously expanding and growing. Despite improvements achieved in stationary/support phases in “small molecule” separations [1], the chromatographic separation of macromolecules remains a major challenge. Over the years, protein chromatography has been developed with a variety of bead-form support/stationary phases including polysaccharides [2], porous silica [3,4], organic resins [5]. While porous silica phases are very effective for small molecule chromatography, they suffer from certain drawbacks in macromolecule separations. Specifically, when the solute molecule sizes are on the order of the pore diameters they tend to become retained in pores, resulting in band broadening that is exaggerated at high mobile phase linear velocities due to slow intra-stationary phase diffusion (aka C-term broadening). Thus, the majority of protein separations are carried out using nonporous, partially porous (e.g. porous shell), and superficially porous phases where short diffusional distances yield improved separation efficiencies [6-9].

Microbore column chromatography is a unified approach in chromatography. The development and application of small inner diameter columns in LC was reported first in 1967 for the separation of ribonucleotide mixtures [10]. Subsequently, Scott and coworkers employed a 1 mm i.d. micropacked column to demonstrate its utility for solvent economy and high resolution using a modified commercially available chromatograph [11]. The advantages of microbore column liquid chromatography are well established in literature [10-16], among them are increased sensitivity, decreased solvent and sample consumption, the ability to operate at relatively high linear velocities at inherently low volume flow rates, and better compatibility with mass spectrometry interfaces as compared to standard 4.6 mm i.d. columns. However, extra column-broadening and low sample loadability are the primary drawbacks of using small-diameter columns. Microbore HPLC is utilized in protein chromatography with the above advantages [17,18]. Southan and coworkers have demonstrated the utility of disposable microbore columns to be adapted to a wide range of analytical and micro-preparative separations with simple construction at low cost [17].

Because there is a high demand for an increased separation performance in the field of proteomics, fiber form alternatives to polymer beads have been investigated as stationary phases in protein chromatography [19-24]. The microbore column format is ideal to assess the separation performance of fiber phase stationary phases, as they can be easily constructed at reduced cost,

making them disposable. The low back pressure characteristics of fibrous stationary phases allow for packing into long microbore columns to enhance chromatographic performance and increased number of identified proteins in a complex mixture (i.e. higher peak capacity). Rapid protein separations can be expected in fiber-packed microbore column chromatography operating at high linear velocities, again at lower volume flow rates used in comparison to standard size columns.

Marcus and coworkers have developed capillary-channeled polymer (C-CP) fibers as a platform for variety of separations, predominantly for macromolecules [25-33]. When C-CP fibers are packed in a column, the fibers interdigitate to form a network-like structure and that enables high fluid transport efficiency with low backpressure characteristics. The hydrodynamic aspects of C-CP columns have been studied with respect to packing density, column inner diameter, and linear velocity in a previous publication [33]. In another study, the zone broadening processes of PP C-CP fiber columns was studied extensively, leading to the optimization of fiber diameter, column dimensions, and packing density (interstitial fraction) for chromatographic separations on analytical (1.2-4.6 mm i.d.) columns [34]. The resulting optimized packing parameters were applied successfully for the separation of a three-protein suite using reversed phase (RP) gradient conditions. Stanelle and coworkers utilized microbore C-CP fiber columns for the separation of small molecules [30]. Those studies illustrated that polypropylene (PP) C-CP fiber microbore columns can operate at

very high linear velocities (up to 50 mm s^{-1}) in a 1000 mm long column while maintaining relatively low backpressures. They also demonstrated that radial compression of C-CP microbore columns enhanced the separation efficiency by decreasing the A-term broadening. These results lay a good foundation for developing PP C-CP microbore HPLC towards analytical scale macromolecule separations.

We describe here the evaluation of the roles of column length, linear velocity, and radial compression of PP C-CP microbore columns on the separation characteristics of a three protein. Polypropylene fibers possess a totally aliphatic surface, thus predominantly hydrophobic interactions control analyte retention, thus driving the RP gradient conditions used here. Previous studies have in fact utilized polyester and nylon-6 C-CP fibers to affect protein separations by ion exchange (IEC) and hydrophobic interaction chromatography (HIC) as well [31,32]. The synthetic protein mixture included, ribonuclease A (~13.7 kDa), cytochrome c (12.3 kDa), and transferrin (77 kDa). Uracil is also used as an unretained, marker compound in the protein mixture to assess the void volume and general fluidic properties of the columns. C-CP fiber microbore columns ranging from 40 – 110 cm long were evaluated, without sacrificing specific permeability. The separation performance of the microbore C-CP columns was evaluated through the resultant chromatographic resolution and peak capacity with respect to linear velocity. The ability to radially compress C-CP microbore columns is an added advantage in comparison to standard metal

columns as it increases the packing uniformity of C-CP fibers. It is believed that the performance of PP C-CP fiber microbore columns suggests great promise in developing high speed, economical and efficient protein separations.

Experimental

Column Construction

The polypropylene (40 μm diameter) C-CP fibers were manufactured in the Clemson University School of Materials Science and Engineering (Clemson, SC, USA) [35], with the fluorinated ethylene propylene (FEP) microbore tubing (0.8 mm i.d.) purchased from Cole-Parmer Instrument Company (Vernon Hills, IL, USA). The microbore columns were packed with the fibers to achieve an interstitial fraction of $\varepsilon_i = 0.67$ as described in previous publications [30,34]. In this case, 480 fibers are in parallel alignment in each column. The ends of fibers were cut flush with the end of the tubing and end fittings and PEEK unions were assembled. End-fittings were purchased from Valco Instruments (Houston, TX, USA). PEEK unions were purchased from (VWR International, West Chester, PA, USA). Radial compression of microbore columns was accomplished by pulling the 1.7 mm o.d. column through a 1.4 mm diameter orifice [30]; effectively reducing the interstitial fraction from ~ 0.67 to 0.62.

Sample Preparation and Solution Delivery

The test mixture containing ribonuclease A (bovine pancreas), cytochrome c (bovine heart), and transferrin (human) at a concentration of $100 \mu\text{g mL}^{-1}$ each and spiked with $10 \mu\text{g mL}^{-1}$ uracil, was prepared in MilliQ water (conductivity $18.2 \text{ M}\Omega \text{ cm}^{-1}$) with 0.1 % HPLC grade trifluoroacetic acid (TFA). The proteins and TFA were purchased from Sigma Aldrich (Milwaukee, WI, USA). The HPLC grade ACN used in mobile phase was purchased from Fisher Scientific (Pittsburgh, PA, USA). Once prepared, the protein solutions were stored at 6°C . The chromatographic system consisted of a Waters Model 717 autosampler (injection volume = $5 \mu\text{L}$), 600S Controller, 616 HPLC pump and Waters 996 photodiode array detector at 216 nm (Milford, MA, USA). Data was collected by the Millennium 32 and Empower 2 Chromatography Manager and further processed and managed in Microsoft Excel (Seattle, WA, USA). The separation of uracil and three proteins was achieved at flow rates ranging from 0.25 to 1.50 mL min^{-1} (representing linear velocities $U_0 = 12 - 75 \text{ mm s}^{-1}$), employing a constant gradient elution program of 80:20 to 50:50 H_2O containing 0.1% TFA (v/v):ACN containing 0.06% TFA (v/v) over 3.5 minutes. Data presented here are the means of triplicate runs, with the error bars in each plot representing a variance of one standard deviation. As a point of reference, column-to-column variations in retention characteristics of microbore C-CP columns are typically $< 5 \text{ \%RSD}$.

Results and Discussion

General Gradient Composition and Linear Velocity Effects on Protein Separation

The separation performance of proteins on C-CP fiber columns is affected by packing density (interstitial fraction), linear velocity, and gradient composition (gradient steepness and organic modifier makeup). Previous studies have shown that the optimum interstitial fraction for both small molecules and protein separations on C-CP fiber columns is ~0.63 [34]. Therefore, microbore columns packed with interstitial fractions of 0.62 - 0.67 were used throughout this study. The optimization of the gradient conditions for the C-CP microbore columns was initiated based on the gradient rates used for protein separations in standard column formats [34]. As demonstrated in the top chromatogram in Fig. 3.1 wherein the gradient was initiated after 2.5 minutes, the three proteins elute across 3.5 min of gradient time, in a solvent window of 80:20 – 50:50 H₂O:ACN. Superimposed on the chromatogram is the Cu²⁺ (absorbance) profile under same gradient conditions as a reference point. Clearly, the three proteins elute during the course of gradient.

The gradient method was modified to decrease the protein on-column residence time by changing gradient elution conditions, 20-50% mobile phase B (ACN with 0.06% TFA) over 3.5 min at a flow rate of 1 ml min⁻¹, with the gradient initiated upon sample injection. As seen in the lower chromatogram of Fig. 3.1, the modified gradient resulted in a faster separation in comparison to the previous method (by 3 min), in fact with improved peak characteristics.

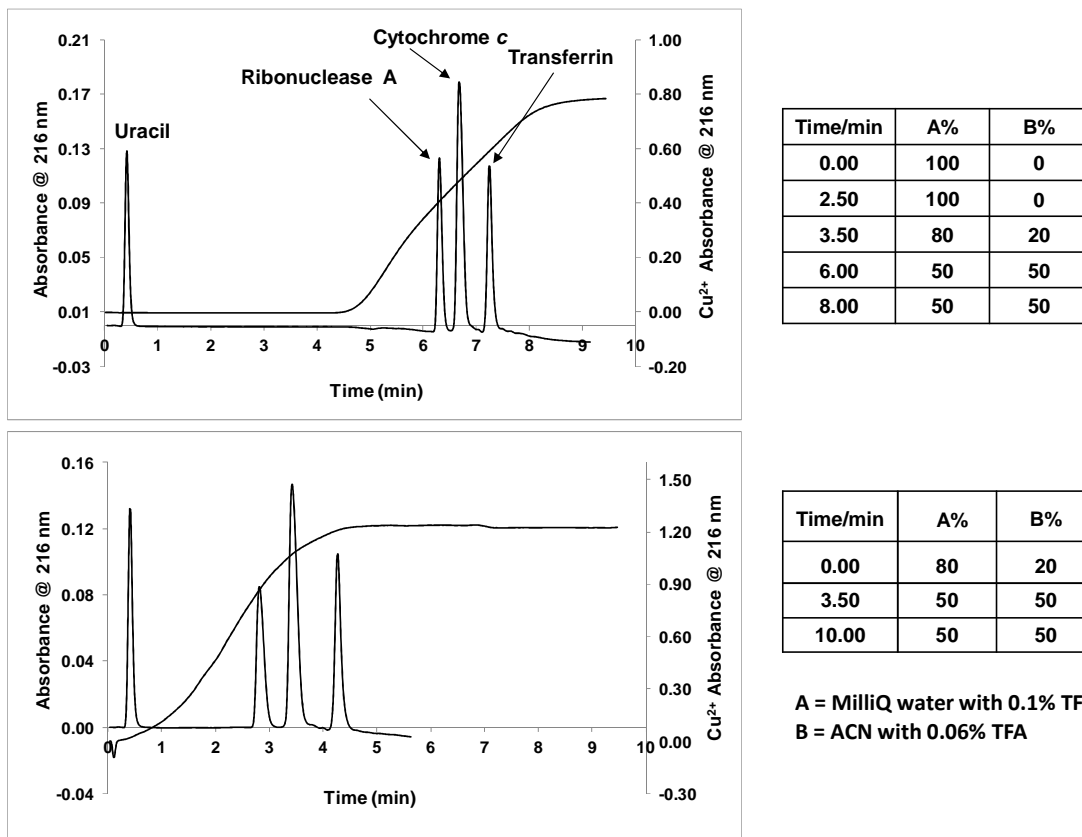


Figure 3.1. Reversed phase separation of uracil and three protein suite (ribonuclease A, cytochrome c and, transferrin) on a PP C-CP fiber packed column (800 mm x 0.8 mm i.d.) at an interstitial fraction of ~ 0.67 . UV detection at 216 nm, volume flow rate = 1 mL min^{-1} , injection volume $5 \mu\text{L}$, mobile phase composition presented in tables.

The gradient conditions used here enabled use of a lower percentage of organic modifier (0.06% vs. 0.08% TFA) and a much steeper gradient (8.57% vs. 1.18% change/min) than in previous protein separations reported with PP C-CP fibers on standard format columns [28]. Based on the elution order (ribonuclease A, cytochrome c, and transferrin), separation of proteins is purely based on hydrophobicity and not a function of molecular weight, reflecting no size exclusion process.

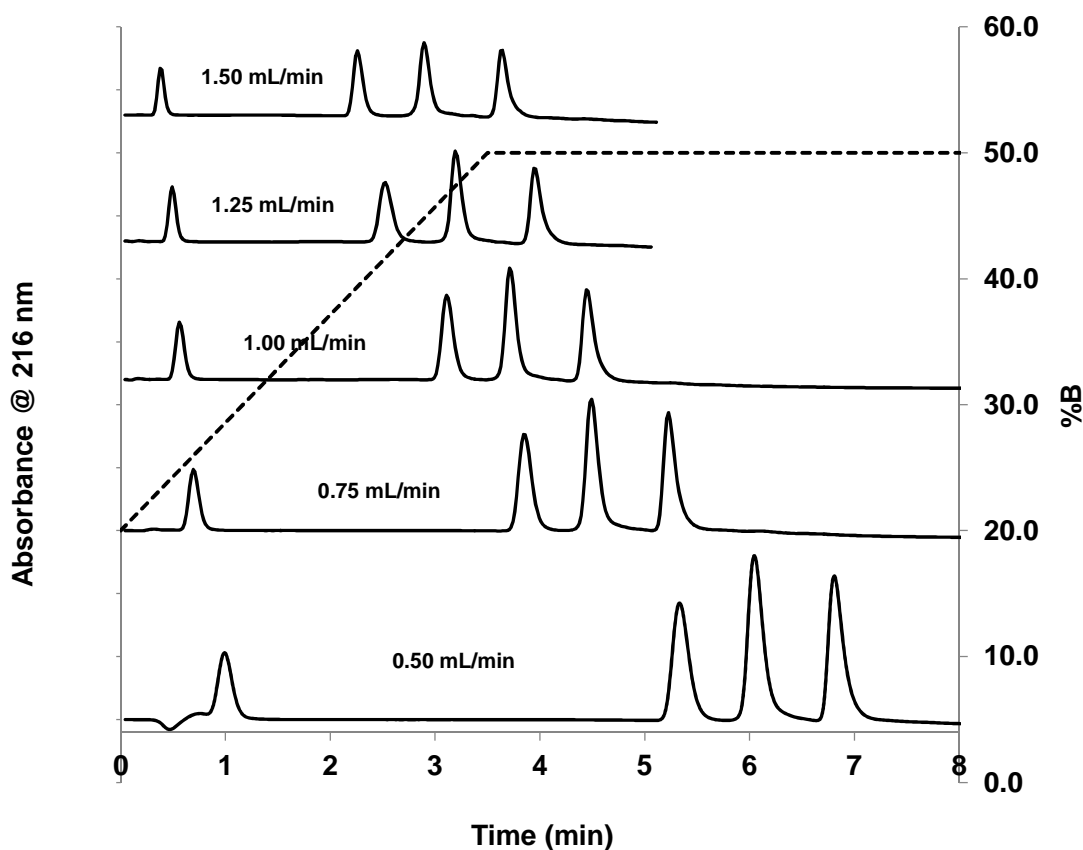


Figure 3.2. Effect of linear velocity (flow rate) of PP C-CP fiber column packed at an interstitial fraction of ~ 0.67 (110 cm x 0.8 mm i.d.) on separation of uracil and three proteins. Mobile phase composition: gradient elution program of 80:20 to 50:50 water containing 0.1% TFA (vol/vol): ACN containing 0.06% TFA (vol/vol) over 3.5 min, injection volume = 5 μL , detection wavelength = 216 nm.

One of the primary challenges in proteomics as well as preparative protein chromatography is achieving high throughput. As demonstrated in Fig. 3.2, baseline separation of three proteins was carried out at linear velocities ranging from 12 – 75 mm s^{-1} based on the flow rates of 0.25 – 1.5 mL min^{-1} under same gradient conditions on an 110 cm long column. Clearly seen, the retention times of the uracil, ribonuclease, cytochrome c, and transferrin solutes are all affected by the linear velocity. In the most straightforward case, the retention times for

the unretained marker compound decrease from ~1.0-to-0.39 min. As noted in previous protein separations on C-CP fiber columns, a unique situation occurs wherein the retention times for the proteins decrease to a far greater extent [28,32]. The total decrease in the protein retention times range from 2.8 min for ribonuclease A to 3.2 min for transferrin. As depicted in the superimposed gradient program, the extent of reduced retention, relative to the gradient, is far greater than would be projected simply by faster elution upon desorption; if the critical solvent composition values remained the same. Indeed, what is seen is a decrease in the solvent strength as linear velocity increases. Other important general observations can be made regarding operation at these very high linear velocities. For example, there is suppression in the absorbance signals of the protein peaks seen at high linear velocities. This is not a reflection of reduced recoveries (as the same effect is seen for uracil), but is a reflection of the limited sampling rate of the detector system. Also seen (and quantified in detail in subsequent discussions), the peak widths for each of the solutes is inversely related to the linear velocity; indicative of the absence of van Deemter C-term limitations [3]. This relationship, and the absence of appreciable peak tailing seen in Fig. 3.2, is a clear indication of the favorable mass transfer characteristics of C-CP fibers (due to an absence of intra-stationary phase diffusion), in contrast to porous phases where mass transfer is limiting. In fact, there is a definite improvement in peak shapes in comparison to the previous protein separations with C-CP fibers in standard format columns (2.1 and 4.6 mm

i.d.) [26,28]. As a point of reference, the peak width of ribonuclease A is decreased by 37% in microbore column separation in comparison to 2.1 mm i.d. standard column at identical volume flow rates (1 mL min^{-1}). This improvement in peak shapes can be attributed in part to the better packing uniformity in microbore columns than in standard format columns.

Effect of Linear Velocity on Peak Widths, Selectivity, and Resolution

In addition to retention characteristics, elution peak widths determine the ultimate resolution and peak capacity of a separation. In gradient chromatography, elution is affected by attaining a critical solvent composition at which point solutes desorb and elute from the column. Different from the case of small molecules, the typical picture of protein elution is that release occurs over a range of solvent concentrations as multiplicities of solute-surface interactions are overcome. As such, there is some relationship between gradient rate and resultant chromatographic peak width. In the case of the chromatograms in Fig. 3.2, the rate was held constant. The peak width of the eluting proteins (4σ) from those chromatograms is plotted in Fig. 3.3 as a function of mobile phase linear velocity. Two important aspects are immediately clear. First, as noted previously, the observed peak widths are roughly inversely related to the mobile phase velocity. These responses reflect the fact that (following desorption from C-CP fiber surface) rapid protein transport down the column minimizes longitudinal diffusion (van Deemter B-term). Protein diffusion coefficients are in the order of $10^{-7} \text{ cm}^2 \text{ s}^{-1}$ [36].

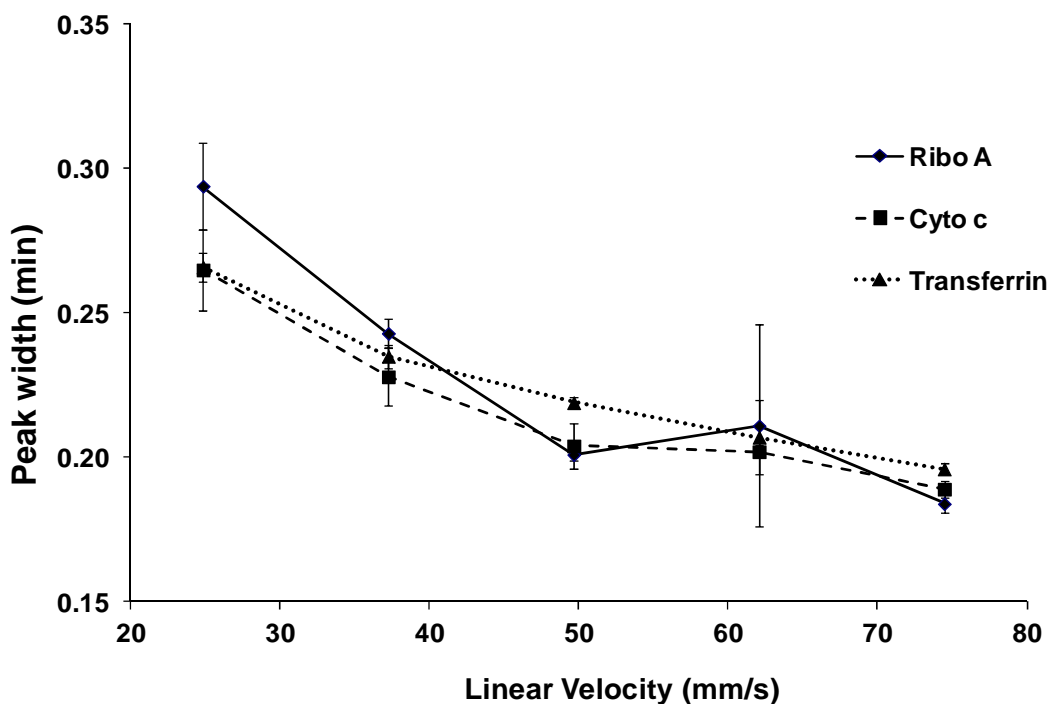


Figure 3.3. Effect of linear velocity of PP C-CP fiber column packed at an interstitial fraction of ~ 0.67 (110 cm x 0.8 mm i.d.) on peak widths of ribonuclease A, cytochrome c, and transferrin. Experimental conditions the same as Fig. 3.2.

Thus within the time domain of the microbore C-CP separations, this process is minimized as linear velocity is increased; as would be expected. Second, the peak widths of the respective proteins are essentially the same, even though their hydrophobicities and molecular weights are different from each other. As predicted through the van Deemter relationships, one would expect that peak widths to increase as U_0 is increased. Stationary phase mass transport (van Deemter C-term), which is a function of molecular weight (to a first approximation) is the primary limitation in the use of porous media in protein separations. None of the typical C-term contributions are evident in the chromatograms of Fig. 3.2 or the peak widths presented in Fig. 3.3.

The selectivity (α) of a separation, also known as the separation factor, is the ratio of retention factors ($\alpha = k_2/k_1$) of two adjacent peaks. The selectivity of a separation is driven by the chemistry of the system, resulting from the combined action of mobile and stationary phases on the solutes [3]. In the case of a gradient separation, the selectivity is dictated by mobile phase composition and the rate of change. Figure 3.4 illustrates the effects of linear velocity on the selectivity for the two protein pairs in the mixture, holding the gradient rate constant. Very different from other stationary phase constructs the selectivity of both the ribonuclease A/cytochrome c and cytochrome c/transferrin pairs increases with mobile phase velocity.

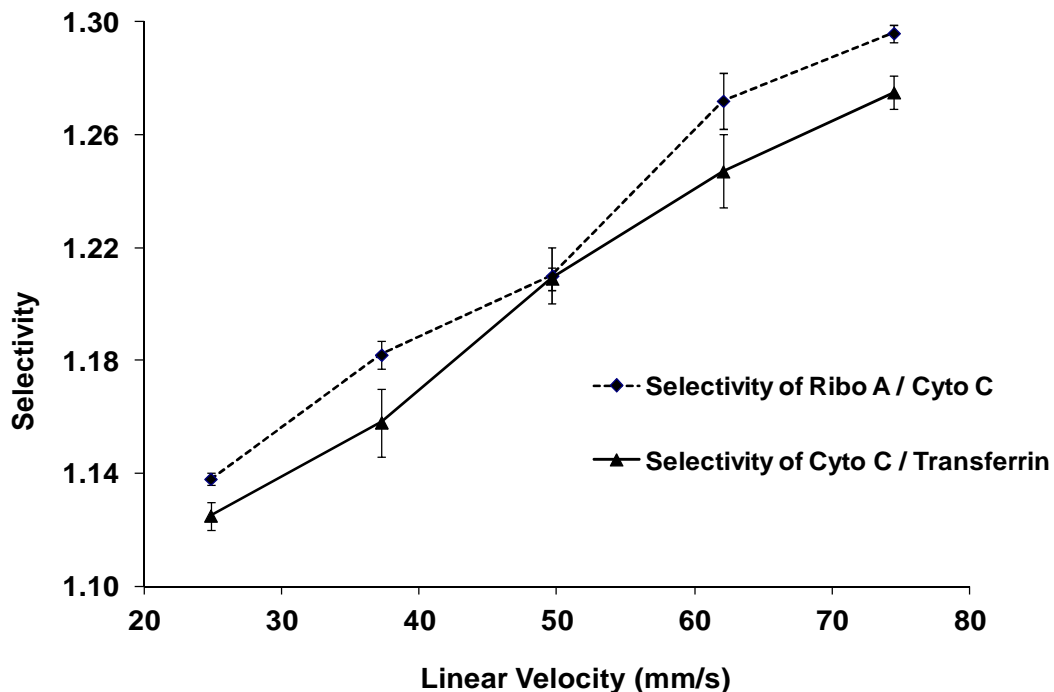


Figure 3.4. Effect of linear velocity of PP C-CP fiber column packed at an interstitial fraction of ~ 0.67 (110 cm x 0.8 mm i.d.) on selectivity of ribonuclease A/cytochrome c and cytochrome c/transferrin pair. Experimental conditions the same as Fig. 3.2.

The selectivity values (1.1-1.3) seen here are comparable to the previous protein separations on standard columns with C-CP fibers [26,28], but the proportional increase in selectivity with linear velocity reflects the fact that the actual retention characteristics change as a function of linear velocity. In literal terms, what is seen is the least-retained proteins experience larger reductions in retention times across the board.

As described in previous works, it is believed that the high shear rates under which the C-CP fiber columns operate contribute to this phenomenon. The shear rate in a two dimensional system is defined as the ratio of linear velocity and gap spacing ($\gamma = U/d$). In case of C-CP fiber phases, this gap is equivalent to inter-fiber gap ($\sim 1\text{-}5 \mu\text{m}$), and thus very high shear rates ($2400 - 75000 \text{ s}^{-1}$) can be achieved with the velocities offered by microbore C-CP columns. Physicochemical studies show that high shear rates result in less surface relaxation of proteins [37-43], and so lower solvent strengths are required for desorption. The data here suggest that this is indeed the case in C-CP fiber protein separations, with the effect most pronounced for the most weakly-bound proteins.

In combination, the smaller peak widths and higher selectivity factors that result from high mobile phase velocities should result in the highest resolution and peak capacity. The results plotted in Fig. 3.5 demonstrate the effect of linear velocity on resolution for the two protein pairs on the 110 cm C-CP microbore column. A distinct difference exists in the separation performance of the C-CP

fiber columns versus most other HPLC stationary phases, as there is a dramatic increase in resolution with increased mobile phase velocities.

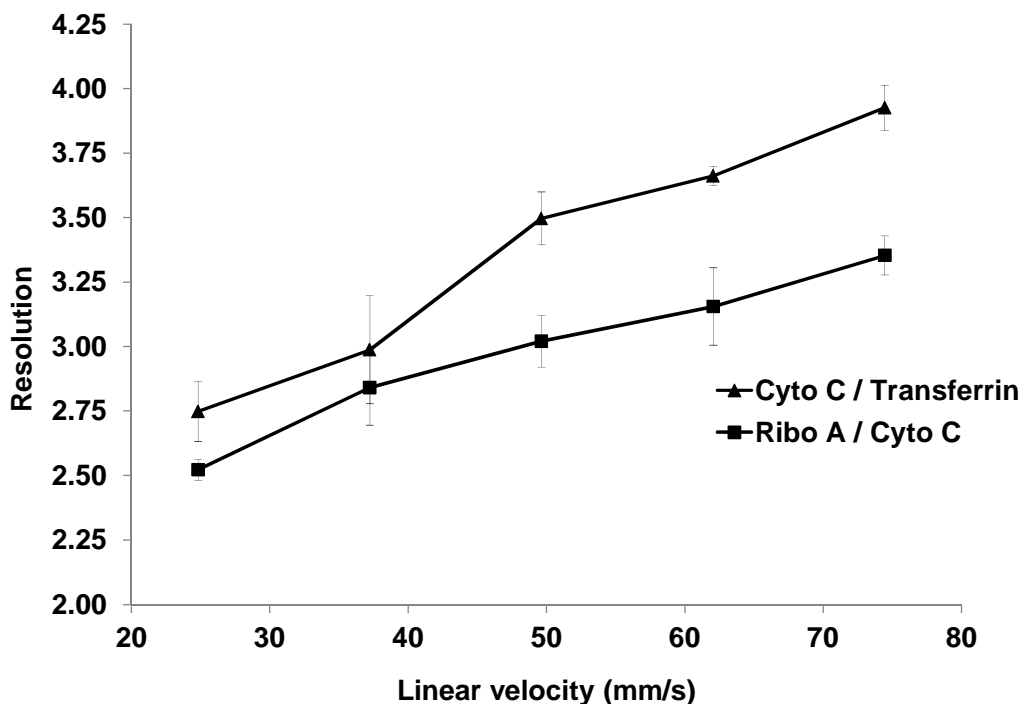


Figure 3.5. Effect of linear velocity of PP C-CP fiber column packed at an interstitial fraction of ~ 0.67 (110 cm x 0.8 mm i.d.) on resolution of ribonuclease A/cytochrome c pair. Experimental conditions the same as Fig. 3.2.

The resultant resolution values are increased from 30-40% when mobile phase linear velocity is increased from 25 to 75 mm s^{-1} . By the same token, the total analysis times have been decreased from ~ 11 min to 4.5 min; a 60% improvement in throughput without any sacrifice in performance.

Effects of Column Length and Linear Velocity on Resolution and Peak Capacity

To a first approximation, chromatographic columns realize higher efficiencies in proportion to the length to the one-half power ($L^{1/2}$) [3]. This improvement is based on the fact that the differences in retention times (Δt_R) are proportional to length, while diffusional broadening (B-term) increase with $L^{1/2}$. Unlike packed-bed and monolithic column formats, the lengths of C-CP fiber microbore columns can be increased (to the 110 cm lengths here) without reaching prohibitively high backpressures. By the same token, greater lengths do not suffer appreciably by overly long separation times and their coincident susceptibility to longitudinal broadening. In order to study the effect of column length on separation performance of the microbore columns, a single 110 cm long column was packed with PP C-CP fibers ($\epsilon_i = 0.67$). The same column was eventually cut into different lengths (40, 60, and 80 cm) and used throughout this study to guarantee the same packing uniformity. During this process, the column was cut at the injection side to minimize additional band broadening as commonly done in length studies of monolithic columns [44]. Consistent operation was verified in the backpressure and the column void volume responses. To that end, it must be emphasized that the changes in column length by a factor of three results in only minimal increases in backpressure, on the order of 40% at a given volume flow rate. Here again, the collinear nature of the C-CP fibers, as opposed to a tortuous path system where pressures scale with the column length.

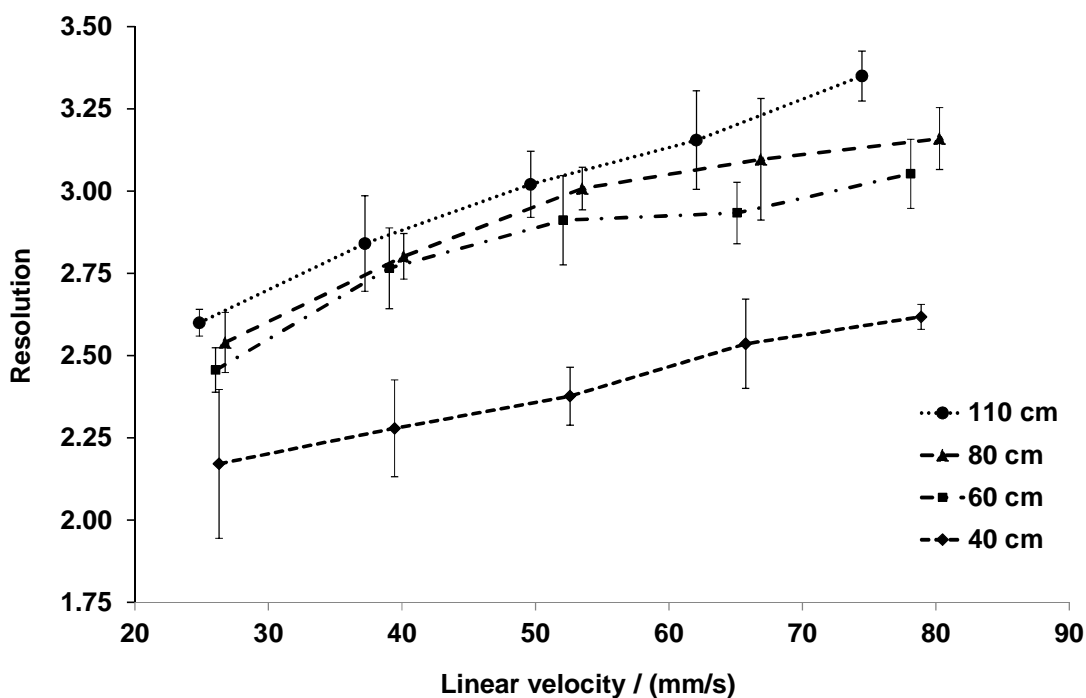


Figure 3.6. Effect of column length and linear velocity of PP C-CP fiber columns packed at an interstitial fraction of ~ 0.67 on resolution of ribonuclease A/cytochrome c pair. Experimental conditions the same as Fig. 3.2.

As demonstrated in the previous studies, the obtained resolution is an excellent reflection on the overall performance of the microbore column proteins separations. Figure 3.6 illustrates the effect of column length on resolution of ribonuclease A/cytochrome c pair for linear velocities ranging from 25 to 80 mm s^{-1} (flow rates 0.50 mL min^{-1} to 1.50 mL min^{-1}). (The response for the cytochrome c/transferrin pair is essentially the same.) As seen in Fig. 3.5, resolution increases with linear velocity irrespective of column length, with longer columns yielding the best performance. The important feature leading to the trend in Fig. 3.6 is that the relative peak width is decreased relative to the overall

retention time as column length increases (as expected). The maximum increase in resolution is observed between the two smallest-size columns, and it is less pronounced when the column length is increased from 60 cm to 110 cm, because the decrease in peak widths becomes minimal. As in the case of packed-bed columns, the longer retention times for the longest columns begin to fall victim to longitudinal broadening effects. In fact, while the other three lengths seem to be approaching some maximum resolution value as a function of U_0 , it appears that the longest column would in fact show greater resolution yet at higher linear velocities. As a final note, the ability to operate with extended length columns with minimal backpressure penalties also minimizes the impact of the classic primary disadvantages of smaller diameter columns, extra column broadening and low sample loadability.

In practical terms, the separation performance of HPLC columns under gradient conditions is best described by the peak capacity (PC). Peak capacity represents the maximum number of theoretical components that can be separated on a column within a given gradient time [45,46]. Different definitions of peak capacity have been reported in literature for gradient separations [47,48]. In the current work, peak capacity PC is calculated from the average peak width w (measured as 4σ) and the gradient time t_g according to Eq. 3.1.

$$PC = 1 + \frac{t_g}{w} \quad (3.1)$$

Mirroring the response for resolution depicted in Fig. 3.6, peak capacities increase uniformly when column length is increased and as the linear velocity is

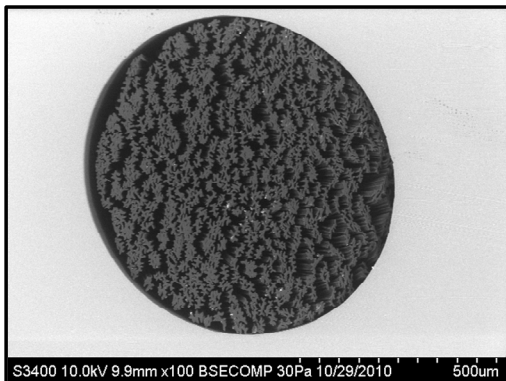
increased, ranging from PC = 12 - 20. According to the theory of gradient elution, it can be assumed that the peak capacity is proportional to the square root of plate number (N) and thereby is proportional to the column length (L) [44,49]. But in this case, the peak capacities are increased by ~1.2 times with column length at the highest linear velocity. The maximum peak capacity (~20) observed in this study is slightly lower in comparison to previous separations carried out with similar proteins on C-CP fiber-packed standard columns [28]. Significantly, those protein separations were performed under much shallower gradient conditions (1.18% vs. 8.57% change/min) than used here.

As another point of comparison, fast protein liquid chromatography (FPLC) is a form of HPLC that utilizes small-diameter stationary phases to achieve high-resolution separations. Kirkland and coworkers have used fused-core silica particles with 160 Å pores for highly efficient, fast separations of peptides and proteins [50]. They have achieved a peak capacity of 530 in 150 min at a flow rate of 0.5 mL min⁻¹ with a gradient rate of 0.54% change/min on coupled HALO peptide/ES-C18 columns (combined length = 250 mm). It is difficult to compare and evaluate the performance of two different column formats in terms of peak capacity. If PP C-CP columns were run under similar gradient rates (extended separation times), and realized peak widths consistent throughout this work, peak capacities on the order of 700 could be achieved; surpassing the peak capacities of most commercially used columns in proteomics. To be clear, this assumption is based on the fact, not unreasonably, that peak widths are dictated

totally by mobile phase broadening of proteins once they are desorbed from the surface. Certainly, this metric must be directly measured.

Radial Compression of Microbore C-CP Fiber Columns

Uniform packing is necessary to minimize the A-term broadening and achieve better separations in all forms of chromatography. Previous hydrodynamic studies carried out with C-CP fiber columns have shown that the optimum packing conditions for both small and macromolecule separations are practically equivalent [30,33,34]. Those studies have demonstrated the lower packing densities (i.e., higher interstitial fractions) can result in greater velocity variances and thus A-term broadening. On the other hand, densely packed columns showed poor separation efficiency due to the crimped channels and disturbed packing uniformity. Applying radial compression to flexible-wall HPLC columns can result in a more uniform bed structure as it also decreases the void volume, thus reducing the A-term broadening [3]. This concept is successfully utilized in microbore columns packed with PP C-CP fibers for small molecule separations [30]. In that study, Marcus et al. observed improved separation efficiency (30%) and increased resolution (36%) in compressed columns in comparison to uncompressed columns having the same number of fibers. Here the goal is to study the effect of radial compression on separation of proteins on microbore C-CP fiber columns. The radial compression was accomplished by pulling the 1.7 mm outer diameter column through a 1.4 mm diameter orifice as described in the previous publication [30].



Uncompressed column



Compressed column

Figure 3.7. Scanning electron micrographs of C-CP microbore columns packed with polypropylene fibers a) uncompressed column, b) radially compressed column. Note that a 500 μm scale is included in both micrographs.

The scanning electron micrographs shown in Fig. 3.7 depict the effects of radial compression on PP C-CP fiber packed 0.8 mm i.d. microbore columns. Each column is packed with 480 polypropylene fibers. In the uncompressed column, fibers are less densely packed near the wall and more inter-fiber void volumes are seen, causing wall effects and larger dispersion of the mobile phase velocities. In the radially-compressed column, the fibers are more densely packed near the wall, also appearing more uniformly packed with a decreased the void fraction. Thus a more homogenous flow distribution is expected in radially-compressed column as wall effects are minimized. Figure 3.8 illustrates the effect of radial compression on the separation of protein mixture at 1 ml min^{-1} on an 800 mm long column. As a result of compression, the interstitial fraction decreased by 5% to a value of $\epsilon_i = 0.62$, which actually is the optimum packing

density for both small molecule and protein separations [34]. Thus a shift in the chromatogram towards lower retention times for uracil and the proteins is seen.

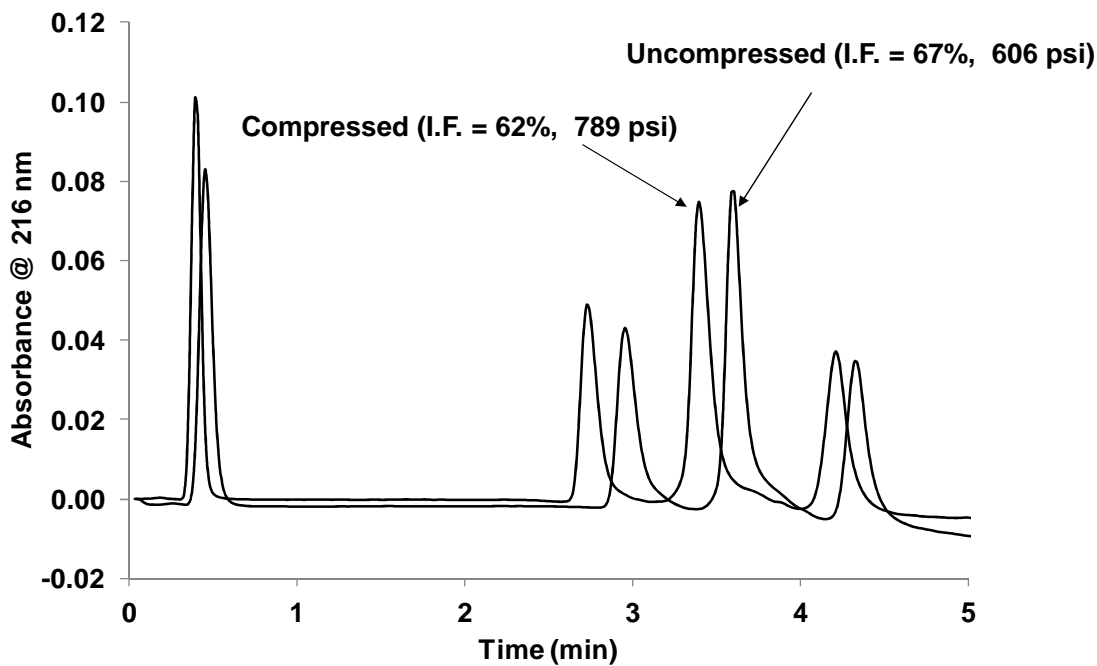


Figure 3.8. Overlay of chromatographs for the separation of uracil and three proteins on uncompressed vs. radially compressed PP C-CP microbore columns (800 mm x 0.8 mm i.d.). Volume flow rate = 1.0 mL min⁻¹. Mobile phase composition: gradient elution program of 80:20 to 50:50 water containing 0.1% TFA (vol/vol): ACN containing 0.06% TFA (vol/vol) over 3.5 min, injection volume = 5 μ L, detection wavelength = 216 nm.

By virtue of the higher fiber density, the linear velocity (U_0) is increased by 4% to a value of 54 mm s⁻¹ at a constant volume flow rate of 1.00 mL min⁻¹. Here, as in the case of the other studies reported here, the increased linear velocity yields reductions in the solvent strengths required for protein elution. This is most easily assessed as the uracil retention time decreases by only 0.06 min, while the proteins exhibit decreases of up to 0.23 min. Here again, the changes in apparent critical solvent concentration are attributed to the increased shear

rates as the inter-fiber spacing is reduced. There is no appreciable change in peak widths or peak asymmetry upon compression. The only disadvantage to note here is the relative increase (30%) in pressure drop in compressed column. Ultimately, the radially-compressed column demonstrated an increased resolution (~14%) for ribonuclease A and cytochrome *c* pair. As in the previous studies, greater improvements would be expected at higher linear velocities.

Conclusions

Polypropylene C-CP fiber packed microbore columns have been evaluated by separating uracil and a three-protein suite over a wide range of linear velocities (12 – 75 mm s⁻¹) under reversed phase gradient conditions. The effect of linear velocity on separation performance was investigated by evaluating the peak widths, selectivity, and resolution for an 80 cm long microbore column. Columns having different lengths (40-110 cm) were also tested with regards to resolution and peak capacity. As expected, longer column (110 cm) outperformed the shorter columns in separation performance. The increase in column length resulted in dramatic improvements in resolution and peak capacity while still maintaining the relatively low pressure drops offered by C-CP fiber phases. The peak capacity is increased by ~1.2 times with column length. Radial compression of microbore columns increases the packing uniformity and decreases the wall effects; thereby more homogeneous mobile phase dispersion is achieved. Although radial compression increases the pressure drop by 30%, it

enables fine-tuning of the packing density and uniformity of C-CP fiber phases within microbore columns.

The results obtained in this study confirm that the microbore C-CP fiber column format is the most effective way of achieving high separation efficiencies for rapid analytical scale protein separations with C-CP fibers. To be clear, the ability to achieve high linear velocities at low flow rates will be important for future applications in LC/MS and proteomics in general. Further developments will focus on more direct comparisons with commercially available columns in terms of affecting high peak capacities on complex protein mixtures. The cost of the base fibers and the ease of column fabrication hold a great deal of promise in terms of practical implementation in targeted separations. Finally, the ability to operate across a range of base fiber chemistries, or functionalization thereof, presents a degree of flexibility that may offer very unique capabilities in terms of proteomics applications.

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CHAPTER FOUR

INITIAL EVALUATION OF LYSOZYME THROUGHPUT AND YIELD ON
NYLON-6 CAPILLARY-CHANNELED POLYMER (C-CP) FIBERS BY ION
EXCHANGE CHROMATOGRAPHY

Introduction

The development of new strategies for simple, high throughput, efficient and low cost downstream processing of commercially important biomolecules has attracted great interest in recent years. Different types of liquid chromatography (LC) are utilized in separation of biomolecules with the sequential use of variety of supports such as silica, organic polymers [1-3], and fibrous supports [4-10]. Separation mechanisms include size exclusion, ion-exchange, adsorption, and affinity chromatographic processes. Ion exchange chromatography (IEC) is extensively used in downstream bioseparations, because it provides a gentle environment for biomolecules near physiological conditions, allowing greater flexibility for optimizing separation conditions in method development stage [11]. Despite the improvements achieved in process-scale protein separations, the major goal in preparative chromatography to isolate the maximum amount of pure yield per unit time (i.e. high sample throughput) as economically as possible remains a major challenge in the protein therapeutics. Therefore it is of high relevance for downstream separation protocols to develop new support/stationary phases for economical and high sample throughput separation strategies.

Several attempts to develop polymer based chromatographic systems for bioseparations have been reported in the literature in recent years. Byers and coworkers have described a prep-scale IEC process using a commercial ion-exchange resin for the separations of four major proteins and lactose from sweet dairy whey [12]. They have found the combined anion- cation-exchange methods and processing cycles need to be modified to reduce the time and improve the economic picture of the scaled-up plant. Ladisch and co-workers used LC columns packed with continuous rolled fabric stationary phase for loading and separating proteins by IEC [13]. Mechanical stability, high selectivity, and recovery were the desirable attributes of this stationary phase for preparative separations although capacities were relatively low. Later, Pinto and coworkers extended the work studied by Wikstrom and Larson [14] and King and Pinto [15], using polysulfone (PS) as the base polymer in randomly packed short-fiber (RPSF) columns for anion exchange separation of BSA and β -lactoglobulin [16]. The results of this study showed that protein adsorption capacities of PS fibers depend on the size of protein, pH and salt concentration. The frontal development studies revealed several interesting aspects regarding downstream processing of proteins. In particular, column throughput and yield is increased when a high concentration of protein is loaded at a low flow rate and eluted at a very high flow rate. The major drawback of the PS fiber is its poor mass transfer characteristics in the porous media during the protein loading; as a result it limits the utilization of favorable low pressure drops at high flow rates.

There has been growing interest in using fibrous support/stationary phases to affect protein separation as an alternative methodology [9,10,13,16-20]. Marcus has recently reviewed the physical and chemical attributes and relevant applications that depict how various fiber based formats are developed to affect bioseparations addressing the limitations of conventional silica phases [21,22]. Natural and synthetic polymer fibers have found use in a variety of chromatographic modes, including reversed phase chromatography (RP), size exclusion chromatography (SEC) and IEC. In most cases, high mobile phase velocities are employed with minimal backpressures, an attractive feature for preparative protein chromatography. These polymer fiber phases suffer from poor plate heights due to their lower porosity/phase ratios, and such are not competitive with silica phases in terms of small molecule separations. However the lack of porosity of fiber phases is advantageous in the separation of macromolecules. Specially, high velocities can be used in loading and elution steps to affect high level of throughputs and yields [13,16,17]. The flow conditions through fiber phases, coupled transport (convective and diffusion) to the surfaces, yield high loading and chromatographic efficiencies at high linear velocities. In addition the low material costs together with feasibility in construction make fiber phases good candidates for rapid and efficient preparative scale bioseparations.

Capillary-channeled polymer (C-CP) fibers are being developed in this laboratory as a platform for macromolecule separations [18-20,23-25]. The C-CP

fibers have eight capillary channels running axially along the entire length of each fiber, which act in concert as an efficient transport medium for pressurized flow, yielding relatively low back pressures and at high velocities. These fluidic properties and the non-porous nature of C-CP fibers (with respect to the physical dimensions of proteins) allow high speed macromolecule separations on C-CP fiber columns. When C-CP fibers are packed in a column, the fibers interdigitate to form a network of parallel plate channels/capillaries resulting average inter-fiber diffusion distances of 1-5 μm . Thus high shear rates ($\sim 500 - 2000 \text{ s}^{-1}$) are expected to play an important role in protein transport to and from the stationary phase. The non-porous nature of C-CP fibers eliminates both size selectivity and mass transfer limitations resulting from intra-stationary phase diffusion. C-CP fibers come in a variety of base polymers; polypropylene, polyester, and nylon. The ability to utilize different functionality of base polymer provides a variety of solute-surface interactions (e.g. hydrophobic, ionic, hydrogen bonding, $\pi-\pi$, etc.) to affect separation. In an effort to separate under non-denaturing conditions, polyester and nylon-6 C-CP fiber columns have successfully been utilized for protein separations by IEC and hydrophobic interaction chromatography (HIC) in this laboratory [23,25]. The limited specific surface area of C-CP phases (\sim single m^2/g) may reduce the equilibrium loading capacities of proteins. However dynamic loading capacities, throughput, and yield are expected to be very favorable in downstream processing of biomacromolecules with C-CP fibers. Since C-CP fibers have already shown promise in analytical scale protein

separations, future studies should be directed towards developing preparative scale separations. Low material costs, ability to operate at high linear velocities with low back pressures, and the absence of mass transfer limitations found in porous phases are the primary advantages of C-CP fibers in developing for large scale macromolecule separations.

We describe here basic studies in the characterization and development of nylon-6 C-CP fiber stationary phases as substrates for the downstream processing of biomacromolecules. An evaluation of adsorption and desorption characteristics of lysozyme on nylon-6 fibers have been investigated with the aim of determining frontal throughput and % yield as described by Pinto et al. [16]. Specifically, the roles of loading and elution cycle, linear velocity, protein concentration, and salt strength (0- 1 M) on throughput and yield are investigated. Reproducibility of results was evaluated on the same column by multiple loading runs to evaluate the column durability. Key comparisons are made to similar studies performed on polymeric short fiber columns. It is believed that the results shown here present a number of potential benefits for the use of C-CP fiber support/stationary phase in downstream processing of biomacromolecules.

Experimental

Column Construction

The nylon-6 C-CP fibers were manufactured in the Clemson University School of Materials Science and Engineering (Clemson, SC, USA)

[26]. For the frontal loading studies, both standard size (150 mm x 2.1 mm i.d.), and microbore (300 mm x 0.8 mm i.d.) columns were packed to achieve approximately similar interstitial fractions ($\epsilon_i = 0.63$) with nylon-6 fibers.

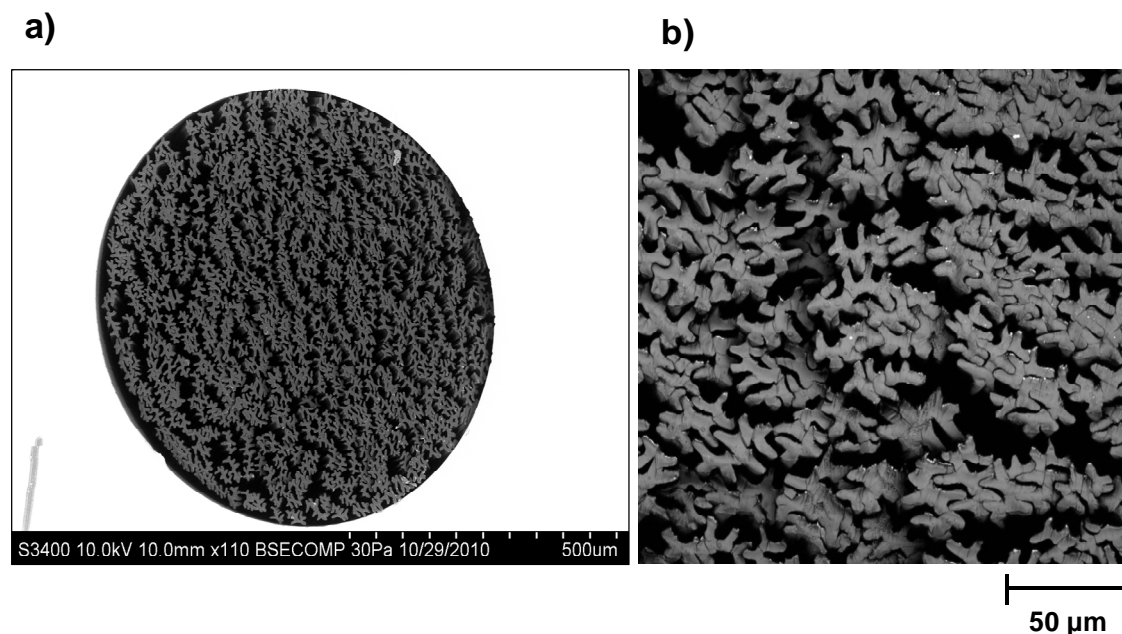


Figure 4.1. a) Scanning electron micrographs of a radially compressed C-CP microbore column packed with polypropylene fibers, b) Optical micrograph of a cross section of a polypropylene C-CP microbore column.

Figure 4.1a and b represents a scanning electron micrograph of a C-CP fiber packed microbore column and an optical micrograph of a cross section of that microbore column respectively. Based on the measurements of fiber perimeter and the number of fibers in the column, specific surface area of standard and microbore columns are $3.83 \text{ m}^2/\text{g}$ and $3.95 \text{ m}^2/\text{g}$ respectively. Note that this approach neglects any degree of “porosity” of fibers.

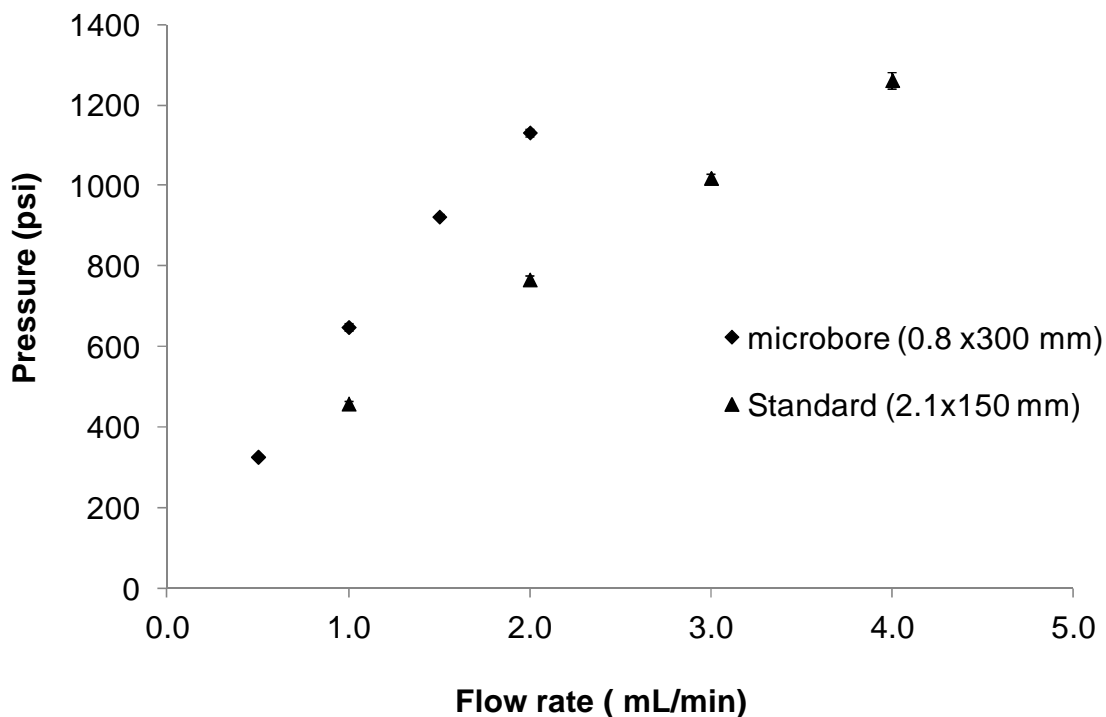


Figure 4.2. Effect of flow rate on backpressure for microbore (0.8x 300 mm) and standard size (2.1 x 150 mm) nylon-6 fiber columns packed at an interstitial fraction of ~0.63.

Figure 4.2 shows the effect of flow rate on backpressure on both column formats when they are packed at a $\varepsilon_i = 0.63$. The packing process of C-CP columns has been described in greater detail previously [27-29]. The C-CP fiber columns were packed by winding the fibers onto a spool and then doubling the fiber over a monofilament and pulling the fiber bundle through the tubing. The ends of fibers were then cut flush with the end of the tubing and frits and end fittings were assembled. The stainless tubing, 10 μm porosity frits, and end-fittings were purchased from Valco Instruments (Houston, TX, USA). The capillary tubing

(microbore) was purchased from Cole-Parmer Instrument Company (Vernon Hills, IL, USA).

Chromatographic System and Operation

The chromatographic system used for the frontal loading experiments consisted of a Waters (Milford, MA) Model 717 auto sampler, 600S Controller, 616 HPLC pump, and Waters 996 photodiode array detector. For these studies protein (lysozyme) absorbance was detected at 280 nm. Data was collected by the Millennium 32 and Empower 2 Chromatography Manager and further processed and managed in Microsoft (Seattle, WA) Excel files. All the data presented here are the means of triplicate runs, with the error bars in each plot representing a variance of one standard deviation.

Chemicals and Reagents

Lysozyme (MW ~14 kDa, geometric size ~5 nm x 3 nm x 3 nm) and NaCl were purchased from Sigma-Aldrich (Milwaukee, WI, USA). High-purity water ($18.2 \text{ M}\Omega \text{ cm}^{-1}$, Barnstead Nanopure, Dubuque, IA), and Tris-HCl buffer (20 mM, Teknova, Hollister, CA), were used as solvents. Stock solutions of lysozyme (0.25 mg/mL) and NaCl (1 M) were made in 20 mM Tris-HCl buffer (pH =8.1). Fresh protein solutions were made daily for frontal loading experiments. The columns were cleaned with guanidinium-HCl (50 μL) (Sigma Aldrich, St. Louis, MO) in between each frontal loading run to make sure that there is no any adsorbed protein left on fiber surface.

Frontal Analysis

The frontal loading experiments were carried out with lysozyme solutions of 0.10 -0.30 mg/mL at 1.00 – 4.00 mL min⁻¹. Frontal throughput and % yield were calculated as described by Singh and Pinto [16]. Throughput (T) and yield (Y) were calculated using equations 4.1 and 4.2, where Q and Q' are the amount of protein recovered and loaded respectively. The loading time (t') and elution time (t'') were obtained from the breakthrough and elution profiles (see Figure 4.3). Amount of protein recovered was calculated using a calibration curve generated from the chromatographic peak area vs. lysozyme concentration.

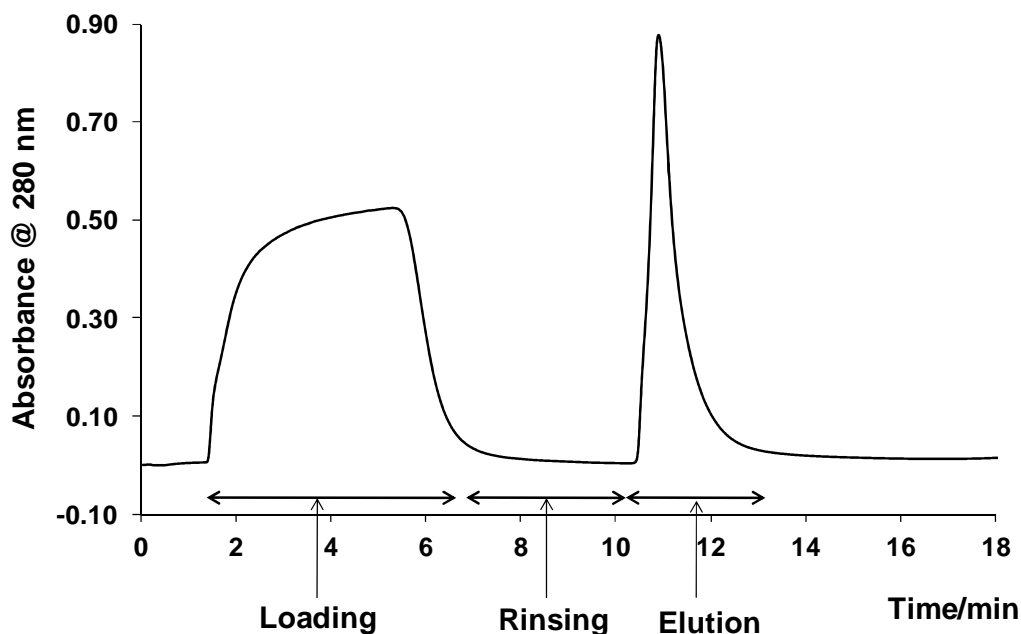


Figure 4.3. Nylon-6 microbore C-CP fiber column response for frontal loading of 0.25 mg/mL lysozyme in 20 mM tris-HCl (pH= 8.1), washing with Milli-Q water for 5 min, and eluting with 1 M NaCl in tris-HCl (pH= 8.1) at a flow rate of 1 mL min⁻¹.

$$\text{Throughput (T) (mg/min)} = \frac{Q}{t'+t''} \quad (4.1)$$

$$\text{Yield (\%Y)} = \frac{Q}{Q'} \times 100\% \quad (4.2)$$

Results and Discussion

Nylon-6 C-CP fibers as a support/stationary phase for downstream processing

The nylon-6 monomer consists of both amine and carboxylic functional groups on an alkyl backbone. This ionizable amine and carboxylic acid end groups naturally present in nylon-6 C-CP fibers allow for hydrophobic interaction (HIC) and ion exchange chromatography (IEC) protein separations as demonstrated in previous studies [23,25]. Previous studies have shown that microbore C-CP fibers columns are the most effective way of achieving high separation efficiencies for rapid analytical scale protein separations with C-CP fibers [30]. This is based on the ability achieve high linear velocities at low volume flow rates with C-CP fiber microbore columns and better hydrodynamics. Thus as an starting point, microbore nylon-6 C-CP fiber columns are used to demonstrate the utility of using C-CP fibers as a platform for downstream processing of biomacromolecules. Once the loading and elution conditions, were optimized, subsequent studies were carried out with standard size columns with the view of scaling up for preparative chromatography. As demonstrated in the scanning electron micrograph of Fig. 4.1a, C-CP fibers are packed in a radially-compressed 0.8 x 300 mm column to achieve an interstitial fraction of $\epsilon_i = 0.63$. This interstitial fraction is chosen as previous studies have shown that this is the

optimal interstitial fraction (packing density) of C-CP fiber columns for both small molecules and protein separations [28,31]. Radially-compressed microbore C-CP columns offer increased and uniform packing density as void volume is decreased upon compression [29]. The optical micrograph of a dry C-CP fiber column in Fig. 4.1b depicts how fibers interdigitate to form a network like structure allowing high fluid transport efficiency (i.e. high velocities (U_o) at low back pressures) which is important in high speed and preparative scale macromolecule separations. This is very evident in Fig. 4.2, where the microbore columns exhibits a backpressure of 1132 psi (7.8 MPa) at 2.00 mL min⁻¹ ($U_o = 106 \text{ mm s}^{-1}$) where as 1262 psi (8.7 MPa) for a standard-size 2.1x 150 mm columns at 4.00 mL min⁻¹ ($U_o = 24 \text{ mm s}^{-1}$). The substantially lower backpressures of C-CP fiber columns are very unique in comparison to the commercial stationary phases. The backpressures show a linear increase with flow rate. According to Leva's equation, which is commonly utilized in prediction of pressure drops versus flow rate, better mass transfer characteristics are expected at high linear velocities for viscous fluids [32].

Frontal loading

In the case of analytical separations, peak capacity is the most important parameter in characterizing the performance of high-speed protein separations. When it comes to preparative scale protein separations, the process throughput (T), yield (Y), and productivity become the parameters of interest. These parameters are derived from frontal development. Frontal development is

commonly used to assess protein adsorption and desorption rates. Pinto and co-workers described a frontal development technique which involves a cycle of three steps, loading of the column to breakthrough, a salt wash to recover the isolated protein, and re-equilibration of the column for the next cycle [16]. In this study, we employ a frontal development method for the determination of throughput and yield of lysozyme on nylon-6 C-CP fiber columns. A similar to that of Pinto's work, but slightly modified strategy is demonstrated in Fig. 4.3, where three steps loading, rinsing, and elution are performed to isolate the protein. Figure 3 illustrates the response of a microbore nylon-6 C-CP fiber column when 0.25 mg/mL lysozyme in tris-HCl (pH = 8.1) is loaded to breakthrough, followed by a rinsing step with milli-Q water for 5 min and elution with 1 M NaCl in tris-HCl (pH = 8.1) at a mobile phase flow rate of 1 mL min⁻¹ ($U_0 = 53.4 \text{ mm s}^{-1}$). There are many published studies on the adsorption of proteins; with the majority of the studies are using bovine serum albumin (BSA) and lysozyme as model proteins [33-37]. Also lysozyme has been shown to maintain its native structure upon adsorption to many hydrophobic surfaces [38-41]. Thus lysozyme is chosen as the model protein for frontal throughput determinations in this study. Unlike in Pinto's studies, aqueous wash step is used in between the loading and elution steps to avoid possible co-elution of loading and elution peaks at high flow rates and to remove any unbound protein and excess buffer. It was found that there is no effect of rinsing time on the frontal throughput or yield (quantified in detail in subsequent discussion). A frontal throughput of 0.08 mg/min per 9.75 mg of

average mass per column and with ~84% yield is observed when 0.527 mg of lysozyme is loaded at 1 mL min^{-1} . These results and the chromatographic response for frontal development observed in smaller diameter microbore nylon-6 C-CP fiber column revealed many interesting features predicting excellent behavior of C-CP fibers in scale-up protein separations. Thus the following discussion is based on the continued optimization steps carried out in terms of salt strength, protein concentration, and linear velocity to achieve maximum throughput and yield with use of larger diameter columns.

Effect of Salt Concentration, Protein Feed Concentration, and Rinse Time on Column Throughput and Yield.

As the first step of optimization, the effect of elution salt strength (0 –1 M) on protein recovery (yield) was evaluated using a microbore column. The resulting frontal loading and elution profiles are illustrated in Fig. 4.4. In all experiments, the loading, washing and elution flow rates are maintained constant at 1 mL min^{-1} ($U_0 = 53.4 \text{ mm s}^{-1}$). The responses in Fig. 4.4 clearly show the typical ion exchange behavior, wherein an increase in salt concentration results in an increase in yield (~10% with 0.005 M vs. ~82% with 1 M NaCl). It is also found that the column throughput is increased when proteins are eluted at high salt concentrations. When salt concentration is increased 10 times from 0.005 M to 0.05 M, a significant increase in elution peak area (throughput and yield) is seen. However, increasing the elution strength from 0.05 to 1 M only increases

by 18% more, thus the effect of salt concentration is minimal when reaching high concentrations.

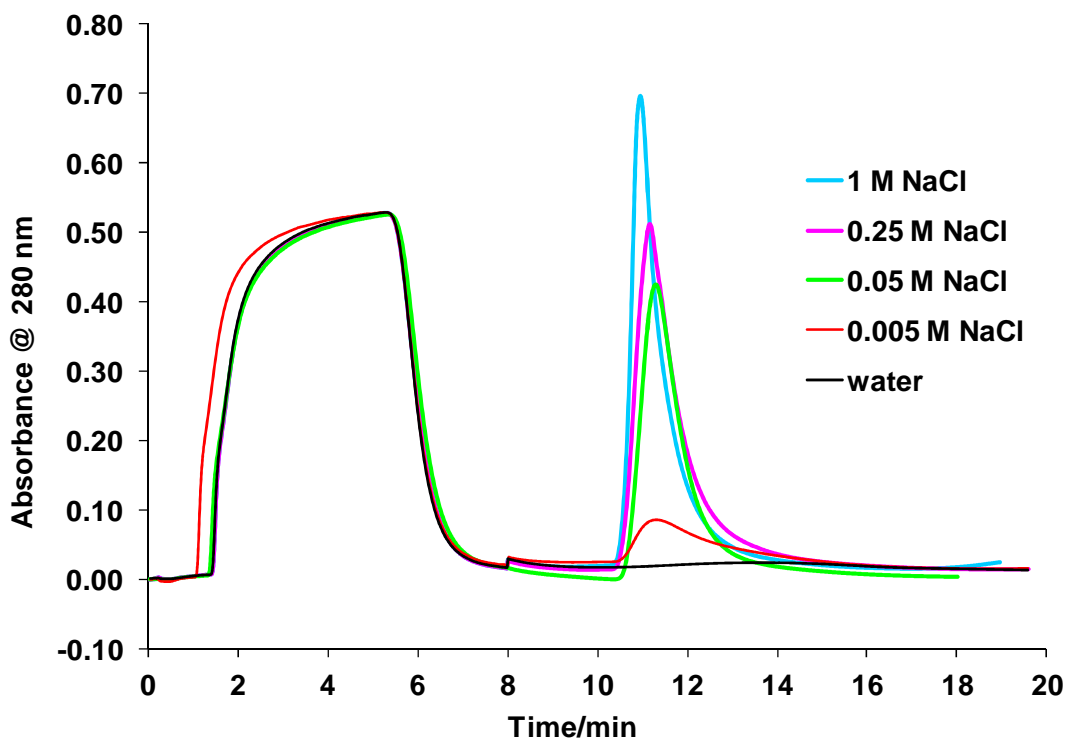


Figure 4.4. Effect of salt concentration (0-1 M) on nylon-6 microbore C-CP fiber column response for the frontal loading of 0.25 mg/mL lysozyme in 20 mM tris-HCl (pH= 8.1), washing with Milli-Q water for 5 min, and eluting with 1 M NaCl in tris-HCl (pH= 8.1) at a flow rate of 1 mL min⁻¹.

A similar response was observed in a study of adsorption and desorption rates of lysozyme onto gel-filled rigid particles by IEC. In that study Carta and co-workers illustrated that the equilibrium becomes less favorable as the salt concentration is increased and at very high concentrations (1 M NaCl), equilibrium limitations are completely removed [37].

It was believed that an aqueous water wash step was needed before the elution step to avoid co-elution of loading and elution peaks, especially at high flow rates. To assess the necessity, the rinsing time was changed from 1 min to 6 min to see if there was any effect on the peak area of the elution peak. In fact, there is no appreciable change in peak area of elution peak with rinse time. Thus rinse time does not affect the frontal throughput or yield during this frontal development method. For simplicity we chose 5 min long rinse time to separate the time gap between the loading and elution profiles so that the calculations are not affected at all with co-elution. Initially, instead of water, tris-HCl buffer was used for the rinsing step, but it did not work efficiently as water. However, as reported in literature a buffer wash is common before elution in frontal analysis for characterizing protein adsorption-desorption behavior by IEC and high performance frontal affinity chromatography [37,42].

In order to test column performance with respect to reproducibility of yield, ten successive frontal loadings were carried out on a single microbore nylon-6 C-CP column at 0.50 mL min^{-1} and the yields were evaluated. The results show that lysozyme yield (93.9%-95.3%, RSD = 0.4%) is not substantially changed over multiple loadings. The columns were cleaned with guanidinium-HCl (50 μL) in between each frontal loading run to make sure that there is no any adsorbed protein left on fiber surface. This response suggests two important characters. One, nylon-6 C-CP fiber columns offer a high level of reproducibility in protein isolation by frontal development as observed in previous protein separations with

C-CP fibers. The second, it is possible to use the same C-CP fiber column for multiple cycles of protein isolation without compromising the loading and recovery efficiencies. These aspects are vital in developing C-CP fiber columns towards preparative chromatography as it has been often stated that the column is the heart of the LC. Scale up from analytical LC to preparative LC is often time consuming and wasteful of materials unless an optimized scale-up strategy is employed. Thus the above results suggest lot of promise in developing C-CP fibers as a platform for preparative chromatography.

For further characterization of microbore nylon-6 C-CP fiber columns, the effect of protein feed concentration on frontal throughput was studied for concentrations ranging from 0.10-0.30 mg/mL lysozyme were evaluated at a flow rate of 1 mL min^{-1} ($U_o = 53.4 \text{ mm s}^{-1}$). As expected, lower feed concentrations took a long time to reach breakthrough and that time is decreased when concentration is increased. The washing and elution cycles were carried out at the same flow rate. As seen in Fig. 4.5, there is a nearly linear increase in frontal throughput with lysozyme feed concentration. The frontal throughput is seen to increase to 0.135 mg/min for 9.75 mg of fiber mass per column by ~2.3 times as the feed concentration is increased by 3 times. Noticeably, the observed yields are independent of lysozyme feed concentration. In all cases a higher yield (87%-90%) was observed. Pinto and co-workers have also seen similar result with increase in throughput as a function of protein feed concentration [16]. However, the frontal throughput (0.058 mg/min) observed for 0.10 mg/mL of

protein is twice that reported in Pinto's paper in which relatively larger size column formats are used.

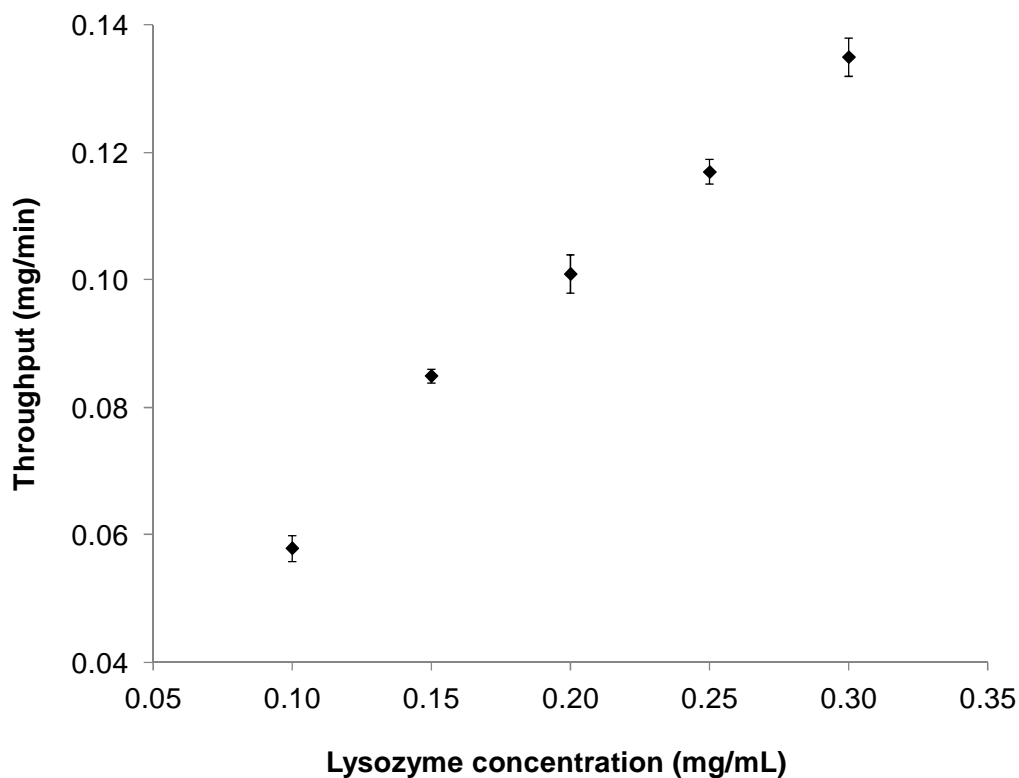


Figure 4.5. The effect of lysozyme feed concentration on column throughput. Loading and elution flow rate =1 mL min⁻¹.

Effect of Flow Rate on Frontal Throughput and Yield

The evaluation of the mobile phase flow rate (velocity) on frontal throughput and yield is very important as it is expected to play a major role in protein adsorption-desorption kinetics. Up to this point, characterization of nylon-6 C-CP fibers was conducted on microbore format as the starting point. The following studies were continued with a standard size (2.1 x 150 mm) column

packed with an identical packing density to that of microbore column. The size of the column format is increased to evaluate the utility of C-CP fiber columns for large scale protein isolations. In order to evaluate the effect of loading and elution flow rate on throughput and yield, both loading and elution flow rates were changed ($1 - 4 \text{ mL min}^{-1}$) at the same time for a feed concentration of 0.25 mg/min . Figure 4.6 shows the column response for frontal loadings at different flow rates as a function of the total mobile phase volume (mL).

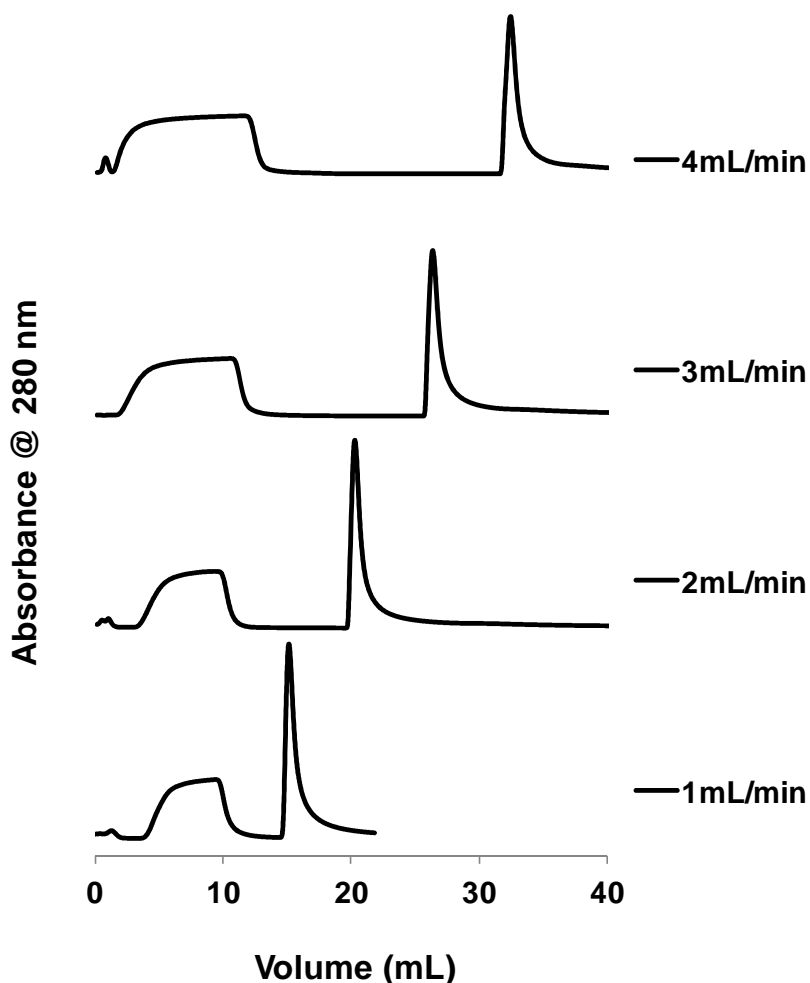


Figure 4.6. The column (2.1 x 150 mm) response for frontal loading of 0.25 mg/mL lysozyme in 20 mM tris-HCl ($\text{pH}= 8.1$), washing with Milli-Q water for 5 min, and eluting with 1 M NaCl in tris-HCl ($\text{pH}= 8.1$) at different flow rates.

As the flow rate is increases from 1 - 4 mL min⁻¹, the breakthrough time is decreases, as expected. One thing to note in Fig. 4.6 is that the increase in spacing between loading and elution profiles at high flow rates is due to the increase in volume of the washing step since a 5 min long aqueous wash step was maintained throughout. Frontal throughput and %yields were calculated for each flow rate and tabulated in Table 4.1. As the flow rate is increased, both the amount of protein loaded and recovered decrease.

Table 4.1. Effect of loading and elution linear velocity on frontal throughput and yield when 0.25 mg/mL lysozyme is loaded on a 2.1 x 150 mm column.

Flow rate (mL/min)	Linear velocity (mm/s)	Amount of protein loaded Q' (mg)	Amount of protein recovered Q (mg)	Throughput T (mg/min)	Yield (%)
1.0	6.0	1.29	1.12	0.117	87.43
2.0	12.0	1.13	0.96	0.188	85.09
3.0	18.0	0.76	0.68	0.200	90.70
4.0	24.0	0.56	0.52	0.213	93.24

However, proteins elute giving a sharp peak at high flow rates in a small time window. As a result, frontal throughput (amount of protein recovered per unit time) of lysozyme is increased appreciably as the flow rate is increased. As illustrated in Table 4.1, the throughput values are increased by ~2x when flow rate is increased by 4x as a result of enhanced mass transfer characteristics at high flow rates. These throughput values are significantly higher than the values

reported for polymeric short-fiber supports under similar flow and feed conditions [16]. A slight increase in %yield (6%) is observed when the flow rate is increased.

The increase in throughput at high loading and elution velocities reveals a number of interesting aspects of C-CP fiber supports. Unlike polymeric short-fiber columns, C-CP fiber supports can accommodate high flow rates to offer high mass transfer characteristics. In fact, the low backpressure characteristics offered by C-CP fibers are an added advantage in this case, allowing operating at high flow rates to maximize both throughput and yield. This can be attributed to the high shear rates generated by C-CP fiber columns at very high linear velocities within narrow interfiber gaps. It is believed at high shear rates ($\dot{\gamma}$), more favorable mass transfer kinetics can be expected during and after the desorption process on PP C-CP fiber surfaces as reported in previous work [19,30]. The shear rate in a two dimensional system is defined as the ratio of linear velocity and gap spacing ($\dot{\gamma} = U/d$). In case of C-CP fiber phases, this gap is equivalent to inter-fiber gap which is approximately 1-5 μm size. Thus very high shear rates (1200 – 4800 s^{-1}) can be achieved with the high linear velocities offered by C-CP columns. According to Leveque's explanation, the concentration gradient over which diffusion from the bulk flow to the surface changes to a much steeper gradient as opposed to a parabolic form. As a result, the boundary layer is decreased with a $\dot{\gamma}^{1/3}$ dependency ($\delta_c \propto 1/\alpha \dot{\gamma}^{1/3}$) allowing enhanced diffusion within the channels towards the fiber surface [43-45]. This combination of effects results in greatly enhanced diffusion, called convective diffusion. This effect is more

pronounced for proteins than small molecules which have larger mobile phase diffusion coefficients. In combination with high throughput and yield, nylon-6 fiber format shows a lot of promise as a platform for downstream processing of biomacromolecules.

Figure 4.7 represents the comparison of column responses for frontal loading of lysozyme and washing at 1 mL min^{-1} , and eluting at flow rates ranging from $1 - 4 \text{ mL min}^{-1}$.

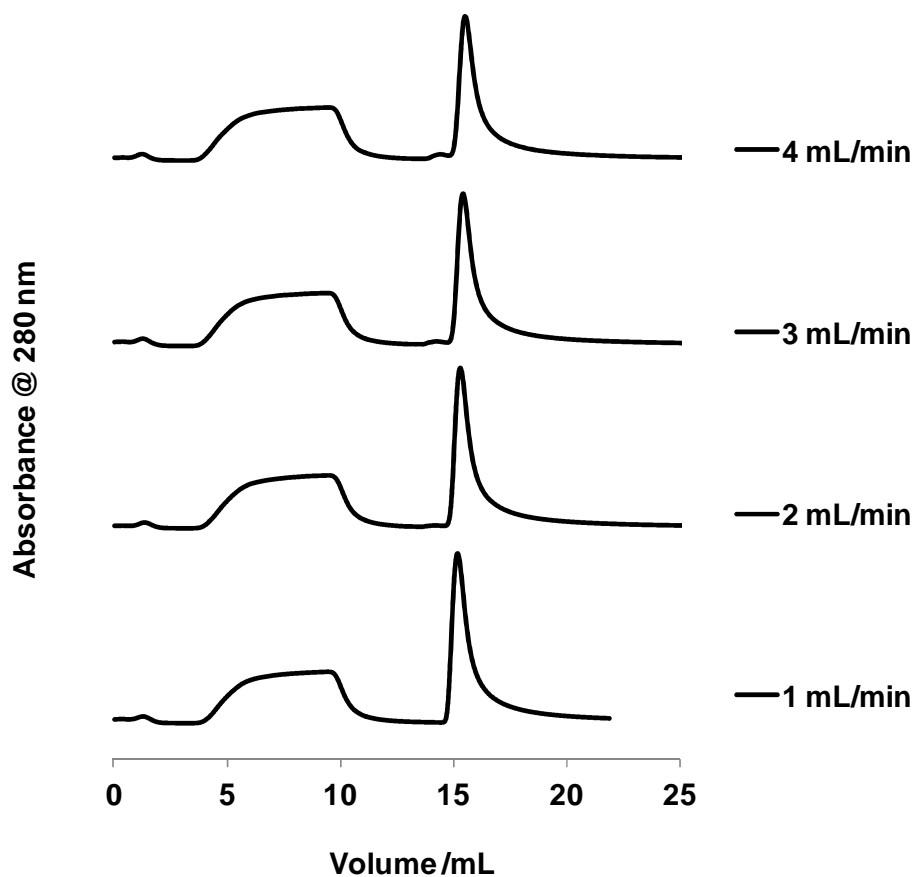


Figure 4.7. The column response ($2.1 \times 150 \text{ mm}$) for frontal loading of 0.25 mg/mL lysozyme in 20 mM tris-HCl ($\text{pH} = 8.1$), washing with Milli-Q water for 5 min , at 1 mL min^{-1} , and eluting with 1 M NaCl in tris-HCl ($\text{pH} = 8.1$) at different flow rates.

In other words the elution flow rate is varied while loading flow rate kept constant to see the effect of elution rate on throughput and yield. As can be seen, the breakthrough volumes appear to be the same, as loading rates are the same. Interestingly, the proteins are recovered at high flow rates in shorter amount of time, once again resulting in higher throughputs and yields. The calculated values for amount of protein loaded, recovered, throughput and %yield are tabulated in Table 4.2. It is very clear in this case that the amount of protein loaded remains consistent as they are loaded under identical flow rates. However, the amount of protein recovered is increased with the rate of elution. Thus lysozyme throughputs and yields are increased at high volume flow rates. Again this response can be explained with regard to high shear rates generated in C-CP fibers.

Table 4.2. Effect of elution linear velocity on frontal throughput and yield when 0.25 mg/mL lysozyme is loaded on a 2.1 x 150 mm column at 6 mm/s.

Elution flow rate (mL/min)	Elution Linear velocity (mm/s)	Amount of protein loaded Q' (mg)	Amount of protein recovered Q (mg)	Throughput T (mg/min)	Yield (%)
1.0	6.0	1.29	1.107	0.116	86.08
2.0	12.0	1.30	1.115	0.156	88.54
3.0	18.0	1.27	1.129	0.178	89.03
4.0	24.0	1.21	1.132	0.196	93.56

As described in above paragraph, under high shear conditions, a very steep concentration gradient is formed over which diffusion from bulk flow to the C-CP fiber surface resulting convective diffusion as described by Leveque [43-45]. This enhanced diffusion leads to high mass transfer characteristics at high linear velocities to yield increased throughput and yields. Therefore a combination of a high feed concentration and high loading and elution velocities offer enhanced frontal throughputs and yields.

Although C-CP fiber columns posses lower surface area ($4 \text{ m}^2/\text{g}$ vs. $70 \text{ m}^2/\text{g}$) in comparison to a commercial Sepharose IEC column, C-CP fiber columns offer other advantages in terms of maximum operational backpressure (3000 psi vs. <100 psi), linear velocity limit 3600 cm h^{-1} vs. $<150 \text{ cm h}^{-1}$) and obviously the cost of manufacturing (\$ 350 vs. \$ 2000). However the loading capacities are not competitive (5 mg/g of fiber vs. 70 mg/mL of media for lysozyme) with commercially used IEC columns.

Conclusion

Nylon-6 C-CP fiber packed columns have been evaluated by frontal development for the initial evaluation of lysozyme throughput and yield by ion exchange chromatography. Microbore nylon-6 C-CP column format was used for primary characterizing steps. Frontal development cycle involved 3 steps, protein loading to breakthrough, an aqueous rinse step, and elution with a salt. It was found that a rinse time of 5 min is necessary to avoid co-elution of loading and elution profiles. The effect of salt concentration was investigated and it was found

high salt concentrations (1 M NaCl) results in increased yields. Also the columns were tested for reproducibility of yields for multiple loading steps. It showed a high level of reproducibility in %yield with 0.4 % RSD. The results showed a combination of high feed concentration and loading/elution at high flow rates are the favorable means of achieving high lysozyme throughput and yield. The role of linear velocity is the key in maximizing throughputs and yields on C-CP fiber supports as they offer high shear rates at high velocities allowing efficient mass transfer characteristics in protein adsorption-desorption process. According to the results of current study, two times higher frontal throughputs (0.213 mg/min per 78 mg of fiber mass/column) were achieved with nylon-6 C-CP fibers in comparison to polymeric short-fiber phases under same flow conditions.

Based on these results, further developments will focus on scaling up for preparative chromatography with comparison to commercially available columns. With the progress achieved so far in protein separations together with low material cost, ease of packing and ability to operate at high flow rates with minimal backpressures make C-CP fibers good candidate for downstream processing of bio-macromolecules.

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CHAPTER FIVE

SUMMARY

The research work presented in this dissertation has described the fluid dynamic evaluation of C-CP fibers as a support/stationary phase in HPLC for macromolecule separations. The long term goal was to develop and characteristics C-CP fibers as rapid, efficient and economical stationary phase for downstream processing of bio-macromolecules. The overall objective of the research includes, studying the hydrodynamic aspects of C-CP fiber column performance for rapid analytical-scale and high throughput preparative scale macromolecule separations. Chapter one outlines the basic introduction to hydrodynamics of chromatography and development of new stationary phases for the separation of macromolecules. The development of C-CP fibers as an alternative platform for macromolecule separations is also described in Chapter one.

Chapter two described the dynamic evaluation of polypropylene (PP) C-CP fibers as a stationary phase in HPLC. Specifically, that the roles of fiber size and shape, linear velocity, interstitial fraction, and column inner diameter play in separation efficiency and resolution of uracil and butylparaben have been investigated. Four PP fiber types, having nominal diameters ranging from 30 to 65 μm , were used as the stationary phase in 250 x 2.1 mm i.d. stainless steel columns for the performance evaluation under isocratic conditions. Optimum flow characteristics, as judged by plate height and resolution, were observed for 40

μm diameter PP C-CP fibers packed at an interstitial fraction of 0.63 under a broad range of linear velocities (~ 2 to 37 mm s^{-1}). The best performing column in terms of plate height and resolution was the 2.1 mm inner diameter out of 1.5, 2.1, and 4.6 mm i.d. sizes. Having determined the best column conditions based on small molecule performance, PP C-CP columns were also evaluated for the separation of a three-protein mixture composed of ribonuclease A, cytochrome c, and transferrin. Results obtained with the bio-macromolecules mixture validate the optimal structural and operative conditions determined with the small solutes (uracil and butylparaben).

Chapter three demonstrated the investigation of the performance of microbore columns utilizing PP C-CP fibers as the support/stationary phase for rapid reversed phase HPLC of proteins. The separation performance of PP fiber packed microbore columns (peak width, peak capacity, and resolution) were evaluated using the separation of a three-protein mixture composed of ribonuclease A, cytochrome c, and transferrin under reversed phase gradient conditions. Opposite the performance of other phases, high velocities observed in microbore column format provide enhanced resolution of the three-protein suite as peak widths decrease with velocity. Increases in column length result in increased resolution as the peak widths remain essentially constant, while the retention times increase. In addition, it was found that the peak capacities increase with column length and linear velocity. Radial compression of the microbore tubing enhances the packing homogeneity and thereby the separation

efficiency and resolution. Radially-compressed columns result in a decrease in interstitial fraction (~5%), but an increase in resolution of ~14% for the ribonuclease A and cytochrome c pair. It is clear that the fluid and solute transport properties of the C-CP fiber microbore columns afford far better performance than obtainable in standard format columns. The ability to achieve high separation efficiencies, on short time scales and low volume flow rates, holds promise for high capacity protein separations in proteomics applications.

Chapter four examined the initial evaluation of nylon-6 capillary-channeled polymer (C-CP) fibers as an alternative ion exchange support/stationary phase for downstream processing of macromolecules. Ionizable amine and carboxylic acid groups naturally present in nylon-6 C-CP fibers allow for ion exchange chromatography (IEC) on the native nylon surface. More specifically, adsorption and desorption characteristics of lysozyme on nylon-6 fibers are investigated. The nylon-6 fibers were packed into standard-size (2.1mm i.d.) and microbore (0.8 mm i.d.) columns allowing the evaluation of the role of linear velocity on pressure drop, frontal throughput, and yield. The frontal development method used here for protein isolation involved three steps, loading of the column to breakthrough, an aqueous wash, and a salt wash to recover the protein. The frontal throughput was evaluated with different salt concentrations (0 – 1000 mM NaCl) and different linear velocities (6 – 24 mm/s). It is observed that the rinse time does not affect the throughput or yield. The observed throughput values are in the range of 0.12 - 0.20 mg/min when 0.25 mg/mL lysozyme is loaded. The

throughput and yield were found to increase with protein feed concentration and linear velocity. Also, higher throughput and yield were found when high concentrations of protein were loaded at a very high linear velocity and eluted at a very high linear velocity. This study will benefit the development of C-CP fibers for downstream processing of biomacromolecules.