2007

Evaluating the effective peak capacity of a saw-tooth gradient for reverse-phase high performance liquid chromatography separation of proteins and peptides

Guimei Cai
West Virginia University

Follow this and additional works at: <https://researchrepository.wvu.edu/etd>

Recommended Citation

Cai, Guimei, "Evaluating the effective peak capacity of a saw-tooth gradient for reverse-phase high performance liquid chromatography separation of proteins and peptides" (2007). *Graduate Theses, Dissertations, and Problem Reports*. 1819.
<https://researchrepository.wvu.edu/etd/1819>

This Thesis is protected by copyright and/or related rights. It has been brought to you by the The Research Repository @ WVU with permission from the rights-holder(s). You are free to use this Thesis in any way that is permitted by the copyright and related rights legislation that applies to your use. For other uses you must obtain permission from the rights-holder(s) directly, unless additional rights are indicated by a Creative Commons license in the record and/ or on the work itself. This Thesis has been accepted for inclusion in WVU Graduate Theses, Dissertations, and Problem Reports collection by an authorized administrator of The Research Repository @ WVU. For more information, please contact researchrepository@mail.wvu.edu.

**Evaluating the Effective Peak Capacity of a Saw-Tooth Gradient for
Reverse-Phase High Performance Liquid Chromatography Separation
of Proteins and Peptides**

Guimei Cai

**Thesis Submitted to the Eberly College of Arts and Sciences at West Virginia
University in Partial Fulfillment of the Requirements for the Degree of**

**Master of Science
In
Chemistry**

Aaron T. Timperman, Ph.D., Chair

Harry O. Finklea, Ph.D.

Ronald B. Smart, Ph.D.

C. Eugene Bennett Department of Chemistry

Morgantown, WV

2007

**Keywords: saw-tooth gradient, peak capacity, proteomics, low molecular weight
human serum, proteins and peptides separation, capillary-RP HPLC-FTICR MS**

Abstract

Evaluating the Effective Peak Capacity of a Saw-Tooth Gradient for Reverse-Phase High Performance Liquid Chromatography Separation of Proteins and Peptides

Guimei Cai

The performance of saw-tooth gradient for comprehensive proteome analysis is evaluated using model proteins and a low molecular weight human serum by reverse-phase high performance liquid chromatography. The peak splitting and number of peptides detected is used to characterize the effective peak capacity of the saw-tooth gradient as a function of solvent strength change ($\Delta\phi$). Peak splitting demonstrated in experimental result as one single peptide or protein detected in more than one saw-tooth step. No peak splitting is observed directly from chromatograms for two model proteins by decreasing the solvent strength change ($\Delta\phi$) from 5.0%, 1.0%, to 0.1%. The numerical comparisons of the peak area, peak width and peak asymmetry from saw-tooth gradients with the same parameter from the linear gradient show no peak splitting for both model proteins. Anthracene does not have peak splitting, but a significant reduction in peak area is observed. It is indicated that the S values proportional with the molecular weight significantly affect the observed effective peak capacities. Peak capacity of the saw-tooth gradient is also explored by a LMW ($\leq 30\text{kDa}$) human serum on an online capillary-HPLC-ESI-FTICR MS by decreasing the $\Delta\phi$ from 1.0%, 0.5%, 0.2%, to 0.1% in the 26.0%-27.0% solvent B range. When the $\Delta\phi$ decreases from 0.5% to 0.1%, the number of protein splitting increases from 5 to 28 while a higher increase in the number of total peptide signals detected is observed from 21 to 67 when $\Delta\phi$ decreases from 1.0% to 0.1%. In fact, more peptide signals are observed with the saw-tooth gradient than the long linear gradient. Moreover, no new proteins are detected in the isocratic holding segment. Overall, the saw-tooth gradient provides a promising first dimension separation technique for a two-dimension separation with an effective peak capacity of several hundred at 0.1% $\Delta\phi$.

Acknowledgements

I would like to gratefully acknowledge the assistant of my advisor, Dr. Aaron Timperman, for guiding and supporting me throughout the process of my courses and research. Through his support and patience, I have learned how to be more independent in scientific thoughts.

I would like to thank my committee members, Dr. Harry O. Finklea and Dr. Ronald B. Smart for their guidance during my graduate career. I would like to acknowledge Jim Lenke for his help in guiding me upon joining the group. And I would also like to thank Ting Zhao, Kathleen Kelly, Brent Resky, Trust Razguzwa, Yuchen Lu and Matt Powell for their help to my work.

I would like to thank my parents who encouraged me to go abroad and to continue my education. Their constant love and support has helped me through this and all of life's endeavors.

I would like to thank my daughter, Audrey, her birth bring a huge blessing for my life and a great encouragement for me to complete my work timely.

Finally, and most importantly I would like to thank my husband, Zhaoyong, for supporting me throughout the entire experience. We together have sacrificed much for me, give up great opportunity in China and go abroad for a higher degree. Without his encouragement, I would not be where I am today.

Table of Contents

Abstract	ii
Acknowledgements	iii
Table of Contents	iv
Table of Figures	vi
Table of Tables	viii
1. Introduction	1
1.1 Research purposes and goals	1
1.2 Separation in proteomics	2
1.3 Multidimensional separation technique	3
1.3.1 Fundamentals and advantages	3
1.3.2 Development of two-dimensional (2D) techniques for peptides and proteins	4
1.3.3 Peak capacity	5
1.4 Separation	7
1.4.1 Background of liquid chromatography/high-performance liquid chromatography	7
1.4.1.1 Principle of reversed-phase high performance liquid chromatography	7
1.4.2 Protein separations by reversed-phase high performance liquid chromatography (RP-HPLC)	10
1.4.2.1 The development of nonporous C18 reversed-phase liquid chromatography separation on proteins and peptides	11
1.4.3 Saw-tooth gradient	13
1.5 Mass spectrometry	15
1.5.1 Electrospray ionization (ESI)	15
1.5.2 Fourier Transform ion cyclotron resonance mass spectrometry	16
2. Experimental	18
2.1. Model proteins separation on analytical-HPLC	18
2.2 Anthracene separation on analytical-HPLC	20
2.3 Gradient shape and column test on nano-HPLC	21

2.3.1 Gradient shape test by different saw-tooth gradients	21
2.3.2 Comparison of protein recovery on three different types of columns	22
2.4 LMW (<30kDa) human serum separation on nano-HPLC-FTICR MS	23
2.4.1 Nano-HPLC-FTICR MS analysis of LMW (<30 kDa) human serum	23
2.4.2 LMW (<30 kDa) human serum mass measurement by protein assay	25
3. Results and Discussion	26
3.1 Model proteins separation on analytical-HPLC	26
3.1.1 Peak splitting and protein recovery test of two model proteins	26
3.2 Anthracene separation on analytical-HPLC	31
3.3 Gradient shape and column test on nano-HPLC	33
3.3.1 Gradient shape test by different saw-tooth gradients	33
3.3.2 Comparison of protein recovery on three different types of columns	39
3.4 LMW (<30kDa) human serum separation on nano-HPLC-FTICR MS	40
3.4.1 Nano-HPLC- FTICR MS analysis of LMW (<30kDa) human serum	40
3.4.2 LMW (<30kDa) human serum mass measurement by protein assay	51
4. Conclusions	53
References	54
Appendix	57

Table of Figures

Figure 1. Dependence of capacity factor k' on mobile phase %B (ϕ) for a small molecule benzene and several peptides or proteins	10
Figure 2. Schematic representation of a protein binding to a reversed-phase HPLC silica-based sorbent	11
Figure 3. Schematic of saw-tooth gradient components	14
Figure 4. Schematic of the mechanism of electrospray ionization	16
Figure 5. Programmed saw-tooth gradients used in peak splitting test by two model proteins on the analytical-HPLC	19
Figure 6. Programmed saw-tooth gradients used in peak splitting test by anthracene on the analytical-HPLC	20
Figure 7. Programmed saw-tooth gradients of four different $\Delta\phi$ for LMW ($\leq 30\text{kDa}$) human serum sample peak splitting studies on the nano-HPLC-FTICR MS	22
Figure 8. Schematic of column with spray tip	24
Figure 9. Chromatograms of model proteins produced by linear and saw-tooth gradients at various $\Delta\phi$ values	28
Figure 10. Illustration for measurement of peak asymmetry	29
Figure 11. Chromatograms of anthracene produced by linear and saw-tooth gradients at various $\Delta\phi$ values	32
Figure 12 (A-B). Total ion chromatograms of two saw-tooth gradients ($\Delta\phi$ is 1.0% and 0.5%) with the gradients superimposed	35
Figure 12 (C-D). Total ion chromatograms of two saw-tooth gradients ($\Delta\phi$ is 0.2% and 0.1%) with the gradients superimposed	36
Figure 13. Schematic of connections from mixing point of Agilent 1100 nano pump to the separation column	37
Figure 14. Protein recovery on three RP-HPLC columns packed with different types of beads	40
Figure 15. Total ion chromatogram of the long linear gradient with the gradient superimposed	44

Figure 16. Total peptide signals detected and protein splitting numbers of four saw-tooth gradients in the 26.0%-27.0% range of solvent B	45
Figure 17. Theoretical comparison of the saw-tooth gradient with two linear gradients in the 45.0-65.0% solvent B range	48
Figure 18. Mass spectra of fractions when $\Delta\phi=0.1\%$ from Figure 12 (D)	50
Figure 19. Standard curve of BSA together with the concentration of LMW ($\leq 30\text{kDa}$) human serum determined by this standard curve	52

Table of Tables

Table 1. Contribution of different band-broadening processes to column plate height H	12
Table 2. Linear gradient used in protein recovery test on the analytical-HPLC	19
Table 3. The detailed setup of long linear gradient for LMW (≤ 30 kDa) human serum sample on the nano-HPLC-FTICR MS	25
Table 4. Comparison of model protein peaks produced by linear and saw-tooth gradients at various $\Delta\phi$ values	30
Table 5. Comparison of anthracene peaks produced by linear and saw-tooth gradients at various $\Delta\phi$ values	33
Table 6. The absorbance maxima value for each tooth or step of the saw-tooth gradient with 0.1% $\Delta\phi$	39
Table 7. Detailed results of total peptide signals detected and protein splitting numbers of four saw-tooth gradients and a long linear gradient in the 26.0%-27.0% range of solvent B	46
Table 8. Measurement of absorbance on different concentration of standard BSA	51
Table 9. The absorbance and corresponding concentration of the LMW (≤ 30 kDa) human serum fraction	52

1. Introduction

1.1 Research purposes and goals

Many biological or environmental samples are extremely complex and cannot be separated adequately, creating a need for the development of improved separations. Recently we reported the development of a saw-tooth gradient that is well suited for use prior to the final stage of a multi-dimensional separation.¹ The overall utility of this method is largely dependent on the effective peak capacity that can be achieved. Although the concept of peak capacity easily translates to all separations, the gradient alternates between negative and positive slopes, making it discontinuous in nature. Thus, an operational approach has been used to evaluate the effective peak capacity for this saw-tooth gradient. For the comprehensive proteomic analyses, detection limits and sample throughput are the main consideration because the detection of low abundance proteins is the main concern. Much current research is focused on improving the detection of low abundance proteins and increasing the total number of proteins that can be identified in comprehensive proteome analyses. The purpose of this work is to determine the extent to which the solvent strength change of each gradient step can be reduced while minimizing peak splitting and maximizing the number of proteins identified. The peak capacity is evaluated by two model proteins to explore the optimum solvent strength change that has minimum peak splitting. The peak splitting on a small molecular weight compound is evaluated to provide supporting information for the peak splitting results on peptides and proteins. The accuracy of the saw-tooth gradient delivery by the produced with the capillary high performance liquid chromatography system interfaced with the MS is evaluated. The protein recovery rate of the nonporous beads packed column is also compared with porous beads packed columns. Finally, the maximum peak capacity of the saw-tooth gradient with minimum peak splitting is further evaluated using a real proteomic sample. The total protein and peak splitting numbers are used to justify which saw-tooth gradient has the maximum peak capacity and minimum peak splitting. The protein loss in the isocratic segment of the saw-tooth gradient is also evaluated by comparing the spectrum with adjacent spectra eluted by saw-tooth steps. The results of the saw-tooth gradient are compared with the

corresponding linear gradient to provide thorough understanding of the properties of the saw-tooth gradient.

1.2 Separation in Proteomics

Proteomics is the high throughput protein analysis from biological system, which emphasizes identification and quantitation of as many components as possible.² Proteins are responsible for almost all processes occurring in an organism, and their abundance and structure are likely to change as a response to disease. Protein complexes are important in signal transduction, metabolism and protein synthesis. A proteome is very complex and changes with the cell type and cell history.³ Proteomics will help us understand the fundamental biology of cells and living systems. This knowledge is very important for finding biomarkers for diseases and determining the underlying changes in molecular biological that lead to diseased states in order to rationally design new treatments.

Serum is an excellent example of a real proteomic sample which has a high content of proteins (60-80 mg of protein/mL), with many of these being secreted and shed from cells and tissues. However, the majority of the serum proteins are composed by six abundant proteins (representing approximately 85% of the total protein mass): albumin, IgG, IgA, transferrin, haptoglobin and alpha-1-antitrypsin.⁴ While most clinically important proteins (~10,000 proteins) have low abundance and have concentrations of 10 orders of magnitude less than the more abundant serum proteins.⁵⁻⁸ This large dynamic range of proteins makes the detection of low molecular weight protein analysis extraordinarily challenging by any separation technique. The most powerful one dimensional column chromatography techniques typically have a peak capacity of a few hundred, where peak capacity is defined as the maximum number of peaks that can be resolved on a given column.⁹ However, the actual number of completely resolved components is always much smaller than the theoretical peak capacity. Peak capacity assumes perfect spacing of all the separated components. In the real world with proteins, spacing is random. This discrepancy between the limited capabilities of one dimensional separations and the complexity of real world samples motivates the development of multidimensional separations, due to the greatly enhanced resolving power they offer.¹⁰

Since the dynamic range of the serum sample is large, it is important to reduce the sample complexity by removing the most abundant proteins using current methods. The low abundance proteins cannot be observed without removal of high abundance proteins. It is difficult to detect lower abundance proteins without effectively removing higher abundance proteins. Affinity chromatography⁷ and ultrafiltration¹¹ are two broadly used techniques to remove the more abundant proteins in serum.

1.3 Multi-dimensional Separation Techniques

1.3.1 Fundamentals and advantages

After depletion of abundant proteins, a separation technique, such as ion exchange^{5, 8, 12}, reverse-phase liquid chromatography,^{7, 13} and/or isoelectric focusing¹⁴⁻¹⁶, can be used as fractionation technique to further reduce the sample into small groups. Then another technique that is orthogonal to the fractionation technique can be used to separate the proteins in each fraction for identification.

A multi-dimensional separation combines two or more modes of separation to complete a single analysis. The benefits of the multi-dimensional separations include increased peak capacity, which is the product of peak capacity of each individual separation dimension. Multi-dimensional separation allows an increased number of components to be detected from complex mixtures. The fundamental requirements of multi-dimensional separations were first established by Giddings.¹⁷ First, all the separation mechanisms must be orthogonal, which means that the separation mechanisms of all the modes used must be different from each other. If not orthogonal, the total peak capacity will be less than the product of the peak capacity of each technique. If the separation modes are orthogonal, the distribution of the components in one dimension does not correlate with the distribution of components in another dimension. Second, any recombination of peaks separated in the first dimension at the beginning of the second dimension decreases the overall peak capacity. Because the peak capacity is the product of the peak capacity of each dimension, adding additional dimensions can increase the peak capacity. However, there have been few reports of multi-dimensional separations with more than two dimensions of separation due to the practical constraints.¹⁰

When performing two-dimensional separations, the sampling rate of the second-dimension separation with respect to the elution speed of the first dimension defines the

extent to which the separation achieved in the first dimension is preserved in the second dimension. Higher sampling rates improve the resolving power and peak capacity of the first dimension. It is suggested that each peak in the first dimension should be sampled at least three times to obtain high two-dimensional resolution.¹⁸ However, higher sampling rates also divide the sample into more fractions, which reduces the S/N and increases the limits of detection for interfaces that combine complete transfer of sample between the first and second dimensions. For comprehensive proteomic analyses, detection limits and sample throughput are the main consideration because the detection of low abundance proteins is the main concern.

1.3.2 Development of two-dimensional (2D) techniques for peptides and proteins

Multi-dimensional separation techniques for biomolecules such as peptides and proteins can be generally summarized into four categories: 2D-gel electrophoresis, LC-LC and LC-CE. 2D gel electrophoresis is a 2D-in-parallel separation technique which separates the analytes over a two-dimensional planar surface. All the others are typically 2D-in-series, in which the separation is carried out by first performing a one-dimension separation, and then sequentially subjecting individual fractions to a second-dimensional separation.^{10, 19}

Two-dimensional gel electrophoresis (2DE) is used routinely for proteome analysis. In 2DE, the first dimension is isoelectric focusing (IEF) which separates the analytes by their *pI* value and the second dimension is sodium dodecyl sulfate polyacrylamide gel sieving electrophoresis (SDS-PAGE) which separates by molecular weight. These two methods are orthogonal to each other. However, it has many limitations. First, the detection of low abundance proteins is restricted by the limited peak capacity and high limit of detections. In proteomics, many proteins that are potential biomarkers are low abundant. Second, the separation of membrane proteins remains challenging because their solubility can not be maintained in the gel. Membrane proteins play a central role in many biological activities. Third, the method is labor intensive, requires high skill level, and is difficult to automate.^{10, 20}

The limitations of 2D-gel can be reduced by 2D separation methods based on liquid chromatography (LC) and/or capillary electrophoresis (CE). Since LC and CE include many different separation modes, many biomolecules that are difficult to separate

by 2D-gel such as hydrophobic, acidic, and basic proteins can be separated by choosing proper modes. Additionally, LC and CE techniques can be interfaced between different separation techniques and coupled with many different detection methods such as UV absorbance, laser-induced fluorescence or mass spectrometry to achieve full automation.¹⁰ Interfacing directly with MS has the additional requirements of having volatile buffer/solvent additives and little or no detergents. Gradient LC can be used to concentrate the sample and improve the detection limits.

The interfacing of two different separation methods such as LC and CE has many restrictions. First, the mobile phase used in the two separation modes must be compatible. Because the two techniques have to be orthogonal, the solvents used for the two techniques have to be chosen carefully to be compatible with all of the separation modes. Second, excessive band broadening may be caused by the extra valves and loops used between columns. This extra column band broadening will decrease the concentration of the low abundance proteins and raise the detection limits. Third, it is required that the separation speed in the second dimension is much faster than that in the first dimension. The total separation time is largely dependent on the speed of the second dimension separation, because each fraction must be separated by the second dimension. According to the third requirement, the comprehensive mode of a LC-LC system is more difficult to develop compared to LC-CE systems, because CE performs faster than LC. Coupling of LC with CE is complicated because there is generally a large difference in peak volume of the LC and the injection volume of the CE. However, this discrepancy could be solved by employing a capillary HPLC column which uses a low flow rate.¹⁹

1.3.3 Peak capacity

As mentioned above, peak capacity is an important figure of merit for two-dimensional separations. It is the measurement of the maximum number of solutes that can be completely resolved between the inert peak and the last eluted peak. To achieve the maximum peak capacity, the resolution between two adjacent peaks should be equal to one. If the resolution between two adjacent peaks is smaller than one, the separation is not complete and the system is incomplete. If the resolution is greater than one, the separation is complete but the system is wasteful. Based on these conditions and the assumption that the plate number is the same for each solute, Giddings⁹ developed the

mathematical expressions for the peak capacity, n_c , for isocratic/isothermal chromatography as in equation 1:

$$n_c = 1 + \frac{\sqrt{N}}{4} \ln(t_n / t_1) \quad \text{Equation 1}$$

Horvath and Lipsky²¹ further developed the peak capacity for gradient LC based on Giddings' work. They assume that all peaks have the same width:

$$n_c = 1 + \frac{\sqrt{N}}{4} \left(\frac{t_n}{t_1} - 1 \right) \quad \text{Equation 2}$$

In both equation 1 and 2, N is theoretical plate number, t_1 and t_n are the retention times of the first and the last peak.

As indicated by equation 2, peak capacity is affected by the separation efficiency and the ratio of the elution time of the first and last peaks. Since the elution time of the last peak is related to the nature of the last solute component, the main focus for optimizing peak capacity is maximizing the plate number. There are many factors that affect the plate number, such as mobile phase velocity, column length, temperature, and the stationary phase. Grushka²² gave a detailed discussion on how these different factors affect the peak capacity.

However, the actual number of completely resolved components is always much smaller than the theoretical peak capacity. It is shown that a random chromatogram will never contain more than 37% of its potential peak capacity and among these peaks only 18% will be completely resolved²³. This discrepancy between the limited capabilities of one-dimensional separations and the complexity of real world samples motivates further development of multidimensional separations, due to the greatly enhanced resolving power they offer.

In this work, the effective peak capacity of a saw-tooth gradient is explored. The saw-tooth gradient can be used as the first dimension in a 2D separation when the separations are performed in series. Measuring the peak capacity is important because the peak capacity of a 2D separation is the product of the peak capacity of each dimension. Therefore, the main objective of this work is to determine if the peak capacity is high enough to be useful for comprehensive 2D proteomics separations. The term effective peak capacity is used because the original definition of peak capacity

assumes use of a linear gradient. The saw-tooth gradient investigated in this work includes positive, negative, and zero slope segments.

1.4 Separation

1.4.1 Background of liquid chromatography/high-performance liquid chromatography

Chromatography is a physical separation technique in which the components are selectively distributed between two immiscible phases: a mobile phase and a stationary phase. According to the mobile phase, chromatographic methods can be classified as: gas chromatography (GC), liquid chromatography (LC), or supercritical fluid chromatography (SFC). The chromatography process is a repeated adsorption and desorption of analyte from the stationary phase as the analyte molecules move through the column. The analytes are separated according to their differential solubility in the stationary phase and the mobile phase.²⁴

Many improvements in liquid chromatography are the result of improvements in stationary phase materials. Improvements in stationary phases provided smaller more uniform particles, and a new technique named high-performance liquid chromatography (HPLC) was established, providing higher separation efficiencies. The free space between packing materials is decreased when smaller particles are used. Therefore, the packing material had a much lower permeability so that it is necessary to use a pump to generate sufficient pressure to produce optimal flow rates. HPLC has better resolving power and speed than the traditional liquid chromatography techniques.²⁵

A classification of the various modes of liquid chromatography includes: normal-phase liquid chromatography, reverse-phase liquid chromatography, ion exchange chromatography, size exclusion chromatography and affinity chromatography. Among these, reverse-phase liquid chromatography is the most common mode for the separations of compounds in chemical, biological, pharmaceutical and biomedical sciences. The popularity lies in the following factors: 1) it has an excellent resolving power, 2) it is convenient to manipulate the chromatographic selectivity by changing mobile phase characteristics, 3) it has high recoveries, and 4) the reproducibility is great.²⁶

1.4.1.1 Principle of reverse-phase high performance liquid chromatography (RP-HPLC)

In reverse-phase liquid chromatography, the separation is carried out on a solid nonpolar stationary phase and a polar mobile phase. The most commonly used nonpolar stationary phase is silica support bonded with hydrocarbon chains, such as C₄, C₈ and C₁₈. The mobile phase is typically a mixture of water with one or more organic solvents, such as acetonitrile, methanol, ethanol and isopropanol. Acetonitrile is preferred in protein separation due to its low viscosity and low ultra-violet cut off (~190nm). An ion-pairing agent, such as acetic acid or heptafluorobutyric acid, is usually added in the mobile phase to reduce the ionic interactions with the silica surface. The retention mechanism of reverse-phase LC is explained best by the solvophobic theory.²⁷ This mechanism assumes that the stationary phase is a uniform layer of a non-polar ligand. When the solute binds to the stationary phase, the surface area of the solute exposed to the mobile phase is reduced. This reduction in exposed surface area of solute to the solvent plays the greatest role in adsorption to stationary phase. Therefore, the mobile phase composition has a greater influence on separation selectivity than the stationary phase in this mode of chromatography.²⁷⁻³¹

Separation of the protein mixture is dependent on the distribution coefficients of the individual species bound to the column. The distribution coefficient (K), also known as the thermodynamic partition ratio, is the ratio of the concentration of the analyte in the stationary phase to its concentration in the mobile phase. It depends only on temperature and the nature of the stationary and mobile phases.

$$K = C_s/C_m \quad \text{Equation 3}$$

Analytes with higher K values are bound more tightly to the column and will elute later in the separation.

The distribution coefficient can also be related to the capacity factor (k'), of the chromatographic system by the phase ratio (β).

$$K = k' \beta \quad \text{Equation 4}$$

The phase ratio is characteristic for the column and relates the volume of the mobile phase to the volume of stationary phase.

$$\beta = V_m/V_s \quad \text{Equation 5}$$

The capacity factor is the ratio of the mass of analyte in the stationary phase to the mass of the analyte in the mobile phase.

$$k' = m_s/m_m \quad \text{Equation 6}$$

The capacity factor depends on the column characteristics (such as the column dimensions and phase ratio) and reflects the binding strength of a molecule to the stationary phase under specific experimental conditions.

In reverse-phase HPLC, the analytes are introduced into the system in an aqueous solution and are eluted by the high organic content mobile phase. The elution can be carried out either by isocratic or gradient conditions. In isocratic elution, the concentration of organic solvent is constant, therefore, the k' value for each analyte is constant. In gradient separation, the organic solvent is increased with time, causing the k' value for each analyte to decrease with time. Gradient elution provides a solution for the general elution problem, which is the separation of a sample with very wide capacity factor in a reasonable time. Compared to isocratic separation, the peaks will elute in less time with more uniform peak widths, improving the detection of latter eluting peaks. These improvements do, however, decrease the resolving power of the separation. The k' value decreases with the increasing solvent strength until the k' is low enough that analyte desorbs from the column. The relation of retention factor with the solvent strength can be represented by:

$$\log k' = -S\phi + \log k_w \quad \text{Equation 7}$$

where ϕ is the volume fraction of organic solvent in mobile phase B, S is a constant, and k_w is the value of k' at zero organic solvent content ($\phi = 0$)³². The relationship of $\log k'$ and ϕ is shown in Figure 1 by a small molecule and several big molecules such as peptides and proteins.³³

Figure 1. Dependence of capacity factor k' on mobile phase %B (ϕ) for a small molecule benzene and several peptides or proteins. Adapted from reference 33.

As shown in Figure 1, the small molecule benzene has small S value, therefore, a small change in ϕ cause a slow change in k' . It is also assesses that all isocratically eluted peaks are broad. However, for large molecules, such as peptides and proteins, their S value is large and a very small change in ϕ will cause a rapid change in k' causing them to elute in sharp peaks. The greater S , the less partitioning there is after analyte desorbs which contributes to the formation of narrow peaks and improvement of sensitivity. During the separation, different analytes progressively elute as the gradient moves through the reverse phase column, with the more hydrophobic analytes eluting later in the run.³⁴

1.4.2 Protein separations by reverse-phase high performance liquid chromatography (RP-HPLC)

In reverse-phase liquid chromatography, proteins are adsorbed onto the hydrophobic surface of the column and eluted out at high organic content of the mobile phase. The elution order is related to the hydrophobicity of the molecule — the more soluble a solute is in an organic solvent, the greater its retention time. It has been reported that the binding of protein molecules to the hydrophobic surface in RP-HPLC

results in the denaturation of protein structures.³⁵ The RP-HPLC separation process drives the protein molecule to unfold to expose its hydrophobic interior surface for better binding with the hydrophobic stationary phase. The protein binds to the reverse-phase surface through the hydrophobic contact region. Proteins separate in reverse phase because the specific analyte-solvent interactions modulate solubility and give different binding strength for different proteins and peptides. The percentage and type of organic modifier is the most important parameter in adjusting the retention of proteins because its inherently easier to adjust the mobile phase than repack the column.²⁴

Figure 2. Schematic representation of a protein binding to a reverse-phase HPLC silica-based sorbent. The protein interacts with the immobilized hydrophobic ligands through the hydrophobic chromatographic contact region.

1.4.2.1 The development of nonporous C18 reverse-phase liquid chromatography separation on proteins and peptides

Researchers have struggled with finding adequate stationary phases for RP-HPLC separation of proteins, due to their large size and structural heterogeneity. The column is an essential part of liquid chromatography and the development of the column packing material, type, size and shape has also gained substantial attention for a long time. Silica is the most common solid support used in HPLC due to its wide availability in different shapes, sizes, and degrees of porosity. It also can withstand a higher pressure than other material such as resins and soft gels. Most importantly, the surface of the silica can be modified with different chemical bonding reactions. With the advent of HPLC, smaller

and spherical porous or pellicular particles were developed to provide higher separation efficiencies.³⁶

When porous beads were introduced, the surface areas of the binding sites and the loading capacity of the column were greatly increased. However, for protein separations, the use of porous particles tends to reduce separation efficiency and increase retention time. Because proteins are large and approach pore size, it is difficult for proteins to diffuse in and out of pores and they can bind strongly in topographical defects that are very close to proteins in size. Two methods can be used to solve this problem. One is increase the pore size to have perfusion particles, and the other is to eliminate the pores.

Column efficiency is measured by the theoretical plate height, H . Minimizing H increases the number of theoretical plates and the separation efficiency. The theoretical plate height was originally related to the linear flow rate (u) by the Van Deemter equation:

$$H = A + B/u + Cu \quad \text{Equation 8}$$

In which A term is eddy diffusion, caused by the different travel paths of the solvent, B term is longitudinal diffusion, and C term is band broadening caused by stagnant mobile phase mass transfer and stationary phase mass transfer. The detailed expression of each term is listed in Table 1.³⁷ It is obvious from Table 1 that the decrease in particle size, d_p , will decrease both terms A and C. Therefore, the theoretical plate height is minimized by using small uniform particle.

Table 1. Contribution of different band-broadening processes to column plate height H

Process	Contributions to plate height
Eddy diffusion (A-term)	$C_e d_p$
Mobile-phase mass transfer (A-term)	$C_m d_p^2 u / D_m$
Longitudinal diffusion (B-term)	$C_d D_m / u$
Stagnant mobile-phase mass transfer (C-term)	$C_{sm} d_p^2 u / D_m$
Stationary-phase mass transfer (C-term)	$C_s d_f^2 u / D_s$

Note: C_d , C_e , C_m , C_s , C_{sm} = plate height coefficients; d_p = diameter of packing particle; d_f = thickness of stationary-phase layer; u = mobile-phase velocity.³⁷

Several other modifications have been made to the Van Deemter equation to improve its validity and utility. The Hawkes equation is one version that works well with packed column chromatography, because it places more emphasis on the C_m term that is usually negligible in the Van Deemter equation.³⁸ This stationary phase mass transfer directly relates to the situation of a liquid stationary phase.

The use of nonporous particles eliminates adsorption in the pores and eliminates the stagnant mobile phase mass transfer in term C of the Van Deemter equation. Thus, the elimination of this mechanism of band broadening improves the separation efficiency and the protein recovery. These advantages are especially pronounced with the use of smaller particles which is explained in the following paragraphs.³⁹

Snyder and Lommen compared the performance of several different columns that were packed with macroporous, porous, and nonporous particles for protein separations. The highest resolving powers were achieved for peptides and proteins with small nonporous particle packed columns. However, the back pressure is increased due to the small size of nonporous particles.^{16, 40}

1.4.3 Saw-tooth gradient

Generally, when a gradient is used by reverse-phase HPLC to separate a complex biomolecular sample such as whole proteins, the mixture will be separated into many unresolved peaks and only part of the samples will be collected for further analysis. To achieve complete sample transfer between the first- and second-dimension, a novel saw-tooth gradient was created in our lab.¹ This saw-tooth gradient is composed of steps that include positive, negative and horizontal linear portions (as shown in Figure 3.). The solvent strength change ($\Delta\phi$) is the solvent difference between two maximum points of two adjacent saw-tooth steps.

Figure 3. Schematic of saw-tooth gradient components

The saw-tooth gradient was compared with a linear gradient and segmented gradient. The difference between a saw-tooth gradient and a segmented gradient is that a negative slope portion is added after an increasing part in saw-tooth gradient. It was determined that the resolving power of the saw-tooth gradient is comparable with that of the linear gradient and superior to the segmented gradient. The results proved that the negative reduces the band broadening caused by the isocratic elution in the segmented gradient and improves the resolving power of the saw-tooth gradient.

In this thesis, a novel saw-tooth gradient is connected in series as the first-dimension for RP-HPLC separation of proteins prior to a second dimension separation. This novel saw-tooth gradient could separate the complex mixture into small subsets, and send the sample subsets to the second dimension for further separation. The protein subsets can be separated by the second dimension, while an isocratic holding period, of arbitral length, holds the rest of the proteins on the column. A low molecular weight human serum sample is used to determine the effective peak capacity of the saw-tooth gradient. In this real sample, the more abundant peptides with greater peak widths will split in multiple fractions before the lower abundance proteins are. Additionally, some peptides will elute near the end or peak of a saw-tooth gradient step and will split into multiple fractions before the peptides that elute in the middle of each step. This peak splitting demonstrated in experimental data is that one single peptide or protein detected in more than one saw-tooth step. Because the peak splitting will decrease the sensitivity of the detection, peak splitting should be minimized. The extent of peak splitting and number of peptides identified is used to characterize the effective peak capacity as a

function of $\Delta\phi$. Mass spectra acquired during the holding period are used for relative quantitation of the amount of proteins that elute during the isocratic holding periods.

1.5 Mass Spectrometry

1.5.1 Electrospray ionization (ESI)

Electrospray ionization (ESI) is a soft ionization technique that is applicable to charged compounds in liquid matrices. The majority of ions produced by electrospray, for large biomolecules, are multiply charged, which greatly extends the mass range of the instrument as the mass spectrometers measure the m/z (mass-to-charge) ratio of an ion. They are only multiply charged if they have a lot of basic functional groups (for low pH at positive ion mode) and a lot of acidic functional groups (for high pH at negative ion mode). In ESI, ions are generated by the drying of charged droplets over micro second time scales, which is relatively long at the molecular level. The large energy required for ionization is applied slowly, avoiding fragmentation. Thermally labile molecules may be ionized without fragmentation which is the characterization of the soft ionization. Thus, ESI has become an essential tool for characterization of large biomolecules, since its introduction by Fenn.⁴¹

A simplified representation of the ESI process is shown in Figure 4. A potential difference, typically in the 1-4kV range, is applied between the spray tip of the capillary and a counter electrode that is separated 1-2mm. This high voltage produces an electrostatic field to disperse the solution from the spray tip into a fine mist of charged droplets. ESI is initiated when coulombic repulsions exceed surface tension. The charged droplets enter the heated capillary and repeated cycles of solvent evaporation and droplet fission eventually generates bare ions in the gas phase. Then the ions enter the mass analyzer for further analysis.⁴²

Figure 4. Schematic of the mechanism of electrospray ionization

1.5.2 Fourier transform ion cyclotron resonance mass spectrometry (FT-ICRMS)

Fourier transform mass spectrometers are well known for their excellent mass accuracy and resolution. Their potential is fully realized when combined with external ionization technique such as electrospray ionization. The principle of the technique is based on ion cyclotron resonance where the ions are analyzed and detected in a cubical or cylindrical cell. This cell mainly contains four plates: two excitation plates and two detection plates. A magnetic field is applied to the cell which cause the ions move perpendicular to its direction. Initially, the orbit radii of ions in the FT-ICR are too small to be detected. A radio frequency potential pulse is applied to the two excitation plates at the resonant frequency of the ions, which excites the ions increasing the radius of their orbit. Ions of different m/z values orbit at different frequencies, and this orbital motion is called the cyclotron motion. The cyclotron frequency is related to the mass-to-charge ratio (m/z) of the ion and is shown in equation 9:

$$f = \frac{zeB}{2\pi m} \quad \text{Equation 9}$$

f is cyclotron frequency (hertz, Hz), z is the net number of electronic charges on the ion, e is the charge per electron (coulombs, C), B is magnetic field strength (tesla, T) and m is the mass of an ion. Ions of lower m/z will have higher cyclotron frequencies than ions of higher m/z . When these ions pass the detection plates, they induce a change in the plates that is measured electronically, providing non-destructive detection. The induced change on the detection plates is measured as a time-domain free-ion decay signal. The time

dependent ion curves represent the detection of all the ions at the same time with different cyclotron frequencies. The Fourier transform converts this time-domain data into a frequency-domain signal. A mass spectrum is generated by relating the frequency is related to m/z by equation 9.⁴³ The combination of Fourier transform with ICR MS also improves the signal-to-noise ratio (S/N) of the analysis. The signal is degraded when the pressure and the number of the ions inside the ICR cell increase. The reason is that the cyclotron frequencies of the trapped ions are shifted because of the increasing of pressure and the population of the ions. These shifts will affect the measured mass values. Therefore, FT-ICRMS is best interfaced with an external ionization source, such as ESI, to maintain the low pressure in the ICR cell.

Mass accuracy is the measurement of the closeness of the observed m/z to the true value. It is very important when measuring complex samples in which many components could have the similar m/z values. Among the modern commercial mass spectrometers, FT-ICR has mass accuracy to 1ppm or lower where others are in the order of tens of parts per million over a specific mass range.

Resolution is also very important for resolving closely spaced signals, especially a complex mixture. From equation 10, it is obvious that resolution (R) is proportional to the magnetic field strength B and observation time T but inverse to m/z .⁴²

$$R \leq \frac{kzBT}{m} \quad \text{Equation 10}$$

Resolution can be improved by increasing the magnetic field strength or the observation time. Since increasing the strength of the magnetic field is expensive, the observation time is always increased to improve the resolution.

The low molecular weight human serum sample used in this work is a complex sample is composed mainly of peptides with at least thousands of different sequences. Therefore, the high resolving power and mass accuracy of FT-ICR mass spectrometer will aid in determining the number of peptides in each saw-tooth step and which peptides are found in multiple fractions. The peptides are normally multiply charged and the high resolving power of the FT-ICR is needed to resolve the isotopic peaks and determine their charge state. This peptide-like signature is used to distinguish the proteins and

peptides in stead of identifying them by sequence matching. The mass spectra are deconvoluted to determine their accurate mass from the numerous peaks present.

2. Experimental

2.1 Model proteins separation on analytical-HPLC

In this section, all the experiments are carried out on an Agilent 1100 series analytical-HPLC (Agilent Technologies Inc., Delaware, OH) equipped with a 20 μ L manual injector, a binary pump, a column heating chamber and a diode array detector (DAD) with a 5 μ L flow cell. The column used is a 1.5 μ m nonporous silica C₁₈ column (NPS ODS-I, 33 $\times$ 3.0 mm, Eprogen Inc., Darien, IL). Mobile phase A contains 5% HPLC-grade acetonitrile, 0.1% (v/v) glacial acetic acid, 0.05% heptafluorobutyric acid (HFBA), and 95% deionized water. Mobile phase B contains 70% acetonitrile, 0.1% (v/v) acetic acid, 0.05% HFBA and 30% deionized water. All the chemicals are purchased from Fisher Scientific (Fair Lawn, NJ) without further processing. The Chemstation software is used to set the flow rate to 0.2 mL/min, the column heating temperature at 60°C, and the DAD wavelength to 215 nm.

Two proteins are used as model proteins for the peak splitting study on the analytical-HPLC system and each is obtained in lyophilized form from Sigma-Aldrich Co. (St. Louis, MO) and used without further purification. These two standard proteins, ribonuclease A (RNase A, from Bovine pancreas) and carbonic anhydrase I (CA1, from human Erythrocytes), represent a low molecular weight protein (RNase A, 16461 Da) and a high molecular weight protein (CA1, 28870 Da) in the low molecular weight ($\leq$ 30 kDa) human serum proteins. Stock solutions for each protein are prepared separately at a concentration of 50 μ M in mobile phase A and stored at 0°C until used. A mixture of the two proteins for protein peak splitting and recovery studies are prepared by diluting both protein concentrations to 1.0 μ M with mobile phase A. The solvent strength change is decreased from 5.0%, to 1.0%, 0.1% to check the peak splitting. The saw-tooth gradients used for peak splitting studies of standard proteins on analytical-HPLC are summarized in Figure 5. The gradient setup details are listed in Appendix 1. Blank run is tested before each sample injection to check the baseline stability.

Figure 5. Programmed saw-tooth gradients used in peak splitting test by two model proteins on the analytical-HPLC. The linear gradient for the protein recovery test is also presented for comparison. The solvent strength changes ($\Delta\phi$) for method standard-1, standard-2, and standard-3 are 5.0%, 1.0% and 0.1% respectively.

Protein recovery test is carried out to provide supporting evidence for the peak splitting test that is justified by the changing of protein peak areas. The linear gradient used in protein recovery experiments increases the mobile phase B from 0% to 100% in 40 min, plateaus at 100% for 5 min, and decreases to 0% in 5 min (as shown in Figure 5 and Table 2). Each sample run is conducted after a water blank run to check the stability of the baseline.

Table 2. Linear gradient used in protein recovery test on the analytical-HPLC

Time (min)	Solvent B (%)
0.00	0.0
40.00	100.0
45.00	100.0
50.00	0.0

2.2 Anthracene separation on analytical-HPLC

In this section, all the experiments are carried out on an Agilent 1100 series analytical-HPLC. The instrument set up and column used are all the same as for the model proteins except that the DAD wavelength is 225 nm.

Anthracene is purchased from Sigma-Aldrich Co. (St. Louis, MO) and used without further purification. A stock solution of 50 ppm anthracene dissolved in acetonitrile is used. The solvent strength change is decreased from 5.0%, to 1.0%, 0.1% to check the peak splitting. The saw-tooth gradient shapes maintain the same as that used for model proteins except that the start point for the first saw-tooth step is adjusted for anthracene to save time. The linear gradient used to provide supporting information is the same as for the model proteins. The saw-tooth gradients used for peak splitting are summarized in Figure 6. The gradient setup details are listed in Appendix 2. Blank run is tested before each sample injection to check the baseline stability.

Figure 6. Programmed saw-tooth gradients used in peak splitting test by anthracene on the analytical-HPLC. The solvent strength changes ($\Delta\phi$) for method anthracene-1, anthracene-2, and anthracene-3 are 5.0%, 1.0% and 0.1% respectively.

2.3 Gradient shape and column test on nano-HPLC

All the following experiments are carried out on an Agilent 1100 nano-HPLC (Agilent Technologies, Delaware, OH) equipped with a nano-pump and a variable wavelength detector (VWD) with a 35 nL flow cell. The experimental conditions are listed as follows: flow rates are 0.7 $\mu\text{L}/\text{min}$ for gradient shape test and 0.5 $\mu\text{L}/\text{min}$ for protein recovery test on three different columns, column temperature is 60°C, and absorbance is monitored at 215 nm. The column temperature is controlled by a heating matt (Model 03125-10 with extension adapter 03122-71) which is set with a temperature controller (standard model: 89000-00) and monitored by a thermocouple probe (Type T, 08113-22). All the heating equipments are purchased from Cole-Parmer Instrument Co. (Vernon Hills, IL). Mobile phase A contains 0.05% heptafluorobutyric acid, 0.1% acetic acid, 5% acetonitrile and 95% deionized water; mobile phase B is 0.05% heptafluorobutyric acid, 0.1% acetic acid, 70% acetonitrile and 30% deionized water.

2.3.1 Gradient shape test by different saw-tooth gradients

The four saw-tooth gradients used for the nano-HPLC-FTICR MS analysis are monitored on the nano-HPLC to get the actual gradient shape.

To measure the maximum peak capacity, the solvent strength of the saw-tooth gradient is decreased to the minimum to find out if the protein will split into more than one saw-tooth steps. The solvent strength change ($\Delta\phi$) of the saw-tooth gradients is decreased from 1.0%, 0.5%, 0.2% to 0.1% (minimum value allowed by the ChemStation software) with the 26.0-27.0% mobile phase B range (Figure 7, detail set up in Appendix 3). The actual gradient shape of those different saw-tooth gradients are tested on the nano-HPLC by a nonporous C18 capillary column (1.5 μm , 25 mm $\times$ 200 μm , beads purchased from Eprogen, Inc.). The capillary column used in the nano-HPLC for gradient shape experiments is called test capillary column and is obtained in a similar process as the separation column. This test capillary column has the same dimension and packing material as the separation capillary column except the spray tip is replaced by an inline-microfilter with a 1.0 μm stainless steel microscreen (void volume is about 109 nL) (Upchurch Scientific Inc., Oak Harbor, WA) to hold the nonporous beads. All the gradient shapes are tested without sample injection.

Figure 7. Programmed saw-tooth gradients of four different $\Delta\phi$ for LMW (≤ 30 kDa) human serum sample peak splitting studies on the nano-HPLC-FTICR MS. The solvent strength changes ($\Delta\phi$) for method serum-1, serum-2, serum-3, and serum-4 are 1.0%, 0.5%, 0.2%, and 0.1% respectively.

2.3.2 Comparison of protein recovery on three different types of columns

Protein recovery is compared on three different columns. The three columns are: Zorbax 300SB-C18 (3.5 μm , 300 $\text{\AA}$, 150 mm $\times$ 75 μm , Agilent Technologies, Delaware, OH), porous C8 column (5 μm Monitor 100 $\text{\AA}$ -Spherical silica beads, 40 mm $\times$ 200 μm , beads purchased from Column Engineering, Inc., Ontario, CA), nonporous C18 column (1.5 μm , 70 mm $\times$ 200 μm , beads purchased from Eprogen Inc., Darien, IL). The experimental conditions maintain the same for all three columns. Sample is 10.0 μM carbonic anhydrase I in mobile phase A and injection volume is 1.0 μL . Linear gradient begins at 0% B at time 0, increases to 100%B by 40 min, remains at 100% for 10 min, and decreases to 0% B in 5 min.

2.4 LMW (≤ 30 kDa) human serum separation on nano-HPLC-FTICR MS

2.4.1 Nano-HPLC-FTICR MS analysis of LMW (≤ 30 kDa) human serum

The LMW (≤ 30 kDa) human serum sample is prepared by ultrafiltration as described in the Harper et al protocol.¹¹ Preparation of the 30 kDa centrifugal filter membranes is performed according to the manufacture's specifications. One milliliter of human serum (From clotted human male whole blood, Sigma-Aldrich, St. Louis, MO) is processed by first dividing the sample into 200 μ L aliquots, then diluting with 120 μ L of deionized water and 20% (v/v) acetonitrile. Proteins are denatured at 40°C for 15 min; then, centrifuged at 9700 rpm for 10 min to remove any precipitated material that could clog the filters. Supernatants are then added to the prepared centrifugal filters and spun at 3760 rpm for 120 min. The filtrates are lyophilized and stored at -80°C until use. The LMW (≤ 30 kDa) human serum samples are prepared by dissolving one vial of the stock in 20 μ L deionized water which is 0.155mg/mL as measured in section 3.4.2. Then the sample is introduced directly into the separation column by using a pressure vessel (pressure is ~ 30 bar). One microliter of sample is injected for each run and the amount of the injection is monitored by a 5 μ L micro-pipette.

The instruments used are an Agilent 1100 nano-HPLC pump which can generate flow rate from 100 nL to 4.0 μ L and a hybrid linear trap quadrupole 7-Tesla Fourier transform ion cyclotron resonance (FTICR) mass spectrometer (Thermo Finnigan, San Jose, CA). The nano-HPLC conditions are listed as follows: flow rate is 0.7 μ L/min and the column temperature is 60°C.

Prior to analysis of samples, LTQ-FTICR MS is tuned and calibrated by a direct infusion of a solution containing 1pmol/ μ L Angiotensin I and Somatostatin in 50% methanol/0.1 M acetic acid. Both peptides are purchased from Sigma Aldrich (St. Louis, MO) without further purification. The experiment parameters used in direct infusion are: spray voltage is 2.5 kV; capillary temperature is 200°C; sample flow rate is 0.5 μ L/min controlled by syringe injector. The spray tip of 5- μ m inner diameter is made from a 360 $\times$ 100 μ m fused silica capillary by a pre-setting program of a laser puller (Model P-2000, Sutter Instrument Co., Novato, CA).

For analysis of low molecular weight (≤ 30 kDa) human serum proteins, the lyophilized sample is dissolved in 20 μL of deionized water and introduced directly onto the separation column by using a pressure vessel at about 30 bar. The amount of the injection is 1 μL as monitored by a micro-pipette. Data is collected with the FTMS over a scan range of 300-2000 m/z using profile mode by the Tune Plus software at a selected resolution of 200,000 and scan time of 1000 ms. Spray voltage is set at 2.5 kV with positive ESI mode. Heated capillary temperature is set at 200°C.

The separation capillary column used in the nano-HPLC-FTICR MS is packed in a capillary with a pulled tip. In the pulling process, a 360×200 μm fused silica capillary (~ 15 cm long) (Polymicro Technologies, L.L.C., Phoenix, AZ) is obtained and the polyimide coating is removed over a small section (~ 1 cm) in the middle to make a window. Next, the spray tip is pulled with a laser puller (Model P-2000, Sutter Instrument Co., Novato, CA) to make a 20 μm inner diameter tip.⁴⁴ During packing, 5 μm porous silica C18 beads (Monitor, 300 Å, Column Engineering, Inc., Ontario, CA) is loaded with the pressure vessel for 1-2 seconds at 1 bar to form a holding frit (~ 1 mm long). Then, the 1.5 μm nonporous C18 beads are loaded into the column with the pressure vessel at 30 bar until it reaches a length of about 2.5 cm long. After packing, the column is conditioned by first injecting 1 pmol Angiotensin I (Sigma-Aldrich, St. Louis, MO) with the pressure vessel and then running a 30 min linear gradient with the HPLC pump. The conditioned columns are stored in mobile phase A until use. The schematic of the spray tip column is shown in Figure 8.

Figure 8. Schematic of column with spray tip

Maximum effective peak capacity of the saw-tooth gradient is explored by decreasing the solvent strength change from 1.0%, 0.5% and 0.2% to 0.1% (as shown in Figure 7.). A long linear gradient is also designed to compare with the saw-tooth gradient of 0.1% $\Delta\phi$. The effective separation range for the long linear gradient from 26.0%-27.0% mobile phase B is designed to contain only the effective separation time (30.4 min, the isocratic part is subtracted) of the 0.1% $\Delta\phi$ saw-tooth gradient. The long linear gradient increases from 0% to 20% in 10 min, from 20% to 26% in 10 min, from 26% to 27% in 30.4 min, from 27.0% to 100% in 4.6 min (as shown in Table 3). The first 20 min of the long linear gradient is designed to decrease the overall gradient time.

Table 3. The detailed setup of long linear gradient for LMW (≤ 30 kDa) human serum sample on the nano-HPLC-FTICR MS.

Long linear gradient	
t (min)	B (%)
0.00	0.0
10.00	20.0
20.00	26.0
50.40	27.0
55.00	100.0

Xtract in Qual Browser (Xcalibur version is 2.0) is used to deconvolute all spectra acquired. The spectrum Xtract settings are: Generate Masses Mode is M (whole molecular weight), resolution at 400 m/z is 200000, S/N threshold is 2 and the mass range is 300.0-2000.0.

2.4.2 LMW (≤ 30 kDa) human serum mass measurement by protein assay

The concentration of the low molecular weight (≤ 30 kDa) human serum sample is measured by the Bio-Rad Protein Assay. This method is based on a dye-binding assay which will result in a color change when different concentrations of protein bind to dyes in the assay. 450 mL Dye reagent concentrate and lyophilized bovine serum albumin are purchased from Bio-Rad for this experiment. Dye reagent concentrate is diluted by adding 25 mL concentrated dye reagent to 100 mL deionized water (ratio is 1:4). Then the diluted dye reagent is filtered by 0.22 μ m pore size Durapore membrane filter paper

(Millipore, Billerica, MA). Lyophilized bovine serum albumin is dissolved in 20 mL deionized water to make a bulk concentration of 1.44 mg/mL. Four standard bovine serum albumin solutions are made from the stock: 0.2, 0.4, 0.6 and 0.8 mg/mL. 5.0 mL of diluted dye reagent is added to 100 μ L of the above standard solutions and each solution is mixed well. The lyophilized LMW (≤ 30 kDa) human serum sample is dissolved in 10 μ L deionized water and five vials of the same samples are added together to get 50 μ L sample for measurement in VU-Vis absorbance spectrophotometer. 2.5 mL diluted dye reagent is added into the 50 μ L sample. All the standards and the sample are incubated at room temperature for 10 minutes and the absorbance is measured at 595 nm. The measurement is carried out on a Smart Spec 3000 Spectrophotometer (Bio-Rad, Hercules, CA). The concentration of the low molecular weight (≤ 30 kDa) human serum sample is obtained from the standard curve by bovine serum albumin.

3. Results and Discussion

3.1 Model proteins separation on analytical-HPLC

3.1.1 Peak splitting and protein recovery test of two model proteins

Each saw-tooth gradient step is characterized by a net change in solvent strength change ($\Delta\phi$) as shown in Figure 3. If each saw-tooth gradient step is treated as a single fraction, decreasing the step size increases the number of fractions that can be collected and the effective peak capacity. To achieve the best limits of detection in the MS, the average peak width should be matched with the $\Delta\phi$. Thus, there is an optimum $\Delta\phi$ value that maximizes the peak capacity and number of proteins or peptides detected while minimizing the peak splitting.

The objective of this work is to determine this optimum $\Delta\phi$ value which will be used in the determination of the effective peak capacity. This optimum $\Delta\phi$ value will be used in place of the separation efficiency in the calculation of the peak capacity. To determine if peak splitting could be observed directly, two protein standards, ribonuclease A and carbonic anhydrase I, are separated at $\Delta\phi$ values of 5.0%, 1.0%, and 0.1% as shown in Figure 9. No attempt is made to align the center of the $\Delta\phi$ of the saw-tooth gradient step with the median $\Delta\phi$ value of the peak. Thus, the median ϕ value of elution of the sample band may or may not be aligned with the positive slope segment of

the saw-tooth step. Figure 9 (B) shows no peak splitting for RNase A. Figure 9 (B) shows three peaks for the second standard, CA1. As observed in Figure 9 (A), CA1 has a small peak at the tail of the main peak which is also present with the linear gradient. The small peak is resolved better in all of the three saw-tooth gradients and is caused by the partially denatured structures of CA1 when binding with the hydrophobic surface of the column.^{45,46} In the linear gradient, a primary peak and an additionally smaller peak are clearly detected. The presence of multiple isoforms for carbonic anhydrase 1 has been noted in the literature.⁴⁷ In the saw-tooth gradient, three peaks are detected, and it is unclear whether the third peak is from another isoform or from peak splitting.

Figure 9. Chromatograms of model proteins produced by linear and saw-tooth gradients at various $\Delta\phi$ values. (A) Separation of RNase A and CA1 using a linear gradient; the retention time of RNase is 16.3 min and the retention time of CA1 is 25.5 min. (B) The peak shape obtained with saw-tooth gradient for solvent strength changes ($\Delta\phi$) of 5.0%, 1.0% and 0.1%. The slope of the linear gradient is the same as all the positive segment of the saw-tooth gradients.

Although peak splitting is not definitely observed, it is possible that some of the sample may not elute in a detectable manner. For instance, a portion of the sample may “bleed off” the column in a broad undetectable peak. Therefore, the peak areas of the standards at a number of $\Delta\phi$ values are compared with the peak area obtained with the linear gradient. The peak area obtained with the linear gradient is assumed to represent complete elution. In addition to incomplete protein recovery, other deleterious effects of the saw-tooth gradient adversely affect peak width and symmetry. Further peak splitting effect is explored on the main peak of RNase and CA1 by comparing the peak area, peak width and asymmetry obtained from the saw-tooth gradients with the same parameters from the linear gradient. Peak asymmetry is measured at the 10% peak height as B/A , where A is the distance from peak front to peak maximum and B is the distance from peak maximum to peak end (Figure 10).³⁴ As can be seen from Figure 10, peaks of good symmetry has a B/A value that equals 1. Peak tailing gives B/A value greater than 1 and peak fronting gives B/A value smaller than 1.

Figure 10. Illustration for measurement of peak asymmetry. Peak asymmetry is measured at the 10% peak height as B/A , where A is the distance from peak front to peak maximum and B is the distance from peak maximum to peak end.

The average values of four experimental results are compared by T-test as in Table 4. There is no significant difference between the saw-tooth and the linear gradient for peak area and peak width for both proteins. However, the asymmetries of the three saw-tooth gradients are all significantly greater than the asymmetry of the linear gradient for RNase A. Although the asymmetry T-test for CA1 is not significant for all three saw-tooth gradients, it does show that the asymmetry value of CA1 is higher than that of the linear gradient. When designing the experiments, the linear gradient and the three saw-tooth

gradients are carried out in the same day under the same experimental conditions with varying orders of data acquisition to rule out the systematical error. Therefore, the tailing of the protein peak in three saw-tooth gradients is due to the specific design of the saw-tooth gradient. Under the linear gradient, the peak tail elutes under high solvent strength and the peak is focused, which helps provide symmetric peaks. For a saw-tooth gradient, the situation is reverse compared with what happens by a linear gradient. The peak front has stronger organic content than the peak tail, which increases peak tailing.

Table 4. Comparison of model protein peaks produced by linear and saw-tooth gradients at various $\Delta\phi$ values. T-tests are carried out on peak area, peak width and asymmetry of each protein to compare the average value of the saw-tooth gradients with the linear gradient.

(A) Comparison of peak areas

Protein	Saw-tooth			Linear gradient		t-test (P-value)
	$\Delta\phi$	Average peak area (mAU·s)	Std dev	Average peak area (mAU·s)	Std dev	
RNase A	5.0%	221	12	222	3	0.93
	1.0%	233	17			0.28
	0.1%	235	12			0.23
CA1	5.0%	478	15	500	17	0.32
	1.0%	508	23			0.74
	0.1%	494	41			0.77

(B) Comparison of peak widths

Protein	Saw-tooth			Linear gradient		t-test (P-value)
	$\Delta\phi$	Average peak width (min)	Std dev	Average peak width (min)	Std dev	
RNase A	5.0%	0.28	0.008	0.29	0.018	0.84
	1.0%	0.29	0.028			0.91
	0.1%	0.28	0.013			0.81
CA1	5.0%	0.29	0.008	0.30	0.013	0.22
	1.0%	0.30	0.005			0.68
	0.1%	0.30	0.013			0.48

(C) Comparison of peak asymmetries

Protein	Saw-tooth			Linear gradient		t-test (P-value)
	$\Delta\phi$	Average peak asymmetry	Std dev	Average peak asymmetry	Std dev	
RNase A	5.0%	3.5	1.4	1.1	0.17	0.02*
	1.0%	4.0	1.1			0.008*
	0.1%	3.5	0.95			0.02*
CA1	5.0%	3.4	0.25	2.6	0.96	0.18
	1.0%	3.3	0.49			0.20
	0.1%	3.3	0.53			0.22

*: Significant

3.2 Anthracene separation on analytical-HPLC

Previous experiments show no peak splitting for proteins. It is suspected that proteins have a very large S value (as indicated in Figure 1) which makes capacity factor (k') changing rapidly with a small change of organic content in solvent B (ϕ) and results in no peak splitting for proteins. However, small molecules such as anthracene have a small S value which makes k' changing slowly with small change of ϕ . Therefore, small molecules are susceptible to split into more than one saw-tooth steps. Anthracene with molecular weight of 178.32 is chosen to represent a small molecule to see if peak splitting can be observed.

The chromatograms of the peak splitting and peak recovery for anthracene are presented in Figure 11 with programmed gradients superimposed. Compared with linear gradient, no peak splitting is observed for anthracene by three different saw-tooth gradients. However, it is noticed that the peak area from three saw-tooth gradients are much smaller than the peak area from the linear gradient. T-tests are carried out on peak area and peak width for each anthracene peak to compare the average value of the saw-tooth gradients with those from the linear gradients. The results are summarized in Table 5. As can be seen from Table 5, the peak areas of anthracene by three saw-tooth gradients are all significantly smaller than the peak area by the linear gradient, which indicates anthracene is not fully recovered in the observable peaks. The peak area of anthracene decreases with the decreasing of the $\Delta\phi$ of the saw-tooth gradient. The peak width of anthracene shows the same trend as that of peak area. Both data indicate that peak splitting occurs when saw-tooth gradients are employed. The reduced peak area of anthracene further confirms the experimental results of the slow change of the capacity factor with organic content in solvent B for small molecules.

Therefore, the saw-tooth gradient is well suited for large biomolecules such as proteins that elute in narrow $\Delta\phi$ ranges. Because proteins elute in narrow $\Delta\phi$ ranges, a relatively high effective peak capacity can be obtained.

Figure 11. Chromatograms of anthracene produced by linear and saw-tooth gradients at various $\Delta\phi$ values with the programmed gradients are superimposed. The solvent strength changes ($\Delta\phi$) for three saw-tooth gradients gradient-2, gradient-3 and gradient-4 are 5.0%, 1.0% and 0.1% respectively. The decreased peak area of the lower molecular weight anthracene is significantly different from the observed results with the protein standards that showed full recovery.

Table 5. Comparison of anthracene peaks produced by linear and saw-tooth gradients at various $\Delta\phi$ values. T-tests are carried out on peak area and peak width to compare the average value of the saw-tooth gradients with that of the linear gradient.

(A) Comparison of peak areas						
Protein	Saw-tooth			Linear gradient		t-test (P-value)
	$\Delta\phi$	Average peak area (mAU·s)	Std dev	Average peak area (mAU·s)	Std dev	
Anthracene	5.0%	1349	33	2008	71	<0.001*
	1.0%	1265	64			<0.001*
	0.1%	1188	30			<0.001*

(B) Comparison of peak widths						
Protein	Saw-tooth			Linear gradient		t-test (P-value)
	$\Delta\phi$	Average peak width (min)	Std dev	Average peak width (min)	Std dev	
Anthracene	5.0%	0.76	0.015	0.51	0.002	<0.001*
	1.0%	0.78	0.013			<0.001*
	0.1%	0.81	0.013			<0.001*

*: Significant

3.3 Gradient shape and column test on nano-HPLC

3.3.1 Gradient shape test by different saw-tooth gradients

An important characteristic of the saw-tooth gradient is that the features created by the changes in sign of the slope or the gradient “teeth” are susceptible to rounding in which the sharp features in solvent strength are lost. This is a main concern when the saw-tooth gradient is used with a capillary column and low flow rates. Gradient rounding is related to the dwell volume and dispersion or loss of the desired shape. Dispersion and gradient rounding can be also caused by the same processes that give rise to band dispersion in transfer capillaries or tubes, including Taylor dispersion caused by the parabolic profile of pressure driven flow and longitudinal diffusion. The dwell volume is defined as the volume between the point at which solvents are mixed and the beginning of the column.⁴⁸ The volume of the mixer plays a major role in the amount of gradient rounding. For most effective retention of the initial gradient shape, it is important to minimize the dwell volume between the pumps and the column inlet. To monitor the gradient shape no injection is made and the gradient shape is followed by monitoring the change in solvent absorbance from the changes in acetonitrile

concentration. Thus, the actual gradient shape could be monitored at the detector, and compared with the programmed gradient. Both the programmed gradient and gradient shape measured by the absorbance detector are shown with the total ion chromatograms (TIC) in Figure 12. As shown in Figure 12 (A-D), the actual saw-tooth gradient steps still provide an incremental increase with the $\Delta\phi$ decreases from 1.0% to 0.5%, 0.2% and 0.1%.

Figure 12 (A - B). Total ion chromatograms of two saw-tooth gradients ($\Delta\phi$ is 1.0% and 0.5%) with the gradients superimposed. In each TIC, the fractions that are used for total protein count and protein overlapping are marked. (A) TIC obtained by saw-tooth gradient serum-1: $\Delta\phi = 1.0\%$, fraction 3 used; (B) TIC obtained by saw-tooth gradient serum-2: $\Delta\phi = 0.5\%$, fraction 4 and 5 used.

Figure 12 (C - D). Total ion chromatograms of two saw-tooth gradients ($\Delta\phi$ is 0.2% and 0.1%) with the gradients superimposed. In each TIC, the fractions that are used for total protein count and protein overlapping are marked. (C) TIC obtained by saw-tooth gradient serum-3: $\Delta\phi = 0.2\%$, fraction 4, 5, 6 and 7 used; (D) TIC obtained by saw-tooth gradient serum-4: $\Delta\phi = 0.1\%$, fraction 4 to 13 used.

The degree of gradient rounding is related to V_M/V_G , where V_M is the volume from mixer to column head, and V_G is the gradient volume.³³ A smaller V_M/V_G causes reduced gradient rounding. Generally, when $V_M/V_G > 0.1$, the gradient is significantly distorted.⁴⁹ For the Agilent 1100 nano-HPLC used here, a built-in splitter, electronic flow control (EFC) which contains an electromagnetic proportional valve and a flow sensor, are used to deliver the nano-scale flow rate to the column while dumping a very large portion of the solution into waste (Figure 13).

Figure 13. Schematic of connections from mixing point of Agilent 1100 nano pump to the separation column.⁵⁰ V_0 is volume from mixing point to the tubing after the filter. V_1 is the volume from EMPV to the flow sensor, V_2 is the connecting tubing from the flow sensor to a zero dead volume Tee connector, and V_3 is the empty tubing before the packing of the separation column.

The flow rate before the splitter is 2.5mL/min and the total volume (V_0) is 398 μ L which make the delivery time very short (9.6 seconds) during this section,⁵⁰ producing a negligible amount of distortion before the EFC. The effective mixing volume includes the volume after EMPV and contains the volume from the EMPV to the column inlet.⁵⁰ V_M can be obtained by the following equation 11:⁴⁹

$$V_M^2 = V_1^2 + V_2^2 + V_3^2 \quad \text{Equation 11}$$

Where V_1 is the volume from EMPV to the flow sensor, V_2 is the connecting tubing from the flow sensor to a zero dead volume Tee connector, and V_3 is the empty tubing before

the packing of the separation column. In this experiment, V_1 is $0.3\mu\text{L}^{50}$, V_2 is $0.18\mu\text{L}$ ($360\text{mm}\times 25\mu\text{m}$ capillary tubing), and V_3 is $3.77\mu\text{L}$ ($120\text{mm}\times 200\mu\text{m}$), yielding V_M of a $3.8\mu\text{L}$. Clearly, V_M is dominated by the empty tubing before the packing in the column. The effective gradient time (t_G) for the saw-tooth gradient with $0.1\%\Delta\phi$ is 70 min and the volumetric flow rate (F) is $0.7\mu\text{L}/\text{min}$. V_G ($49\mu\text{L}$) can be calculated from the following equation 12, which is $49\mu\text{L}$.

$$V_G = t_G \times F \quad \text{Equation 12}$$

The gradient rounding of V_M/V_G is 0.08. The saw-tooth gradient with $0.1\%\Delta\phi$ is still in allowable range without severe rounding by the nano-HPLC as shown by the actual gradient shape in Figure 12 (D).

A more detailed numerical analysis of the saw-tooth gradient rounding for $0.1\%\Delta\phi$ is presented in Table 6. It is noticed that the pump delivery is stabilized after the first few saw-tooth steps following the long linear start. The absorbance differences between two adjacent peak points are calculated from the third “teeth” where the pump delivery is stabilized. The average value of absorbance is 0.016 mAU with std. dev. of 0.005 and relative standard deviation of 31%. Although the gradient rounding is substantial for $\Delta\phi=0.1\%$, as shown in Figure 12 (D), there is a consistent increase in $\Delta\phi$ with each saw-tooth step once the gradient stabilizes, as shown in Table 6. The large relative standard deviation indicates that the pump is working near the limit of the minimum $\Delta\phi$ for which it can produce consistent increases in solvent strength. The maximum peak capacity experiment with serum in the range of 26.0%-27.0% solvent B is designed to begin at the third “teeth” when the pump delivery is stable.

Table 6. The absorbance maxima for each tooth or step of the saw-tooth gradient for a 0.1% $\Delta\phi$. The average and standard deviation of the absorbance difference between two adjacent peak points is calculated.

serum-4 gradient ($\Delta\phi = 0.1\%$)		
B (%)	Absorbance (mAU)	Absorbance difference between peak points (mAU)
26.1	4.963	—
26.2	4.975	0.012
26.3	4.981	0.006
26.4	4.999	0.018
26.5	5.018	0.019
26.6	5.042	0.024
26.7	5.060	0.018
26.8	5.072	0.012
26.9	5.087	0.015
27.0	5.110	0.023
		Average: 0.016 mAU
		Std dev: 0.005 RSD: 31%

Two other important limitations of the HPLC system for the saw-tooth gradient is the number of points that can be set and the precision to which the gradient can be set. The maximum number of points that can be set in the Chemstation software is 50, limiting the maximum number of peaks of saw-tooth steps to 15. The maximum settable precision of the gradient is one-tenth of a percent, which limits the minimum $\Delta\phi$ to 0.1%.

3.3.2 Comparison of protein recovery on three different types of columns

Theoretically, columns packed with nonporous beads provide better separation efficiency for protein separation than porous beads. The small nonporous beads decrease eddy diffusion and improve the separation efficiency. In order to verify that the nonporous beads perform better than the porous beads with low molecular weight serum sample, columns packed with nonporous and porous beads are compared. Figure 14 presents the results of the protein recovery tests on three reverse phase columns with different types of beads or packing material. The retention times of CA1 on three columns are around 70 min as shown in Figure 14. It is observed that the retention time of the CA1 is far behind the end point of the gradient. This discrepancy is caused by the extra volume from the autosampler added between nano-pump and the column. However, there is a huge

difference between the protein recoveries between nonporous bead packed column and the porous ones. The protein peak intensity on the nonporous bead column is more than 10 fold of that of porous columns. The higher protein recovery of the nonporous C18 column indicates it is the best among the three columns. This result is consistent with the previous observations that nonporous beads provide greater separation efficiency for proteins by eliminating inner-pore diffusion.^{16, 51-53}

Figure 14. Protein recovery on three RP-HPLC columns packed with different types of beads. Nonporous beads packed column has the highest protein recovery rate among three types of columns. The dead time is about 5 min.

3.4 LMW (≤ 30 kDa) human serum separation on nano-HPLC-FTICR MS

3.4.1 Nano-HPLC-FTICR MS analysis of LMW (≤ 30 kDa) human serum

Here, the effective peak capacity with minimum sample splitting is evaluated using the low molecular weight fraction of human serum. The sample is prepared by ultrafiltration of the whole human serum. Human serum contains on the order of ten thousand proteins with a dynamic range of protein concentration that spans nine orders of magnitude, making it a complex sample with components having a wide range of

concentrations. The LMW (≤ 30 kDa) human serum contains many low molecular weight components, especially peptides. The peptides are not identified by sequence determination; instead, the molecular weight is used to distinguish among different peptides.

Initially, it is anticipated that the number of components that are split between multiple fractions will increase as the $\Delta\phi$ value decreases, and an optimum value will be observed after which excessive peak splitting will diminish the ability to detect additional proteins. The effects of decreasing the magnitude of the $\Delta\phi$ value on the peak splitting and the total number of proteins identified are investigated. As the $\Delta\phi$ value decreases, it is expected that peak splitting will increase and that peak splitting will eventually cause a decrease in the number of proteins identified. It was hypothesized that $\Delta\phi$ values significantly narrower than the average peak width will divide the components in multiple fractions, decreasing the S/N in the MS. Because the endpoints of each saw-tooth gradient step are arbitrarily set, components that elute at these $\Delta\phi$ values are expected to split into two fractions. Broader peaks are expected to split into multiple fractions before narrower peaks split. Thus some peak splitting is anticipated. In this real sample, differences in peak width are caused by differences in relative abundance and by differences in the absorptive properties of the peptides. The objective of many proteomic experiments is to identify as many proteins as possible from a complex sample and the most difficult proteins and peptides to detect are the low abundance proteins and peptides. Therefore, the effective peak capacity should be characterized with signals of lower intensity and the $\Delta\phi$ value at which these peaks split corresponds to the peak width term in the calculation of the peak capacity. It should be noted that while splitting of the higher intensity signals increases as the $\Delta\phi$ value decreases, the number of protein identification increases over the range tested. It should be noted that the LMW (≤ 30 kDa) human serum sample provides a realistic and stringent test because peptide have lower molecular weight values than that of proteins.

Separations are performed for $\Delta\phi$ values of 1.0%, 0.5%, 0.2%, and 0.1%. The total ion chromatogram of the eluted proteins and the programmed gradients are shown in Figure 12. Due to the instrumental limitation of a maximum of 50 data points, a range of

26.0% to 27.0% solvent B was focused on. A capillary column is used for the separation to increase the separation efficiency and to use a format that is consistent with standard proteomic methods. An electrospray interface is used to inject the peptides directly into the MS, and the peptide masses are measured at high mass accuracy (<2 ppm). The peptide signals are identified using an automated deconvolution routine and the identifications are checked manually. Manual checking of the identifications returned by the deconvolution routine revealed many signals that could not be validated due to low signal to noise and a lack of the characteristic isotopic peaks. When the deconvoluted masses are compared within the triplicate runs it is found that the signals that could not be manually validated did not appear in the multiple runs. Upon comparing the triplicate runs, only the signals that are common to all three of the chromatograms are considered real and are reported in Figure 16 and Table 7. This approach provided a means to select reliable matches in a rapid and repeatable manner. The TICs are shown in Figure 12 (A-D) indicate that groups of peptides clearly elute in each step of the saw-tooth gradient and appear as well defined peaks. After elution of the peak associated with each saw-tooth gradient step, the peptides are largely held on the column during the holding period. As the $\Delta\phi$ of each saw-tooth gradient step is reduced, the area of the corresponding peak decreases, because the amount of protein eluted with each step decreases.

Using linear gradient as a standard, the total protein and the protein splitting numbers by four saw-tooth gradients are compared in the solvent strength range of 26.0%-27.0% mobile phase B (Figure 16 and Table 7). For a saw-tooth gradient with 1.0% $\Delta\phi$, only one fraction (fraction 3 in Figure 12 (A)) is contained in this range, therefore, only total protein numbers are counted for this saw-tooth gradient. For saw-tooth gradient with 0.5% $\Delta\phi$, two fractions (fraction 4 and 5 in Figure 12 (B)) are used for total protein and overlapping protein numbers counting. There are four fractions for saw-tooth gradient with 0.2% $\Delta\phi$ (fraction 4 to 7 in Figure 12 (C)) and ten fractions for saw-tooth gradient with 0.1% $\Delta\phi$ (fraction 4 to 13 in Figure 12 (D)).

It can be seen that the number of peptides split in more than one fractions increases from 5 to 28 (or 24) as the $\Delta\phi$ value decreases from 0.5%, and 0.2% to 0.1%. This increased splitting is not advantageous, but the total number of peptides increases

from 30 to 59 and 24 to 67 as the $\Delta\phi$ value decreases (as shown in Figure 16 and Table 7).

For saw-tooth gradients with a 0.1% $\Delta\phi$, the mobile phase B only increases 1.0%, one may suspect that if the result of this saw-tooth gradient is the same as a similar linear gradient, then it has no sense to waste such a long time (66.4 min include isocratic part) to get the same result as a linear gradient. The saw-tooth gradient provides unique capabilities. However, the saw-tooth gradient could adversely affect the S/N of the peak. The linear gradient is used as the standard to compare the peaks from the saw-tooth gradient to determine if the peaks are adversely affected. For saw-tooth gradient, some gradient character is retained compare to the similar linear gradient. The extra time includes the negative slope part and the isocratic part. Since the isocratic part acts as a holding period to carry out a second dimensional separation and the length can be set arbitrarily, it is not counted as the effective separation time. The effective separation time only contains the positive slope part and the negative slope part which is 30.4 min for saw-tooth gradient with a 0.1% $\Delta\phi$. The total protein count range for the long linear gradient is also 26.0%-27.0% of mobile phase B which is 28.2-58.6min after adding the dwell time (Figure 15).

Figure 15. Total ion chromatogram of the long linear gradient with the gradient superimposed. The fractions that are used for total protein count and protein overlapping are marked, total $\Delta\phi = 1.0\%$, 26.0%-27.0% solvent B range is used.

The total peptide signals detected using the long linear gradient is also presented in Figure 16. The total peptide signals detected by the 0.1% $\Delta\phi$ saw-tooth gradient (59 and 67, as indicated in Table 7) is larger than that of the long linear gradient (54 and 58, as indicated in Table 7). A loss in the peak capacity is not observed when compared with a long linear gradient. In fact, the number of peptide signals detected during the steps of the saw-tooth gradient was greater for the saw-tooth gradient than the corresponding long linear gradient.

Because the primary objective of proteomic experiments is protein or peptide identification, the saw-tooth gradient with a $\Delta\phi$ of 0.1% provides the best overall performance. Unfortunately, instrumental limitations prevent the programming of gradient steps smaller than 0.1%, prohibiting the analysis of smaller steps. However, previous experiments indicate that 0.1% is near the instrumental limit of the HPLC nano pump. This instrumental limitation could be overcome by narrowing range of solvents A and B. Therefore, over a range of 0 to 100% solvent B, 1000 fractions could be collected

for a saw-tooth gradient with a $0.1\%\Delta\phi$. However, the proteins and peptides do not elute over the entire range which makes the actual fractions or groups of proteins that can be collected on the order of a few hundred.

Figure 16. Total peptide signals detected and protein splitting numbers of four saw-tooth gradients in the 26.0%-27.0% range of solvent B. Each series corresponds to peptides signals common to all three runs of a triplicate set. Data of two triplicate sets are presented. Total protein of the long linear gradient at the same separation time range with $0.1\% \Delta\phi$ saw-tooth gradient is also presented for comparison.

Table 7. Detailed results of total peptide signals detected and protein splitting numbers of four saw-tooth gradients and a long linear gradient in the 26.0%-27.0% range of solvent B. Numerical data plotted in Figure 16.

Group 1 result — in black

Gradient type	Saw-tooth				Linear
$\Delta\Phi$	1.0%	0.5%	0.2%	0.1%	1.0%
Proteins in 1 fraction	25	25	24	31	54
Split in 2 fractions	—	5	14	17	—
Split in 3 fractions	—	—	4	6	—
Split in 4 fractions	—	—	1	1	—
Split in 5 fractions	—	—	0	2	—
Split in 6 fractions	—	—	—	2	—
Split in 7 fractions	—	—	—	0	—
Split in 8 fractions	—	—	—	0	—
Split in 9 fractions	—	—	—	0	—
Split in 10 fractions	—	—	—	0	—
total splittings	0	5	19	28	—
total proteins	25	30	43	59	54

Group 2 result — in grey

Gradient type	Saw-tooth				Linear
$\Delta\Phi$	1.0%	0.5%	0.2%	0.1%	1.0%
Proteins in 1 fraction	21	19	29	43	58
Split in 2 fractions	—	5	17	17	—
Split in 3 fractions	—	—	2	3	—
Split in 4 fractions	—	—	1	2	—
Split in 5 fractions	—	—	0	0	—
Split in 6 fractions	—	—	—	0	—
Split in 7 fractions	—	—	—	1	—
Split in 8 fractions	—	—	—	1	—
Split in 9 fractions	—	—	—	0	—
Split in 10 fractions	—	—	—	0	—
total splittings	0	5	20	24	—
total proteins	21	24	49	67	58

A surprising result is that the total number of peptide signals detected with the saw-tooth gradient is slightly more than the number of peptide signals detected with the long linear gradient. Further comparisons are needed to better understand this improvement in the number of peptide signals detected. As presented by Figure 17, a saw-tooth gradient with 5.0% $\Delta\phi$ is compared with two linear gradients in the 45.0-65.0%

solvent B range. Linear gradient-1 is increased from 0 to 100% solvent B in 40min, producing the same 2.5%/min slope as the saw-tooth gradient and only takes 8min to bring solvent B from 45.0-65.0%. Linear gradient-2 is increased from 0 to 45% solvent B in 18min, 45% to 65% in 20min, and 65% to 100% in 2 min. It has the same effective separation time (20min from 45% to 65% solvent B) as the saw-tooth gradient. Linear gradient-1 has 2.5%/min slope, which is the same slope as the positive slope segment of the 5.0% $\Delta\phi$ saw-tooth gradient. Linear gradient-2 has the same total time and solvent strength change as the 5.0% $\Delta\phi$ saw-tooth gradient and has a slope of 1.0%/min. The total time for the 5.0% $\Delta\phi$ saw-tooth gradient is calculated for the positive and negative slope segments only without the isocratic portion. The isocratic portion is not included because the length of the holding period is arbitrary. Compared with linear gradient-1, the saw-tooth gradient has the same slope as linear gradient-1 but a longer separation time which increase the peak capacity. As to linear gradient-2, the saw-tooth gradient has the same separation time but a steeper slope which could improve the sensitivity. Therefore, the saw-tooth gradient has a better resolving power and signal to noise ratio compared to the corresponding linear gradients which explain the results of the LMW (≤ 30 kDa) human serum on the series saw-tooth gradients. With the decreasing of $\Delta\phi$, the separation time of saw-tooth gradient is elongated with a steeper slope as compared with corresponding linear gradients to provide better resolution and sensitivity. These improvements lead to the detection of more peptide and protein signals in the sample.

As presented previously, proteins have large S values and only can observe splitting with saw-tooth gradient. While peptides of small molecular weight have small S value and making them more susceptible to peak splitting. The masses of the signals detected are less than 5kDa.

Figure 17. Theoretical comparison of the saw-tooth gradient with two linear gradients in the 45.0-65.0% solvent B range. Saw-tooth gradient has a 2.5%/min slope with 20 min total effective separation time. Linear gradient-1 has the same slope as the saw-tooth gradient (2.5%/min), while linear gradient-2 has the same separation time as saw-tooth gradient.

Another concern is whether proteins or peptides elute isocratically during the holding period. The mass spectra of the 0.1% solvent strength saw-tooth gradient from Figure 12 (D) are analyzed to determine the extent of protein loss in the isocratic part. Fraction 4, fraction 5 and the isocratic holding segment between fraction 4 and 5 are explored. Their mass spectra are presented in Figure 18. After deconvolution of the spectra in the isocratic segment, the peptide masses are compared with the fractions right before and after it. It is observed that proteins found in this holding period are either already appeared in its previous or appear in later fractions. Four representative peak spectra are presented: m/z : 772.57; m/z : 719.80; m/z : 847.57; and m/z : 822.68. The monoisotopic masses for each m/z after deconvolution are also presented in Figure 18. The spectra show some elution of the peptides during the isocratic portion of the gradient,

at levels that are roughly between 10-20% of the peak intensity during the saw-tooth elution. This level of isocratic elution is not significant and a second dimension separation could be carried out during this period with negligible loss peptide signal intensity.

Figure 18. Mass spectra of fractions when $\Delta\phi = 0.1\%$ from Figure 12(D). Peptides signals detected in fraction 4 are labeled with “*” while proteins identified in fraction 5 are labeled with “#”. A) Mass spectrum of fraction 4; B) Mass spectrum of isocratic part between fractions 4 and 5; C) Mass spectrum of fraction 5. Note changes in the intensity scale. D) Four representative isotopic distributions of peptides from isocratic segment are listed with their monoisotopic masses: (1) m/z : 772.57; (2) m/z : 719.80; (3) m/z : 847.57; (4) m/z : 822.68.

3.4.2 Measure of the total protein content in the LMW (≤ 30 kDa) human serum fraction

To determine the total protein content in the human serum sample, the Bio-Rad protein assay colorimetric method is used. A calibration curve is made by measuring the absorbance of different concentrations of the standard, BSA, by the Smart Spec 3000 Spectrophotometer. As shown in Table 8, the absorbance is plotted as a function of concentration in Figure 19. The absorbance of the LMW (≤ 30 kDa) human serum fraction is measured for three samples, and the concentration of the sample is obtained using the calibration curve. The average concentration of the sample is 0.309 mg/mL (Table 9). Because the total proteins concentration is very low for the LMW (≤ 30 kDa) human serum to be measured by protein assay, the measured concentration of the LMW (≤ 30 kDa) human serum sample dissolved in 10 μ L is appropriate for the measurement. The concentration of the LMW (≤ 30 kDa) human serum sample for LC-FTMS experiments is dissolved in 20 μ L to avoid long cleaning time of the less hydrophobic peptides and proteins. The total peptides and proteins concentration measured for the sample is 0.155 mg/mL. Since only 1 μ L sample is injected into the capillary column, the mass of proteins for nano-LC-FTMS analysis is 0.155 μ g.

Table 8. Measurement of absorbance on different concentration of standard BSA

Concentration of BSA (mg/mL)	0.2	0.4	0.6	0.8
Absorbance	0.254	0.461	0.629	0.815

Figure 19. Standard curve of BSA together with the concentration of LMW (≤ 30 kDa) human serum determined by this standard curve.

Table 9. The absorbance and corresponding concentration of the LMW (≤ 30 kDa) human serum fraction. The average concentration is obtained from the three sample measurements.

Sample #	1	2	3	Average Conc. (mg/mL): 0.309
Absorbance, y	0.378	0.358	0.354	
Conc. (mg/mL), x	0.325	0.304	0.299	

4. Conclusions

The performance of saw-tooth gradient is explored by two model proteins and a real proteomic sample. Peak splitting is studied by two model proteins on analytical-HPLC. No peak splitting is observed for both proteins by decreasing the solvent strength change from 5.0%, 1.0%, to 0.1%. However, the peak tailing is more severe for the saw-tooth gradient than for the linear gradient. A small molecular weight anthracene is used for peak splitting by saw-tooth gradients with $\Delta\phi$ decreases from 5.0% to 1.0% and 0.1%. The obvious peak splitting explained the theoretical prediction that saw-tooth gradient works great for large molecules such as proteins. The protein recovery is compared among three different columns: porous C8, porous C18 and nonporous C18. Nonporous C18 column has the best protein recovery among three columns. Peak capacity of the saw-tooth gradient is explored by a real proteomic sample on nano-HPLC-FTICR MS by decreasing the solvent strength change from 1.0%, 0.5%, 0.2%, to the minimum of 0.1% in the 26.0%-27.0% solvent B range. The total peptide signals detected and peptide splitting number are both increased with the decreasing solvent strength change. However, the total peptide signals detected number increases more rapidly than the peptide split numbers. A loss in the peak capacity is not observed when compared with a long linear gradient. Moreover, protein loss is little in the isocratic part of the saw-tooth gradient. Consider the primary objective of the proteomic experiment is protein or total peptide signals detected, the saw-tooth gradient with a $\Delta\phi$ of 0.1% provides the best overall performance. With the saw-tooth gradient, a slightly greater number of peptide signal is detected, allbut at a cost of time. However, it does provide a holding period that can be used for a downstream operation, such as a second dimension of separation. The sample injection amount is substantially decreased by the capillary column which is also important for the analysis of biology samples. Overall, the saw-tooth gradient provides a promising first-dimension separation technique for peptides and proteins without a complex instrumental setup.

References

- (1) Morris Daniel, L., Jr.; Sutton Jennifer, N.; Harper Robert, G.; Timperman Aaron, T. *Journal of proteome research* **2004**, 3, 1149-1154.
- (2) Liebler, D. C. *Introduction to Proteomics: Tools for the New Biology*; Humana Press, 2002.
- (3) Issaq, H. J. *Electrophoresis* **2001**, 22, 3629-3638.
- (4) Zolotarjova, N.; Martosella, J.; Nicol, G.; Bailey, J.; Boyes, B. E.; Barrett, W. C. *Proteomics* **2005**, 5, 3304-3313.
- (5) Adkins, J. N.; Varnum, S. M.; Auberry, K. J.; Moore, R. J.; Angell, N. H.; Smith, R. D.; Springer, D. L.; Pounds, J. G. *Molecular and Cellular Proteomics* **2002**, 1, 947-955.
- (6) Anderson, N. L.; Anderson, N. G. *Molecular and Cellular Proteomics* **2002**, 1, 845-867.
- (7) Martosella, J.; Zolotarjova, N.; Liu, H.; Nicol, G.; Boyes, B. E. *Journal of Proteome Research* **2005**, 4, 1522-1537.
- (8) Tirumalai, R. S.; Chan, K. C.; Prieto, D. A.; Issaq, H. J.; Conrads, T. P.; Veenstra, T. D. *Molecular and Cellular Proteomics* **2003**, 2, 1096-1103.
- (9) Giddings, J. C. *Anal. Chem.* **1967**, 39, 1027-1028.
- (10) Evans, C. R.; Jorgenson, J. W. *Analytical and Bioanalytical Chemistry* **2004**, 378, 1952-1961.
- (11) Harper, R. G.; Workman, S. R.; Schuetzner, S.; Timperman, A. T.; Sutton, J. N. *Electrophoresis* **2004**, 25, 1299-1306.
- (12) Millea, K. M.; Kass, I. J.; Cohen, S. A.; Krull, I. S.; Gebler, J. C.; Berger, S. J. *Journal of Chromatography, A* **2005**, 1079, 287-298.
- (13) Jin, W.-H.; Dai, J.; Li, S.-J.; Xia, Q.-C.; Zou, H.-F.; Zeng, R. *Journal of Proteome Research* **2005**, 4, 613-619.
- (14) Tu, C.-J.; Dai, J.; Li, S.-J.; Sheng, Q.-H.; Deng, W.-J.; Xia, Q.-C.; Zeng, R. *Journal of Proteome Research* **2005**, 4, 1265-1273.
- (15) Li, R.-X.; Zhou, H.; Li, S.-J.; Sheng, Q.-H.; Xia, Q.-C.; Zeng, R. *Journal of Proteome Research* **2005**, 4, 1256-1264.
- (16) Zhu, Y.; Lubman, D. M. *Electrophoresis* **2004**, 25, 949-958.
- (17) Giddings, J. C. *Anal. Chem.* **1984**, 56, 1258A-1270A.
- (18) Murphy, R.E., S., M.R., Foley, J.P. *Anal. Chem.* **1998**, 70, 1585-1594.
- (19) Stroink, T.; Ortiz, M. C.; Bult, A.; Lingeman, H.; de Jong, G. J.; Underberg, W. J. M. *Journal of Chromatography, B: Analytical Technologies in the Biomedical and Life Sciences* **2005**, 817, 49-66.
- (20) Griffin, P. R. C., J.A.; Hood, L.E. and Yates, J.R., III. *International Journal of Mass Spectrometry and Ion Processes* **1991**, 111, 131-149.
- (21) Horvath, C. G., Lipsky, S.R. *Anal. Chem.* **1967**, 39, 1893.
- (22) Grushka, E. *Anal. Chem.* **1970**, 42, 1142-1147.
- (23) Davis, J. M., Giddings, J.C. *Anal. Chem.* **1983**, 55, 418-424.
- (24) Niessen, W. M. A. *Liquid Chromatography-Mass Spectrometry*, Second ed.; Marcel Dekker, Inc.: New York, 1999.

- (25) Lough, W. J., Wainer, I.W. *High Performance Liquid Chromatography: Fundamental Principles and Practice*, First ed.; Blackie Academic & Professional, an imprint of Chapman & Hall: Glasgow, 1995.
- (26) Aguilar, M. I. *HPLC of Peptides and Proteins: Methods and protocols*; Humana Press Inc.: Totowa, New Jersey, 2004.
- (27) Horváth, C., Melander, W. *J. Chromatogr. Sci.* **1977**, *15*, 393-404.
- (28) Snyder, L. R., Glach, J.L., Kirkland, J.J. *Practical HPLC Method Development*; John Wiley & Sons, Inc.: New York, 1988.
- (29) Horváth, C., Melander, W., Molnár, I., Molnár, P. *Anal. Chem.* **1977**, *49*, 2295-2305.
- (30) Horváth, C., Melander, W., Molnár, I. *Anal. Chem.* **1977**, *49*, 142-153.
- (31) Horváth, C., Melander, W., Molnár, I. *J. Chromatogr.* **1976**, *125*, 129-156.
- (32) Snyder, L. R., Dolan, J.W.. *High-Performance Gradient Elution: The Practical Application of the Linear-Solvent-Strength Model*; A John Wiley & Sons, Inc., .
- (33) Snyder, L. R., Dolan, J.W.. *High-Performance Gradient Elution: The practical application of the linear-solvent-strength model*; John Wiley & Sons, Inc.: Hoboken, New Jersey, 2007.
- (34) Robards, K., Haddad, P.R., Jackson, P.E. *Principles and Practice of Modern Chromatographic Methods*; Academic Press Inc.: San Diego, 1997.
- (35) Kunitani, M., Johnson, D. *J. Chromatogr.* **1986**, *371*, 313-333.
- (36) Majors, R. E. *American Laboratory* **2003**, *October*, 46-54.
- (37) Snyder, L. R., Kirkland, J.J. *Introduction to Modern Liquid Chromatography*, 2nd ed.; Wiley-Interscience: New York, NY, 1979.
- (38) Hawkes, S. J. *J. Chem. Educa.* **1983**, *60*, 393-398.
- (39) Banks, J. F., Gulcicek, E.E. *Anal. Chem.* **1997**, *69*, 3973-3978.
- (40) Lommen, D. C., Snyder, L.R. *LC-GC* **1993**, *11*, 222-232.
- (41) Yamashita, M. F., John B. *Journal of Physical Chemistry* **1984**, *88*, 4451-4459.
- (42) Dass, C. *Principles and Practice of Biological Mass Spectrometry*; A John Wiley & Sons, Inc., Publication: New York, 2001.
- (43) Barrow, M. P., Burkitt, W.I., Derrick, P.J. *Analyst* **2005**, *130*, 18-28.
- (44) *LCQ Series*, Revision A ed.; Thermo Finnigan LC and LC/MS Division: San Jose, California, 2002.
- (45) Lau, S. Y. M., Taneja, A.K., Hodges, R.S. *J. Chromatogr.* **1984**, *317*, 129.
- (46) Kerlavage, A. R., Weitzmann, C.J., Hasan, T., Cooperman, B.S. *J. Chromatogr.* **1983**, *266*, 225.
- (47) Osborne, W. R. A. a. T., R. E. *Archives of Biochemistry and Biophysics* **1984**, *230*, 222-226.
- (48) Dolan, J. W. *LCGC* **2000**, *18*, 1228-1232.
- (49) Quarry, M. A., Grob, R.L., Snyder, L.R. *J. Chromatogr.* **1984**, *285*, 1-18.
- (50) *In Performance characteristics of the Agilent 1100 series nanoflow LC system for MS, Technical overview*; Agilent Technologies, 2004.
- (51) Zhu, K.; Miller, F. R.; Barder, T. J.; Lubman, D. M. *Journal of Mass Spectrometry* **2004**, *39*, 770-780.
- (52) Hamler, R. L.; Zhu, K.; Buchanan, N. S.; Kreunin, P.; Kachman, M. T.; Miller, F. R.; Lubman, D. M. *Proteomics* **2004**, *4*, 562-577.

- (53) Chong, B. E.; Lubman, D. M.; Miller, F. R.; Rosenspire, A. J. *Rapid Communications in Mass Spectrometry* **1999**, *13*, 1808-1812.

Appendix

Detail program of different saw-tooth gradients used in this thesis

1. Programmed saw-tooth gradients used in peak splitting test by two model proteins on the analytical-HPLC.

Standard-1		Standard-2		Standard-3	
$\Delta\phi=5.0\%$		$\Delta\phi=1.0\%$		$\Delta\phi=0.1\%$	
t (min)	B (%)	t (min)	B (%)	t (min)	B (%)
0.00	0.0	0.00	0.0	0.00	0.0
14.00	35.0	15.20	38.0	15.80	39.5
15.00	30.0	16.20	33.0	16.80	34.5
19.00	30.0	20.20	33.0	20.80	34.5
23.00	40.0	22.60	39.0	22.84	39.6
24.00	35.0	23.60	34.0	23.84	34.6
28.00	35.0	27.60	34.0	27.84	34.6
32.00	45.0	30.00	40.0	29.84	39.7
33.00	40.0	31.00	35.0	30.84	34.7
37.00	40.0	35.00	35.0	34.84	34.7
43.00	55.0	44.20	58.0	44.88	59.8
44.00	50.0	45.20	53.0	45.88	54.8
48.00	50.0	49.20	53.0	49.88	54.8
52.00	60.0	51.60	59.0	51.92	59.9
53.00	55.0	52.60	54.0	52.92	54.9
57.00	55.0	56.60	54.0	56.92	54.9
61.00	65.0	59.00	60.0	58.96	60.0
62.00	60.0	60.00	55.0	59.96	55.0
66.00	60.0	64.00	55.0	63.96	55.0
70.00	100.0	67.00	100.0	65.00	100.0

2. Programmed saw-tooth gradients used in peak splitting test by anthracene on the analytical-HPLC.

Anthracene-1		Anthracene-2		Anthracene-3	
$\Delta\phi=5.0\%$		$\Delta\phi=1.0\%$		$\Delta\phi=0.1\%$	
t (min)	B (%)	t (min)	B (%)	t (min)	B (%)
0.00	0.0	0.00	0.0	0.00	0.0
10.00	25.0	11.60	29.0	11.96	29.9
11.00	20.0	12.60	24.0	12.96	24.9
15.00	20.0	16.60	24.0	16.96	24.9
19.00	30.0	19.00	30.0	19.00	30.0
20.00	25.0	20.00	25.0	20.00	25.0
24.00	25.0	24.00	25.0	24.00	25.0

28.00	35.0	26.40	31.0	26.04	30.1
29.00	30.0	27.40	26.0	27.04	25.1
33.00	30.0	31.40	26.0	31.04	25.1
34.00	100.0	33.00	100.0	33.00	100.0

3. Detailed setup of programmed four saw-tooth gradients for LMW (≤ 30 kDa)

human serum sample peak splitting studies on the nano-HPLC-FTICR MS. Total protein count and protein overlapping are compared in the 26.0%-27.0% solvent strength of mobile phase B.

serum-1		serum-2		serum-3		serum-4	
$\Delta\phi = 1.0\%$		$\Delta\phi = 0.5\%$		$\Delta\phi = 0.2\%$		$\Delta\phi = 0.1\%$	
t (min)	B (%)	t (min)	B (%)	t (min)	B (%)	t (min)	B (%)
0.00	0.0	0.00	0.0	0.00	0.0	0.00	0.0
10.00	25.0	10.00	25.0	10.00	25.0	10.00	25.0
11.00	20.0	11.00	20.0	11.00	20.0	11.00	20.0
15.00	20.0	15.00	20.0	15.00	20.0	15.00	20.0
17.40	26.0	17.20	25.5	17.20	25.5	17.20	25.5
18.40	21.0	18.20	20.5	18.20	20.5	18.20	20.5
22.40	21.0	22.20	20.5	22.20	20.5	22.20	20.5
24.80	27.0	24.40	26.0	24.40	26.0	24.40	26.0
25.80	22.0	25.40	21.0	25.40	21.0	25.40	21.0
29.80	22.0	29.40	21.0	29.40	21.0	29.40	21.0
32.20	28.0	31.60	26.5	31.50	26.3	31.44	26.1
33.20	23.0	32.60	21.5	32.50	21.3	32.44	21.1
37.20	23.0	36.60	21.5	36.50	21.3	36.44	21.1
39.60	29.0	38.80	27.0	38.60	26.5	38.48	26.2
40.60	24.0	39.80	22.0	39.60	21.5	39.48	21.2
44.60	24.0	43.80	22.0	43.60	21.5	43.48	21.2
47.00	30.0	46.00	27.5	45.70	26.8	45.52	26.3
48.00	25.0	47.00	22.5	46.70	21.8	46.52	21.3
52.00	25.0	51.00	22.5	50.70	21.8	50.52	21.3
54.40	31.0	53.20	28.0	52.80	27.0	52.56	26.4
55.40	26.0	54.20	23.0	53.80	22.0	53.56	21.4
59.40	26.0	58.20	23.0	57.80	22.0	57.56	21.4
61.80	32.0	60.20	28.5	59.90	27.3	59.60	26.5
62.80	27.0	61.20	23.5	60.90	22.3	60.60	21.5
66.80	27.0	65.20	23.5	64.90	22.3	64.60	21.5
69.20	33.0	67.40	29.0	67.00	27.5	66.64	26.6
70.20	28.0	68.40	24.0	68.00	22.5	67.64	21.6
74.20	28.0	72.40	24.0	72.00	22.5	71.64	21.6

76.60	34.0	74.60	29.5	74.10	27.8	73.68	26.7
77.60	29.0	75.60	24.5	75.10	22.8	74.68	21.7
81.60	29.0	79.60	24.5	79.10	22.8	78.68	21.7
84.00	35.0	81.80	30.0	81.20	28.0	80.72	26.8
85.00	30.0	82.80	25.0	82.20	23.0	81.72	21.8
89.00	30.0	86.80	25.0	86.20	23.0	85.72	21.8
93.00	40.0	90.80	35.0	90.20	33.0	87.76	26.9
94.00	35.0	91.80	30.0	91.20	28.0	88.76	21.9
98.00	35.0	95.80	30.0	95.20	28.0	92.76	21.9
102.00	45.0	99.80	40.0	101.20	43.0	94.80	27.0
103.00	40.0	100.80	35.0	102.20	38.0	95.80	22.0
107.00	40.0	104.80	35.0	106.20	38.0	99.80	22.0
113.00	55.0	110.80	50.0	112.20	53.0	105.80	37.0
114.00	50.0	111.80	45.0	113.20	48.0	106.80	32.0
118.00	50.0	115.80	45.0	117.20	48.0	110.80	32.0
124.00	65.0	121.80	60.0	123.20	63.0	116.80	47.0
125.00	60.0	122.80	55.0	124.20	58.0	117.80	42.0
129.00	60.0	126.80	55.0	128.20	58.0	121.80	42.0
135.00	100.0	130.00	100.0	130.00	100.0	125.00	100.0