Development of Immobilized Metal Affinity Chromatography for the Isolation and Selective Enrichment of Phosphopeptides

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A Thesis Submitted to the Eberly College of Arts and Science at West Virginia University in partial fulfillment of the requirements for the degree of

Master of Science

In

Chemistry

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Keywords: Phosphopeptides, Immobilized Metal Affinity Chromatography

Abstract

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Protein structure and function are often regulated by covalent modifications to the protein. One of the most important of these modifications is phosphorylation, which can control many cellular processes. The sites of phosphorylation must be determined to fully understand the protein's function and regulatory events, which can be challenging because of the low stoichiometry of the phosphorylated regulatory proteins.

Several strategies for enrichment of phosphopeptides prior to analysis have been developed, however, Immobilized Metal Affinity Chromatography (IMAC) is the most widely used method. Online IMAC-ESI-MS has been developed with ammonium hydroxide as the elutor. Gradient elution from the IMAC column demonstrates that the phosphopeptides were found to desorb from the IMAC slowly, leading to broad peak widths. By increasing the temperature, the elution from the IMAC column was more rapid, which made the peak widths more narrow.

Dedication

In loving memory of my Uncle Dean, Aunt Joy, and cousin J. R. whose loss I will always suffer but whose life I will celebrate.

Acknowledgments

First and foremost, I would like to take this opportunity to thank my advisor, Dr. Aaron Timperman, for helping me to become a better scientist. Through his support and patience, I have learned how to be more independent and self-reliant in my thoughts and ideas and I have learned to be more systematic in my scientific approach.

I would like to thank my committee members, Dean F. L. King and Dr. R. B Smart for their guidance during my graduate career. I would like to acknowledge Jim Lenke for his patience and help in designing experiments. And I cannot forget my graduate peers, who made this an enjoyable experience.

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

Lastly, I would like to thank my husband, Brian, who was a constant support throughout the entire experience. Without his encouragement, I would not be where I am today.

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1 Introduction

1.1 Protein Phosphorylation

Protein structure and function are often regulated by covalent modifications of the protein. One of the most important of these modifications is phosphorylation, which can control many cellular processes, such as cell growth, cell division, cell differentiation, signal transduction, gene expression, cytoskeletal regulation, and apoptosis.[1,2] When normal cellular control of a signaling or regulatory pathway is lost, i.e. protein phosphorylation and dephosphorylation, diseases such as cancer and diabetes result.[3] The sites of phosphorylation must be determined to understand the protein's function and regulatory events fully, which is challenging because of the low stoichiometry of the phosphorylated regulatory proteins. The stoichiometry refers to the amount of the phosphorylated form compared to the non-phosphorylated form. The presence of the phosphopeptide in lower abundance than the non-phosphorylated peptides consequently makes phosphopeptide selective enrichment necessary to reduce the number of non-phosphorylated peptides considering the phosphopeptide mass spectrometric response may be suppressed in the presence of other peptides.

Covalent modifications to proteins, such as phosphorylation, control enzyme activities in the cell membranes, ribosome, mitochondrion, and nucleus.[4] When a phosphate group attaches to an amino acid residue, enzyme activity is regulated, leading to either synthesis or degradation. Phosphorylation is observed on many amino acids; though it most commonly occurs on the serine, threonine, and tyrosine residues, acting as a reversible switch in cellular processes through the addition or removal of the phosphate

groups.[1] Some tumor viruses can induce cancer by means of stimulating excessive phosphorylation of tyrosine residues on proteins that control cell proliferation.[5]

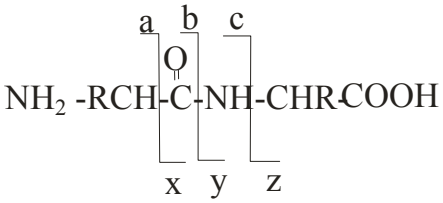
Traditional strategies in the determination of phosphorylation sites involve radiolabeling proteins with [^{32}P] adenosine triphosphate (ATP), either *in vivo* or *in vitro*,[6] followed by gel electrophoresis and enzymatic digestion of proteins. The resulting peptides are then subjected to HPLC fractionation and Edman degradation.[1] The traditional techniques are tedious, use significant amounts of phosphorylated protein, and a large amount of radioactivity. Alternative approaches, based on immobilized metal affinity chromatography (IMAC) for the selective enrichment of phosphopeptides, have been developed to overcome these limitations.

1.2 Mass Spectrometry

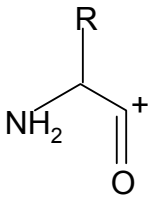
In recent years, mass spectrometry (MS) has become a powerful tool in the determination of post-translational modifications to proteins. Given that the covalent modification of the polypeptide by the phosphate is measured as an increase in molecular mass, the sensitivity and specificity of MS make it an attractive option.[2] Normally, the purified and digested proteins are examined by single stage MS using either of two soft ionization techniques for large molecules, (1) matrix assisted laser desorption mass ionization (MALDI) or (2) electrospray ionization (ESI). MALDI is used to identify phosphopeptides based on the differences in the mass of the phosphopeptide and the mass of the nonphoshorylated peptide in the spectrum before and after treatment with phosphatase (80 Da difference).[1] Regardless of the method utilized, HPLC separation prior to MS aids in the reduction of ion suppression effects (phosphopeptide MS response is less intense compared to its unphosphorylated counterpart), though ESI-MS alone is

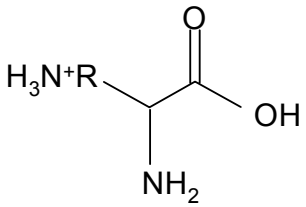
capable of direct analysis while MALDI-MS requires the laborious collection of HPLC fractions. A further benefit is the ease of coupling HPLC to ESI-MS utilizing compatible solvent systems and thus minimizing sample handling.

Mass spectrometric analysis can be simply described as two stages. Stage one, or single stage MS, measures all peptide masses, whereby the ion intensity is plotted versus the mass to charge ratios (m/z ratios), generating a mass spectrum. The second stage, tandem MS, isolates an ion of interest, usually based on intensity, in the first stage. The isolated ion undergoes collisions with neutral gas molecules and fragments in a process named collision induced dissociation (CID).[7] Low energy CID, <100eV, utilized in the following experiments, results in fragments of a positively charged peptide along its backbone. In low energy CID, fragments are also observed for ions that have lost water and ammonia. The fragment ion intensities are measured, and the spectrum is referred to as a CID spectrum. In a CID spectrum, the relative abundance of the fragment ion is also plotted versus its m/z ratio. The fragmentation ion spectra is used to determine the sequence of the peptide by observing the amino acid residue difference between the two different types of fragment ions, b- and y- ions.[8] In Figure 1, the peptide is shown before and after fragmentation. Lines are drawn between the possible fragmentation locations. The b-ion contains the N-terminus of the peptide ions while the C-terminus is included in the y-ion. Different fragment ions produced by the CID process can be isolated and fragmented. Multiple stages of tandem mass spectrometry, MS^n, provide further structural information.



Peptide Before Fragmentation





B-Ion (N-terminus) Y-Ion (C-terminus)

Figure 1 – B- and Y- Ions.

The most simple and straightforward MS approach to peptide identification would be measurement of the mass to charge of the peptide, however, the mass alone does not give sufficient information for unambiguous identification (i.e. structure, location of phosphorylation). Tandem mass spectrometry (MS^2) is an attractive technique, providing information on the mass, structure, and often the location of phosphorylation on the peptide. Tandem MS is approached in two different ways depending on the amount of known information and is described below.

If the sample's mass, sequence, and phosphorylation sites are known, as is the case with standards and targeted analytes, a targeted analysis is performed. The mass spectrometer is programmed to specifically isolate and fragment the ions having a particular mass to charge ratio while ignoring all other peptides. The advantage of this

type of analysis is that the peptide of interest is targeted for sequence analysis of a phosphopeptide whose ESI-MS response may be weak.[1]

If the sample is an unknown (mass, sequence, and phosphorylation site not known), then data dependent scanning is required. A full MS scan is recorded and a particular ion of interest is selected (in most cases, it is the most abundant) to be fragmented in the collision cell.[7] In phosphopeptide MS analysis, the neutral loss of phosphate (as HPO_3 or H_3PO_4) is a favored fragmentation event which suppresses the peptide backbone cleavage that can be useful for sequence determination. The loss of phosphate is more dominant when the peptide is singly charged, however, the fragmentation information can be obtained if enough data for positive identification of the peptide can be collected to distinguish the weaker backbone fragment ions.[1] Manually sequencing the spectra is an option, but due to the large number of spectra which are typically acquired, this method is a difficult and time-consuming task. Thus, a database-searching program, such as Sequest, can aid in the sequencing of proteins and peptides.

1.3 Separation Techniques

A separation technique such as high performance liquid chromatography (HPLC) allows for the on-line separation of peptides prior to MS detection and is easily interfaced to ESI-MS. The performance of HPLC-ESI-MS has advanced greatly with the use of lower flow rates, on the order of nanoliter/minute, allowing a longer measurement time of the analyte at unchanged signal, due to the dependence of concentration to signal in electrospray.[9,10,11] Even though the separation capabilities and MS techniques are powerful, phosphorylated peptides may be difficult to detect without prior selective

enrichment simply from the fact that phosphorylation does occur at low stoichiometric levels; therefore, sample preparation is extremely important. Negative ion mode MS, limited by lower sensitivity, could be used to analyze the phosphopeptides since the phosphopeptide adds a negative charge to the peptide.[12] However, alkaline pH for the HPLC solvent system is required for optimum sensitivity in negative ion mode causing an inherent problem when coupled to reverse phase packing materials as they are unstable in basic conditions.[13,14] Reverse phase chromatography is problematic when analyzing phosphopeptides considering that phosphopeptides tend to be relatively hydrophilic and may elute in the void volume.[2] Therefore, several different strategies for the selective enrichment of phosphopeptides prior to analysis have been developed. The sample preparation enrichment techniques can be classified as categories that include: (1) HPLC fractionation, limited by sample handling and hydrophilic phosphate loss, (2) antibody enrichment, limited by effective immunoprecipitation due to difficulty in relative tight binding of the antibody-phosphorylated serine and threonine residues, (3) chemical tagging of phosphorylation sites, limited by its inability to enrich for proteins containing phosphotyrosine, and (4) Immobilized Metal Affinity Chromatography (IMAC), limited by its non-specific interactions but selective for all phosphorylated residues.[1] IMAC was chosen for the selective enrichment of phosphopeptides due to its non-discrimination and because the non-specific interactions limitations can be overcome. Additionally, IMAC can be performed on-line with MS analysis, thereby minimizing sample handling and loss.

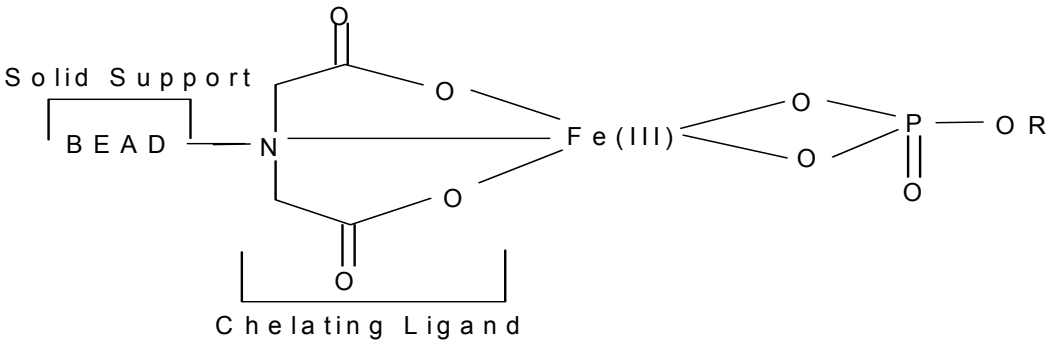
1.4 Immobilized Metal Affinity Chromatography

Metal chelate affinity chromatography has been widely used for the separation of proteins since its introduction in 1975 by Porath and his coworkers.[15] The most commonly used ligand is iminodiacetic acid (IDA) and is usually covalently coupled to agarose, sepharose, or poly(styrene-divinylbenzene) (PS-DVB). In the beginning, Zn^{+2}, Ni^{+2}, and Cu^{+2} were the predominant metals that were employed in immobilized metal affinity chromatography (IMAC).[16] Porath and his coworkers further extended their work in 1983 by observing that Al^{+3}, Fe^{+3}, In^{+3}, and Ga^{+3} displayed different adsorption properties towards proteins when immobilized.[17] In 1985, Sulkowski determined that the accessible electron-donating groups on the proteins were coordinated to the chelated and immobilized metal ions.[16] For example, metals such as Al^{+3} or Fe^{+3} have a tendency to coordinate OH^-, O_2, and F^-, electron-rich "bases", which easily donate electrons from their filled *p* orbital to the empty *d* orbital of the hard metal, thereby forming a metal ligand complex.[16] Pearson defines "hard" metals as those metals having tightly bound electrons in the outer shell and a *d* orbital that is empty and of rather low energy. The most typical hard metals used in IMAC are Ga^{+3}, Ca^{+2}, La^{+3}, Al^{+3}, Ti^{+4}, and Fe^{+3}, whose similar physical properties include a large positive charge and a small ionic radius.[16] The fact that phosphoserine has a binding constant above 10^{13} for Fe^{+3} made it a target for Fe^{+3} – phosphopeptide complex research.[18]

IMAC is the most widely used method for the selective enrichment of phosphopeptides from mixtures due to the selectivity of the metal-phosphate interaction.[1,4] In this technique, metal ions are bound to a chelating support such as nitriloacetic acid (NTA) or iminodiacetic acid (IDA) sepharose resin.[2] Phosphopeptides

undergo an electronic interaction with the immobilized metal ions (Fe^{+3} or Ga^{+3}). Two coordinate bonds are formed between the metal-phosphopeptide while the carboxyl groups will form only a single bond with the immobilized metal ion, contributing to the high affinity the phosphopeptides have for the immobilized metal ions.[4] Ga (III) exhibits the ability to isolate a multiply phosphorylated peptide and release the peptide at lower hydroxide concentrations than the Fe (III); however, Fe (III) is more selective for monophosphorylated peptides.[19,20] Clearly, the differences in the selectivity and affinity of Ga (III) and Fe (III) need further investigation.

In Figure 2, the proposed interaction of the chelating ligand with the Fe^{+3} and phosphopeptide is shown. A weaker ligand, water, is expelled as the phosphopeptide is adsorbed to the chelated metal.[4]



R=Peptide

Figure 2 – Proposed Interaction of the Chelating Ligand – Fe^{+3} - Phosphopeptide[4].

The removal of the phosphopeptide from the immobilized metal ion has not been investigated fully in terms of classes of elutors, specific elutors, or elution mechanisms, but several options are available for the elution of the phosphopeptides from the Fe (III)-

IMAC column which include (1) competitive elution with a Fe (III)-binding molecule, (2) pH gradient, or elution with a (3) magnesium or (4) phosphate ion.[4]

Sensitive detection of phosphopeptides requires minimization of phosphopeptide losses. Several limitations leading to possible loss of phosphopeptides include the phosphopeptide's inability to bind to the metal, difficulty in eluting the multiply phosphorylated peptides, and non-specific interactions.[1] Precaution has been taken in this research to minimize the non-specific interactions that will be described in more detail in Section 1.9.

Many IMAC applications for the selective enrichment of phosphopeptides have been found, including the separation of numerous low abundance phosphopeptides, information on the biological functions of phosphorylation, sequence specificity of the protein kinase, and protein turnover.[4] Cao and Stults have successfully coupled IMAC to capillary electrophoresis/electrospray ionization-mass spectrometry (IMAC/CE/ESI-MS) to selectively preconcentrate low picomole level phosphopeptides prior to identification.[21] On-line IMAC-ESI-MS of phosphopeptides has been shown to decrease sample loss compared to off-line experiments. In exchange for decreased sample loss in Nuwaysir and Stult's report, decreased signal to noise was observed since the Sepharose beads were not stable at the high pH required.[22]

1.5 General Chromatography

Chromatography is a separation process based on partitioning between a mobile and stationary phase that is dependent on their intrinsic physical properties. The different physical and chemical characteristics of the analyte being separated result in different

degrees of partitioning, which causes differential migration. The partition or distribution coefficient, K, is dependent on the composition of the stationary and mobile phase, the analytes properties and temperature, and is given by the following equation:

$$\mathbf{K = [C_s]/ [C_m]} \qquad \textbf{Equation 1}$$

$\mathbf{[C_s]}$ = Concentration in the stationary phase

$\mathbf{[C_m]}$ = Concentration in the mobile phase

Equation 1, a characteristic physical property of the analyte, is a thermodynamic parameter due to the interconversion of the analyte between the mobile and stationary phases.[23]

The capacity factor (also known as the partition ratio), k', is defined as the ratio of analyte masses, **m**, in the stationary and mobile phases, ($\mathbf{m_s}$ and $\mathbf{m_m}$ respectively):

$$\mathbf{k' = (m_s)/ (m_m)} \qquad \textbf{Equation 2}$$

The smaller the k' value, the closer to the void volume the substance will elute. The void volume, also referred to as dead volume, is the volume needed to move a completely unretained analyte from the injector to the detector. Consequently, the larger the k', the more interaction the analyte has with the column, and the longer its retention time.

The distribution coefficient and the capacity factor can be related through the phase ratio, **B**, which can also be described as the openness of the column. The following equations relates these terms:

$$\mathbf{K = k'B} \qquad \textbf{Equation 3}$$

$$\mathbf{B = (V_m/V_s)} \qquad \textbf{Equation 4}$$

$\mathbf{V_m}$ = Volume in the mobile phase

$\mathbf{V_s}$ = Volume in the stationary phase

Separating mixtures with a large range of k' values under isocratic conditions is problematic and is referred to as the General Elution Problem (GEP). The broad peak widths and difficulty determining the end of the elution associated with GEP can be overcome using gradient elution.

1.5.1 General Gradient Chromatography

In gradient elution, the composition of the mobile phase changes during a run to allow a large range of k' values to be separated with improved efficiency compared to an isocratic run. Regardless of the type of chromatography, the gradient will begin with a weak-eluting solvent (high k') and the solvent strength will increase. As a result of the changing solvent strength, the k' value will decrease causing the analyte to desorb from the column with an increasing percentage of elution buffer, consequently giving more narrow bands and increased sensitivity for every band.[23] In gradient elution, the strongly retained analyte will exhibit a shorter time on the column compared to isocratic elution conditions.

When considering solvents for gradient elution, a few parameters must be taken into consideration. First, the two chosen solvents must be fully miscible. Second, a linear gradient from Solvent A to Solvent B is run through the column, where compounds with a large range of k' values can be separated utilizing the steepest gradient that provides the best retention. Lastly, sufficient time for column re-equilibration must be allowed for reproducible chromatography.

Optimization of gradient separations requires consideration of three main variables: (1) initial percentage of Solvent B, (2) final percentage of Solvent B, and (3)

gradient slope or gradient time (%B/min).[24] The initial percentage of Solvent B must be low so the k' value is high at the beginning of the run allowing the analytes to be fully retained on the column. The final percentage of Solvent B must be of sufficient strength to elute all of the compounds off the column. As gradient slope increases, the peak height increases and analysis time decreases, however, gains are inherently limited by a simultaneous decrease in peak capacity. A shallow gradient slope will suffer from an increased time in analyte column retention, which will lower peak height and consequently cause a loss in sensitivity.[23] All three variables need to be optimized for good gradient separations.

1.6 Gradient Elution in Immobilized Metal Affinity Chromatography

The selectivity of Fe (III)-IMAC is based on the electronic interaction between the Fe^{+3} and the phosphate ($H_2PO_4^-$). The strength of this interaction is enhanced by the formation of two coordinate bonds between the Fe^{+3} and the phosphate ($H_2PO_4^-$) group. Analytes that possess this interaction will remain in the stationary phase much longer than those that do not.

Several options are available for successful gradient elution in IMAC. Salt gradients[4] and pH gradients[18,25, 26,27,28,29,30,31] are the two most common types employed to date. In addition to salt and pH gradients, an increasing gradient of NH_3 run in combination with an increasing pH gradient has been used in IMAC gradient elution.[32] By combining increasing gradients of both NH_3 and pH, a better separation has been achieved than if the NH_3 was solely employed. In this work, an increasing pH gradient elution method utilizing ammonium hydroxide solution was used to elute the phosphopeptides from the Iron (III)- activated IMAC column prior to MS analysis.

1.7 Kinetics in IMAC Separations

The gradient elution from the IMAC column resulted in broad peaks, probably caused by slow kinetics. Although these kinetics have not been studied in great detail, some work has been done to show the importance in IMAC separations, especially with respect to reversibility and speed.[33] It has been found that sufficient time must be allowed for both the adsorption and desorption process to obtain maximum capacity or to avoid partial elution.[17] The proper solvent strength is of great importance because isocratic behavior has occasionally been observed, although no experimental data has been reported to demonstrate clearly that the equilibria does occur quickly.[17]

In this work, manipulation of the temperature during IMAC elution to change desorption was a focus. This provides insight into the role of kinetics in band broadening of separations performed at various temperatures. Elution from the IMAC column at a higher temperature resulted in higher separation efficiencies compared to elution at lower temperature, indicating that the peak width is kinetically limited and that IMAC elution benefits from higher temperature during desorption.

1.8 Electrospray Ionization Mass Spectrometry

Electrospray ionization (ESI) is a soft ionization technique and was introduced by Fenn and coworkers in 1985.[7] ESI allows molecular ions to be observed in the mass spectra because there is little fragmentation in the ion source. Collision induced dissociation (CID) of multiply charged species allowed Hunt, et. al. to interpret amino acid information based on overlapping fragment information.[34,35] An improvement in both desolvation and in ionization efficiency is observed by lowering the flow rates in ESI. It has been proposed that the smaller droplets form a stable spray at lower flow

rates.[36] At extremely low flow rates, in the range of 1-200 nL/min, ESI is referred to as nanospray or nano-ESI. Low flow rates require a smaller spray needle diameter, thus reducing the size of the Taylor cone and resulting droplets.

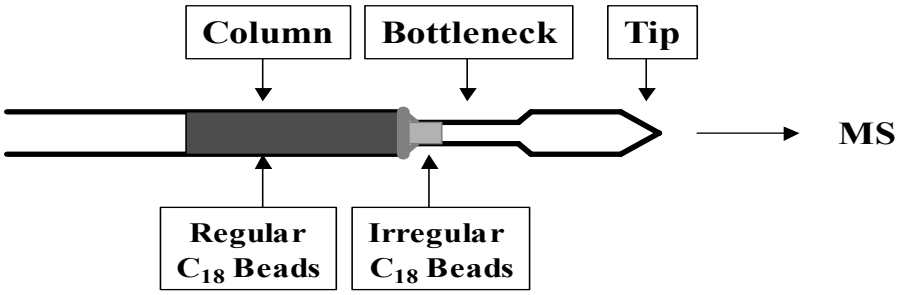


Figure 3 – Block Diagram of Column, Bottleneck, and Tip.

Figure 3 is a block diagram of the column, bottleneck, and tip, which are one piece of fused silica to reduce the dead volume in the capillary (Polymicro Technologies, Phoenix, AZ). For reverse phase, the inner diameter is typically 50 or 75 micron. The tip and bottleneck are pulled in house with a CO_2 Laser Puller (Sutter Instrument Company, Novato, CA). A bottleneck has been pulled near the tip to retain the beads and to aid in flow restriction to the mass spectrometer. The bottleneck has an inner diameter of ~5 micron and YMC irregular C_{18} beads (between 5-20 micron in size), (YMC Corp., Morris Plains, NJ), are packed into this restriction to act as a frit before the regular C_{18} beads (YMC Corp., Morris Plains, NJ) are packed. All columns are packed at 1000 psi via a pressure vessel unless otherwise stated. The solvents used for packing the C_{18} columns are 80% n-propanol and 20% acetonitrile. The spray tip is pulled with the laser puller into a tip with an inner diameter of 2-3 micron.

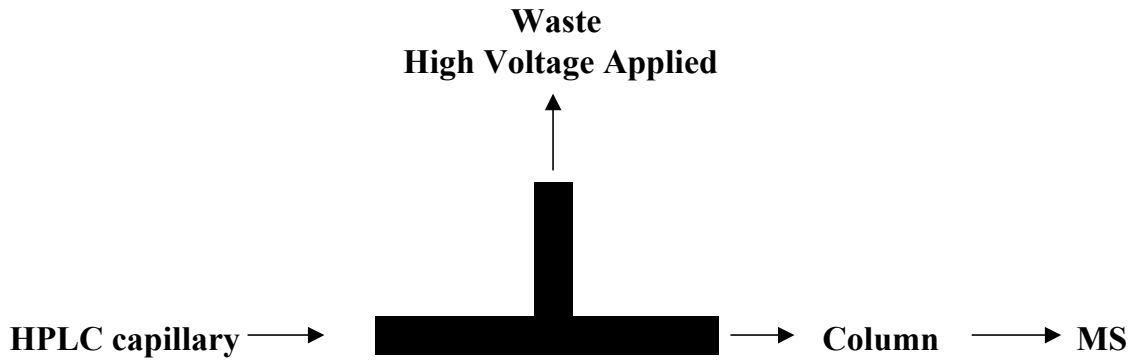


Figure 4 – Block Diagram of Nanospray Ionization.

A block diagram of the flow splitter unit is shown in Figure 4. A T-fitting allows a capillary from the HPLC, a column, and a waste capillary to be connected. The HPLC solvent gradient is introduced through the tube labeled "HPLC capillary". The waste capillary is where the voltage is applied to the spray tip to minimize the uptake of electrolysis bubbles. The waste capillary is also used to reduce the flow to the mass spectrometer.

1.9 Research Objectives

The focus of this research project was to utilize IMAC for the selective enrichment of phosphopeptides in an efficient and simple manner. The main objectives in this research project were as follows:

- Reduce the non-specific interactions
  - Acidic Interactions
  - Hydrophobic Interactions
  - Ionic Interactions
- Explore whether peptides could be gradient eluted off of the IMAC
- Explore the kinetics of the IMAC gradient elution and improve by heating/cooling

An efficient IMAC-MS method was developed and non-specific interactions were reduced, which decreased noise and separation efficiencies. Non-specific interactions with the chelating ligand, the POROS MC solid support, and the immobilized Fe(III) were minimized by esterifying the acidic amino acid residues,[37] adding salt to reduce the ionic interactions, and using a high organic percentage in solvent A to minimize the hydrophobic interactions with the solid support. Salt was added to the solvent system to bind to the possibly ionized solid support in order to minimize peptide binding that occurred. An experiment utilizing a detergent prewash was used to simulate an IMAC separation with a hydrophilic solid support so that a high percentage of organic in solvent A could be avoided. Online IMAC was used to elute phosphopeptides directly into the MS at levels as low as 500 femtomoles with a simple binary solvent system. Gradient chromatography was used to elute the phosphopeptides from the Fe (III)-activated IMAC column prior to MS analysis, however, the broad peak shape led to the manipulation of kinetics during the peptide desorption from the IMAC column.

2 Experimental

2.1 Phosphopeptide Standard

The objective was to develop a standard protocol to ensure appropriate methods were utilized for the identification of phosphopeptides. An amino acid sequence, DLDVPIPGRFDRRVpSVAAE, from the cAMP-dependant protein kinase (PKA) regulatory subunit type II (RII) labeled peptide (Biomol, Plymouth Meeting, PA), was used as the phosphopeptide standard to test whether or not the phosphoserine could be detected with tandem MS. The phosphopeptide stock was made up to a final concentration of 0.5 μg/μL with deionized water (Barnstead 18 MΩ Water Purifier,

Ontario, Canada) and frozen. For sample analysis, 1 microliter of phosphopeptide stock was dissolved in HPLC solvent A to give a final phosphopeptide concentration of 1 pmol/μl. One microliter of a $1*10^{-6}$ μM standard was loaded on a C_{18} column (360 μm OD × 75 μm ID, Polymicro Technologies, Phoenix, AZ) packed in house. ODS-AQ C_{18} (YMC Corp., Morris Plains, NJ) 5 μm beads with a 300 Å pore size were used. The tip was purchased, (360 μm OD × 50 μm ID, 15 μm tip ID, New Objective PicoTip, Woburn, MA). The solvents used for HPLC solvent A were 0.2% acetic acid (99.99+% purity, Aldrich, Milwaukee, WI), 0.005% heptafluorobutyric acid (99% purity, Aldrich, Milwaukee, WI), and 4% acetonitrile (HPLC grade, Fisher, Fair Lawn, NJ), made up in deionized water. HPLC grade acetonitrile was used as solvent B and the flow rate was measured at 350-400 nL/min. The gradient employed is composed of linear segments between the time points which correspond to acetonitrile percentages and is shown below.

Time (Minutes)	% Acetonitrile
0	4
2	4
4	15.5
17	46.7
20	62.3
21	83.2
30	4

Table 1 – Gradient Elution Parameters.

An LCQ DECA XP (Thermo Finnigan, San Jose, CA) ion trap MS was used to allow for the sequencing of the phosphopeptide.

2.2 Removal of Fe^{+3} with Tiron and Ammonium Hydroxide from the IMAC column

To determine if the Fe^{+3} was removed from the column during the elution process, a colorimetric indicator dye was used. A metal chelating colorimetric indicator, Eriochrome C, EC, (Sigma, St. Louis, MO) was used to test and see whether Tiron, (IUPAC name 4, 5-dihydroxy-1,3-benzenedisulfonic acid, disodium salt; Hydrate), (Sigma, St. Louis, MO) and ammonium hydroxide would remove the Fe^{+3}, EC, or both. EC forms a complex with the Iron (III)-activated IMAC column. The column appears blue in color when the Fe^{+3}-EC exists in the complexed form, but appears red in color when no metal is bound. The column used was 360 μm OD × 200 μm ID (Polymicro Technologies, Phoenix, AX) and was packed in house with POROS 20 Micron Metal Chelate Affinity Packing (Perceptive Biosystems, Framington, MA). The capillary column was rinsed with 50 mM ethylenediaminetetraacetic (EDTA) acid, disodium salt (Fisher, Fair Lawn, NJ), deionized water, 100 mM ferric chloride (Aldrich, Milwaukee, WI), 0.67 μg/μL EC, and 80% acetonitrile for 10 minutes each. Once the EC was loaded, the column appeared blue in color due to the EC-Fe^{+3} complex. The elution was performed with 10 mM Tiron solution, pH 10, for 10 minutes, followed by a deionized water wash. To determine if the Fe^{+3} was removed from the column, EC was once again loaded and the column rinsed with deionized water. The column did not appear blue in color, indicating that the 10 mM Tiron does remove the Fe^{+3} from the column. The removal efficiency is not known considering that this is a qualitative experiment.

The same experiment was repeated using the same conditions as above; however, instead of elution with 10 mM Tiron, 2 M ammonium hydroxide, pH=10.0, was used.

The ammonium hydroxide solution does elute the EC-Fe^{+3} complex off of the column. This experiment was repeated again with 1 M ammonium hydroxide, pH=10.0, and once again, the EC-Fe^{+3} complex was eluted off of the column and was tested by rinsing the column with the EC after elution conditions to see if the blue color reappeared. In both cases, the blue color did not reappear. All experiments were repeated with the same column, indicating that the elutor did not affect the bead.

2.3 Peptide Esterification

Non-specific binding from the -COOH group must be minimized in order to selectively isolate phosphopeptides. The –COOH can be converted to the methyl ester; which does not bind metals, to minimize non-specific interactions. Methyl esters were made by the following conditions: in a small glass vial with stirring, 1 mL of anhydrous methyl alcohol (99.8%, anhydrous, Aldrich, Milwaukee, WI, #32241-5) and 160 µl of acetyl chloride (99+%, Aldrich, Milwaukee, WI, #23957-7) were added slowly to make the 2 M methanolic HCl reaction mixture as this reaction is extremely exothermic. The 2 M methanolic HCl reaction mixture must rest for 5 minutes before adding to a 1 nmol level of the dehydrated peptide mixture. The peptide mixture contained: angiotensin I Human (Sigma, St. Louis, MO), [Tyr $(PO_3H_2)^4$] – angiotensin II Human (Calbiochem,San Diego, CA), RII labeled phosphopeptide (Biomol, Plymouth Meeting, PA), MAP (177-189) pT/pY diphosporylated peptide (Biomol, Plymouth Meeting, PA). From the 2 M methanolic HCl solution, 100 µl was added to the tube of dehydrated peptides and this reaction was allowed to stand for 2 hours at room temperature, and subsequently dehydrated. It was then reconstituted in 0.1% acetic acid and separated by reverse phase HPLC-MS. The peptide sequence was obtained manually from the spectra.

2.4 Comparison of Esterified Peptides and Non-Esterified Peptides on Reverse Phase Beads

The efficacy of using methyl esters to minimize non-specific binding through the carboxylic acid groups was investigated by comparing the elution of an esterified peptide and its non-esterified counterpart on a polymeric reverse phase column. A polymeric reverse phase column was constructed from a slurry of ~500 mg of 5 μm polystyrene-divinylbenzene (PS-DVB) beads (Hamilton Polymeric HPLC Support, Reno, Nevada, PRP-1) in approximately one ml of acetonitrile. The slurry was stirred while packing the column in house via a pressure vessel. A 0.1% ammonium hydroxide rinse followed by a blank gradient sufficiently equilibrated the column. The peptide mixture used in this experiment was angiotensin I human, [Tyr $(PO_3H_2)^4$] – angiotensin II human, RII labeled phosphopeptide, and the MAP (177-189) pT/pY diphosporylated peptide. The esterified and non-esterified peptide experiments were performed separately. Solvent A was 0.1 M ammonium hydroxide and solvent B was 70% acetonitrile in 0.1M ammonium hydroxide. The gradient employed was 0-100% Solvent B in 30 minutes. A targeted analysis was performed in which the MS was programmed to hold the angiotensin-II-phosphate b_6^{+1} ion, m/z = 564.10 (non-esterified peptide) and m/z = 578.10 (esterified peptide), which allowed for the sequencing and manual verification of the peptides.

In contrast to silica based C_{18}, PS-DVB was used as the reverse phase material due to its stability at basic pH. Coupling of the IMAC to the PS-DVB was prohibited by the high organic content of the solvent system that was required by the IMAC to minimize the hydrophobic interactions with the POROS MC solid support. However, the

peptides were shown to separate with the use of basic pH solvent conditions on the PS-DVB reverse phase packing material.

2.5 Investigation of non-specific hydrophobic interactions with POROS hydrophobic solid support

To investigate non-specific hydrophobic interactions with the POROS MC solid support, the peptides were eluted with an organic solvent. To eliminate interactions with the Fe^{+3}, the peptides were loaded directly on the POROS MC-IDA solid support with no immobilized metal ion. Five hundred fmols of seven different peptides were loaded on the column via a pressure vessel at 500 psi. The peptides used were angiotensin I human, [Tyr $(PO_3H_2)^4$] – angiotensin II human, RII labeled phosphopeptide, MAP (177-189) pT/pY diphosporylated peptide. In addition, three synthesized peptides, SLQPRSHpSV, RVApSPTSGV, and YNpSLDYAK, were included in this experiment. Solvent A was 0.1 M acetic acid and Solvent B was 70% acetonitrile (ChromAR, Mallinckrodt, Paris, KY) in 0.1 M acetic acid. A linear gradient was used from 0-100% Solvent B in 40 minutes. The MS was programmed to execute targeted analysis.

2.6 Minimizing the Hydrophobic Interactions with the POROS Hydrophobic Solid Support

As with all bonded phase packing materials for chromatography, the IMAC material used consists of a solid support to which a functional ligand is attached. The functional ligand loading is less than a monolayer, leaving portions of the solid support exposed to the solution. Under the low organic concentrations, hydrophobic analytes will bind to the hydrophobic solid support in a mechanism similar to reverse phase. The degree of hydrophobic interaction was investigated by allowing the peptides to interact

with the solid support when no immobilized metal ion was loaded. The degree of hydrophobic interaction was decreased with increasing percentages of acetonitrile (25% & 80%) in the loading buffers using 25% acetonitrile as the control. An IMAC column was constructed from a piece of 360 μm OD × 200 μm ID and packed with the POROS MC slurry as described before. The column was rinsed prior to use with 50 mM EDTA, pH=8.2, and deionized water for 10 minutes each. The appropriate loading buffer, 80% or 25% acetonitrile, was rinsed through the column for 10 minutes prior to loading the protein digest. A β-casein tryspin digest was used so both hydrophobic and hydrophilic peptides were present. One picomole of protein digest was loaded on the column in the appropriate loading buffer, 80% or 25% acetonitrile. The HPLC solvents used in this experiment were 0.1 M acetic acid in deionized water for Solvent A and 70% acetonitrile in 0.1 M acetic acid. The MS was programmed to execute targeted analysis.

2.7 Minimizing Hydrophobic Interactions with the POROS Solid Support with a Non-Ionic Detergent Prewash

Another way to further minimize the non-specific hydrophobic interactions with the POROS solid support is to coat the hydrophobic surface with a non-ionic detergent such as Nonidet P-40 (NP-40). Using organic to minimize the non-specific hydrophobic interactions could cause problems in the second dimension; therefore, a detergent prewash could be substituted if a second dimensional separation following IMAC could be realized. Experiments performed with and without a prior NP-40 detergent column wash were used to compare whether a detergent prewash would decrease absorbance, indicating a reduction in non-specific hydrophobic interactions.

The IMAC columns for the HPLC-UV experiments were prepared with Tefzel tubing. The Tefzel tubing was used to monitor the column's color change with the addition of the iron (III) chloride solution and also provided a visual indication when the column needed replacement. The inner diameter of the Tefzel tubing (Upchurch Scientific, Oak Harbor, WA) that was used was 0.02", equal to ~508 micron. The frit that was used in conjunction with the Tefzel tubing was a high pressure biocompatible inline filter with a 100% PEEK frit (Upchurch Scientific, Oak Harbor, WA). The POROS MC packing solution and conditions were the same as above. The column was packed to a length of ~7 cm.

The IMAC column was prepared as before with a few minor differences. All of the following washing steps were performed on the pressure vessel and all solutions were loaded for ten minutes except for the loading of the sample, which was measured by volume. The column was rinsed via the pressure vessel at 100 psi. In both experiments, the column was rinsed with 10-minute washes of (1) EDTA, (2) deionized water, (3) iron (III) chloride, and (4) 20% acetonitrile or 100% deionized water. In the experiment with the detergent, the column was washed with an additional solution of 0.03 mM NP-40 for 10 minutes. Six nanomoles of the sample mixture containing [Tyr $(PO_3H_2)^4$] – angiotensin II human, RII labeled phosphopeptide, and MAP (177-189) pT/pY diphosporylated peptide, was loaded on the IMAC column in solvent A, 20% acetonitrile or 100% water. Solvent B was 10 mM sodium phosphate, 0.02% sodium hydroxide, pH 9. A 4-100% B gradient in 17 minutes was performed prior to UV detection.

2.8 Online Immobilized Metal Affinity Chromatography

Online IMAC is an attractive method for selective enrichment due to the high affinity that the phosphopeptides have for Fe^{+3} and because sample losses are minimized. Second dimensional separations could be used, however, there are difficulties with compatibility. For example, reverse phase chromatography is not typically suitable for the second dimension due to its inability to handle the basic conditions, however, a polymeric stationary phase that could withstand high pH (PS-DVB) could be used if a hydrophilic IMAC solid support could be found. Anion exchange chromatography is not suitable because the stationary phase is not protonated at high pH (pKa=9.4) so no charge would be present to attract anions. Cation exchange also suffers from the pH limitation because the peptides will have little charge at high pH. Normal phase (NP) chromatography could be used in theory, however, silica-based NP chromatography cannot withstand the high pH conditions and alumina-based NP chromatography suffers from the acidic components being stongly retained, sometimes irreversibly, on the alumina.[23] An optimum IMAC stationary phase for this application would have a hydrophilic solid support with the same chelating ligand (IDA), ~20μm particle size, and would be stable at basic pH. An IMAC stationary phase with these conditions is currently not available. Limitations caused by the non-specific interactions with the POROS MC solid support, i.e. using a high organic percentage to minimize the hydrophobic interactions, prohibited direct coupling to reverse phase. Online-IMAC-MS was designed because a simple binary solvent system, consisting of the high organic percentage (necessary to minimize hydrophobic interactions) and ammonium hydroxide,

was found to elute the peptides from the activated IMAC column directly into the MS, thus minimizing sample handling.

A 360 μm OD × 200 μm ID capillary was packed in house with POROS 20 μm Metal Chelate Affinity Packing. A series of washes was performed at low pressure, ~ 500 psi. First, the column was washed with 50 mM ethylenediaminetetraacetic (EDTA) acid, disodium salt, (Fisher, Fair Lawn, NJ, BP120-1) pH=8.2 for 10 minutes to remove any metal that may be bound to the column. The column was rinsed with deionized water for 10 minutes to remove the EDTA solution and then activated with 100 mM iron (III) chloride (anhydrous; Aldrich, Milwaukee, WI, #45164-9) for 10 minutes, binding Fe^{+3} to the column. The column was equilibrated with a 1:1:1 solution of methanol (HPLC grade, Fisher, Fair Lawn, NJ), acetonitrile, and deionized water, pH 9 with ammonium hydroxide (ACS grade, Sigma, St. Louis, MO, A-6899) for 10 minutes. The 1:1:1 solution, pH 9, was also the peptide loading buffer and was 66% organic to minimize non-specific hydrophobic interactions. A peptide mixture was loaded on the column comprising approximately 1 picomole of the following peptides: angiotensin I human, DRVYIHPFHL, [Tyr $(PO_3H_2)^4$] – angiotensin II human, DRVpYIHPF, RII labeled phosphopeptide, DLDVPIPGRFDRRVpSVAAE, and a MAP (177-189) pT/pY diphosporylated peptide, DHTGFLpTEpYVATR. Five hundred femtomoles of each peptide were loaded on the column, followed by the direct connection of the column to the HPLC. Solvent A was an 80% acetonitrile solution made to volume with deionized water. Solvent B was comprised of a 50:50 mixture of acetonitrile and 10% ammonium hydroxide (0.74 M) at a pH of 10, as reported earlier by Carr et al.[38] A linear gradient

from 0-100% Solvent B in 30 minutes was performed. Peptide identification was based on mass to charge values since all peptides had previously been sequenced from MS data.

The same experiment was performed with the esterified counterparts of the four peptides listed above. The column was washed via the pressure vessel at 500 psi with 50 mM EDTA at a pH of 8.2, deionized water, 100 mM iron (III) chloride, and 80% acetonitrile for ten minutes each. Five picomoles were loaded on the IMAC column. Solvent A remained the same (80% acetonitrile), however, Solvent B was increased to 1 M ammonium hydroxide in 80% acetonitrile, pH 10.4. The gradient and tandem MS method remained the same.

2.9 IMAC Kinetics

A series of experiments was designed that would allow the column temperature to be regulated while monitoring the peak shape of the peptides by placing the IMAC column in a temperature regulated water bath during elution. Three different column temperatures were used, 0° C, ~25° C (room temperature), and 60° C and three different sets of peptides were used to monitor the peak shape. The three sets of peptides were (1) three synthesized phosphopeptides differing by only one phosphorylation site, (2) four peptides from the online-IMAC experiment, and (3) four esterified peptides from the online-IMAC experiments. The general outline of the experiment is as follows:

2.9.1 IMAC Column Preparation for the HPLC-UV

The IMAC-HPLC experiments that used the UV detector also used larger diameter columns that were compatible with the flow cell, ~11 μL. The Tefzel tubing apparatus and packing conditions are described in Section 2.7, but with a few minor differences. All of the following washing steps were performed on the pressure vessel

and were ten minutes in length except for the loading of the sample, which was measured by volume. The column was washed via the pressure vessel at 100 psi. The metal ion was stripped with the 50 mM EDTA solution at a pH=8.2. The IMAC column is then washed with deionized water and activated with 100 mM ferric (III) chloride solution. Following activation with Fe^{+3}, the column is re-equilibrated with Solvent A. As mentioned above, the samples; (1) 10 nanomoles each for the synthesized mono-, di-, and tri-phosphopeptides (30 nmol total), (2) 2 nanomoles each for the 4 peptide mix (8 nmol total), and (3) 2 nanomole each for the esterified 4 peptide mix (8 nmol total) were loaded in the 80% acetonitrile. The sample was loaded and measured by the use of a calibrated pipette (Drummond Scientific Company, Broomall, PA).

2.9.2 HPLC Solvents and Gradient

Choosing the HPLC solvents left few options in this experiment considering that the solvents had to be capable of elution off of the IMAC and optically transparent at 220 nm. Solvent A contained 80% acetonitrile and solvent B contained sodium phosphate, monobasic (Fisher Scientific, Fair Lawn, NJ), 0.02% sodium hydroxide (Fisher Scientific, Fair Lawn, NJ), pH equal to ~9 for solvent B and both A and B provided the least amount of absorbance noise. The concentration of sodium phosphate was 50 mM for the experiment shown in Figure 18 and was reduced to 20mM sodium phosphate for the experiments in Figures 19, 20, and 21. The concentration of sodium phosphate was reduced to 10mM for the experiment shown in Figure 22.

A linear gradient was used from 0-100% B in 17 minutes with a ten minute Solvent A wash following the gradient rise.

2.9.3 Temperature Conditions

The sample was injected onto the column at room temperature prior to the column being placed in the water bath. Ten minutes were allowed for temperature equilibration of the column before the separation began. Prior to the sample injection, a sample blank was injected and the background chromatogram measured at each temperature.

2.9.4 Samples used in HPLC Experiments

Different sets of peptides were used in the kinetics experiments to explore whether the peak shape could be altered with temperature and if the different peptides would coelute. Specific phosphopeptides were synthesized at the University of Virginia Biomolecular Research Facility, Charlottesville, VA. The sequences of these peptides were chosen for many different reasons, the most obvious being that the peptide sequence contained no acidic residues except at the C-terminus so methyl esters did not need to be made prior to analysis. A site of phosphorylation was the only difference between the three different peptides listed below.

ATApSTTASSTAR (Mono-phosphorylated peptide)

ATApSTTASpSTAR (Di-phosphorylated peptide)

ATApSTTApSpSTAR (Tri-phosphorylated peptide)

These three peptides were run on the HPLC-MS and manually sequenced for verification prior to being used in these experiments. They will be referred to as (1) MDT.

The other two sets of peptide mixtures used in these experiments contained angiotensin I human, [Tyr $(PO_3H_2)^4$] – angiotensin II human, RII labeled phosphopeptide, and the MAP (177-189) pT/pY diphosporylated peptides, and were referred to as the (2) 4 peptide mixture, and their esterified counterparts, referred to as the (3) methyl ester mixture.

2.9.5 HPLC Instrumentation

The HPLC employed was an Agilent 1100 Series (Agilent Technologies, Waldbronn, Germany). A binary gradient at 1 mL/min was used for all the HPLC experiments.

An Agilent 1100 Series Variable Wavelength Absorbance Detector was used to detect the amido moiety, which has an absorbance max of 214 nm. A wavelength of 220 nm was used in all IMAC kinetics experiments to avoid noise from the solvents.

2.10 Different Organic Percentage Alters Retention on Column

Results from the IMAC kinetics experiments (Figure 18) demonstrated that at more concentrated elutor conditions, the three synthesized peptides eluted with the same number of peaks as they had phosphorylation sites, indicating that the conformation of the peptide was changing. A possible explanation for this was that the organic modifier that was needed to minimize the hydrophobic interactions could change the peptide conformation. Different percentages of organic modifier were used to investigate the effects of the organic modifier on peptide retention.

The conditions used in this experiment were similar to the IMAC kinetics experiments. Three different experiments were run varying only by the percentage of organic used in the column wash, the sample loading buffer, and solvent A. The 0.02 inch ID Tefzel column packed with 7 cm POROS MC was prepared as before with 10-minute washes of (1) EDTA, (2) deionized water, (3) iron (III) chloride, and (4) acetonitrile at three different percentages, 25%, 50%, and 80%. Ten nanomoles of the synthesized tri-phosphopeptide, **ATApSTTApSpSTAR,** was loaded on the IMAC column in the same percentage of acetonitrile as it was washed in. Solvent A was an aqueous solution of acetonitrile at 25%, 50%, or 80%. Solvent B remained the same in

all three experiments, 10 mM sodium phosphate, 0.02% sodium hydroxide, pH=9. The gradient employed was 0-100% B in 17 minutes and the absorbance detector was used.

2.11 Minimizing Non-Specific Ionic Interactions to the Solid Phase

In the IMAC kinetics experiment, non-specific binding was observed when the esterified angiotensin peptide with no phosphorylation site was run on the HPLC-UV. Determining whether the non-specific ionic interactions to the solid support were present was accomplished by adding salt to the gradient. A low concentration, 7.5 mM, of sodium chloride was added to solvent A, 80% acetonitrile, to minimize any ionic interactions that could be present.

The conditions used are similar to the IMAC kinetics experiments, as described above. Tefzel tubing was packed in house with POROS MC to approximately 7 cm. The column was prepared as before with washes of (1) EDTA, (2) deionized water, (3) iron (III) chloride, and (4) 80% acetonitrile, 7.5 mM sodium chloride, for 10 minutes each on the pressure vessel. Four nanomoles of the esterified samples, angiotensin I Human, [Tyr $(PO_3H_2)^4$] – angiotensin II Human, RII labeled phosphopeptide, and the MAP (177-189) pT/pY diphosporylated peptide, were loaded separately on the column via the pressure vessel. The solvents used were 80% acetonitrile, 7.5 mM sodium chloride for solvent A and 10 mM sodium phosphate, 0.02% NaOH, pH 9 for solvent B. A linear gradient was used from 0-100% B in 17 minutes and UV absorbance detection was employed. The results were compared to those from separate runs of the esterified peptides with no salt in the gradient.

3 Results

3.1 Phosphopeptide Standard

The phosphopeptide standard was important for method validation in terms of fragmentation patterns. Sufficient fragmentation for the (1) peptide backbone and the (2) phosphate loss are needed to positively identify the peptide. Figure 5 is a mass range peak chromatogram of the (+3) charge state and the (+2) charge states of the mono-serine phosphopeptide.

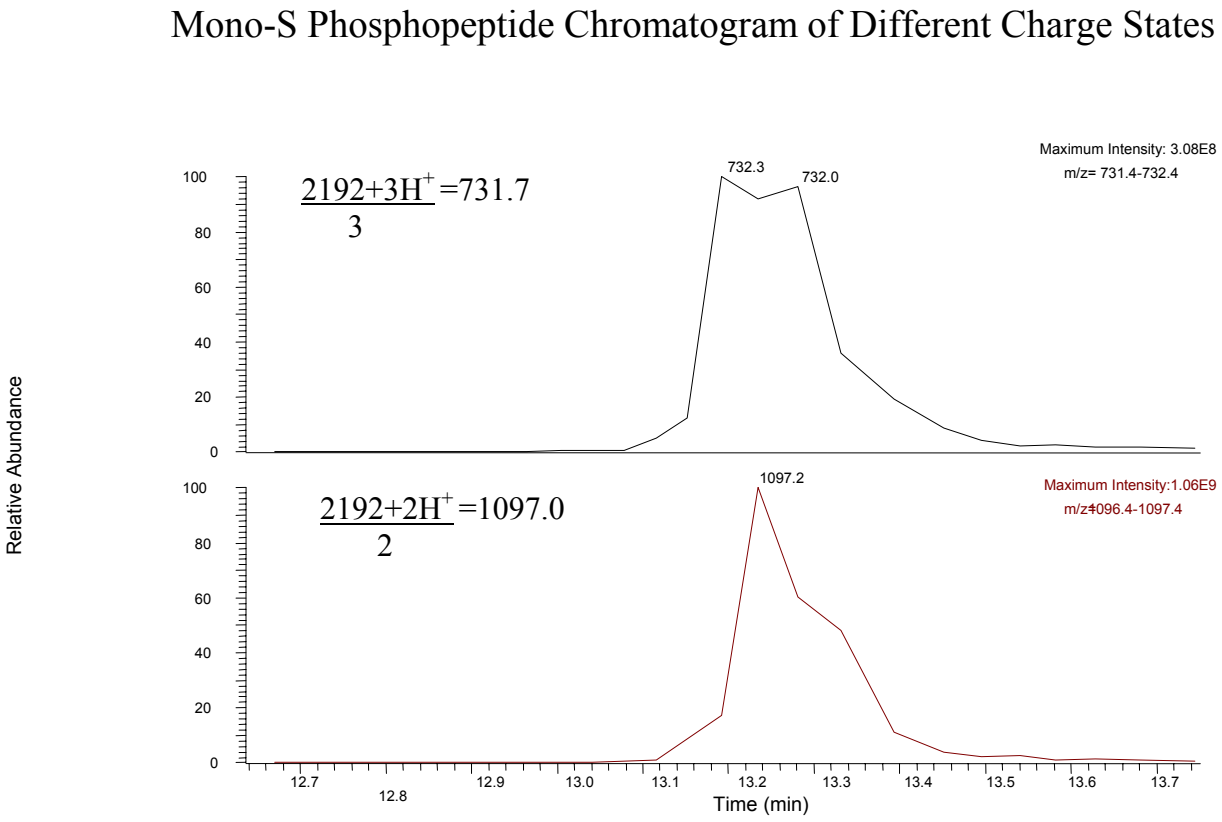


Figure 5 – Mono-S Phosphopeptide Chromatogram of +2 and +3 Charge States from a 1 pmol/µLstandard.

The manual sequencing of phosphopeptides can be difficult due to insufficient peptide fragmentation information leading to gaps in the sequence. B- and y- ion relative abundance is reduced when the phosphate loss is present.[1] In addition, the charge state distribution aids in the verification of the molecular weight of the peptide. Figure 5 provides further verification by showing the elution of two charge states of the Mono-S phosphopeptide.

A CID spectrum and sequencing results from the phosphopeptide experiment are shown next to illustrate manual sequencing. The scan, acquired at 13.17 minutes, is a mass spectrum of the (+3) charge state. The ion fragments were assigned for the high intensity peaks. Below the spectrum, the corresponding b- and y- ions are listed above and below the sequence.

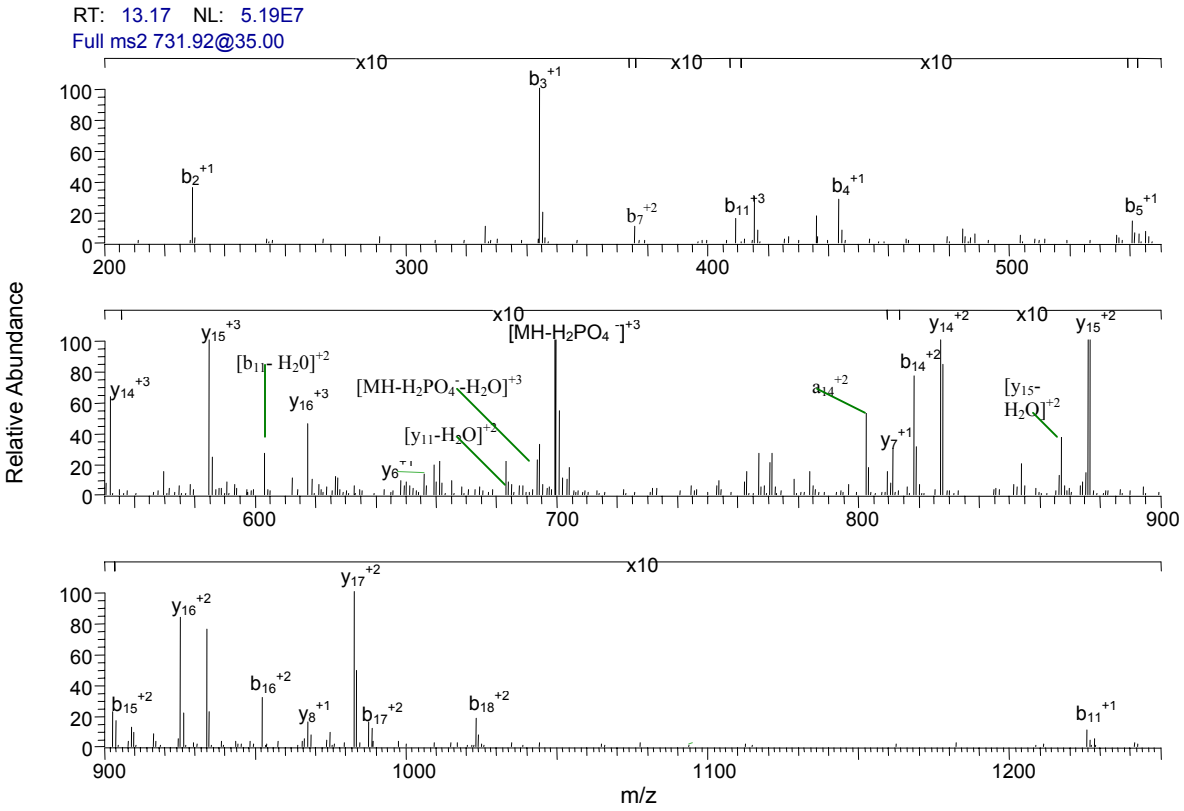


Figure 6-a) Mass Spectrum of Mono-S Phosphopeptide.
Selected CID spectrum shown is $[M+3H^{+}]^{+3}$=731.92.

b_1	b_2	b_3	b_4	b_5	b_6	b_7	b_8	b_9	b_{10}	b_{11}	b_{12}	b_{13}	b_{14}	b_{15}	b_{16}	b_{17}	b_{18}	b_{19}
D	**L**	**D**	**V**	**P**	**I**	**P**	**G**	**R**	**F**	**D**	**R**	**R**	**V**	**pS**	**V**	**A**	**A**	**E**
y_{19}	y_{18}	y_{17}	y_{16}	y_{15}	y_{14}	y_{13}	y_{12}	y_{11}	y_{10}	y_9	y_8	y_7	y_6	y_5	y_4	y_3	y_2	y_1

Figure 6- b) Identified B- and Y-Ion Series for Mono-S Phosphopeptide.
The underlined amino acid residues have been positively identified in Figure 6-a.

The positively identified amino acids are underlined above and in the following figures. The above spectrum is a magnification so as to be able to see the lower

abundance ions. The lines and numbers that appear above the spectra are the amount of magnification at that particular point in the spectrum. The $[MH\text{-}H_2PO_4^-]^{+3}$ peak is an indication of the phosphate loss that readily occurs during the collision induced dissociation in the ion trap. It is evident that there are gaps in the sequencing, which can be attributed to many different things. First, the Oxygen – Phosphate bond is the weakest in the sequence and will be the first bond broken, resulting in a small population of other ions, and making it difficult to sequence completely. A way to avoid this small population and therefore increase sequence information would be to run an MS^3 experiment. The proline inserts even more complications due to its N-terminus directed fragmentation.

3.2 Peptide Esterification

The peptides were esterified to eliminate the non-specific interactions from the carboxylic acid moieties with the chelated metal complex. The following mass spectra are the methyl ester sequences. The b- and y- ions are shown in the spectra with a corresponding label and charge. Following each spectrum, Figures 7b, 8b, 9b, and 10b are shown to illustrate the positively identified amino acid residues with the corresponding b- and y- ion series. The underlined amino acid has been positively identified in the mass spectrum. In each spectrum, the b_1-ion is absent because it cannot proceed through the cyclic intermediate that is required to give product ion spectra.

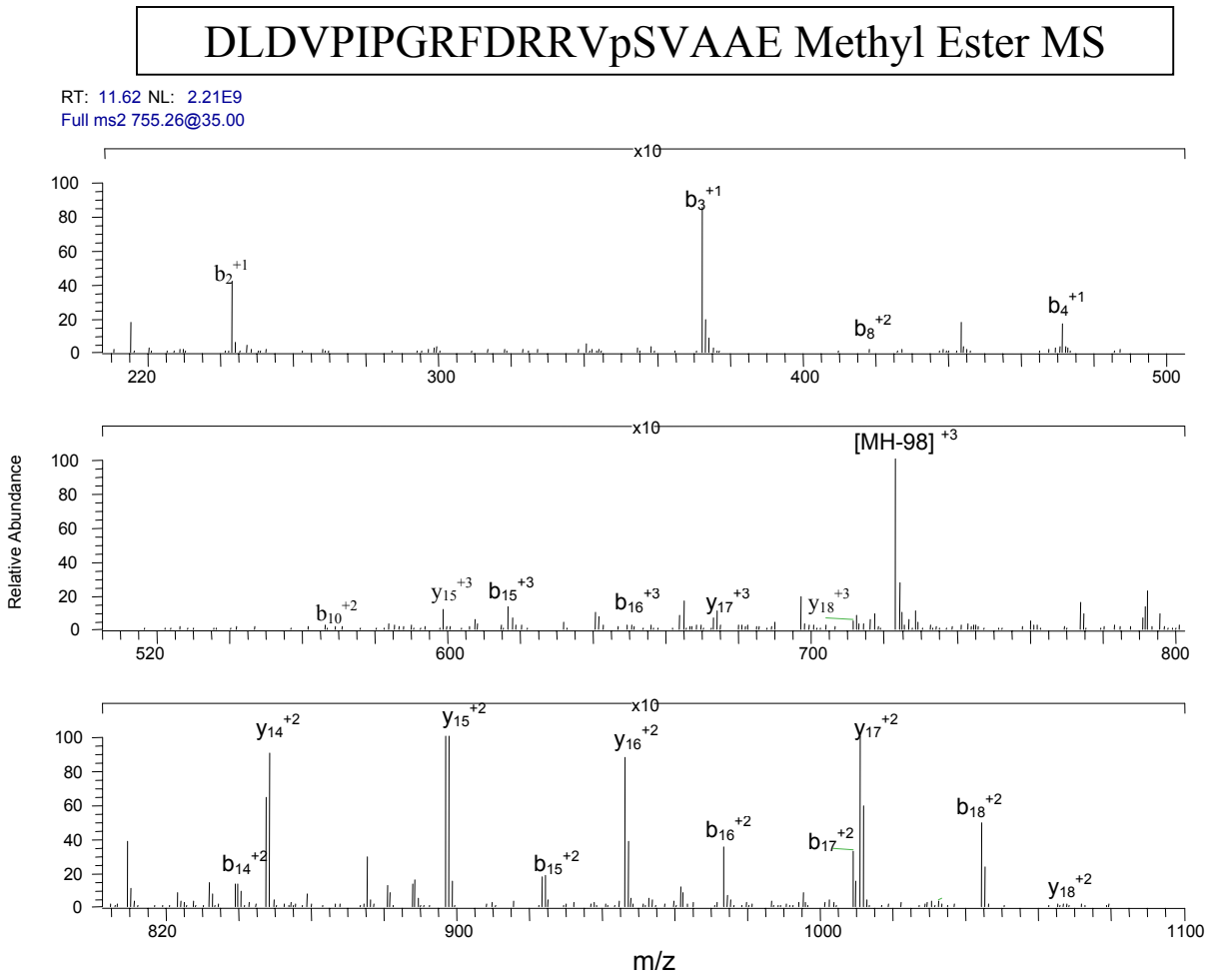


Figure 7-a) Mass Spectrum of Methyl Ester DLDVPIPGRFDRRVpSVAAE phosphopeptide.
Selected CID spectrum shown is $[M+3H^{+}]^{+3}$=755.26.

b_1	b_2	b_3	b_4	b_5	b_6	b_7	b_8	b_9	b_{10}	b_{11}	b_{12}	b_{13}	b_{14}	b_{15}	b_{16}	b_{17}	b_{18}	b_{19}
D	**L**	**D**	**V**	**P**	**I**	**P**	**G**	**R**	**F**	**D**	**R**	**R**	**V**	**pS**	**V**	**A**	**A**	**E**
y_{19}	y_{18}	y_{17}	y_{16}	y_{15}	y_{14}	y_{13}	y_{12}	y_{11}	y_{10}	y_9	y_8	y_7	y_6	y_5	y_4	y_3	y_2	y_1

Figure 7-b) Identified B- and Y-Ion Series for Esterified Mono-S Phosphopeptide.
The underlined amino acid residues have been positively identified in Figure 7-a.

The proline residue often directs fragmentation to its N-terminal side so the b- and y- ion series is less complete.[7] In this spectrum, y_{15}^{+2} is one of the most abundant ions in

the spectrum, which agrees with this theory. Also, Figure 7 is magnified to show the lower abundance ions. The most abundant ions in the spectrum have been assigned, indicating that the increased ion population of the phosphate loss overshadows the other ions in the spectrum.

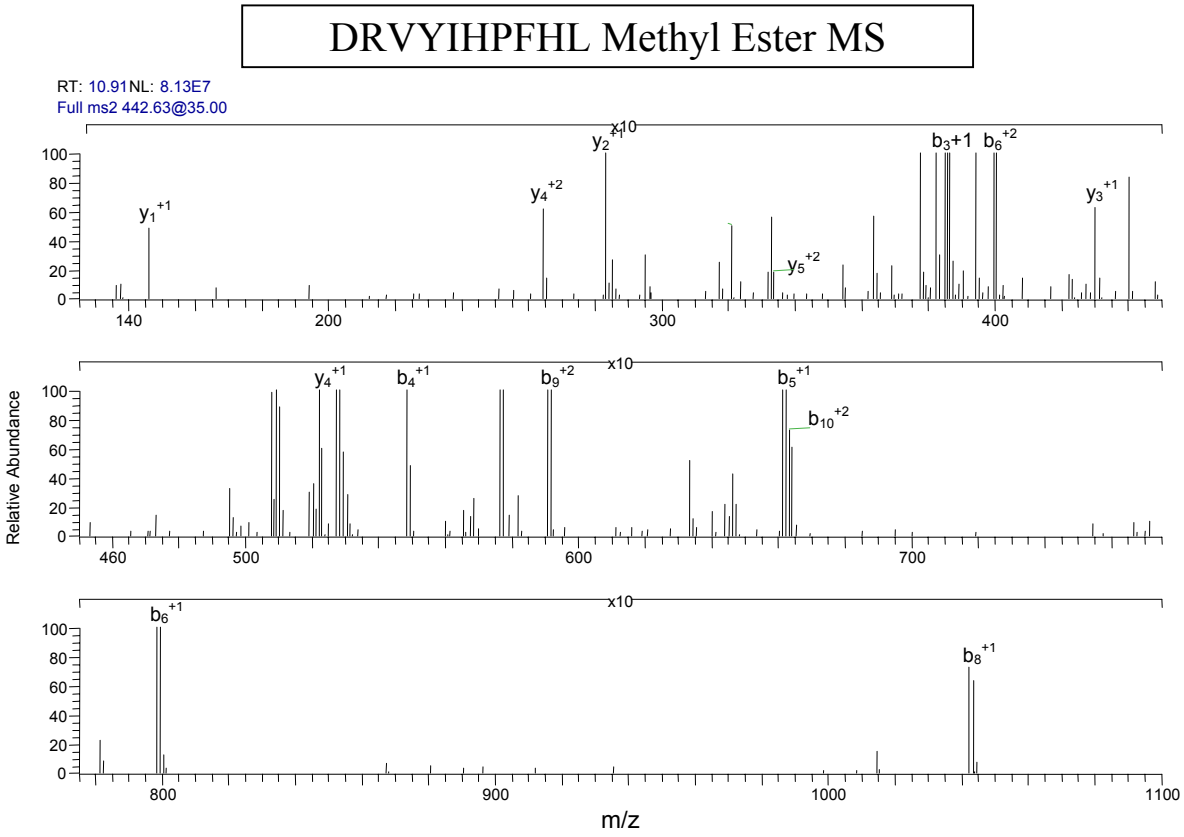


Figure 8-a) Mass Spectrum of Methyl Ester DRVYIHPFHL peptide.
Selected CID spectrum shown is $[M+3H^{+}]^{+3}$=442.63.

b_1 b_2 b_3 b_4 b_5 b_6 b_7 b_8 b_9 b_{10}

D R V Y I H P F H L

y_{10} y_9 y_8 y_7 y_6 y_5 y_4 y_3 y_2 y_1

Figure 8-b) Identified B- and Y-Ion Series for Esterified Angiotensin.
The underlined amino acid residues have been positively identified in Figure 8-a.

The angiotensin standard is shown in Figure 8a due to its use in Sections 3.7 and 3.8. In this case, the internal proline fragment does not affect the sequencing. This sequence is more complete due to the absence of phosphate allowing the ion population of the peptide backbone to be more uniform.

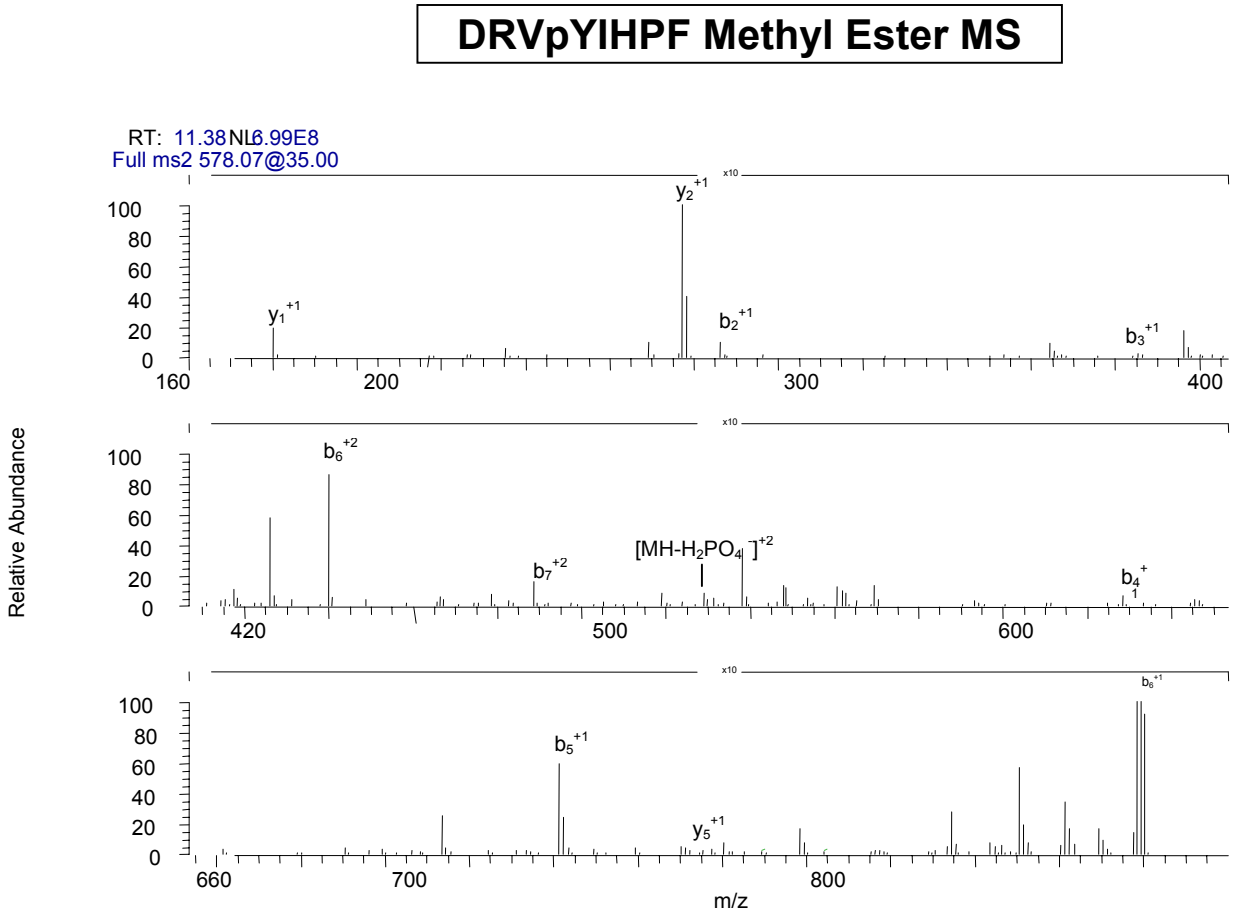


Figure 9-a) Mass Spectrum of Methyl Ester DRVpYIHPF phosphopeptide.
Selected CID spectrum shown is $[M+2H^{+}]^{+2}$=578.07.

b_1 b_2 b_3 b_4 b_5 b_6 b_7 b_8

D R V pY I H P F

y_8 y_7 y_6 y_5 y_4 y_3 y_2 y_1

Figure 9-b) Identified B- and Y-Ion Series for Esterified Angiotensin-II-Phosphate.
The underlined amino acid residues have been positively identified in Figure 9-a.

The angiotensin-II-phosphate peptide is almost completely sequenced except for the Aspartic Acid residue on the N-terminus.

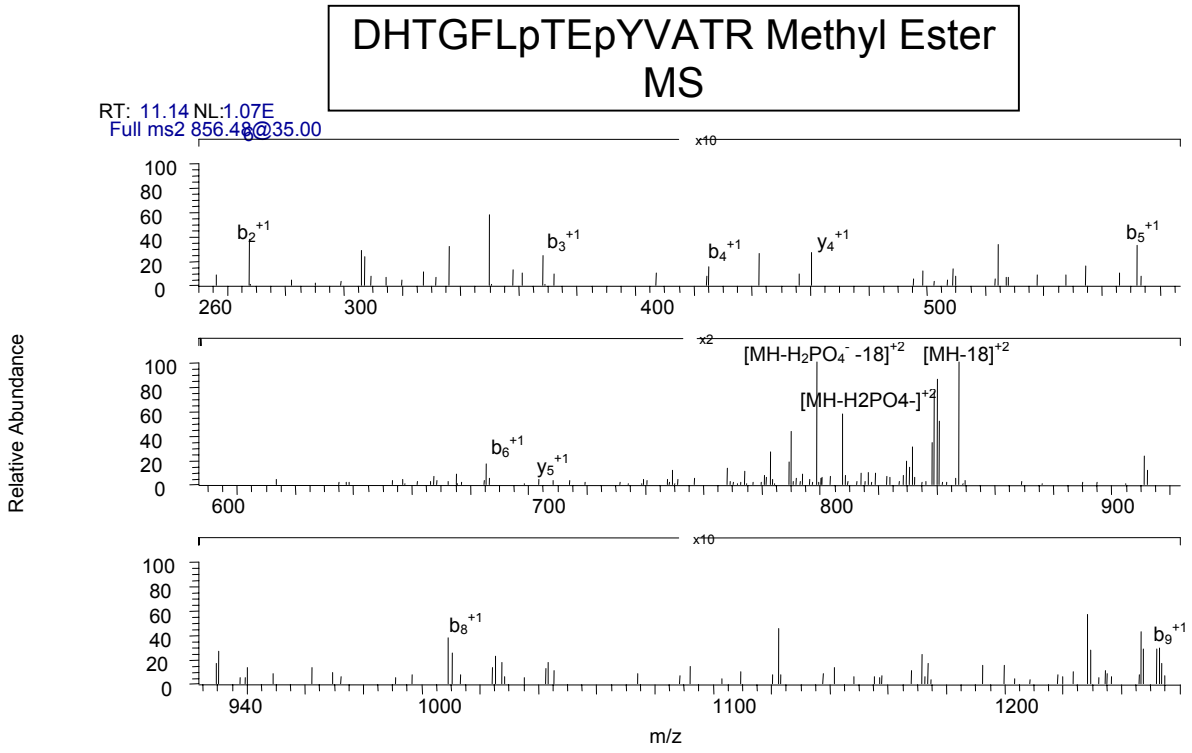


Figure 10-a) Mass Spectrum of Methyl Ester DHTGFLpTEpYVATR phosphopeptide.
Selected CID spectrum shown is $[M+2H^+]^{+2}$=856.48.

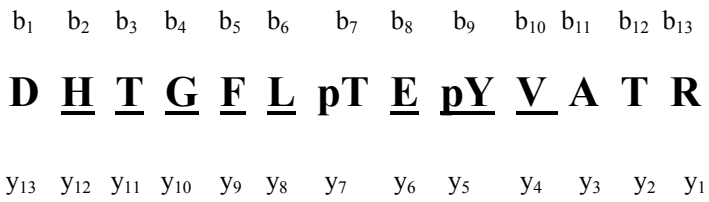


Figure 10-b) Identified B- and Y-Ion Series for Esterified Di-Phosphorylated Peptide.
The underlined amino acid residues have been positively identified in Figure 10-a.

The above sequence is complete, although there are not enough peaks for manual sequencing. The loss of phosphate is observed and it is evident in this mass spectrum that the loss of water is the predominant peak.

3.3 Comparison of Esterified Peptides and Non-Esterified Peptides on PS-DVB

The results below indicate that the esterified angiotensin-II-Phosphate does elute later (approximately 5 minutes) than the non-esterified angiotensin-II-Phosphate on a PS-DVB reverse phase column. The methyl ester does add more hydrophobicity to the chain, which contributes to increased retention time. The difference in molecular weight can be attributed to the substitution of the -H group with a $–CH_3$ group. In the figure below, the addition of 28 (two methyl ester additions) divided by 2 (charge state of this particular peptide) gives a difference of 14, which is shown by the difference in molecular weight between the two peaks.

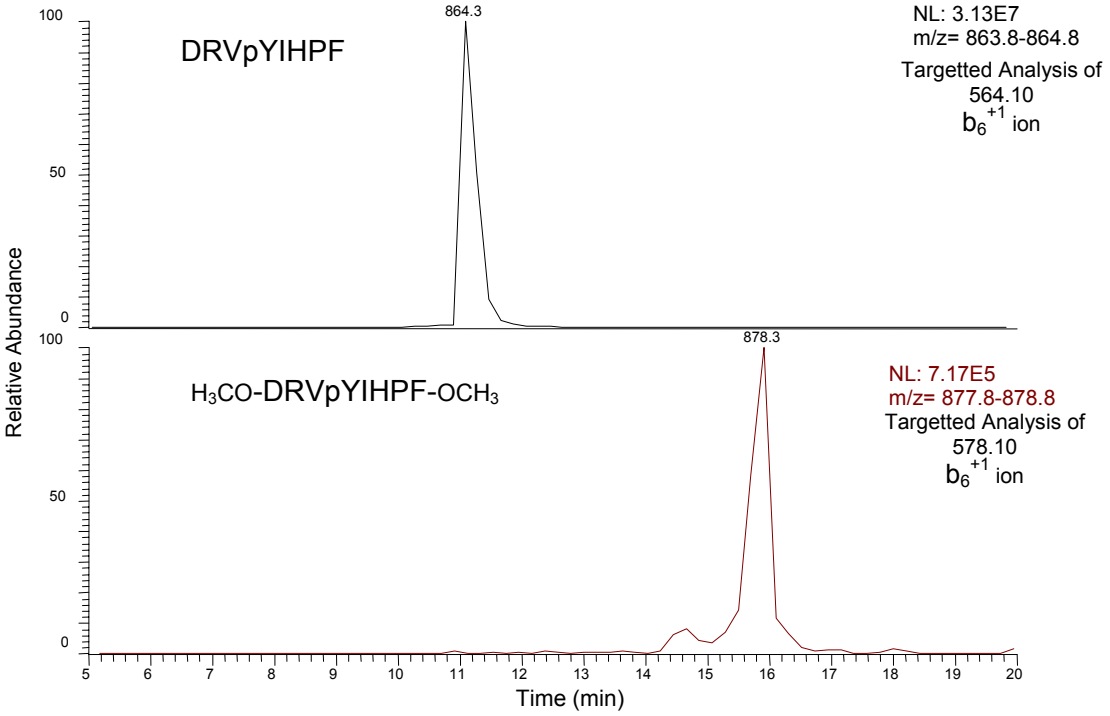


Figure 11 – RP Separation of Angio-II-Phosphate and Esterified Angio-II-Phosphate on PS-DVB.

500 fmol of each peptide was loaded on PS-DVB, gradient eluted from 0-100% Solvent B in 30 minutes, and targeted MS analysis was performed. Solvent A was 0.1 M NH_4OH and Solvent B was 70% ACN in 0.1M NH_4OH. NL is the maximum intensity value attained in the chromatogram.

3.4 Investigation of non-specific hydrophobic interactions with the POROS MC hydrophobic solid support

Non-specific interactions with the POROS-IMAC solid support must be minimized. To prove that the peptides interact hydrophobically with the POROS solid support, they were eluted with an organic gradient from the uncharged IMAC column. A targeted analysis was performed for the mass ranges shown in Figure 12. The noise in

the chromatograms is due to the lack of an intense peak in most scans that would significantly change the y-axis range. The broad peaks indicate that multiple mechanisms of interaction are occurring.

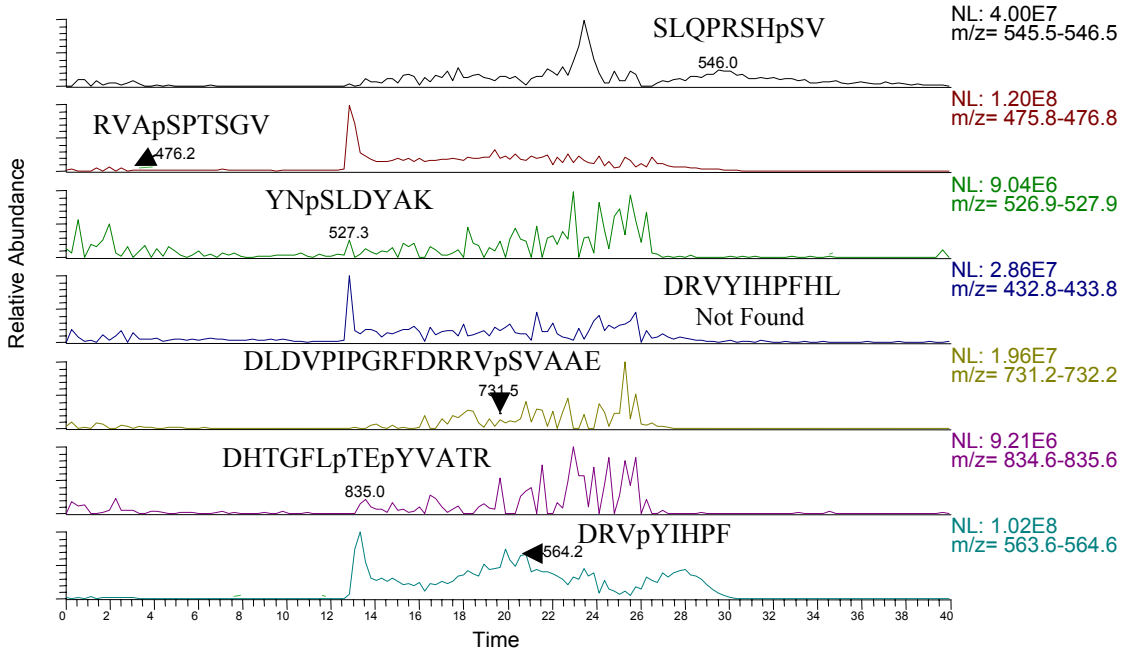


Figure 12 – Phosphopeptide Elution From POROS Hydrophobic Solid Support using Organic.

The point at which there appears an arrow or a mass to charge ratio is where the peptide was positively identified on the basis of overlapping charge states and manual sequencing. 500 fmol of each peptide was loaded onto POROS MC (no Fe^{+3}). Solvents used were Solvent A – 0.1 M Acetic Acid and Solvent B – 70% ACN in 0.1 M Acetic Acid. NL represents the maximum intensity value attained in the chromatogram.

Notice that the non-phosphorylated angiotensin peptide is not found in the above run, however, the angiotensin peptide was observed when the experiment was repeated, thereby indicating that the peptide did not load on the column. Because the peptide mix contained known standards and all peptides had been manually sequenced before, identification was done by matching the known m/z to the CID fragment ions and manually verifying the CID. The table below shows the expected elution order (from a

previous C_{18} separation) and the actual elution order of the peptides. The elution order could be different from the previous C_{18} separation due to more than one mechanism of separation occurring, for example, interactions with the IDA ligand.

Peptide	Elution Order, Expected	Elution Order, Actual
SLQPRSHpSV	1	6
RVApSPTSGV	2	1
YNpSLDYAK	3	2
DRVYIHPFHL	4	Not Detected
DLDVPIPGRFDRRVpSVAAE	5	4
DHTGFLpTEpYVATR	6	3
DRVpYIHPF	7	5

Table 2 – Expected and Actual Elution Orders of the 7 Peptides from an Uncharged IMAC stationary phase.

3.5 Minimizing Hydrophobic Interactions with the POROS Hydrophobic Solid Support

Iron (III) chloride was not loaded on the column to explore whether a higher percentage of acetonitrile could minimize the hydrophobic interactions with the solid support. The β-Casein protein digest used contained a range of both hydrophobic and hydrophilic peptides. The digest was loaded in two different conditions, 80% and 25% acetonitrile loading buffer. The peaks shown are listed by their elution order, shown in Figure 13.

1 FQpSEEQQQTEDELQDK, +2 Charge State, 1031.7

2 AVPYPQR, +1 Charge State, 830.5

3 VLPVPQK, +1 Charge State, 780.5

4 EMPFPK, +1 Charge State, 748.4

5 GPFPIIV, +1 Charge State, 742.5

6 DMPIQAFLLYQEPVLGPV, +2 Charge State, 1094.0

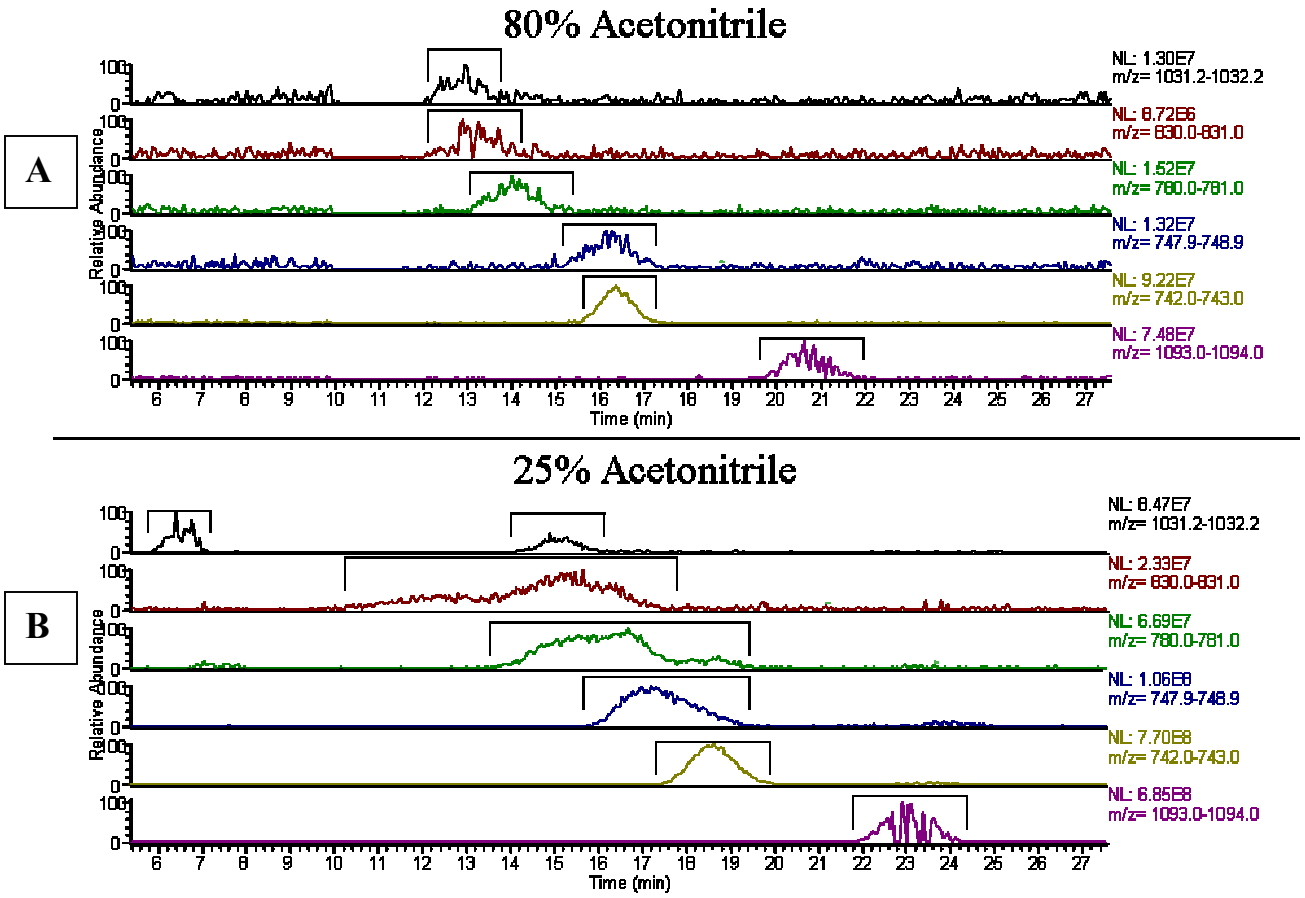


Figure 13 – Comparison of 6 β-Casein peptides in 80% and 25% Acetonitrile Loading Buffer to Minimize Hydrophobic Interactions.

One pmol of protein digested with tryspin was loaded on the column in: **A**) 80% ACN loading buffer or **B**) 25% ACN loading buffer. Solvents used were 0.1M Acetic Acid for Solvent A and 70% ACN in 0.1M Acetic Acid for Solvent B. Between the different loading buffers, the maximum intensity values (NL) were compared and the brackets above the peaks represent the increase in peak width for the 25% ACN experiment.

Figure 13 illustrates that the hydrophobic interactions are decreased and that the peak widths are narrower when the peptides are loaded in 80% acetonitrile. The peptides were identified based on their charge state envelope.

Peptide	80% Acetonitrile	25% Acetonitrile
FQpSEEQQQTEDELQDK	0.73	9.3
AVPYPQR	0.47	6.7
VLPVPQK	1.3	18
EMPFPK	0.50	18
GPFPIIV	4.6	99
DMPIQAFLLYQEPVLGPV	7.5	83

Table 3 –Approximate Peak Areas of β-Casein Peptides Utilizing 80% and 25% Acetonitrile to Aid in the Reduction of Hydrophobic Interactions with the POROS Solid Support.

All approximate peak area values have units of $1*10^7$ counts*min.

Table 3 compares the relative peak areas of the β-Casein peptide in the 80% and 25% acetonitrile loading buffer. The peptides that were loaded in the 80% acetonitrile loading buffer had less overall peak area when compared to the 25% acetonitrile loading buffer, indicating that 80% acetonitrile does minimize the hydrophobic interactions with the POROS solid support.

The results, while encouraging, indicate that the peptides are still interacting with the POROS solid support or the chelating ligand when being loaded in the 80% acetonitrile. This interaction could be attributed to ionic interactions between the chelating ligand and the peptides. The 80% and 25% acetonitrile washes were both at a pH=6, which is above the pKa of the carboxylic acid groups of the IDA, so they will be negatively charged, and low enough to allow the basic residues to remain protonated. Due to the pH of the solvent system, another ionic interaction that could have occurred is the deprotonated acidic residues (or phosphorylated peptide) attraction with the protonated nitrogen on the chelating ligand. A possible solution would be to add salt to the 80% acetonitrile loading buffer to aid in minimizing the ionic interactions, which will be shown in a later experiment. Because the 80% acetonitrile was shown to minimize the

hydrophobic interactions, it was used as solvent A in the majority of the remaining experiments shown.

3.6 Minimizing Hydrophobic Interactions with the POROS Solid Support with Non-Ionic Detergent Prewash

As a preliminary means to test the ability of a hydrophilic support to reduce hydrophobic binding to allow the use of aqueous buffers, the POROS IMAC beads were coated with a non-ionic detergent prior to the separation. A non-ionic detergent, NP-40, was used to coat the column during a pre-wash. Prior to the separation, the excess detergent was rinsed from the column.

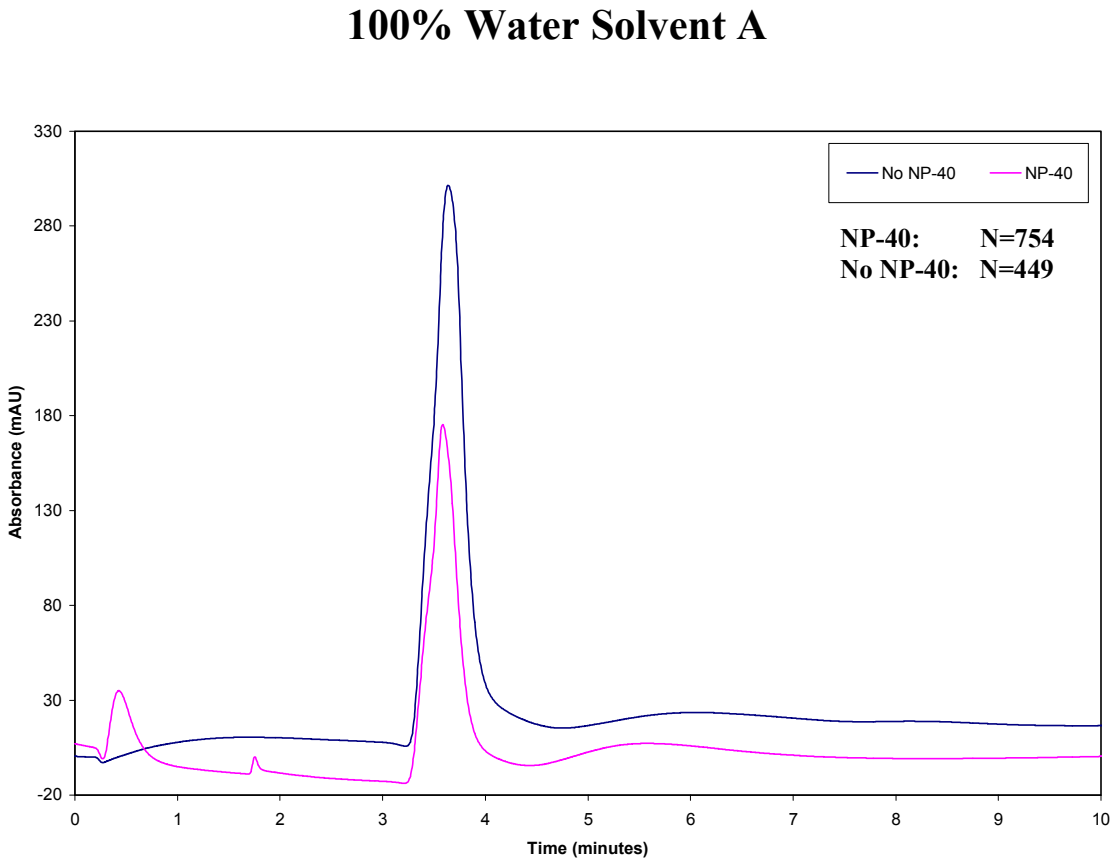


Figure 14 – Three Peptide Mixture with and without an NP-40 Detergent Prewash To Minimize Hydrophobic Interactions with the POROS Solid Support.

The activated Fe^{+3}-IMAC column was loaded with [Tyr $(PO_3H_2)^4$] – angiotensin II human, RII labeled phosphopeptide, and MAP (177-189) pT/pY diphosporylated peptide. This chromatogram is an overlay of two different experiments on the HPLC with UV Detection. Solvent A – 0% Acetonitrile (100% deionized water), Solvent B – 10 mM Sodium Phosphate, 0.02% Sodium Hydroxide, pH=9. Calculated separation efficiencies, N, are shown for both peaks.

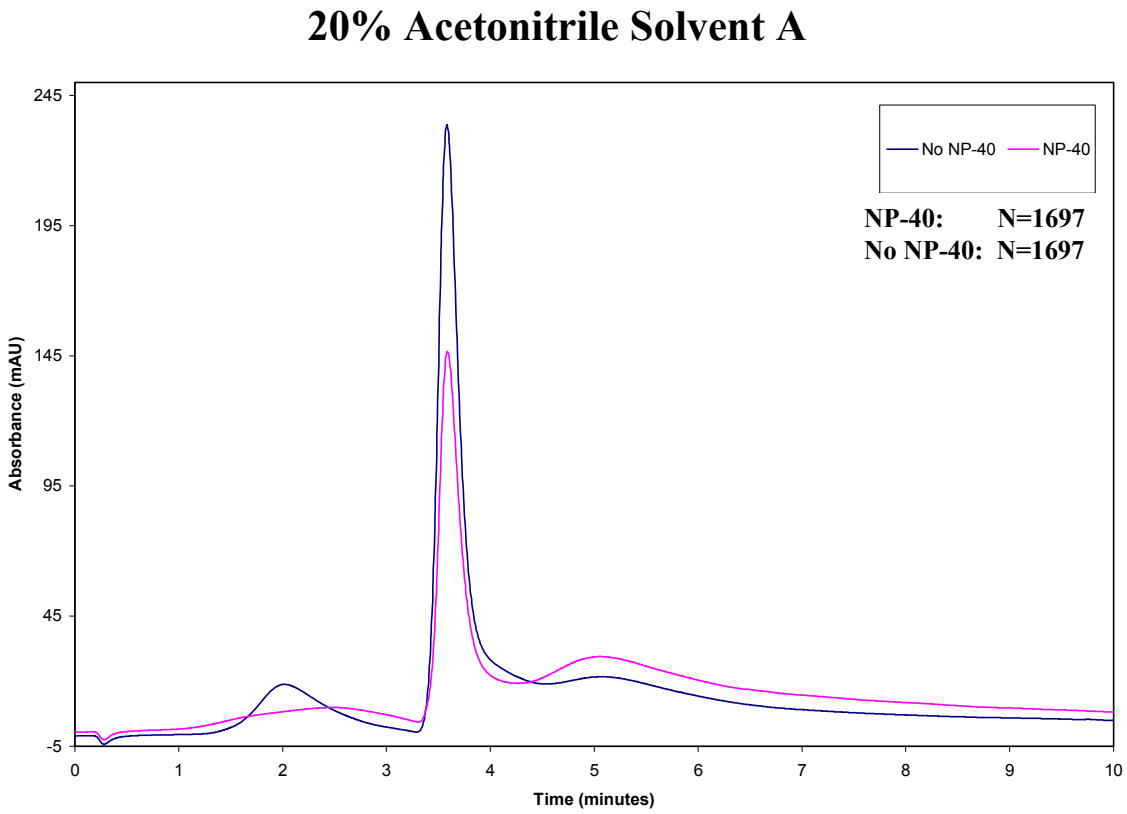


Figure 15 - Three Peptide Mixture with and without an NP-40 Detergent Prewash To Minimize Hydrophobic Interactions with the POROS Solid Support.

The activated Fe^{+3}-IMAC column was loaded with [Tyr $(PO_3H_2)^4$] – angiotensin II human, RII labeled phosphopeptide, and MAP (177-189) pT/pY diphosporylated peptide. This chromatogram is an overlay of two different experiments on the HPLC with UV Detection. Solvent A –20% Acetonitrile, Solvent B – 10 mM Sodium Phosphate, 0.02% Sodium Hydroxide, pH=9. Calculated separation efficiencies, N, are shown for both peaks.

Figures 14 and 15 show the elution of the peptide mixture with and without a detergent prewash. The absorbance of the peptide mixture without a detergent prewash was much greater than the absorbance of the peptide mixture with detergent, indicating that the NP-40 could be changing the environment of the peptide, causing an absorbance change or reducing the peptide- Fe^{+3} interaction. It is important to note that the separation efficiencies are the same in the 20% acetonitrile experiments, indicating that

the detergent prewash is not present due to the increased level of organic. In the 0% acetonitrile experiments, the separation efficiency of the NP-40 prewash was much higher indicating the hydrophobic interactions were minimized. It shows a more hydrophilic solid support can provide decreased hydrophobic interactions with an aqueous mobile phase.

3.7 Online Immobilized Metal Affinity Chromatography

The chromatogram below is an illustration of the gradient elution from the Fe^{+3}-activated IMAC column. The most notable characteristics are the broad peaks. A mixture of 4 peptides was used and the diphosphorylated peptide was not detected.

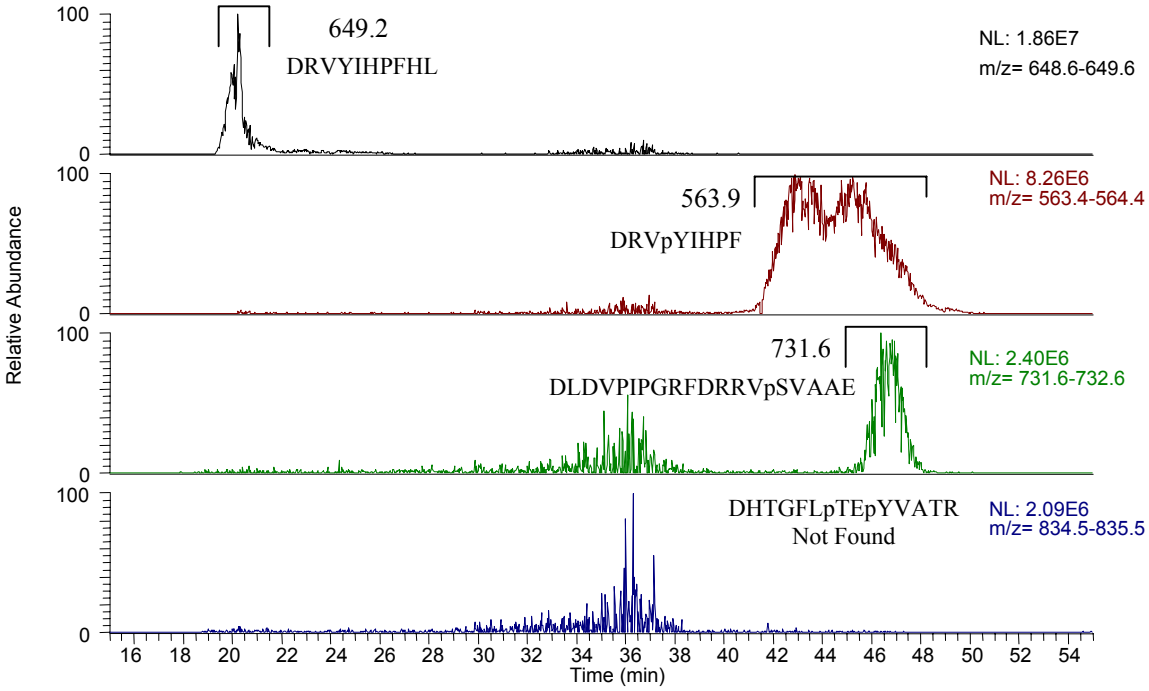


Figure 16 – Gradient Elution from activated Fe^{+3} –IMAC directly into the MS.

500 fmol of each peptide was loaded and eluted with Solvent A-80% ACN and Solvent B-50:50 ACN/10% Ammonium Hydroxide (0.74 M). All peptides were detected with the exception of the di-phosphorylated peptide (see text). NL is the maximum intensity attained in the chromatogram.

Table 4 shows the number of potential phosphorylation and acidic binding sites.

Peptide	Number of Binding Sites ($H_2PO_4^-$)	Number of Binding Sites (Acidic)	Elution Order Predicted & Actual	
DRVYIHPFHL	0	2	1	1
DRVpYIHPF	1	2	2	2
DLDVPIPGRFDRRVpSVAAE	1	5	3	3
DHTGFLpTEpYVATR	2	3	4	No Elution

Table 4 – Number of Potential Binding Sites (Phosphorylation and Acidic) in 4 Peptides.

No elution of the di-phosphorylated peptide could be attributed to the fact that the phosphate forms two coordinate bonds with the metal ion. The carboxyl groups will only form a single bond [4], thus providing the diphosphorylated peptide with more binding

strength than the mono- and non-phosphorylated peptides. The strong binding strength of the di-phosphorylated peptide-Fe^{+3} explains the absence of the elution of the di-phosphorylated peptide with the 0.74 M ammonium hydroxide elution conditions. However, due to the increased concentration of the ammonium hydroxide to 1 M, the diphosphorylated peptide did elute, as seen in Figure 17.

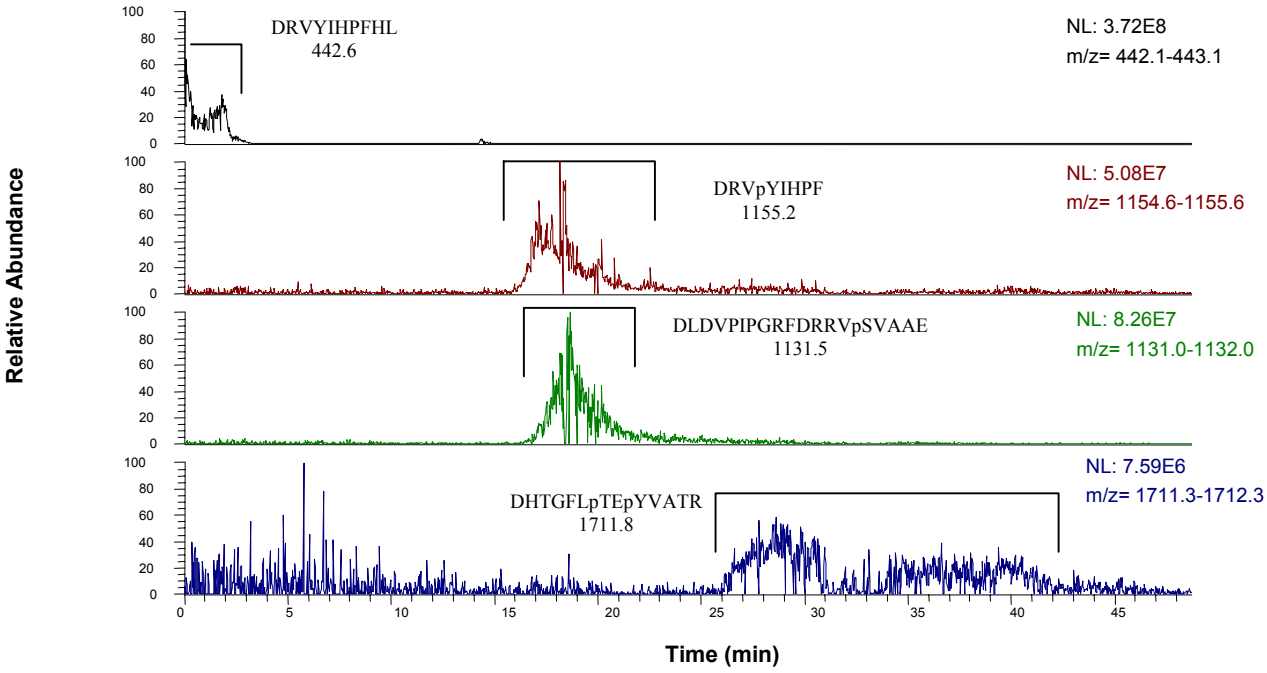


Figure 17 – Gradient Elution of Esterified Peptides from activated Fe^{+3}-IMAC directly into the MS. 5 pmol of each esterified peptide was loaded and eluted with Solvent A – 80% Acetonitrile and Solvent B – 1M Ammonium Hydroxide in 80% Acetonitrile, pH 10.45. All peptides were detected with the 1M Ammonium Hydroxide. NL is the maximum intensity attained in the chromatogram.

The chromatogram of the gradient elution of the esterified peptides from the IMAC is shown above. All four peptides elute in the order predicted from Table 4. Since the hydroxyl of the carboxylic acid groups have been converted to methyl esters,

the only potential binding site that should be present is the Fe^{+3} – phosphate interaction. The angiotensin peptide eluted in the void volume, which was expected because it had no functional groups to complex the Fe^{+3}. The two peptides that contained one phosphorylation site eluted at the same time. With the increased solvent strength, the elution of the di-phosphorylated peptide is observed due to the increased concentration of ammonium hydroxide, however, it is evident that this is not a well resolved mixture and the peaks are very wide. To improve the quality of the separation and the detection, the peak width must be decreased.

3.8 IMAC Kinetics

The purpose of the next section is to investigate if slow desorption kinetics are the main contributors to the broad peaks. The gradient elution of the IMAC directly into the MS demonstrated that while the peptides did elute, they have extremely broad peak widths. To investigate the kinetics, the column temperature was regulated by a water bath.

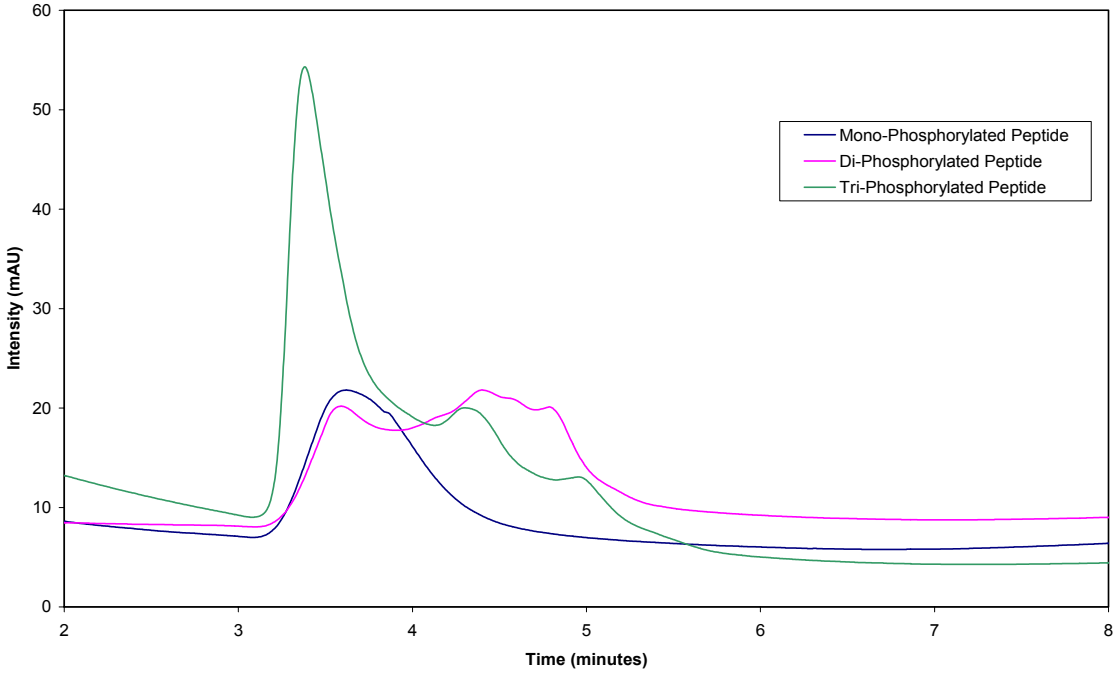


Figure 18 – Three Phosphopeptides, MDT, Separately at Room Temperature.

This chromatogram is an overlay of three separate experiments where 10 nanomoles of each peptide was loaded on the activated Fe^{+3}-IMAC column and detected by UV. Solvent B was 50 mM Sodium Phosphate and 0.02% Sodium Hydroxide, pH~9.

Ten nanomoles of (1) mono-, (2) di-, and (3) tri- phosphopeptides were loaded on a Fe^{+3} activated IMAC column and eluted separately at room temperature to illustrate that the more phosphorylation sites present in the peptide, the greater its retention time. The data above shows that when more phosphate is present, more time is required to break the iron (III) – phosphate bonds. Also, Figure 18 illustrates that as the number of phosphorylation sites increases, the number of peaks increase. This behavior is indicative that different peptide conformations may exist. The organic solvent in the mobile phase could have affected the peptide retention, which may reflect a combination of steric factors and nonelectrostatic interactions,[39] which will be addressed in Section 3.9. These three peptides also appear to elute at the same time, which is shown in the next

chromatogram of the synthesized peptide mixture at varying temperatures.

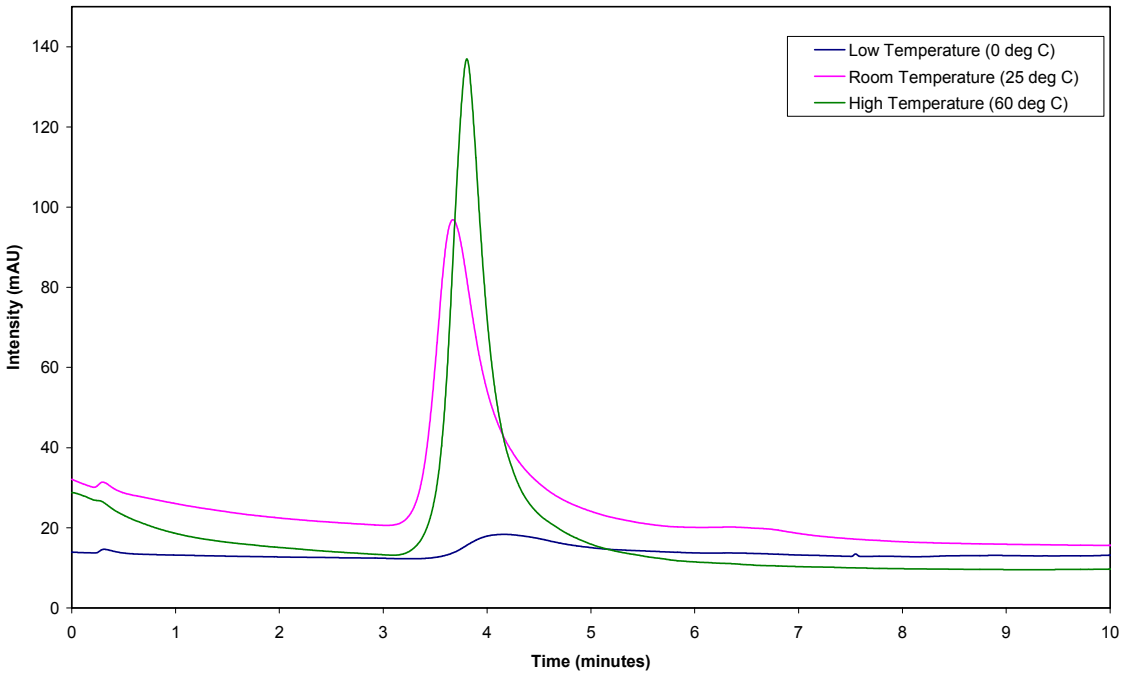


Figure 19 – Mixture of Three Phosphopeptides, MDT, at 3 Different Elution Temperatures.

This chromatogram is an overlay of three separate experiments where 30 nanomoles of the peptide mixture was loaded on the activated Fe^{+3}-IMAC column and detected by UV. Solvent B was reduced to 20 mM Sodium Phosphate, 0.02% Sodium Hydroxide, pH~9. Calculated separation efficiencies, N, are shown for the peaks.

Figure 19 demonstrates that narrower and more symmetric peaks are obtained at higher temperatures. The three separate experiments are each representative of 30 nanomoles total synthesized phosphopeptide. Low temperature slowed the desorption kinetics. The 0° C peak does not return to the baseline and continues to elute very slowly thereby causing a lower separation efficiency, N=115. However, while the peak shape improved between runs, a single peak was observed demonstrating little selectivity, which would be detrimental for the online MS analysis. Solvent B was decreased to 20 mM Na_2HPO_4, 0.02% NaOH at pH 9 to decrease the slope and provide a lower final

strength because the phosphopeptides in the preceding experiments (Figure 18) had been coeluting.

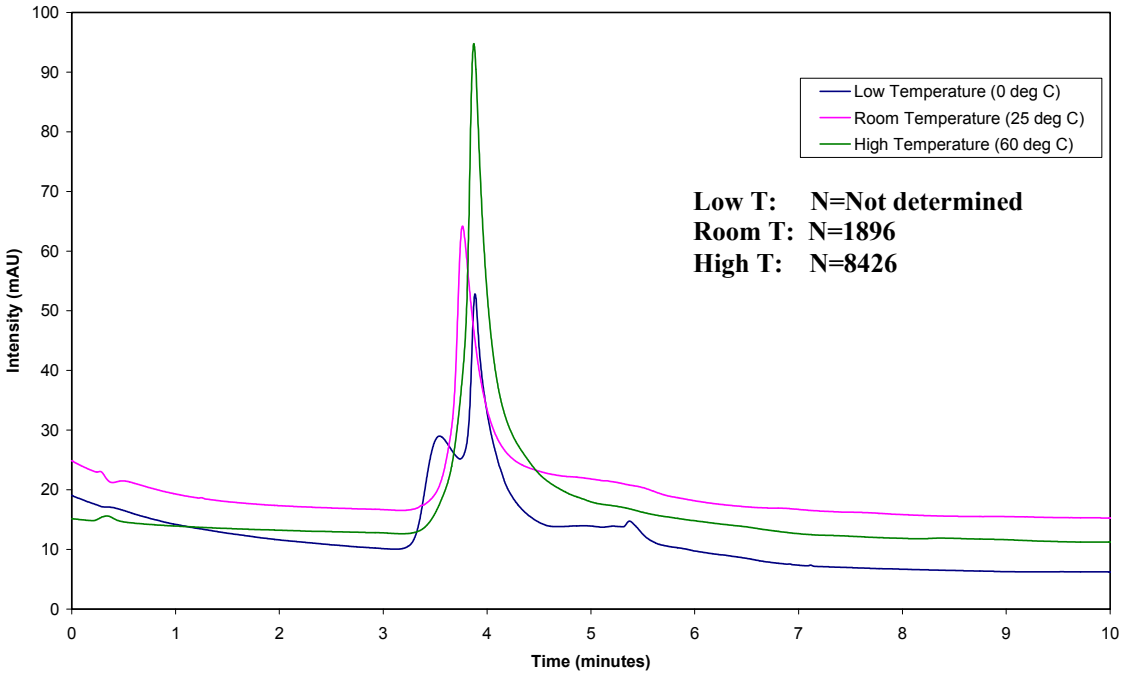


Figure 20 – Mixture of Peptides at 3 Different Elution Temperatures.

This chromatogram is an overlay of three separate experiments where 8 nanomoles of the peptide mixture was loaded on the activated Fe^{+3}-IMAC column. Solvent B was 20 mM Sodium Phosphate, 0.02% Sodium Hydroxide, pH~9. Calculated separation efficiencies, N, are shown for the room and high temperature peaks but could not be determined for the low temperature peak due to peak split.

The chromatogram in Figure 20 is further evidence that the peptide mixture peak shape is more symmetrical at higher temperature. Each run contains 8 nanomoles total peptide mixture which includes angiotensin I human, [Tyr $(PO_3H_2)^4$] – angiotensin II human, RII labeled phosphopeptide, and the MAP (177-189) pT/pY diphosporylated peptide. These peptides have many potential binding sites in comparison to the preceding experiments, four phosphorylation and numerous carboxylic acid-binding sites in each mixture. The peak at low temperature is not symmetrical in shape; however, the peaks

are more symmetrical at higher temperature, which agrees with the data shown in Figure 19. The separation efficiency of the low temperature peak could not be determined due to the split peak, possibly caused by the mixed mode of separation from the phosphate and carboxylic acid moieties. Elevated temperatures cause higher separation efficiencies, as previously observed in Figure 19, but are accompanied by a decrease in the column selectivity.

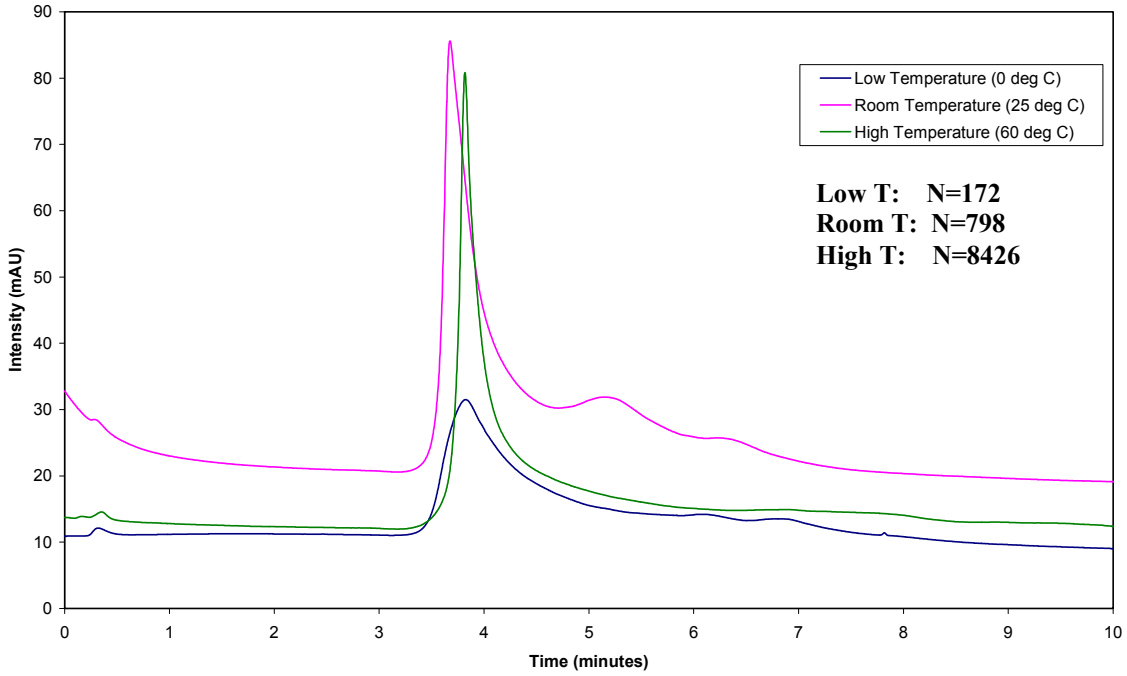


Figure 21 – Mixture of Esterified Peptides at 3 Different Elution Temperatures.

This chromatogram is an overlay of three separate experiments where 8 nanomoles of the peptide mixture was loaded on the activated Fe^{+3}-IMAC column and detected by UV. Solvent B was 20 mM Sodium Phosphate, 0.02% Sodium Hydroxide, pH~9. Calculated separation efficiencies, N, are shown for the peaks.

Figure 21 shows that the esterified peptides elute in a more symmetrical peak at higher temperature. Each run contains 8 nanomoles of total esterified peptide. The

peptide mixture contains angiotensin I human, [Tyr $(PO_3H_2)^4$] – angiotensin II Human, RII labeled phosphopeptide, and the MAP (177-189) pT/pY diphosporylated peptide and these peptides have been esterified and verified by MS prior to being analyzed. Binding should be indicative of the Fe(III)-Phosphate bond because the carboxylic acid groups should be esterified, however, the methyl ester synthesis efficiency is not 100%. Note that the lower temperature peaks have a larger number of distinct peaks while the high temperature conditions cause the peak to be narrow. The general trend seen in Figure 19 and Figure 20 agrees with the data in Figure 21, both in terms of peak shape and separation efficiencies. The high temperature peak is much narrower than the peaks at lower temperatures. The low temperature peak shapes in Figure 21 are more symmetrical in shape when compared to Figure 19 and Figure 20. As temperature increases, random molecular motion and the average kinetic energy of the molecules increases causing faster desorption from the column, which would subsequently result in a more narrow peak shape at higher temperatures.

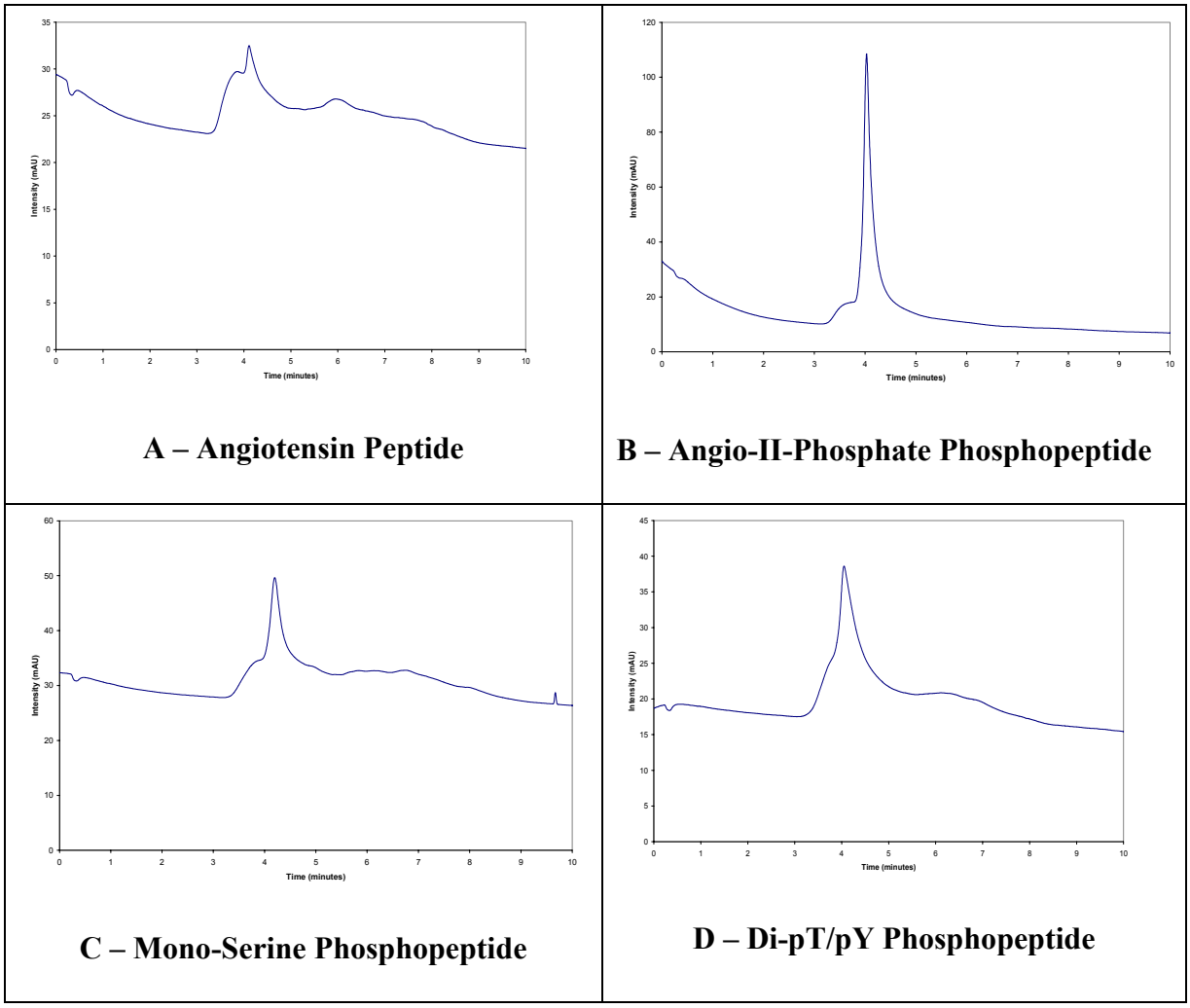


Figure 22 – Esterified Peptides Eluted Separately at Room Temperature and Detected by UV.

The chromatograms are separate experiments where 4 nanomoles of each esterified peptide was loaded on the activated Fe^{+3}-IMAC column and detected by UV. Solvent B was reduced to 10 mM Sodium Phosphate, 0.02% Sodium Hydroxide, pH~9. All chromatograms were background subtracted.

The previous results illustrate that the methyl ester peptides do bind to the IMAC column. The amount of peptide loaded on the Fe^{+3}-activated IMAC column was 4 nanomoles, which was less than in the previous experiments due to the limitation of the methyl ester synthesis. These experiments were performed at room temperature. Solvent B was once again cut in half to 10 mM sodium phosphate. A small amount of angiotensin was detected even though it should have contained no phosphate or acidic

binding sites. Two possible explanations exist to explain the detection of angiotensin. First, non-specific ionic interactions with the solid support could be occurring, allowing the esterified angiotensin possible binding sites. The second possibility can be attributed to the fact that the methyl ester synthesis is not 100% efficient, so a fraction of peptide did have the –COOH moiety through which non-specific binding could occur. It is important to note that the methyl ester derivatization experiment was scaled up (from 1 nmol to 20 nmol) to produce a sufficient amount of peptide to run on the HPLC-UV. The corresponding non-esterified peptide did appear in the mass spectrum when the peptides were verified on the LC-MS, therefore, a fraction of the angiotensin peptide could have been binding through the non-esterified acidic residue.

3.9 Different Organic Percentage Alters Retention on Column

Changing the percentage of acetonitrile does change the peptides' retention on the column, which could possibly be due to changing peptide conformations. The organic solvent in the mobile phase could affect the peptide retention by changing the peptide conformation. An increase or decrease in the peptide retention may indicate that a combination of steric factors and nonelectrostatic interactions are occurring.[39] In Alpert and Andrews report, it was observed that peptides with identical charges at a given pH were resolved through addition of organic solvent to the mobile phases, indicating that additional factors such as hydrogen bonding, charge distribution, conformation, and other non-specific interactions could be contributing to peptide retention.

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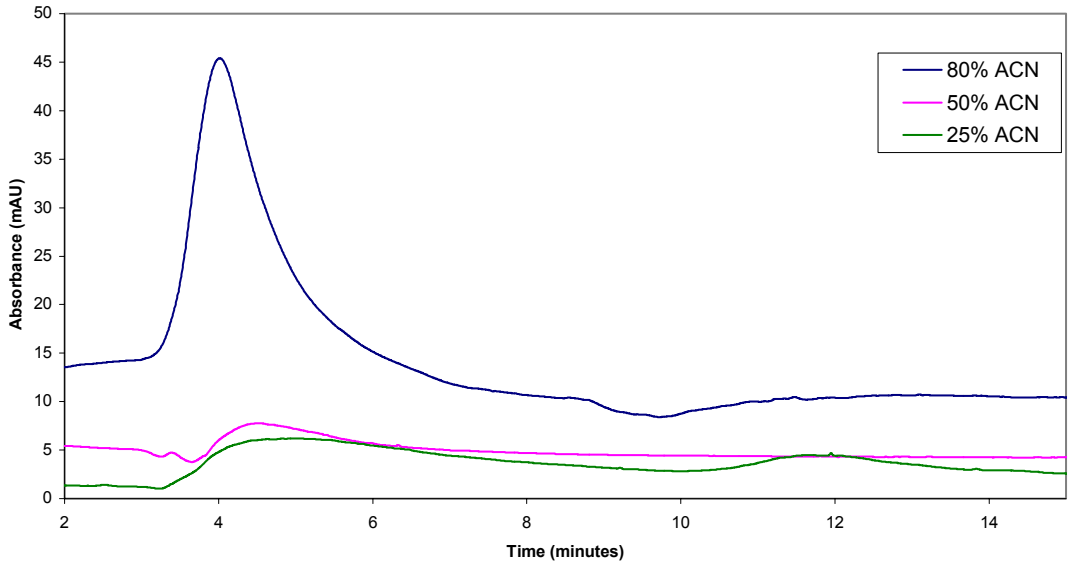

Figure 23– Elution of Tri-Phosphorylated Peptide from the activated Fe^{+3}-IMAC column with Different Percentages of Acetonitrile in Solvent A.

This chromatogram is an overlay of three different runs on the HPLC-UV. Solvent A was (1) 25%, (2) 50%, (3) 80% Acetonitrile and Solvent B was 10 mM Sodium Phosphate, 0.02% NaOH, pH=9.

Figure 23 illustrates that the lower the percentage of acetonitrile, the longer time it takes for the peptide to elute, which could be caused by the peptide being more extended and less folded in solution. Elution was not observed in the 25% acetonitrile experiment while the 80% acetonitrile trial elutes in a symmetrical peak shape. Figure 23 demonstrates that the percentage of organic solvent does change the peptide conformation that plays a role in the adsorption/desorption to the column. However, the results are inconclusive due to two possible competing processes occurring, hydrophobic interactions and conformational changes. The peptide did elute in the higher organic percentage, in agreement with what Alpert and Andrews previously observed with increasing acetonitrile and decreasing retention time.[38] However, this experiment would be most successful with a hydrophilic bead surface.

3.10 Minimizing Non-Specific Ionic Interactions to the Solid Phase

In section 3.8, Figure 22, it was noted that some esterified angiotensin bound to the column. As there were no clear binding sites, it was postulated that binding was a result of ionic interactions (carboxylate) with the chelating ligand (IDA) because the Fe^{+3} was removed from the column. Determining whether non-specific ionic interactions were present was accomplished by adding a low concentration of salt to the gradient. The esterified peptides were used because they had less potential to bind to the column and the results are shown below.

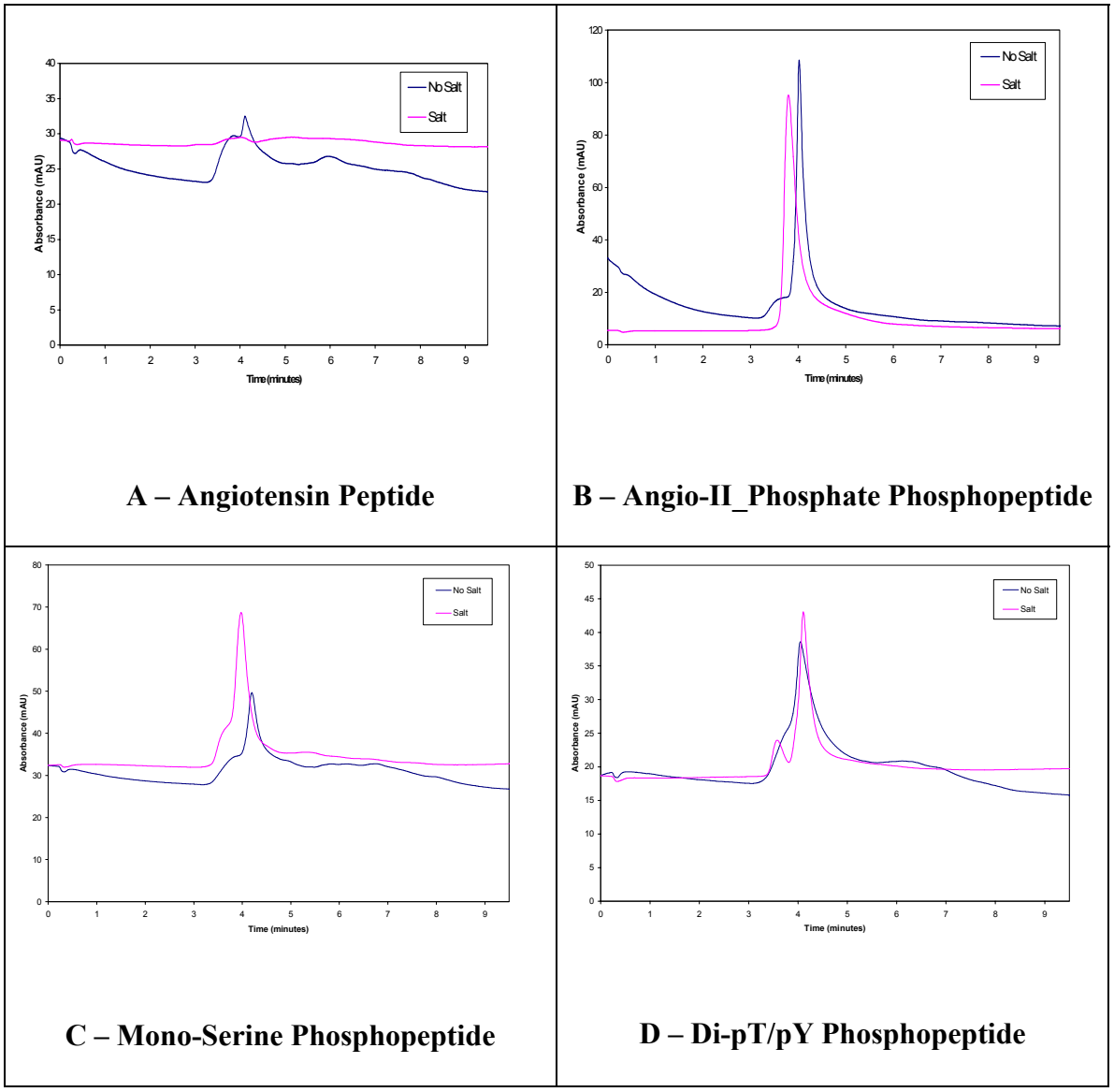

Figure 24 – UV Detection of Esterified Peptides with and without Salt to determine if Ionic Interactions are present.

Salt Gradient: Solvent A – 80% Acetonitrile, 7.5 mM Sodium Chloride, Solvent B – 10 mM Sodium Phosphate, 0.02% NaOH, pH=9. Non-Salt Gradient: Solvent A – 80% Acetonitrile, Solvent B – 10 mM Sodium Phosphate, 0.02% NaOH, pH=9. All chromatograms were background subtracted.

Figure 24 indicates that non-specific ionic interactions are present due to the narrower peak shapes observed with the salt. Upon addition of the salt, the non-specific binding of the angiotensin has been reduced to a non-detectable level, and it appears as if

the other peptides peak shape is narrower, indicating that some of the absorbance that was previously seen was possibly due to non-specific ionic interactions.

4 Conclusions

Phosphopeptides, while occurring in sub-stoichiometric amounts, can be isolated and identified even though detection is difficult due to the fact that phosphorylation does occur at low stoichiometric levels and the MS response of a phosphopeptide can be obscured compared to its unphosphorylated counterpart. In the case of a β-Casein protein for this development work, only one phosphopeptide could be sequenced while the quadruply phosphorylated peptide could not be found presumably due to its high negative charge, which is in agreement with what others have observed.[22]

Immobilized metal affinity chromatography is a logical means for the selective enrichment of phosphopeptides, however, non-specific interactions with the poly(styrene-divinyl benzene) solid support and IDA ligand must be avoided. The presence of hydrophobic interactions was confirmed by the elution of the phosphopeptides off of the POROS-IDA stationary phase when no Iron (III) is present and these interactions were minimized with acetonitrile. The acidic interactions were more difficult to avoid. Methyl esters were successfully synthesized and sequenced, however, the efficiency was not 100%, which meant that the free acid form was still present as an impurity. A low concentration of salt in solvent A and the loading buffer reduced non-specific ionic interactions that were previously observed with the solid support.

Gradient elution online to the MS can be performed, although it was extremely slow when gradient eluted directly into the MS with ammonium hydroxide. Manipulation of the kinetics, made possible by regulating the column temperature during

elution with a water bath, demonstrated that the phosphopeptides could be eluted with a more symmetrical peak shape at higher temperatures. Sodium phosphate at different concentrations was used as the elution solvent in the HPLC in support of compatibility purposes. The peptides in these experiments coeluted, but the temperature could be used to control the peak shape. Effects of ions on selectivity and separation efficiencies vary. For example, when ammonium hydroxide is used, decreased separation efficiency is compensated by an increase in column selectivity. In contrast, sodium phosphate sees an increase in separation efficiency but decreased column selectivity.

5 Future Work

In the interest of time and for the ease of separation, the optimization of online IMAC-MS needs to be realized. The next step in this project could be the online IMAC mass spectral analysis with the aid of heat to decrease the peak width. This could be done with the aid of a water jacket and a temperature controlled water bath, however, preliminary work indicates that many challenges lie ahead for this experiment in terms of solvent choice. Ammonium hydroxide was the solvent that was previously used, however, it suffers from a low boiling point and a frit is not currently available that can withstand heating of the ammonium hydroxide. An appropriate solvent for this application would be a relatively strong base that could withstand heating and would be MS compatible, i.e. no salts.

Also, once better separation results are realized with gradient elution, a second dimension to the separation needs to be found. The optimum separation after the IMAC would be reverse phase because of its MS compatibility, however, the problems override the benefits because the IMAC must be eluted in organic and in base. The high

percentage of organic necessary to minimize the hydrophobic interactions is the largest problem since that is the elutor for the reverse phase separation. If development of a hydrophilic IMAC bead could be found, second dimensional reverse phase could be realized since organic solvent would not be required for IMAC elution and PS-DVB could be used to handle IMAC's basic elution conditions. To date, none have been located.

The mechanism by which the adsorption and desorption occurs is unclear. Future investigation could provide a detailed understanding and better control of selectivity. The elution mechanism most commonly described is competitive elution with a Fe (III)-binding molecule. Different solvents could be studied and different conditions employed to explore this mechanism. It appeared that the sodium phosphate coeluted the phosphopeptides off the stationary phase while the phosphopeptides did not coelute when ammonium hydroxide was used. This mechanism needs to be studied in depth.

Optimum conditions for the IMAC separation need to be realized as phosphorylation is linked to many diseases such as diabetes and cancer, therefore, location of phosphorylation sites could be an important key in the research and development of causes and cures.

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