Methods to Isolate Protein from Detergent-containing Solutions for Proteome Analysis

by

Andrew M. J. Crowell

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Abstract

The use of the ionic detergent sodium dodecyl sulfate (SDS) to assist in the solubilization of protein samples can be highly beneficial in the proteomics workflow. SDS is also a key component in a number of mass-based protein separation techniques such as SDS-PAGE and GELFrEE. Unfortunately, mass spectrometry (MS) based approaches are not compatible with SDS. Protein precipitation with organic solvent is an effective means of depleting contaminants such as sodium dodecyl sulfate (SDS). However, inconsistent and widely varying yields have limited the use of organic solvent precipitation as a front-end purification strategy ahead of MS identification.

This thesis provides an in-depth characterization of protein recovery through acetone precipitation. Through the use of a colorimetric protein assay, the recovery of acetone precipitation is assessed for a variety of sample conditions. It was determined that by increasing the ionic strength of the solution, the efficiency of acetone precipitation can be significantly improved. These results cannot be explained by the current model of protein precipitation. A model of ion-pairing in high organic solvents is proposed to explain this trend. An improved protein precipitation protocol is proposed, using an increased amount of ionic buffer to ensure proper protein precipitation efficiency.

Protein loss during the removal of the supernatant is a known cause of sample loss. To overcome this issue, the use of a filter cartridge to separate the organic solvent from the protein pellet is proposed. Here, a newly developed disposable two-stage spin cartridge, termed the ProTrap XG, is used to facilitate protein precipitation ahead of bottom-up MS analysis. In this device, protein precipitation subsequent enzyme digestion and peptide cleanup occur in a semi-automated manor. High SDS removal (99.75%) efficiency and high protein recovery (>80%) were found to be possible with this device. The device was applied to proteome characterization of rat kidneys experiencing a surgically induced ureteral tract obstruction, revealing several statistically altered proteins, consistent with the morphology and expected pathophysiology of the disease.

Overall, this work provides evidence that acetone precipitation is an effective method to deplete SDS ahead of MS analysis. This concept is demonstrated by coupling gel-eluted liquid fractionation entrapment electrophoresis and acetone precipitation for the analysis of the low molecular weight proteome of plasma. The methods presented to improve protein recovery from acetone precipitation and the development of a filtration unit to streamline the precipitation protocol will help facilitate the use of acetone precipitation as a purification strategy ahead of protein analysis by MS.

List of Abbreviations and Symbols Used

2-D PAGE	Two-dimensional gel electrophoresis
ACN	Acetonitrile
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CID	Collision-induced dissociation
CMC	Critical micelle concentration
CMW	Chloroform/methanol/water
Da	Dalton
DAVID	Database for Annotation, Visualization and Integrated Discovery
dH_2O	Distilled water
DTT	Dithiothreitol
EC_{50}	Half maximal effective concentration
ECD	Electron capture dissociation
ESI	Electrospray ionization
ESI-MS	Electrospray ionization mass spectrometry
ETD	Electron-transfer dissociation
FA	Formic acid
FWHM	Full width at half maximum
GELFrEE	Gel-eluted liquid fractionation entrapment electrophoresis
HILIC	Hydrophilic interaction chromatography
HPLC	High performance liquid chromatography
IAA	Iodoacetamide
ICAT	Isotope-coded affinity tag
iTRAQ	Isobaric tags for relative and absolute quantitation
KDS	Potassium dodecyl sulfate
LB	Luria broth
LC	Liquid chromatography
LC-MS/MS	Liquid chromatography coupled to tandem mass spectrometry
LMW	Low molecular weight
LoD	Limit of detection
m/z	Mass to charge ratio

MALDI	Matrix-assisted laser desorption/ionization
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MudPIT	Multidimensional Protein Identification Technology
MW	Molecular weight
MWCO	Molecular weight cut-off
nESI	Nanoelectrospray ionization
NSERC	Natural Sciences and Engineering Research Council
NSHRF	Nova Scotia Health Research Foundation
PBS	Phosphate-buffered saline
pI	Isoelectric point
PSMs	Peptide-spectrum matches
PTFE	Polytetrafluoroethylene
PTMs	Post-translational modifications
RIPA	Radioimmunoprecipitation assay
RP	Reversed phase
SCX	Strong cation exchange
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SILAC	Stable isotope labeling by amino acids in cell culture
SPE	Solid phase extraction
TFA	Trifluoroacetic acid
Tris or Tris-HCl	tris(hydroxymethyl)aminomethane

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1. Chapter 1: Introduction

1.1 Preface

Proteomics involves the qualitative and quantitative study of proteins, expressed in a biological sample at a defined physiological state. Current protein identification strategies rely on the use of tandem mass spectrometry coupled with liquid chromatography (LC-MS/MS). LC-MS/MS strategies allow for the identification of thousands of proteins in a single experiment. However, biological samples must undergo a number of preparation steps prior to LC-MS/MS. These preparation steps may introduce additives or induce protein loss, which can limit the success of an LC-MS/MS experiment. One such additive, sodium dodecyl sulfate (SDS), is particularly useful for solubilizing proteins. However, SDS interferes with downstream analysis and is difficult to remove. Protein precipitation has been found to be a highly effective method for SDS removal [1]. Historically, protein precipitation has been used as a method to purify and concentrate proteins for over a hundred years. However, the yields from protein precipitation are reported to be non-quantitative and highly variable. This thesis provides insight into protein precipitation using the organic solvent acetone. The overall objective of this work is to provide a highly consistent and effective method for SDS removal. Background information and experimental methods are presented in the first two chapters. In the third chapter, an improved methodology for acetone precipitation is provided. The fourth chapter examines the ability of a two-stage spin cartridge to facilitate protein precipitation. In the final chapter, acetone precipitation was coupled to gel-eluted liquid fractionation entrapment electrophoresis (GELFrEE), as a method to isolate and study the low molecular weight (LMW) proteome of human plasma.

1.2 Introduction to Proteomics

1.2.1 The Importance of Proteomics

Cells function through the transcription of their DNA into RNA, which is then translated into proteins, the workhorse biomolecules of the cell. Proteins facilitate biological activity inside and outside of the cell. This includes cell signaling, cell growth, and cell death. The identity and amount of proteins expressed by a cell is therefore representative of all the biological processes occurring within the cell. The proteome is defined as the entire set of proteins expressed by a specific organism. Proteomics is the study of the proteome of a system to provide insight into the biological functions occurring within the system. The function of proteins is made more complicated by the presence of post-translational modifications (PTMs) such as glycosylation and phosphorylation. Through the characterization and quantitation of proteins in a biological sample in a given physiological state, molecular identifiers of that state can be determined. These "biomarkers" may provide new clinical testing for the diagnoses of disease.

The discovery of protein biomarkers is a promising area of proteomics, potentially leading to advancement in diagnostic testing of a number of diseases. The National Institute of Health defines biomarkers as a molecule which can be "…objectively measured and evaluated as an indicator of a normal biological process, pathogenic process, or pharmacologic responses to therapeutic intervention" [2,3]. Ideally biomarkers may be used in either the diagnosis or the prognosis of the disease [4]. A number of protein biomarkers have made it into clinical practice, such as Pro2PSA for prostate cancer and HE4 for Ovarian cancer [5]. There has been a great deal of interest in biomarker discovery using plasma as a source of protein for biomarker discovery [3].

The advantage of human plasma biomarker discovery stems from its non-invasive method of collection, and the large volumes of sample that can be collected at one time. Plasma has been described as the most widely collected biological sample for the analysis of health, with hundreds of millions of samples being collected yearly for clinical diagnosis [6]. Plasma contains a number of sub-proteomes from the tissues and cells found throughout the body. These tissues and cells are believed to secrete proteins into the blood for transportation and removal. These secreted proteins are believed to be present in the plasma at low concentrations. However the analysis of these less abundant proteins is made complicated by the presence of a large abundance of overly concentrated proteins such as albumin, and immunoglobulins. These proteins mask the presence of the less abundant proteins, reducing the number of proteins identified. The most abundant proteins in plasma are present at concentrations of up to thirty grams per liter. Other plasma proteins have been found to have concentrations lower than one picogram per liter [7,8]. Most exploratory LC-MS/MS approaches for protein identification are limited to a dynamic range under five orders of magnitude [9], which is significantly less than the massive dynamic range of plasma proteins which span over eleven magnitudes. For this reason, methods to decrease the dynamic range through either enrichment of the low abundant proteins or depletion of the overabundant proteins are required. A novel method of depleting the plasma proteome by molecular weight is presented in Chapter 5.

1.2.2 Bottom-up Proteomics and Top-down Proteomics

Two methodologies exist to identify proteins by tandem mass spectrometry (MS/MS). One methodology, termed “bottom-up” proteomics, involves the analysis of peptides obtained from a protein sample. In this methodology proteins are chemically [10] or

enzymatically [11] cleaved into peptides for identification. The most common method is through the use of the proteolytic enzyme trypsin. This enzyme selectively cleaves at the C-terminal end of the amino acids lysine and arginine, forming peptides in a reproducible and predictable fashion. It is noted that when the amino acid proline is present at the N-terminal of lysine or arginine, cleavage is potentially prevented [12]. The digestion of proteins into peptides ahead of LC-MS/MS has the benefit of reducing the charge states of the ions, reducing the chemical complexity of the analytes, and allowing for tandem mass spectrometry through collision-induced dissociation (CID). Peptide fragmentation by CID produces predictable fragment spectra containing mainly y^+ and b^+ ions (see Figure 1.1D for nomenclature) through cleavage of the amide bond as described by the mobile proton hypothesis [13,14].

Peptides are identified through LC-MS/MS by a method known as peptide matching (described in Figure 1.1). This methodology matches the MS/MS fragmentation pattern of the peptide ions to theoretical peptide sequences obtained from a protein database [15]. Bottom-up proteomics is a widely favored technique for the analysis of proteins in biological samples due to its ease of use. It is currently possible to characterize thousands of proteins per experiment using this methodology. For example Webb *et al.* identified 4269 proteins isolated from yeast, through the coupling of strong cation exchange (SCX) and reversed phase (RP) chromatography to LC-MS/MS (termed Multidimensional Protein Identification Technology, MudPIT) [16]. However, this experiment required 15 hours of MS time, due to the extensive amount of peptide separation that is required. Newer and faster scanning LC-MS/MS instruments have

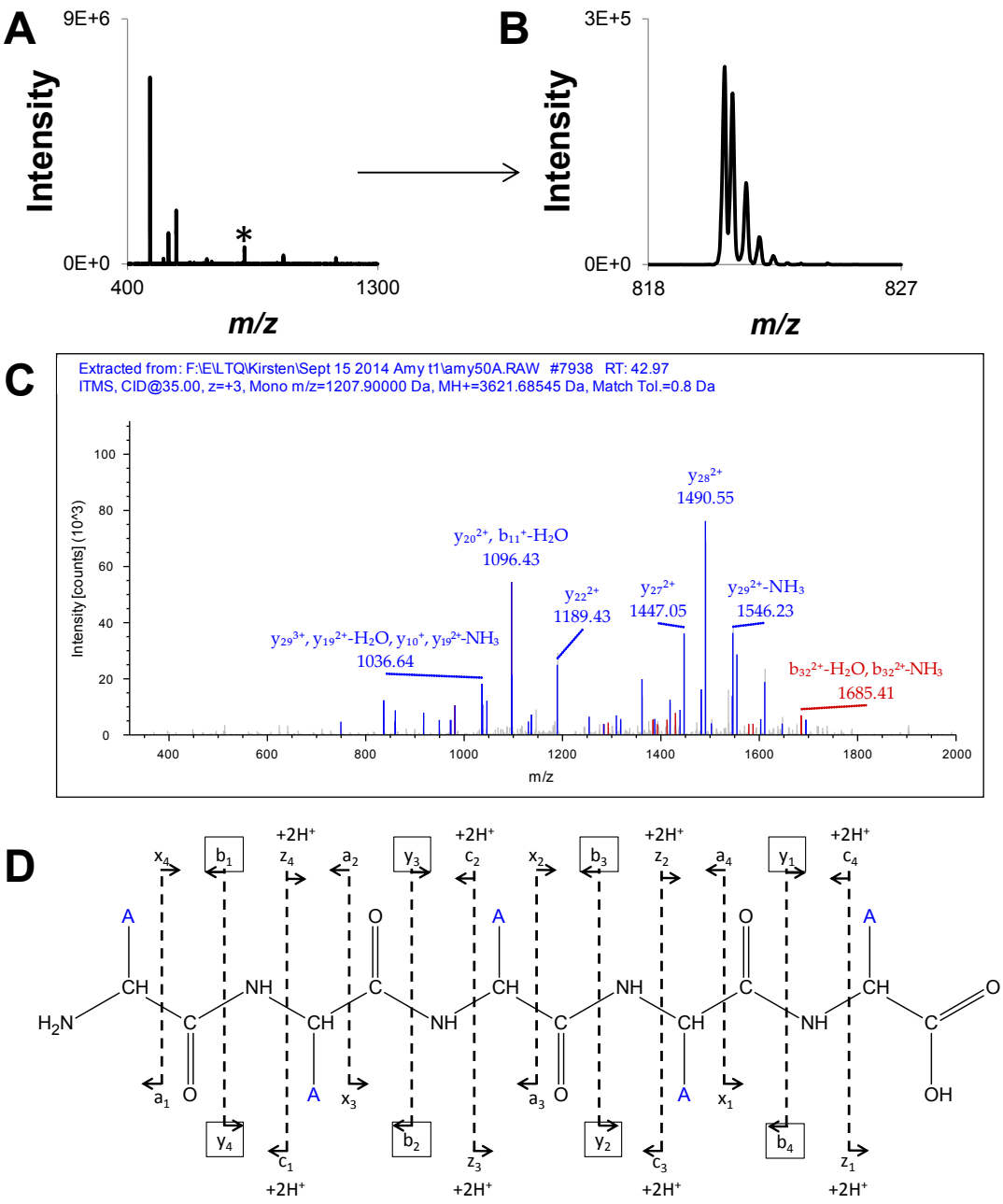

Figure 1.1: Overview of the peptide matching for bottom-up peptide identification. (A) The mass spectrum of all eluting ions is obtained. (B) One ion is isolated (indicated by *). (C) The ion is subject to CID fragmentation to obtain a tandem mass spectrum. MS/MS spectra obtained using Proteome Discoverer 1.4. Low energy CID fragmentation of tryptic peptide ions generally produces y^+ and b^+ ions as shown in (D). Following peptide identification, the resulting amino acid sequences are used to identify possible proteins from a database.

significantly decreased the amount of separation and time required for protein analysis, allowing for the identification of 3977 yeast proteins in a 1.3 hour run [17]. However, due to the overall complexity of the peptide sample, only a fraction of the peptides are identified. For this reason, most proteins are identified with only a fraction of their amino acid sequence [18]. This limits the ability of bottom-up proteomics to detect and quantify any PTMs in the system. The modified peptides are not always identified in the sample. To overcome this, PTM enrichment strategies are required. However these techniques prevent the comparison of PTM modified proteins to their non-modified form.

An alternative approach for protein identification, top-down proteomics, involves the direct analysis of intact proteins by LC-MS/MS. Proteins vary greatly in molecular weight, hydrophobicity, charge, and solubility, which make both sample preparation and protein analysis difficult. In comparison, the majority of peptides are more soluble and less variable in molecular weight than proteins. The main advantage of top-down proteomics is derived from its ability to detect post-translational modifications (PTMs) on proteins [19]. Top-down methodologies allow for the ability to distinguish multiple PTMs on a single protein, and their locations on said given protein. The term "proteoform" has been coined to describe proteins containing varying PTMs. A proteoform is defined as a group of proteins which are expressed from a single gene [20]. Top-down proteomics possess a greater ability to distinguish and detect proteoforms compared to bottom-up proteomics. By examining the intact proteins by MS, it is possible to distinguish between different proteoforms through differences in both parent mass and the fragmentation pattern. However top-down proteomics requires the use of high resolution mass spectrometry (MS) instruments to allow for the distinguishing of the

different charge states of the highly charged proteins. Tandem mass spectrometry of intact proteins is made complicated by the difficulty in both fragmenting proteins, as well as the large number of fragment ions which can occur upon fragmentation. With the development of higher resolution instruments [20,21], powerful software [22,23], and electron capture dissociation (ECD) and electron transfer dissociation (ETD) fragmentation methods, [24,25] top-down proteomics is growing in popularity.

1.2.3 Electrospray Ionization and High Performance Liquid Chromatography

Mass spectrometers allow the separation and detection of gas phase ions according to their mass to charge ratio (*m/z*). However, peptide/protein identification is not possible without a method to form gas phase ions from the aqueous sample. The development of matrix-assisted laser desorption/ionization (MALDI) [21,22] and electrospray ionization (ESI) [23] has led to a massive growth in MS based proteomics. As shown in Figure 1.2, ESI operates through the application of high voltage to a capillary tip containing the sample. The overall ESI process has been reviewed in depth [24–27]. In short, the application of voltage causes a buildup of charge in the capillary. The solution forms a cone like structure known as the Taylor cone at the tip of the capillary due to electrostatic repulsions between charged ions at the liquid-vapor interface. Small charged solvent droplets are ejected from the capillary tip. These droplets undergo solvent evaporation and therefore shrink bringing the ions closer together. Once the repulsive forces between ions overcome the surface tension of the solvent (known as the Rayleigh limit) the shape of the droplet is disrupted causing the formation of many smaller droplets. Ions are ejected into the gas phase from these droplets by one of two models: (1) charge residue model or (2) ion evaporation model. In the charge residue model the droplets continue to

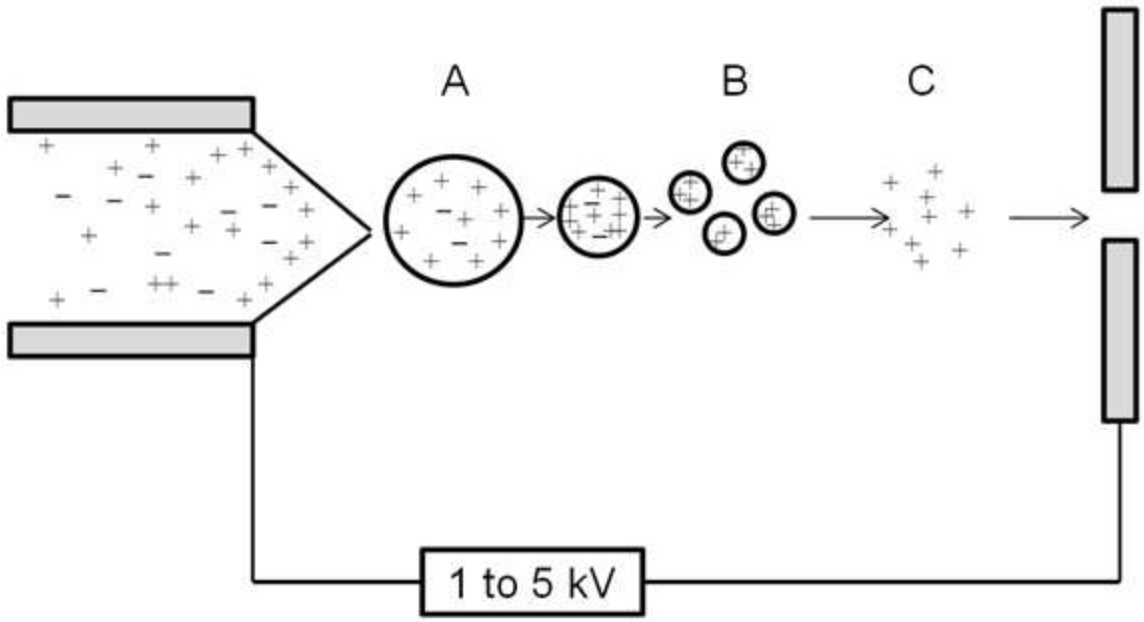

Figure 1.2: Electrospray ionization allows for the formation of charged gas phase ions from aqueous solution through the application of voltage to a capillary tip. (A) Charged droplets are formed and undergo evaporation, causing a decrease in droplet size. (B) Disruption of the charged droplet occurs once the Rayleigh limit is reached. (C) Gas phase ions are produced as described by the charged residue or ion evaporation model. Ions are draw into the MS inlet for analysis.

undergo evaporation and disruption until only one ion remains in the droplet; further evaporation of the solvent forms gas phase ions. In the ion evaporation model, the ions are ejected from the surface of the droplet once the droplet reaches a small enough size. This occurs when the forces acting on the ion overcome the surface tension. The resulting gas phase ions are then drawn towards the MS inlet through a combination of an electrostatic attraction and a pressure gradient. Most proteomics profiling experiments are now carried out through the use of nanoelectrospray ionization (nESI) [28,29], a modified form of ESI which makes use of low flow rates and a small capillary diameter for improved sensitivity.

The ionization efficiency of electrospray and nanoelectrospray is far from 100%. Efficiency is dependent on the flow-rate, the size of the capillary tip, the pH of solution, the solution composition, and the analyte of interest [30]. nESI efficiency has been found to range from 1% to 12% according to these variables [31]. A disadvantage of electrospray ionization is the presence of a saturation effect which occurs when too many charged ions are present in the droplet. The charged ions compete for space upon the surface of the solvent droplet. When the droplet is over saturated with ions, not all ions can be present on the surface. The ions which occupy the middle of the droplet are lost during ionization. To prevent ion suppression from occuring, a method of simplifying the sample is required prior to infusion into the MS instrument. This can be done by sample fractionation. The most common method of sample fractionation, ahead of MS, is reversed phase high performance liquid chromatography (HPLC). Reversed phase HPLC separation allows for the fractionation of analytes based on their hydrophobicity through the interaction with a hydrophobic stationary phase and a mobile organic/aqueous phase.

The main advantage of HPLC is that it can be easily coupled to ESI (or nESI) allowing for online separation ahead of MS analysis. This allows for increased automation, reducing cost and increasing sample throughput.

1.2.4 Protein Quantitation

The power of LC-MS/MS is not limited to the identification of proteins in a biological system, but can be extended to the quantitation of the individual proteins within these samples. Through quantitation of the expressed proteins in a system one can map changes in biological function. A number of techniques have been developed to allow for relative quantitative comparison of proteins between biological samples. These techniques can be broken into two distinct groups: isotopic labeling and label-free methods. A number of different isotopic labeling systems have been developed such as isotope-coded affinity tags (ICAT) [32], isobaric tag for relative and absolute quantitation (iTRAQ) [33], and stable isotope labeling by amino acids in cell culture (SILAC) [34]. These approaches involve the chemical modification of samples with a "heavy" or "light" isotopic label, after which the samples are combined and analyzed by LC-MS/MS. Quantitation is then carried out through the comparison of the label intensities.

Label-free approaches avoid the use of chemical labels. Two label-free methodologies exist: (1) targeted and (2) non-targeted. Targeted label-free approaches are used when the protein of interest is known. Targeted approaches commonly rely on the use of single reaction monitoring (or multiple reaction monitoring) to isolate and quantify proteins/peptides of interest. These methodologies have the advantage of shorter gradient times, higher sensitivity, and higher specificity [35]. This methodology however, cannot be used for proteomic biomarker discovery experiments, which requires

information on all peptides/proteins in a sample. Non-targeted approaches attempt to provide quantitative information on all proteins in a sample. One such method, known as spectral counting [36], has become a favored approach for label-free bottom-up quantitation due to its ease of use. Spectral counting allows the estimation of relative protein abundance through the comparison of the number of unique and redundant peptides assigned to each protein. Peptides of higher abundance are detected more often by LC-MS/MS. This means that proteins with a higher abundance will have a higher number of peptides assigned to them. Quantitation by spectral hits allows for simple and straightforward quantitation of proteins between non-pooled samples. However, this quantitation method assumes that the samples subject to LC-MS/MS are equal in all other factors. Therefore consistent and reliable sample preparation is of the upmost importance for proper quantitation.

1.3 Sodium Dodecyl Sulfate

1.3.1 Protein Solubility

Sodium dodecyl sulfate is an ionic surfactant, commonly used to improve protein solubility ahead of LC-MS/MS analysis [37]. Protein solubility in polar solvents is reliant on the tertiary structure of the protein. A model of protein solubility is shown in Figure 1.3. Proteins adopt a structure such that the hydrophobic amino acids are "shielded" from the polar solvent. The charged polar amino acid groups are exposed and allowed to favorably hydrogen bond with the solvent molecules. This arrangement allows for a more entropic system. The insides of a protein have been described as consisting more of a solid than liquid [38], and containing minimal free space with the exception of empty "voids" that exist within the tertiary structure [39]. In aqueous

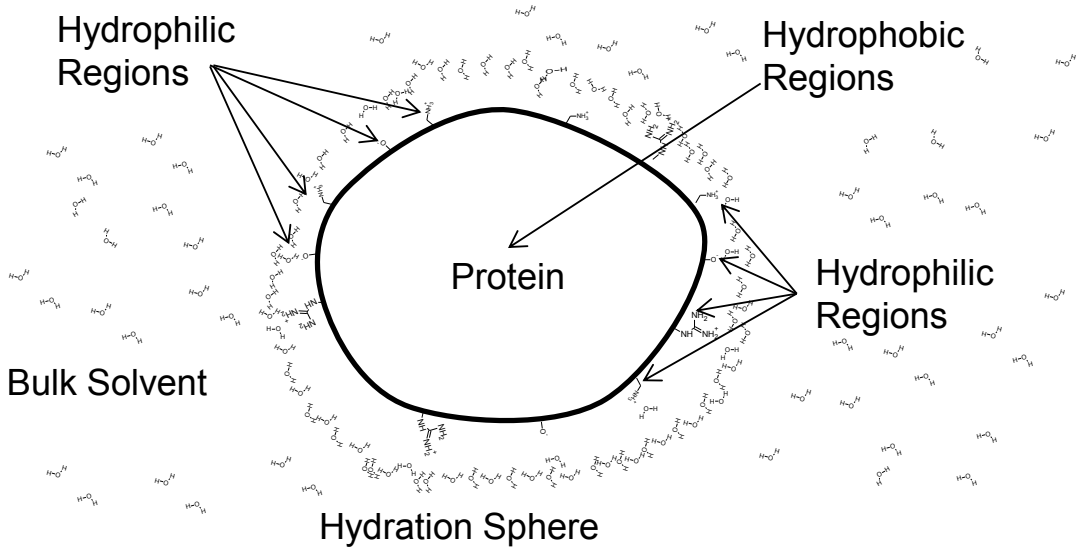

Figure 1.3**:** Diagram of a protein in aqueous solution. The protein's tertiary structure allows for the exposure of hydrophilic amino acids while shielding the more hydrophobic regions. The hydration sphere, an ordered water structure, is formed around the protein.

solution, the exposed hydrophilic region allows the formation of an ordered hydration layer that shields the electrostatic regions of the protein. At low ionic strength, the shielding of the protein by the hydration layer can be described by the Debye-Hückel theory, in which the protein is surrounded by ions of opposite charge to its net ionic charge. This shielding decreases protein-protein interactions, and increases protein solubility. Overall protein solubility in a system can be described by equations 1.1 to 1.3 [40] where s_2 is the solubility of the protein, $s_{2,w}$ is the solubility of the protein in pure water, Z is the overall net charge, ε is the electronic charge, N is Avogadro's number, D is the dielectric constant, R is the universal gas constant, T is the temperature, a is the radius of the ionic cloud, and I is the ionic strength.

$$\ln\left(\frac{s_2}{s_{2,w}}\right) = \frac{Z^2\varepsilon^2 N\kappa}{2DRT(1+\kappa a)} \quad (1.1)$$

$$\kappa = \sqrt{\frac{8\pi N\varepsilon^2}{1000DkT}} * \sqrt{I} \quad (1.2)$$

$$I = \frac{1}{2}\sum_i C_i Z_i^2 \quad (1.3)$$

It should be noted that this equation only accounts for the electrostatic forces of the protein and not any other interactions. A more complete mathematical model to predict protein solubility in aqueous systems has been described elsewhere [41].

Many proteins are poorly soluble in aqueous solution due to the presence of a large number of hydrophobic regions on the protein. The addition of SDS to a sample improves the solubility of proteins. When present above the critical micelle concentration (CMC), SDS monomers form spherical structures known as micelles. A diagram of SDS micelles can be seen in Figure 1.4. The CMC for SDS is 0.0082 M in pure water at 25°C [42], with micelles containing 62 molecules (MW of ~18 kDa) [43].

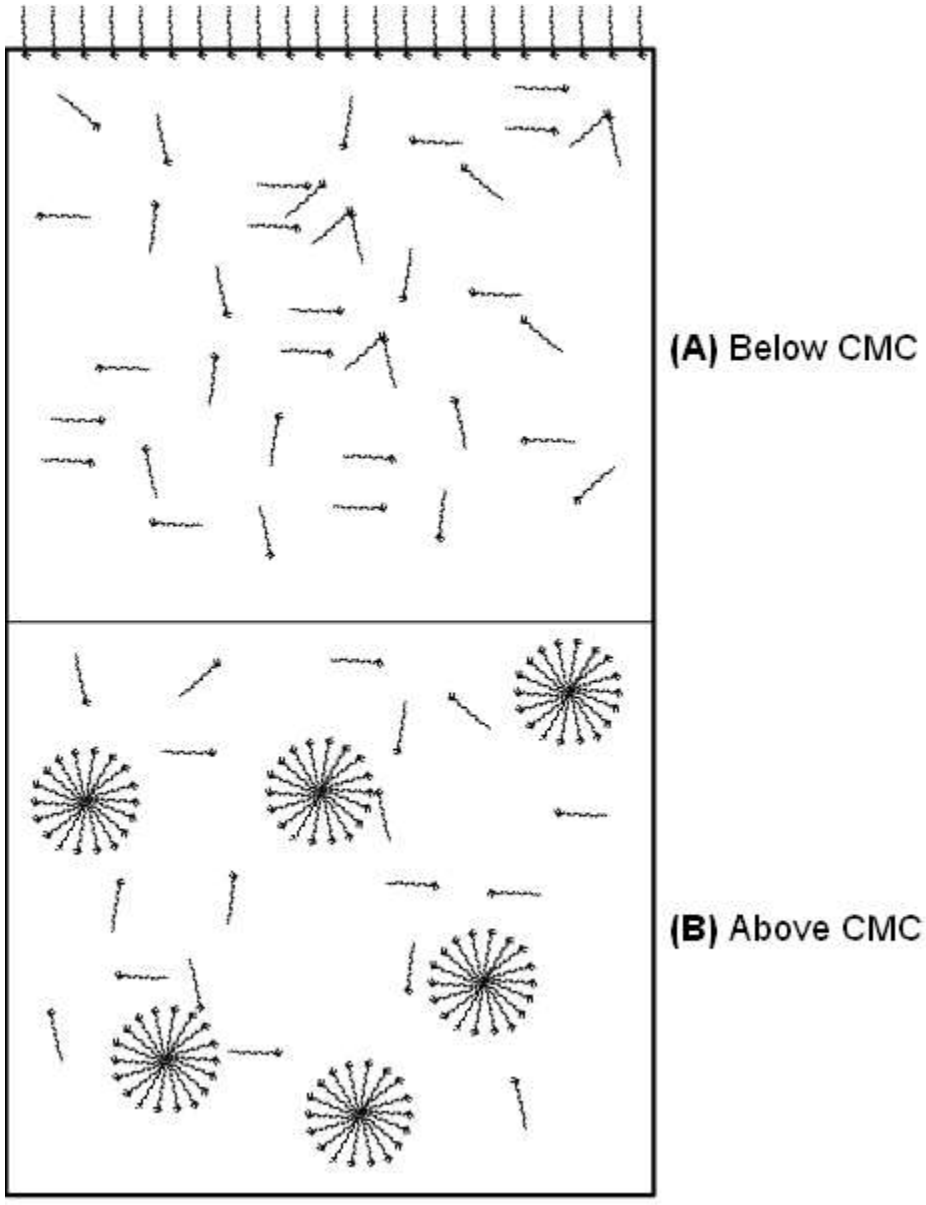

Figure 1.4: Behavior of SDS in aqueous solution. (A) At concentrations below the SDS critical micelle concentration, monomers exist in solution. (B) At concentrations above the CMC, SDS monomers aggregate and form micelles.

The CMC is highly dependent on solvent conditions including ionic strength, temperature, and pH. SDS monomers bind to the protein *via* electrostatic and hydrophobic interactions. Above 0.5 mM, SDS was found to bind to proteins at a constant ratio of 1.4 to 1 by mass [44]. At SDS concentrations above the CMC, SDS micelles begin to form on the protein around the original monomer. This causes protein denaturation, which disrupts the tertiary, quaternary, and secondary structure of the protein. This is due to replacement of previously favored intra-protein hydrophobic interactions to the more favored SDS-protein interactions. The increase in protein solubility is due to the incorporation of the insoluble portions of the protein into the core of a SDS micelle [45].

1.3.2 Incompatibility of SDS to LC-MS/MS

Surfactants, though highly beneficial in the solubilization and fractionation of proteins, cannot be easily incorporated into the proteomics workflow. SDS is especially problematic as it is incompatible with tryptic digestion [46], reversed phase separation [47] and ESI [1]. The effect of SDS on peptide identification by LC-MS/MS was examined by Botelho *et al.* This work determined that 0.01% SDS is sufficient to disrupt LC-MS/MS analysis (1 μg, in 10 μL) [1]. Vieira *et al.* found that the presence of SDS (>0.01% SDS) caused increased retention of proteins and peptides on a C18 reversed phase column [47]. This increased retention causes a decrease in the resolving power of the column. An increase in peak width was also noted. Interference to RP separation is speculated to be caused by the SDS bound proteins, strongly binding to the resin due to the strong SDS-resin binding affinity. High concentrations of SDS have also been shown

to decrease MALDI ionization [48] and tryptic digestion [49]. Taking these observations into account SDS must be depleted below 0.01% prior to LC-MS/MS analysis.

Rundlett and Armstrong fully examined the suppression effect of SDS on ESI-MS in positive mode [50]. They explained the phenomenon of SDS ion suppression as a consequence of the excess anionic surfactant at the liquid-air interface of the solution. SDS monomers prefer to exist at the surface of the solution, allowing their hydrophobic tails to extend into the vapor phase while the polar head group is located in solution. This preference creates a buildup of negatively charged molecules at the solutions surface. Rundlett and Armstrong suggested the presence of SDS detrimentally affects ionization efficiency in two ways [50]. The first is in the disruption of the Taylor cone formation. As described previously, the Taylor cone is formed by the electrostatic repulsions between charged ions at the liquid-vapor interface. The presence of the negatively charged SDS causes a shielding effect for the positive charges, decreasing the overall repulsive force affecting each ion. The second interference occurs after the initial solvent droplet is formed. The presence of SDS molecules in the droplet causes a decrease in ionization due to electrostatic attractions between the SDS molecules and the positively charged ions. This attractive force must be overcome before the Rayleigh limit can be reached. Although SDS is incompatible with LC-MS/MS analysis, it is beneficial for a number of front-end sample preparation steps such as protein extraction [48-50], and protein separation [60].

1.3.3 Protein Extraction Using Detergent Lysis Methods

The extraction of proteins from a biological sample is one of the most important steps of a proteomic analysis. Biological samples may contain cell membranes or cell walls,

which complicates the analysis of the proteins. Proteomic analysis cannot occur until the proteins are extracted into solution. Many methodologies exist to isolate proteins from biological samples, and both mechanical [51] and chemical methods [52] to disrupt the cell wall/membranes have been developed. Detergent based cell lysis is a type of chemical lysis, which relies on the addition of a detergent to disrupt cell walls and/or cell membranes. The use of detergents to extract proteins, is an effective, easy, and low cost method of protein extraction [52–54].

The extraction of the entire proteome from a biological sample is complicated by the large difference in hydrophobicity between proteins. Membrane proteins are a classification of proteins that are associated with the cell lipid bilayer. These proteins are normally hydrophobic and have poor solubility in aqueous solution, making their extraction and analysis difficult. It is estimated that out of all the proteins in the mammalian genome, 15 to 39% are classified as membrane proteins [55–57]. In addition, membrane proteins represent two thirds of protein targets for potential drugs due to their accessibility [58]. Detergent lysis methods ensure the proper extraction and solubilization of membrane proteins from biological samples allowing for a complete proteome analysis. Additionally, the addition of detergents also removes all interfering lipids previously bound to the protein [59]. The addition of SDS (and heat) allows for the efficient and straight forward extraction of both insoluble and soluble proteins from a cell system [54]. Due to SDS incompatibility with downstream LC-MS/MS analysis a number of MS friendly detergents have been developed [47,49,60,61] but have not been found to be more effective than SDS. An SDS based proteomics workflow is presented in Chapter 4 for the analysis of protein extracted from tissue samples.

1.3.4 Gel Eluted Liquid Fractionation Entrapment Electrophoresis

Mass based separation of proteins by electrophoresis relies on the presence of SDS to provide constant charge to mass across all proteins. Protein separation by electrophoresis was originally carried out through the use of sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). In this method, proteins are separated in a porous gel using electrophoresis. As proteins migrate through the gel, the larger proteins move more slowly, allowing the smaller proteins to move ahead. Once separation is complete, proteins can be visualized through staining. The disadvantage of this method is that the recovery of intact proteins from the polyacrylamide gel is difficult. The most common method of obtaining the sample from the gel is through digestion of the proteins and subsequent extraction [62].

Gel-Eluted Liquid Fraction Entrapment Electrophoresis (GELFrEE) was developed by Tran *et al.* [63] in 2008. It allows for the recovery of intact proteins following molecular weight electrophoretic separation. This technique relies upon the same principals of SDS-PAGE, as separation occurs through the use of a porous gel column and electrophoresis. The process of protein separation can be seen in Figure 1.4. Proteins migrate through the column separating by molecular weight due to differences in protein velocity through the porous media. The larger proteins migrate more slowly through the gel allowing the lower molecular weight proteins to reach the end of the column first. The proteins then migrate off the end of the gel column into solution. In this manner, proteins are not separated by distance (SDS-PAGE) but by the time they require to migrate through the gel column. The proteins are collected after set time intervals. Overall, this methodology allows for the mass based separation of proteins,

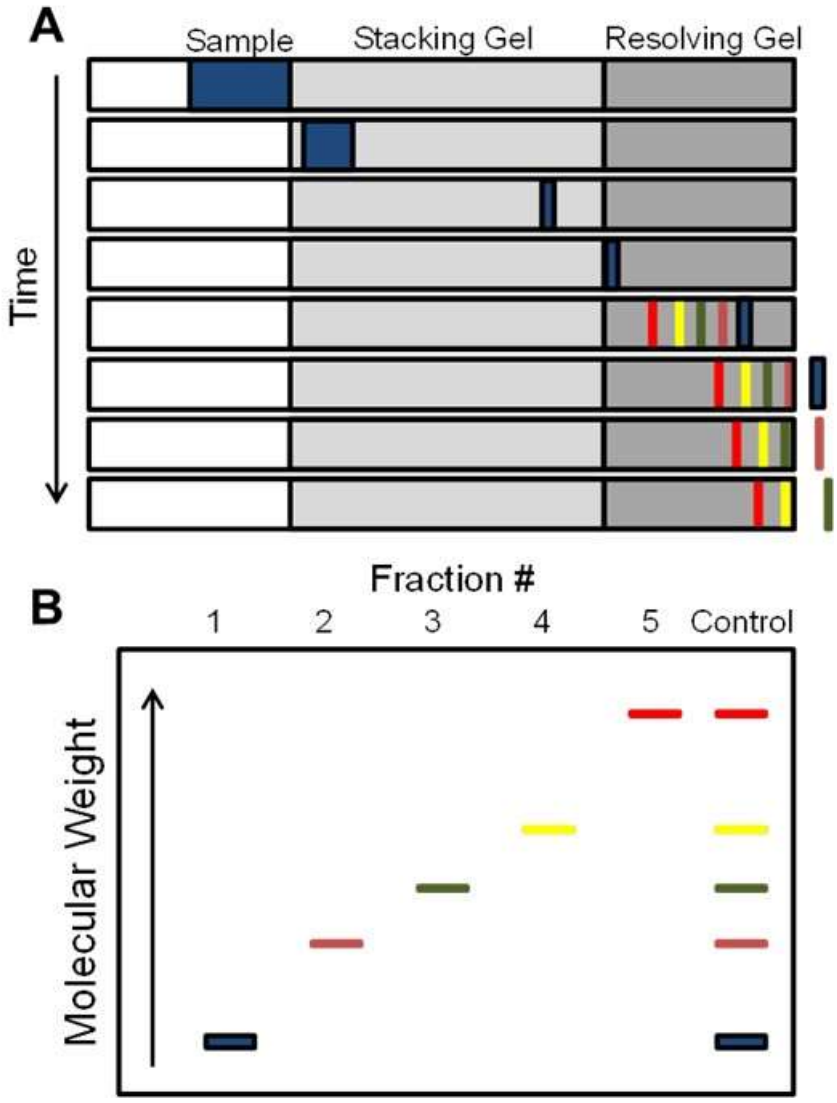

Figure 1.5: (A) Diagram of GELFrEE separation; protein migration through the gel column by electrophoresis as a function of time. A protein sample is loaded into the system. The stacking gel reduces the width of the sample improving separation. Proteins separate by molecular weight as the proteins migrate through the resolving gel. Proteins migrate off the end of the gel to be collected. (B) The SDS-PAGE visualization of the GELFrEE fractions. As can be seen the low molecular weight proteins are in the lower fractions.

allowing for the collection of different fractions in aqueous solution. However, the protein fractions are contaminated with SDS, which is present in the electrophoretic buffer. For LC-MS/MS analysis to occur the SDS must first be removed. In Chapter 4, the ProTrap XG is coupled to GELFrEE fractionation, allowing for semi-automated protein preparation following protein separation.

1.3.5 Removal of SDS from Proteomic Samples

The benefit of using surfactants such as SDS in the proteomics workflow can be gained through the removal of the additive prior to LC-MS/MS analysis. A number of methods have been developed and used for the removal of SDS from proteomics samples. These include dialysis [44], ultrafiltration [64], solid phase extraction [65], and protein precipitation [1]. SDS can be removed either at the intact protein level or at the peptide level (post digestion). Removal at the intact level allows for protein manipulation and top-down analysis, however, it normally requires the addition of another solubilization agent to prevent protein loss through precipitation. This is not the case when depleting SDS from peptide samples. Peptides are normally water-soluble and therefore do not require the addition of any solubilization agents. However, SDS concentrations must be reduced or diluted below 0.1% before protein digestion can occur [66]. Following digestion, SDS must further be removed to levels below 0.01%.

The perfect SDS removal technique would provide quantitative protein yield and complete removal of SDS from the proteomic sample. However, such a technique does not currently exist. A classic method of SDS removal is through the use of a dialysis membrane to separate the larger protein from the low molecular weight SDS contaminant [44]. Dialysis is a separation technique based on the passive diffusion of low molecular

weight contaminants across a molecular weight cut off membrane. The contaminants move from the higher concentrated sample to the lower concentrated buffer. Proteins which are of high molecular weight cannot pass through the membrane and are therefore retained in the sample. However, this technique is does not remove protein bound SDS and is time consuming, difficult to automate, and may result in sample loss due to interactions with the filter.

To overcome the limitations of passive diffusion, the use of a molecular weight cut-off (MWCO) membrane in a centrifugal cartridge was proposed. This method relies on centrifugal force to draw the sample through the filter reducing the time needed for separation to occur. By filtering the sample through these MWCO filters, the high molecular weight proteins are retained on the membrane, while the low molecular weight contaminants flow through with the solvent. The protein is then digested above the filter, and the resulting peptides are recovered. Sickmann *et al.* reported the use of this technique to fractionate human cerebrospinal fluid [67]. A protein recovery of 70% was estimated. It should be noted that these samples did not contain SDS. Manza *et al*. applied a Millipore 5 kDa MWCO filter to purify a number of protein standard and test samples contaminated with a large salt concentration, SDS (Laemmli buffer), or acid [68]. Manza found that although digestion and subsequent MS analysis was possible from the technique, full SDS depletion did not occur.

Wiśniewski *et al.* proposed a "a universal sample preparation protocol" based on ultracentrifugation [64]. In this protocol the MWCO membrane is washed with 8 M urea to remove the excess SDS remaining in the cartridge. However, the SDS may be bound to the protein or may exist as a micelle which is too large to pass through the membrane.

The addition of urea helps to prevent this by weakening SDS-protein interactions and disrupting micelle formation. Wiśniewski *et al*. quantified protein yields ranging from 47-50% [69]. In this work they showed higher peptide yield and containment removal when larger pore size filtration units are used. However, it was noted that filtration units from within the same batch have large variation. All units were tested prior to use and only those with least variations where used. Wiśniewski *et al*. later quantified a protein recovery of 76% from a 50 µg *E. coli e*xtract using a MWCO filter of 30 kDa and a second protein digestion step [70]. They attributed the protein loss to both the non-retained proteins (<5 kDa) and the peptide fragments that could not pass through the filter (>5 kDa). The use of urea is required to fully remove SDS from the samples. Other sample additives, such as the detergent deoxycholic acid, have been used to replace urea in order to deplete the protein bound SDS [71]. Although this technique was reported as a "universal sample preparation method" [64] quantitative yield has yet to be reported.

Solid-phase extraction (SPE) techniques rely on the differences in chemical properties of detergents and peptides/proteins in order to separate them by their affinities to different solid supports. These techniques are usually more suited to detergent removal at the peptide level due to decreased recovery when working with intact protein samples. Size exclusion can be used to remove SDS at the intact level, however it is highly ineffective [72] and not truly a solid-phase extraction technique. Most SPE techniques rely on peptide level separation. These normally rely on the difference in charge between a peptide (positive) and SDS (negative). Using anion exchange chromatography, SDS can be bound to the support allowing for SDS-free peptides to be collected. Visser *et al.* proposed the use of an online anion exchange SDS trapping

column to remove the detergent from tryptically digested protein samples [73]. However sample loss was observed. Alternatively, cation exchange chromatography, allows for the binding of the peptide to the support while the SDS washes through. The peptide is then collected using a salt gradient. Sun *et al.* proposed a protocol to remove and fractionate digested samples using a SCX column [65]. High recovery (~90%) and efficient SDS removal was observed.

Although only a handful of detergent removal techniques were listed, the general trend of non-quantitative protein recovery can be seen. Other techniques include potassium dodecyl sulfate (KDS) precipitation [74], hydrophilic interaction chromatography (HILIC) [75], ion-pair extraction [76], electrophoresis [77], Pierce Detergent Spin Removal Columns (Pierce, Rockford, USA), Detergent-OUT™ (Millipore, Billerica, USA), and many others. None of these approaches allow for the effective removal of SDS at the intact level without protein loss. An alternative approach is required to separate proteins from the interfering detergent. The use of protein precipitation, using the organic solvent acetone, can effectively isolate proteins from SDS contaminated solutions.

1.4 Protein Precipitation

1.4.1 Overview

Protein precipitation involves the removal of proteins from solution through the addition of a precipitating agent. Commonly salts [78], organic solvents [72], acids [79], or polymers [80] are used. Once the proteins are made insoluble they can be separated from the contaminated supernatant and subject to further analysis. The classic work by Hofmeister and Lewis, measured the ability of a variety of salts to induce protein

precipitation using egg white and other protein sources [81]. It was noted that the precipitating ability of a salt is dependent on (1) the type of salt, (2) the type of protein, (3) the concentration of protein, and (4) the concentration of salt. The advantage of salt precipitation over other methods is the ability to selectively precipitate proteins from a solution. For example, Jiang *et al.* applied 50% ammonium sulfate to selectively precipitate all proteins but albumin from plasma [82]. This technique has never successfully been applied for the removal of SDS from protein samples.

Another strategy of protein precipitation involves the addition of organic solvents to the protein sample. This method of precipitation has been carried out with a variety of water miscible solvents, including ethanol [83], acetonitrile [84], methanol [85], and acetone [72]. This thesis will focus on the use of acetone precipitation as a front-end purification procedure. Acetone has been shown to have a less denaturing effect on proteins than alcohols [86]. Acetone precipitation was first described in 1920 [87]. Since then it has been applied to a number of protein experiments including purification prior to two-dimensional gel electrophoresis (2-D PAGE) [88], metabolite isolation [89], and SDS detergent removal [1,72]. In 1990, it was estimated that 80% of all protein experiments contained a protein precipitation step [90]. However, protein precipitation using organic solvents has not gained much traction as a front-end purification strategy ahead of LC-MS/MS. This may be due to the limited effectiveness of the technique as a purification strategy ahead of 2-D PAGE, and its widely reported low protein recovery.

1.4.2 Theory of Organic Solvent Precipitation.

Protein precipitation works through the manipulation of solvent conditions to decrease the solubility of the protein. Figure 1.6 shows the typical workflow used for protein

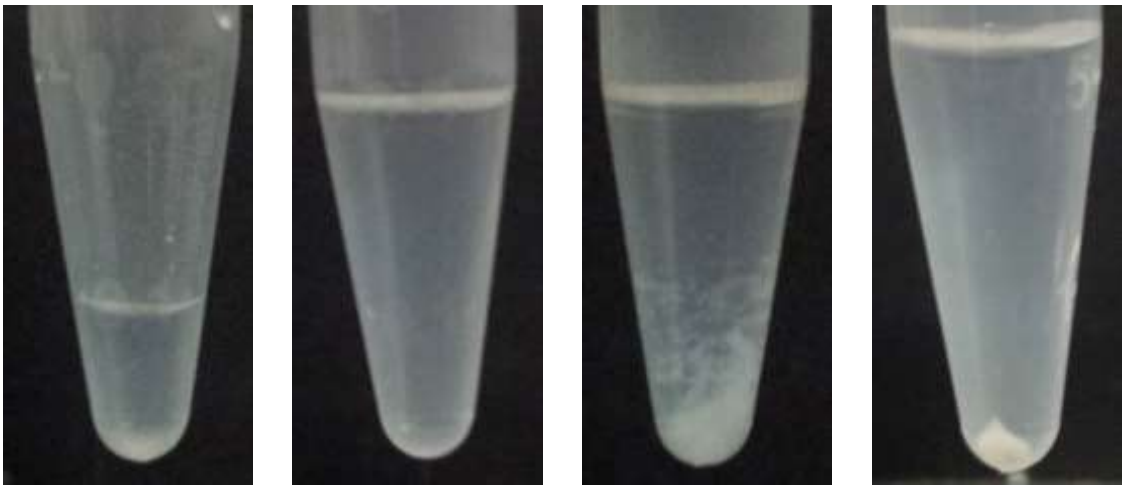

Figure 1.6: Overview of protein precipitation. (A) A non-precipitated protein sample. (B) Addition of precipitating agent (acetone). (C) Protein precipitating from solution. (D) Insoluble protein pellet isolated by centrifugation.

precipitation. Following precipitation, the aggregated protein is then collected, free from interfering contaminants that remain dissolved in the solvent. Precipitation agents change the solvent conditions to lower the solubility of the protein. This is done through a combination of increasing the protein-protein interactions (electrostatic or hydrophobic attractions) and decreasing the solvation ability of the solvent. Protein solubility in aqueous solution is known to depend on the type of protein and the solvent conditions of the system, including ionic strength, pH, dielectric constant, and the composition of the solvent [91]. An overview of protein solubility is described in Section 1.3.1.

To provide an optimized protein precipitation strategy, one must first understand the principals behind solvent precipitation. It is noted that current knowledge of organic solvent precipitation is mostly empirical [86]. Precipitation induced through the addition of organic solvent, at high concentrations, is thought to be caused by a decrease in the dielectric constant of the solution. Water has a high dielectric constant (~70) while most organic solvents are much lower (~20) [92]. The dielectric constant is a measure of the permittivity of a solvent, which relates to the electric field strength of two charges in the medium. As the amount of organic solvent is increased the dielectric is decreased. A decrease in the dielectric of a solution causes two effects. The first is an increase in the magnitude of electrostatic interactions between species in the solution as described by Coulombs law. The second effect is that the solvating power of the hydration sphere is decreased. It has been shown that water is more favored to bind to ethanol than a protein, meaning that water molecules which once shielded the protein will be occupied binding to the organic solvent molecules [93]. Therefore, the water molecules provide less protein-protein shielding as they are occupied hydrating the organic solvent ions. When

the interactions between the proteins overcome the interactions of the proteins with the solvent, precipitation occurs [93]. These interactions include van der Waals interactions, hydrophobic interactions, and electrostatic forces. Some believe that it is either the electrostatic or dipolar forces which cause aggregation [86,90], while others believe that the driving forces are predominately van der Waals forces [93]. Protein clusters continue to grow as more protein molecules are attracted to it. Once these clusters are above a certain size they can be removed using centrifugation or filtration.

High concentrations of organic solvent have been used to induce global precipitation of all proteins from a sample. However it is possible to induce selective protein precipitation, by incorporating a lower concentration of organic solvent and controlling the pH of the solution [86,93–97]. Proteins have reduced solubility at a pH equal to their intrinsic pI due to the decrease of electrostatic charge on the protein's surface. This decreased charge results in a domination of protein-protein interactions over protein-solvent interactions causing the proteins to be less soluble in solution. Although proteins have a reduced solubility at their pI, they are not necessarily insoluble [93]. Through the addition of low concentrations of organic solvent, the solubility can be further reduced, causing protein precipitation [86]. This allows for the selective removal of proteins according to isoelectric point. This is the basis of a technique known as Cohn fractionation of blood plasma [97]. Cohn fractionation allows for the isolation of serum albumin, gamma globulin, fibrinogen, thrombin, and other blood proteins from plasma in large quantities.

Protein precipitation through the use of organic solvents is known to be biased towards molecular weight [86]. The higher the molecular weight of a protein, the lower

the concentration of organic solvent required to induce precipitation [84]. Furthermore it has been previously shown that low molecular weight proteins may not precipitate during acetone precipitation [98]. Merrell *et al.* [99] examined the organic phase after the precipitation of plasma with 60% acetonitrile. They found a number of proteins in the 1-10 kDa range, with the majority of proteins being under 2 kDa (>90%) [99]. This result clearly reveals a molecular weight bias for organic solvent precipitation.

The addition of acetone to protein samples is performed at low temperatures. This is done to prevent protein denaturation which has been found to occur rapidly above 10°C [86]. The denaturation effect of organic solvents is due to the more favorable interaction of the hydrophobic regions of the protein with them when compared to water. This decreases the entropic loss which occurs when the protein unfolds, promoting denaturation. The use of cold temperatures reduces the conformational flexibility of proteins, preventing the organic solvent from accessing the hidden hydrophobic regions. Acetone has been found to be less denaturing than ethanol for protein precipitation [86,92].

1.4.3 Reported Protein Recovery from Acetone Precipitation

Protein recovery from acetone precipitation has been shown to be dependent on a number of factors including protein concentration [100], sample additives [100], and the amount of organic solvent used to induce precipitation [60]. A number of studies have examined the use of acetone as a purification strategy ahead of 2-D gel electrophoresis with limited success due to an increased background streaking compared to other purification strategies [82,101–103]. This increased background streaking is attributed to non-complete removal of salt from the sample [102]. Although acetone precipitation is not

highly effective at removing NaCl, it has commonly been used to remove SDS prior to digestion/analysis [1,72,100].

The efficiency of acetone precipitation to remove SDS was examined by Botelho *et al.* [1] In this report acetone was compared to another precipitation strategy, which uses a mixture of chloroform, methanol, and water to induce precipitation. It was determined that acetone precipitation could deplete SDS to levels below 0.01% after the use of one additional wash step (2% SDS starting concentration). This report did not provide absolute quantitation for protein recovery but found both precipitation methods to provide similar results.

The literature reports highly variable protein yield from acetone precipitation. Protein recovery ranging from anywhere between 40% to near 100% has been reported. For this reason alone, a more detailed examination of the technique is required. Low recoveries ranging from 40 to 50% were obtained by Sickmann *et al.* when purifying human cerebrospinal fluid. However, Yaun *et al.* found recoveries of 94% when applying acetone precipitation to a similar sample of human cerebrospinal fluid. No significant difference in protocol is reported between the two experiments; both groups used 80% acetone overnight at -20°C. Centrifugation at 4°C was used to collect the protein pellet with an addition of two wash steps. The difference in recovery may be due to unreported variables thought unimportant. These may include differences in supernatant removal (removal by pipette or by decanting), original sample volume, concentration, or wash volume.

Thongboonkerd *et al.* measured the protein recovery from urine using a number of precipitation techniques, varying the concentration of precipitating agent used [104].

It was reported that while increasing the concentration of acetone (from 10% to 90%) improved protein recovery, an overall yield of only 40% could be obtained. However, precipitation was only allowed to occur for 10 minutes, which may not be an adequate time frame. Most acetone precipitation procedures allow for overnight incubations, prior to isolation of the protein pellet. Barritault *et al.* reported that the required incubation time for optimal recovery was dependent on protein concentration [100]. He found acetone precipitation provided high yield (>95%) in a short period of time for samples containing high concentrations of protein but required a far greater time (overnight) to provide similar yields for more dilute samples. It should be noted that the "dilute samples" in this work are of higher concentration (*i.e.* 1 g/L) than those of interest to this thesis (*i.e.* 0.1 g/L). Allowing for ample time for protein precipitation to occur is important in obtaining high protein recovery from acetone precipitation.

Another factor that affects the efficiency of protein precipitation is the amount of precipitating agent used. The work by Thongboonkerd *et al.* found that all organic precipitation techniques improve in efficiency as more precipitating agent was used [104]. Prior to this work, Thongboonkerd *et al.* compared the use of 50% acetone and ultrafiltration to isolate proteins from urine [98]. It was noted that basic and hydrophobic proteins were lost during acetone precipitation. Srivastava *et al.* employed 50% acetone to enrich gamma-crystallin from human eye lenses. The hydrophobic, low molecular weight (20 kDa) protein remained soluble in a 50% acetone solution [105]. Ashri *et al.* examined the effect of acetone concentration on isolating proteins from plasma [106]. It was found that 70% acetone was sufficient to induce near quantitative precipitation of the proteins. Lin *et al.* determined that an 85% acetone solution produced

optimal precipitation, however 80% was only marginally lower in efficiency [60]. Puchades reported a 80% protein yield from 80% acetone precipitation using SDS-PAGE as a quantitation method [72].

1.4.4 Peptide Modification by Acetone

Acetone is known to cause peptide modifications if present following protein digestion. Simpson *et al*. described a +40 Da peptide modification that is introduced to the sample upon the addition of acetone to the peptide mixture [107]. They correlate this modification to peptides which contain a glycine residue as the second amino acid in the sequence. They found modifications occurred within one hour of the start of the reaction with a rate constant of $0.29 \pm 0.01\ h^{-1}$. There was no evidence to suggest that acetone can modify the protein prior to digestion. To overcome this issue one must ensure complete solvent removal following acetone precipitation. This is easiest to do using a filtration cartridge as described in Chapter 4, where the solvent can be completely removed by centrifugation.

1.4.5 The Effect of Salt on Acetone Precipitation

The current theory of solvent precipitation described above has been found to be limited in the explanation of organic solvent precipitation. It should also be noted that the current theory is mainly based on empirical observations [90] and not rigorous calculations. Previous work by Dr. Mark Wall[1] and Dr. Alan Doucette[1] has shown that the precipitation of bovine serum albumin (BSA) in acetone is not possible without the addition of SDS (as shown in Figure 1.6A). As can be seen from this data, protein recovery is minimal (~5%) prior to the addition of 0.1% SDS. This trend was later

[1] Department of Chemistry, Dalhousie Univesity, Halifax, N.S.

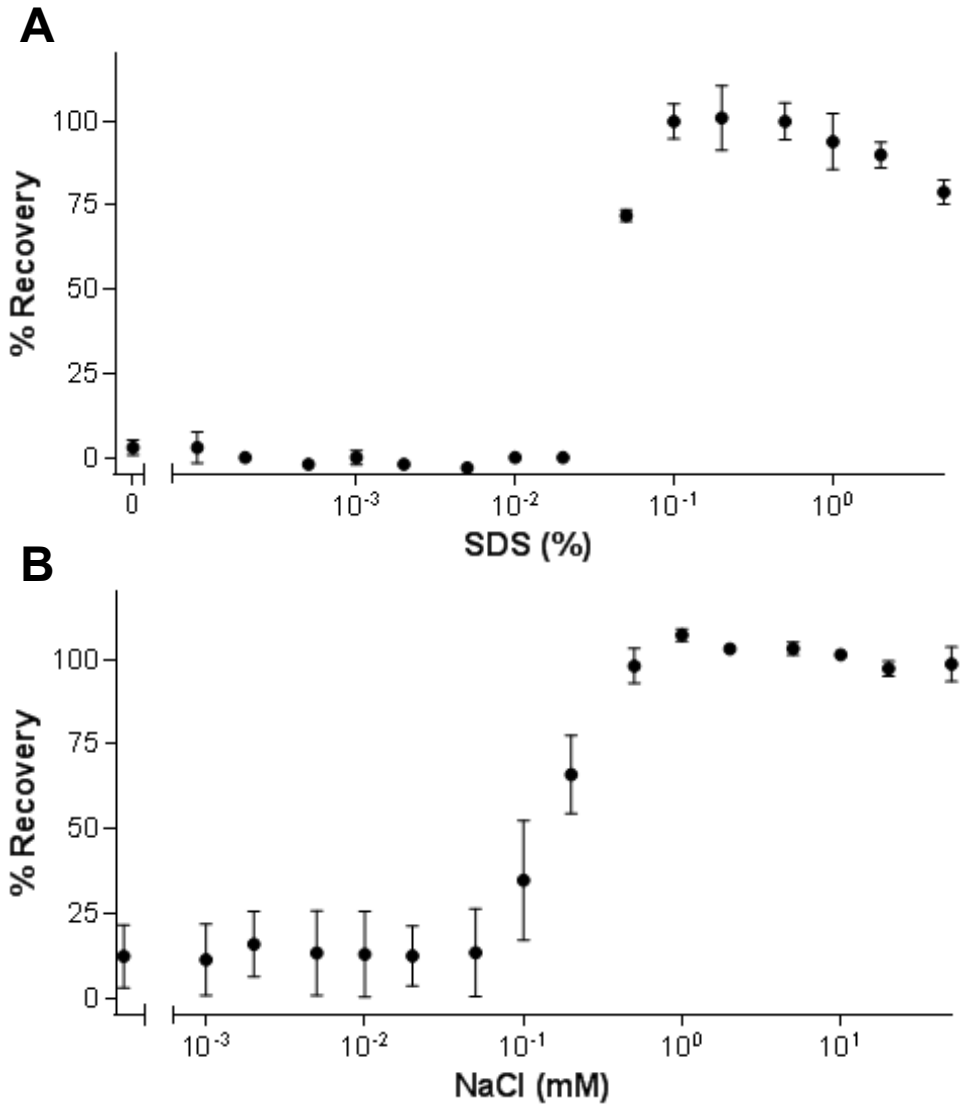

Figure 1.7: (A) Protein recovery obtained from acetone precipitation of 1 g/L BSA as a function of SDS concentration. (B) Protein recovery obtained from acetone precipitation of 0.1 g/L BSA as a function of sodium chloride concentration. Figure recreated and used with permission from Dr. Alan Doucette, Department of Chemistry, Dalhousie University, Halifax, N.S.

extended to the addition of sodium chloride as shown in Figure 1.6B[1]. Later work examined the effect of different concentrations of acetone and salt on the precipitation of BSA [108]. These observations of low protein recovery in 80% acetone have not been previously explained. This thesis provides a potential explanation of this new empirical data (Chapter 3). The overall effect of these observations on the acetone precipitation protocol is also examined.

1.5 Research Objectives

At this current stage in proteomics, no so-called "universal" strategy for front-end sample preparation exists. This thesis, attempts to lay the foundation for a robust sample preparation protocol for protein analysis. Through the addition of the ionic detergent, SDS, protein can be effectively extracted and solubilized from biological samples. SDS can then be removed using acetone precipitation prior to LC-MS/MS analysis. Most biological samples can be analyzed by LC-MS/MS using this simple protocol. This thesis provides effective strategies to improve acetone precipitation, allowing for effective SDS removal with high protein recoveries. In Chapter 3, it is shown that near quantitative protein yields can be obtained through the addition of an ionic buffer prior to acetone precipitation. This observation is explained through a model of ion pairing in organic solvents. A method for high and consistent protein recovery following acetone precipitation through the use of a filtration unit is described in Chapter 4. In this chapter a semi-automated bottom-up protocol is presented for the analysis of proteins samples containing SDS. In Chapter 5, SDS is used in the preservation of plasma samples prior to low molecular weight enrichment through the use of GELFrEE MW separation. SDS

[1] This data was obtained by the author of this thesis, prior to the beginning of this thesis.

removal following GELFrEE is carried out through the use of acetone precipitation allowing for the identification of proteins by LC-MS/MS.

2. Chapter 2: Materials and Methods

2.1 Materials

Individual proteins including lysozyme (cat #L6876), trypsin (cat # T6763), β-lactoglobulin B (cat # L8005), ubiquitin (cat # U6253), α-casein (cat # C6780), invertase (cat # I4504), myoglobin (cat # M0630), cytochrome C (cat # C7752), carbonic anhydrous (cat # C3934), ribonuclease B (cat # R7884), and bovine serum albumin (BSA, cat# A9418) were purchased from Sigma (Oakville, Canada). Lyophilized bovine plasma (cat # P4639), and lyophilized human plasma (cat # P9523) were purchased from Sigma (Oakville, Canada). Sodium chloride (cat # S2830) was obtained from ACP chemicals (Montreal, Quebec). The bicinchoninic acid (BCA) protein assay was obtained from Pierce (Rockford, IL, USA). Protease inhibitor cocktail (cat# S8820), agar (cat# A5306), phosphate buffered saline tablets (PBS, cat# P4417), glycine (cat# G8898), sodium deoxycholate (cat# D6750), tergitol-type NP-40 (cat# NP40S), glycerol (cat# G7893), premixed Luria broth (LB) powder (cat# L3022), tetramethylethylenediamine (cat# T9281), silver nitrate (cat# 209139), sodium thiosulfate (cat# S6672), formalin (cat# F1635), sodium bicarbonate (cat# S2150), trifluoroacetic acid (TFA, cat# T6508), and formic acid (FA, cat# 94318) were obtained from Sigma-Aldrich (Oakville, Canada). Tris(hydroxymethyl)aminomethane (Tris, cat# 161-0719), sodium dodecyl sulfate (SDS, cat# 161-0301), iodoacetamide (IAA, cat# 163-2109), dithiothreitol (DTT, cat# 161-0611), ammonium persulfate (cat# 161-0700), Quick Start™ Bradford reagent (cat# 500-0201), bromophenol blue (cat# 161-0404), 2-mercaptoethanol (cat# 161-0710), and urea (cat# 161-0731) were purchased from

Bio-Rad (Hercules, USA). Milli-Q grade water was purified to 18.2 MΩcm. HPLC grade solvents, acetone (cat# BP2403), isopropanol (cat# A451), and chloroform (cat# C606) were obtained from Fisher Scientific (Ottawa, Canada). LC-MS grade solvents acetonitrile (ACN, cat# A955), and methanol (cat# A452) along with ACS grade acetic acid (cat# 351271-212), and acrylamide (cat# BP14021) were from Fisher Scientific (Ottawa, Canada). The rat proximal tubule cells NRK-52E were a gift from Dr. Dawn MacLellan[1] and were grown according to manufacturer's instructions (American Type Culture Collection, Burlington, Canada). *Escherichia coli* (*E. coli,* strain K12) was obtained as a gift from Dr. Andrew Roger[2].

2.2 Protein Sample Preparation and Isolation

2.2.1 *Escherichia coli* Growth

E. coli was grown and harvested according to established protocols [109]. All glassware underwent sterilization and all work was carried out under sterile conditions. In brief, cells were plated on a 1.5% Agar LB plate and stored at 4°C until use. Cells were replated every 4 weeks to ensure proper growth. For proteomics experiments, cells were then transferred to a 15 mL tube containing 4 mL of LB liquid media (1 g/L LB powder). The tube was incubated at 37°C in a shaking incubator (Sanyo, Watford, UK) and allowed to grow until solution was opaque (4-6 hours). The cells were then transferred to 200 mL of LB media. Cells were grown to an optical density at 600 nm of ~1.0 at 37°C (with shaking). Cells were isolated by centrifugation for 15 min (5 000 ×*g*). The cells were washed with PBS, followed by two washes of distilled water (dH_2O) to remove any traces of LB media. Isolated cells were frozen (-20°C) prior to protein extraction.

[1] Department of Pathology, Dalhousie University, Halifax, NS, Canada
[2] Department of Biochemistry and Molecular Biology, Dalhousie University, Halifax, NS, Canada

2.2.2 Protein Extraction from *Escherichia coli*

In chapter 3, water soluble *E. coli* proteins were extracted in pure water to ensure the resulting protein extract contained minimal salts or other non-protein species. In this protocol, the bacterial cells (Section 2.2.1) were suspended in dH_2O and heated to 95°C for 5 min. The cooled suspension was then subject to multiple passes through a 26 gauge syringe, to lyse the cells. Solid materials were separated by centrifugation at 16 000 ×*g* for 10 min. The concentration of the resulting *E. coli* proteome was determined using Bradford assay (using BSA as a calibrant). Samples were frozen (-20°C) prior to use.

In chapter 4, protein extraction from *E. coli* was carried out through the use of either a French press apparatus or detergent lysis. For the LC-MS/MS examination of the single GELFrEE fractionation in chapter 4, extraction occurred through SDS detergent lysis. In brief, the bacterial cells were suspended in 1% SDS and boiled for 5 min at 95°C, followed by cooling on ice. The remaining cell debris and DNA was removed by centrifugation at 16 000 ×*g* at 4°C for 30 minutes. All other protein extractions used in this thesis was carried out through the use of a French press apparatus (Aminco, Rochester, NY). The isolated *E. coli* cell pellet from was suspended in water and general protease inhibitor. The suspension was subject to two passes in a French press at 10 000 psi to rupture the cell membrane. The resulting slurry was centrifuged at 2 300 ×*g* for 15 min (4°C) to remove the cell debris. Protein concentration from both methods was determined using a BCA assay (BSA as a standard). The samples were frozen at -20°C prior to use.

2.2.3 Membrane and Cytosolic Protein Enrichment from *E. coli*

Membrane and cytosolic protein enrichment was carried out as described by Wu *et al.* with modification [110]. The French press extract from Section 2.2.2 was subject to ultracentrifugation at 118 000 ×*g* (55 min, 4°C) to separate the insoluble membrane proteins from the soluble proteins. The resulting pellet was suspended in water and subject to a second ultracentrifugation step at 166 811 ×*g* (40 min, 4°C), to remove any remaining soluble protein. The resulting membrane pellet was dissolved in 1% SDS with heating (95°C, 5 min). The supernatant, obtained from the first ultracentrifugation step, was labeled as the cytosolic protein fraction. Protein concentration of both fractions was determined using the BCA assay (BSA as standard). The samples were frozen at -20°C until further use.

2.2.4 NRK-52E Osmotic Lysis

NRK-52E cells were a gift from Dr. Dawn MacLellan[1]. The cells were pelleted at 400 ×*g* for 5 minutes in a bench top centrifuge. Two washes of PBS buffer were used to remove any remaining cell media. Cells were suspended in 1 mL of ice cold water and the sample was held on an ice bath for 10 minutes to complete osmotic lyses. Cellular debris was separated through centrifugation at 16 000 ×*g* for 10 min. The resulting concentration of the cellular protein extract was determined by Bradford assay compared to a BSA standard.

2.2.5 Preparation of Kidney Samples

An animal model of antenatal hydronephrosis has been reported [111]. Kidney obstruction was introduced to three Sprague-Dawley weanling rats by the complete ligation of the left ureter with a 6.0 proline suture (Ethicon, Skillman, NJ) by Dr. Dawn

[1] Department of Pathology, Dalhousie University, Halifax, NS, Canada

MacLellan[1]. Animals were anesthetized through inhalation of isoflurane. The left ureter of control rats (n=3) was similarly exposed but not manipulated. All animals were housed individually in metabolic cages (Thermo Fisher Scientific, Burlington, ON) until sacrificed at 21 days post-surgery. The kidneys were harvested, flash frozen in liquid nitrogen by Dr. Dawn MacLellan[1], and Dr. Dennis Orton[1]. The kidneys were stored at -80°C until extraction. Protein extraction was conducted through the use of a modified radioimmunoprecipitation assay (RIPA) buffer [112], containing 150 mM NaCl, 0.5% sodium deoxycholate, 1% NP-40, 0.5% SDS, 50 mM Tris-HCl (pH 8.0). Proteins were extracted through the addition of the buffer at a ratio of 50:1 volume by weight. The slurry was subject to homogenization on ice for 30 sec using a Pelletpestle™ (Fisher Scientific, Ottawa, ON). Samples were allowed to incubate on ice for 10 min. The samples were then subject to centrifugation at 23 000 ×*g* (30 min, 4°C) to remove any insoluble particulates. Samples were diluted with 1% SDS to a final concentration of 0.1 g/L (by BCA assay).

2.3 Vial-based Precipitation Protocols for LCMS

2.3.1 Vial based Acetone Precipitation

Acetone precipitation was carried out in vial format, as described previously [1]. For experiments in Chapter 3, protein samples were prepared in water along with a specified concentration of sample additive (described in the results). For experiments in Chapter 4, acetone precipitation was carried out with samples containing 100 mM Tris-HCl and 1% or 2% SDS unless otherwise specified in the results. The total volume of sample plus acetone was maintained at 500 µL. For example, precipitation in 80% acetone involves addition of 400 µL organic solvent to 100 µL protein solution. For acetone precipitation

[1] Department of Pathology, Dalhousie University, Halifax, NS, Canada

in Chapter 5, samples were maintained at a final volume of 1.5 mL (1.2 mL acetone and 0.3 mL sample). Samples were incubated overnight at -20°C, and then centrifuged at 16 000 ×*g* for 20 minutes to isolate the protein pellet. The supernatant was carefully removed with a pipette, leaving behind less than 20 µL of solution. An additional washing step was performed, through the addition of 400 µL of cold acetone to the pellet (a 1.5 mL wash was used in Chapter 5). The bulk of the supernatant was removed, following centrifugation. Residual acetone was removed from the pellet by air-drying in a fume hood. For experiments that examined the organic supernatant, the original supernatant fraction was subject to solvent evaporation in a Speedvac (Thermo Savant, Burlington, ON).

2.3.2 Vial based Organic Solvent Precipitation

Ethanol and acetonitrile protein precipitation was carried out in a similar fashion to acetone precipitation (Section 2.3.1). Protein samples were prepared in water along with the specified concentration of sodium chloride. The organic solvents were added to the samples, at a 4:1 ratio and allowed to incubate overnight at 4°C. The protein pellet was isolated through centrifugation at 16 000×*g* for 20 min followed by a 400 µL wash step with the appropriate solvent. Residual solvent was removed from the pellet by evaporation under vacuum.

2.3.3 Urea Assisted Protein Digestion

Conventional tryptic digestion following protein precipitation was carried out as described previously [1]. Protein was solubilized through addition of 20 µL of 8 M urea. Three rounds of sonication (10 min per round) in an ultrasonic cleaner (VWR International, Mississauga, ON) was used to disrupt the protein pellet and improve

protein solubility. Next, 65 µL of 50 mM Tris-HCl (pH 8.0) was added, along with 5 µL of 200 mM DTT (in 50 mM Tris pH8) with incubation at 37°C for 60 min. Alkylation was carried out through the addition of 10 µL of 200 mM IAA (30 min, room temperature, dark). Trypsin was added at a mass ratio of 1:50 and incubated overnight in a 37°C water bath (~16 hours). The resulting peptide digest was acidified with 10% TFA (10 µL) to a final volume of 110 µL. Samples were desalted using a one-step reversed phase HPLC method, described in Section 2.6.3, immediately following digestion and acidification. Peptide samples in chapter 5 were desalted using the SPE cartridge as described in Section 2.4.5.

2.4 Two-Stage Filtration Cartridge

2.4.1 Construction of the Two-Stage Spin Cartridge

The two-stage spin cartridge used in chapter 4 was designed and built in-house. Molded plastic pieces were designed by Dr. Mark Wall[1], Dr. Alan Doucette[1], and Robert Warner[2]. The raw plastic pieces were composed of polypropylene and were molded in bulk by Robert Warner[2]. Unassembled pieces are shown in Figure 2.1 containing: (1) the upper filtration cartridge, (2) the lower SPE housing, (3) the SPE housing sealing plug, and (4) the removable plug. The assembled device fits within a conventional 2 mL micro centrifuge tube (Fisher Scientific, Toronto, ON) and can be capped with the vials lid (referred to as the "cap"). The upper filtration cartridge houses a 3/16" diameter, 0.45 µm polytetrafluoroethylene (PTFE) membrane (Lubitech Technologies, Shanghai, China) at the base (cut from a larger 46 mm membrane disk). As shown in Figure 2.2, the

[1] Department of Chemistry, Dalhousie University, Halifax, N.S.
[2] Department of Mechanical Engineering, Dalhousie University, Halifax, NS, Canada

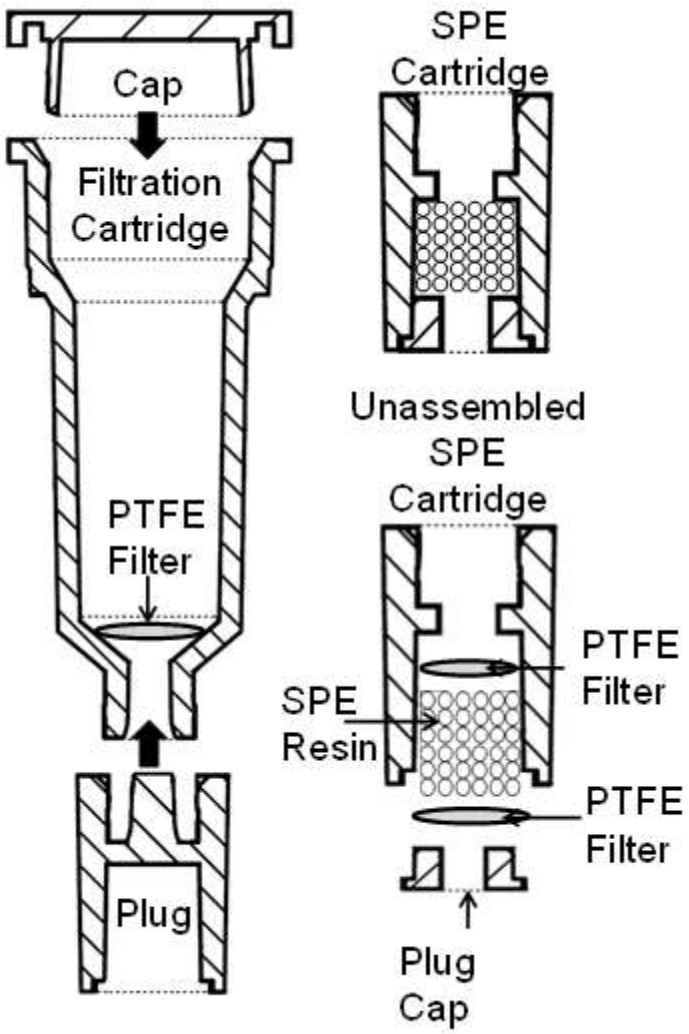

Figure 2.1: Diagram of the two-stage spin cartridge, the "ProTrap XG. Included pieces are: filtration cartridge, plug, SPE cartridge, and filter unit cap. The unassembled SPE cartridge is also shown. Figure recreated and used with permission from Dr. Alan Doucette, Department of Chemistry, Dalhousie University, Halifax, N.S.

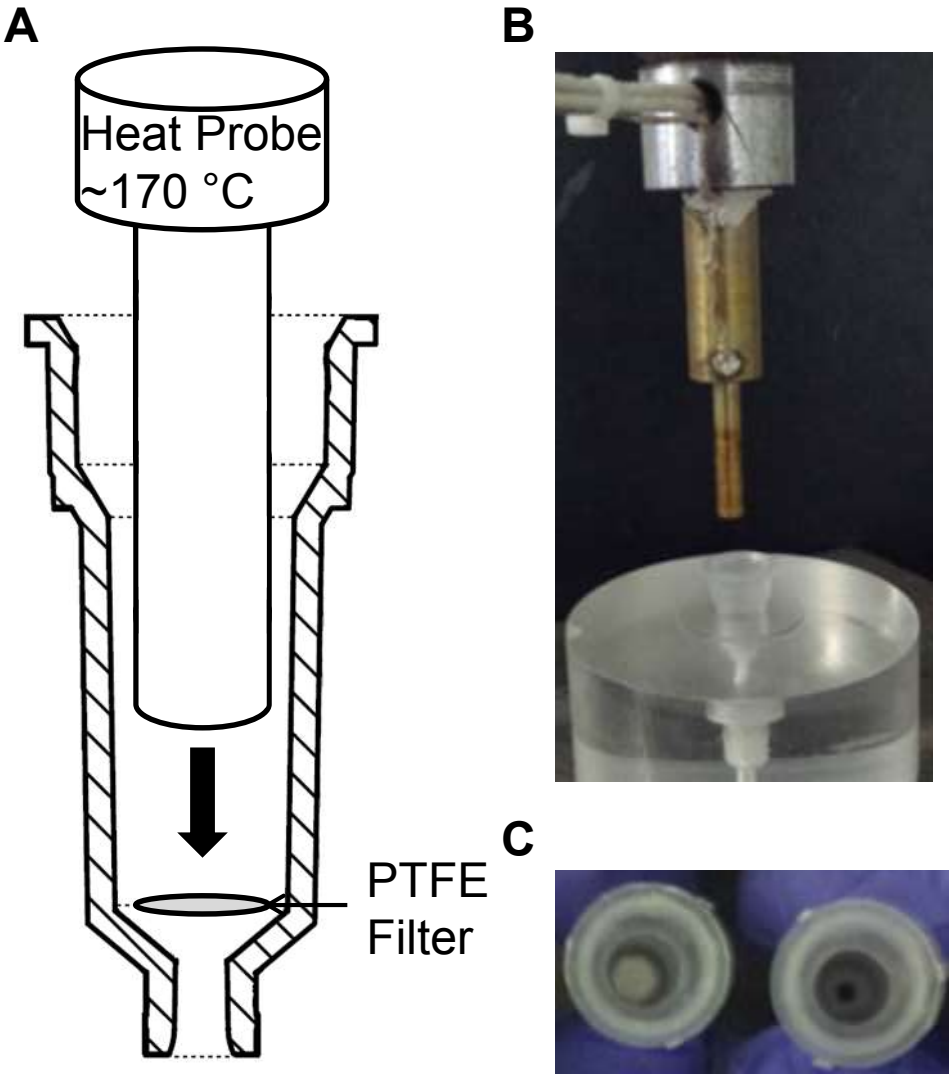

Figure 2.2: Assembly of the upper filtration cartridge. (A) PTFE filter is placed into unit; heat probe (~170°C) is lowered onto the filter to create seal. (B) Picture of heat probe assembly attached to standard drill press. (C) Image of the filter unit with and without membrane in place. Figure recreated and used with permission from Dr. Alan Doucette, Department of Chemistry, Dalhousie University, Halifax, N.S.

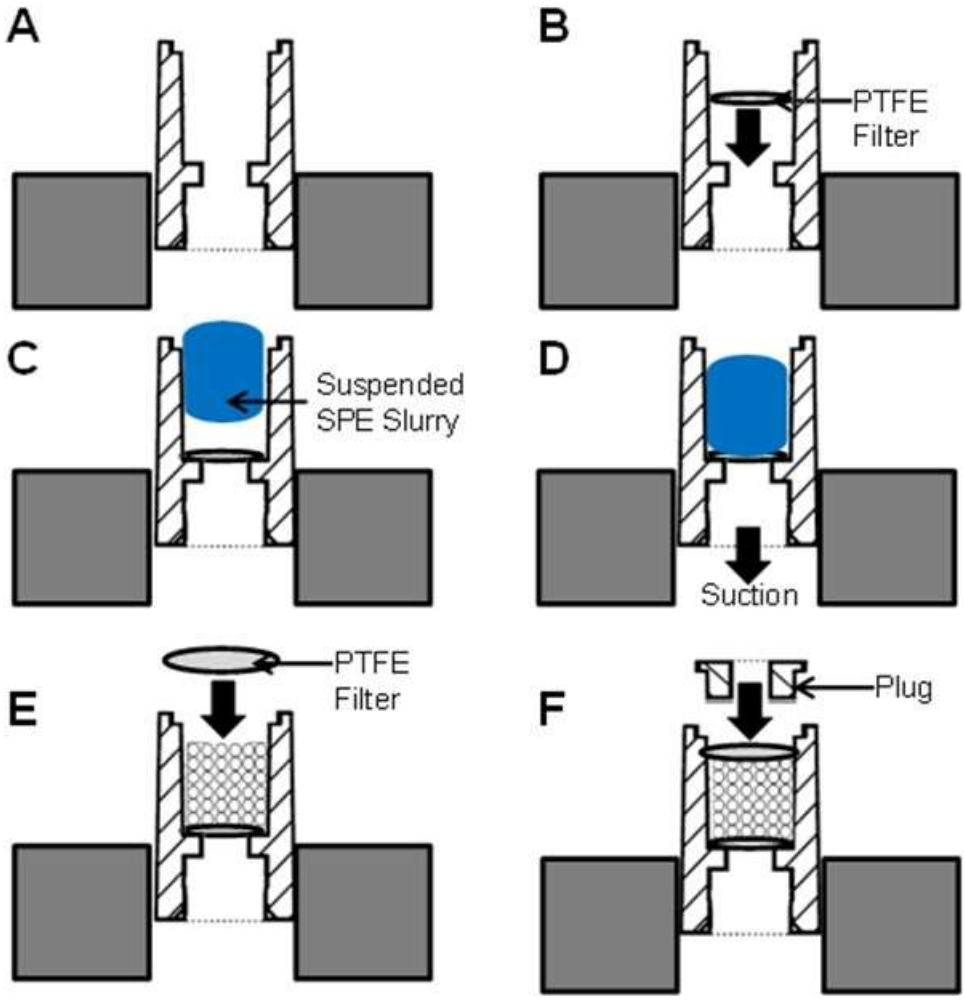

Figure 2.3: Overview of the construction of the SPE cartridge. (A) The cartridge casing is placed into the custom suction unit with the suction turned off. (B) The 5/32" lower membrane filter is placed into the housing. (C) The suspended SPE bead slurry is pipetted into the chamber. (D) The suction is turned on and any excess solvent is removed. (E) The suction is turned off and a second PTFE membrane (3/16") is placed on top of the resin. (F) The plug is used to cap the material into place. Figure recreated and used with permission from Dr. Alan Doucette, Department of Chemistry, Dalhousie University, Halifax, N.S.

membrane was melted into place using a heat-probe attached to a standard drill press. The probe is heated to 170°C. The probe was lowered onto a filtration cartridge with membrane previously placed inside as described in Figure 2.2A. With minimal pressure, the membrane was melted into place for 10 seconds. All upper cartridges were visibly inspected for proper filter placement and melting prior to use. When attached, the plug seals the hole beneath the PTFE membrane. It is suggested that attachable plugs not be reused to ensure proper sealing of the filtration cartridge. The filtration cartridge can be sealed through the use of a standard 2 mL vial cap. The SPE cartridge was assembled through manual manipulation of the SPE material held in place by two filtration disks. An overview of the process is shown in Figure 2.3. The solid phase material was suspended in methanol or ethanol at a 3:1 ratio of solvent to media. A 5/32", 0.22 µm filtration disk (cut from a larger disk, Life Technologies, Burlington, ON) was placed at the top of the SPE housing as shown in Figure 2.3B. The suspended media (R2 resin, 50 µm, 500-10000 Å, Applied Biosystems, Burlington, ON) was pipetted carefully into the SPE housing (Figure 2.3C). Excess solvent was removed through the use of suction leaving the dried SPE material as described in Figure 2.3D. A second 3/16" membrane disk was placed on top the media as shown in Figure 2.3E. The cartridge was sealed with the SPE cartridge plug (Figure 2.3F). All pieces were sealed in a plastic bag to prevent dust contamination prior to use.

2.4.2 Acetone Precipitation in the Upper Filtration Cartridge

Acetone precipitation was carried out in the upper filtration cartridge with plug attached to the base to seal the vessel. A 100 µL aliquot of the sample was added to the cartridge followed by 400 µL of acetone to induce protein precipitation. The cartridge was capped

and stored overnight at -20°C. The cartridge was placed in a 2 mL microfuge tube and centrifuged for 1 min at 400 ×*g* to pellet the protein. The filtration cartridge was turned upside down and the plug was removed. The system was spun briefly (400 ×*g* for 10 seconds) to remove any material from the cap. The cap was then removed and the cartridge was spun at 400 ×*g* for 2 min, allowing the solvent to pass through the cartridge filter. The filter containing the protein pellet was washed through the addition of 300 µL of acetone and removed by centrifugation (400 ×*g* for 2 minutes). It is not possible to completely dry the filter through centrifugation so the filter was allowed to air dry for 10 to 30 minutes. After recapping the cartridge, the plug was reattached and the protein pellet was subject to resolubilization and digestion as described in Section 2.4.4.

2.4.3 Chloroform Methanol Water Precipitation and FA solubilization

Chloroform/methanol/water (CMW) precipitation was carried out with the plug attached to the base of the upper filtration cartridge. A 50 µL aliquot of the sample was added to the cartridge followed by 200 µL of methanol, 50 µL of chloroform and 150 µL of water to induce protein precipitation. The system was subjected to centrifugation for 1 min at 400 ×*g* to pellet the protein. The plug was removed and the system was briefly centrifuged (400 ×*g* for 10 seconds) to remove any trace liquids from the cap. The cap was then removed and the cartridge was spun at 400 ×*g* for 2 min to remove the remaining solvent. A 200 µL wash of acetone was used to remove residue solvent from the precipitation. It was found that protein recovery was reduced if methanol was used as a wash. The plug was reattached and the protein dissolved using 100 µL of 1% SDS for BCA analysis. Formic acid resolubilization occurred through the addition of 20 µL of 80% formic acid and sonication for 10 minutes. After sonication the sample was diluted

with water to a final concentration of 16% FA and centrifuged. The liquid was subject to evaporation under vacuum. The proteins were resolved using SDS-PAGE as described in Section 2.5.3.

2.4.4 Urea Assisted Digestion In the Two-stage Spin Cartridge

Protein was resolubilized in the upper filtration cartridge with plug attached through addition of 50 µL of 8 M urea with three rounds of sonication (10 min per round) in an ultrasonic cleaner. Next, 162.5 µL of 50 mM Tris-HCl (pH 8.0) was added, along with 12.5 µL of 200 mM DTT with incubation at 56ºC for 30 min, followed by 25 µL of 200 mM IAA (30 min, room temperature, dark). Trypsin was added at a mass ratio of 1:50 (final total volume 250 µL) and incubated overnight in a 37ºC water bath. The resulting peptide digest was acidified with 10% TFA (25 µL) and diluted to with 225 µL of 5% ACN/water 0.1% TFA (~500 µL final volume). Peptides were then desalted using the SPE cartridge prior to analysis as described in Section 2.4.5.

2.4.5 Online SPE Desalting of the Peptide Sample

The online SPE cartridge allows for easy removal of salt from the digested peptide sample. The sample was passed through a pre-primed SPE cartridge. Priming of the SPE cartridge was performed using 500 µL of: methanol; 2× 50% ACN/0.1% TFA water; 2×5% ACN/0.1% TFA water (400 ×*g*, 2 min). This step ensures both the complete wetting and cleaning of the bead material. The filter of the upper cartridge requires wetting with 2-5 µL or methanol or ethanol prior to attachment of the SPE cartridge to ensure solvent can flow through without resistance. To prevent protein loss from leaking wetting of the filter was carried out post digestion following the removal of the plug by turning the cartridge upside down. For peptide de-salting, the SPE cartridge was then

attached to the upper filtration cartridge containing the digested protein and spun at 400 ×*g* for 3 min to pass the sample through the SPE cartridge. The flow through was returned to the same upper filtration cartridge and subjected to a second pass through the SPE cartridge as described above. The SPE cartridge was washed with three 500 µL additions of 5% ACN/0.1% TFA water (400 ×*g*, 2 min). It has been determined that two washes of 5% ACN/0.1% TFA water (600 ×*g*, 2 min) provide equal salt removal. Peptides were eluted with 2×300 µL of 50% ACN/0.1% TFA water or other specified solvents (400 ×*g*, 2 min). Samples were dried in a SpeedVac under vacuum and reconstituted in 0.1% TFA water for peptide quantitation by LC-UV (Section 2.6.3), or in 0.1 % formic acid, water for LC-MS/MS analysis (Section 2.7.1).

2.5 Gel Electrophoresis

2.5.1 Traditional GELFrEE

The *E. coli* whole protein extract and the cytosol fraction were mixed with Laemmli gel loading buffer [113] stored as a 5× stock (0.1 g/L SDS, 125 mM tris, pH 6.8, 20% glycerol, 5 g/L bromophenol blue, 10% 2-mercaptoethanol) stored at -20 °C. The samples were then heated to 95ºC for 5 minutes and subjected to GELFrEE fractionation (100 µg per column × 8 columns) as described previously [63] using a 12%T column (1 cm, 127 µL) coupled with a 4% stacking column (2.35 cm, 300 µL) in a glass tube (4 mm diameter). An in-house apparatus was used to perform the protein separation (described [63]). Gel running buffer contained 25 mM Tris, 191 mM glycine, and 0.1 % SDS, pH 8.3. The first fraction was collected after the dye front had fully migrated from the gel column. The remaining fractions were collected using the following timing cycle: 5×1 min, 5×2 min, 3×10 min, 3×15 min (17 fractions in total). The system was run at a

constant voltage of 240 V until 10 minutes into the collection of the fractions. After 10 minutes voltage was increased to 300 V. SDS-PAGE visualization of 1/5 of a fraction was carried out to verify protein separation efficiency. Samples were collected using 100 µL running buffer in the sample chamber. The buffer chamber was washed with 200 µL of running buffer between fractions.

2.5.2 GELFrEE Enrichment and Fractionation of LMW Plasma Proteins

Human and bovine plasma standards were dissolved in 1% SDS and then mixed with Laemmli sample buffer with or without the reducing agent 2-mercaptoethanol. The plasma samples were then heated to 95ºC for 5 minutes and subject to GELFrEE fractionation using a 17 %T resolving gel column. Two column diameters were tested: (1) 4 mm and (2) 6 mm for plasma loading capacity. For the 4 mm column, samples were diluted to a final volume of 25 µL prior to loading. A larger volume (50 µL) was loaded into the larger columns. For the 35 kDa separation the resolving gel was 1.2 cm in length, while all other separations occurred using a resolving gel length of 1 cm (volume: 300 µL for 6 cm tubes and 127 µL for 4 cm tubes). The stacking gel was prepared at standard length (2.35 cm) using a volume of 300 µL (4 cm) or 708 µL (6 cm) for all experiments. The system was run at a constant voltage of 200 V. Once the dye front migrated off the gel column voltage was increased to 240 V for the rest of the separation. The first fraction was collected after the dye front had fully migrated from the gel column. The remaining fractions were collected as described in Table 2.1. Samples were collected using either 100 µL (4 mm tubes) or 300 µL (6 mm tubes) running buffer in the sample chamber. The buffer chamber was washed with 400 µL of running buffer between fractions. Samples were acetone precipitated and dissolved in

either 30 µL of Laemmli sample buffer (for SDS-PAGE, Section 2.5.3) or 50 µL of 8 M urea (for protein digestion, Section 2.3.3). This large quantity of buffer and a significant amount of time (~4-5 hours at room temperature) was required for the samples to dissolve due to the presence of a large amount of glycine following acetone precipitation. Following tryptic digestion the samples were subject to purification using the SPE cartridge as described in Section 2.4.5.

Table 2.1: Collection timing used for GELFrEE separation of low molecular weight proteins from plasma samples.

	Gel Column Diameter	Fraction #											
		1	2	3	4	5	6	7	8	9	10	11	12
Collection Timing (min)	4 mm column	1	1	1	1	2	2	2	2	2	5	5	5
	6 mm column	1	1	1	1	1	1	2	2	2	5	5	5

2.5.3 SDS-PAGE for Protein Visualization

Samples were resolved on a Bio-Rad Mini-Protean® system using a 1 mm, 12% T resolving gel (4% T stacking) in accordance with the classic Laemmli protocol [113]. Samples were mixed with Laemmli gel loading buffer. Samples were heated to 95ºC for 5 minutes or 20 minutes at 60ºC (in Chapter 3) prior to SDS-PAGE analysis. Protein were resolved at 180 V using standard running buffer (described in Section 2.5.1) until the loading dye front was run off the gel. Protein bands were visualized through silver staining [114,115] or Coomassie stain (Bio-Rad, Hercules, USA). Images were developed using a digital camera.

2.6 Assays

2.6.1 BCA Protein Assay for Determining Protein Recovery

For protein recovery calculations following protein precipitation, either 100 µL or 250 µL of 1% SDS was added to the dried pellet and/or evaporated supernatant fractions. Protein resolubilization was assisted through sonication (2×20 min) in an ultrasonic cleaner followed by overnight incubation at room temperature. Proteins were quantified through the Pierce BCA assay with minor modification. The assay was performed through addition of 300 µL of BCA working reagent (50:1 'reagent A' to 'reagent B') to either 20 or 50 µL of sample. The sample was diluted in an effort to assay 2 to 3 µg of protein. The solution was incubated in a 60°C hot water bath for 30 min then cooled in a room temperature water bath for 30 min. Calibration curves were constructed using the respective proteome or protein standard. Calibrants were prepared in 1% SDS over a concentration range 0.25 to 3.0 µg per 50 µL. Measurements were recorded at 562 nm using an Agilent 8453 Spectrometer (Santa Clara, USA), using a 1 cm glass cuvette. Protein recovery was calculated as the fraction of protein recovered in the pellet, relative to a non-precipitated control.

2.6.2 Methylene blue SDS assay

A methylene blue spectrophotometric assay was employed to quantify the SDS remaining in samples following precipitation [116]. The protein pellets were resuspended in 200 µL of water and the trace amounts of SDS were quantified. In short, 200 µL of methylene blue reagent (250 µg methylene blue, 50 g sodium sulfate, and 10 mL concentrated sulphuric acid in water per liter of reagent) was added to the 200 µL of sample and mixed. Chloroform was then added (800 µL) to extract the SDS bound methylene blue

complex from the aqueous phase. The samples were vortexed for at least 1 minute to ensure proper extraction. The sample was centrifuged for 15 minutes at maximum speed and the aqueous layer was completely removed. The remaining water was removed through the addition of an absorbing agent (sodium sulfate). The absorbance was measured at 651 nm with an Agilent G1103A UV/Vis spectrophotometer in comparison to a SDS calibration curve from 0.2 µg to 2 µg SDS.

2.6.3 LC-UV Peptide Assay and Online Desalting Method

Peptides samples from both vial-based precipitation strategies and the two-stage spin cartridge workups were quantified using liquid chromatography coupled to UV detection (LC-UV) [117]. This method was also used to desalt samples prior to LC-MS/MS analysis. All peptides samples in Chapter 4 which were not purified by SPE and all samples in Chapter 5 were subject to offline desalting. Peptide clean-up and quantitation was carried out on a Agilent 1100/1200 hybrid system containing an auto sampler with a 100 µL sample loop, diode array detector equipped with a 50 nL flow cell, and fraction collector. All peptide analysis was accomplished with an in-house packed column (1 mm × 50 mm) containing 5 µm Waters Spherisorb S5 OD52 C18 beads (Milford, MA, USA) at a flow rate of 100 µL/min. Peptide samples were loaded onto the column by the auto sampler and eluted after the injection peak with a gradient step of 0.1 minutes at 8 minutes from 5 to 85 % acetonitrile/0.1% TFA. The peptides elute in one peak which can be manually integrated with Excel (Microsoft, Calgary, AB) to provide peptide quantitation compared to a control/calibration curve from 0.5 µg to 10 µg. Purified samples are subject to evaporation under vacuum in a SpeedVac and stored at -20ºC prior to LC-MS/MS analysis.

2.7 Protein Analysis by Mass Spectrometry

2.7.1 Peptide Sequencing on a Linear Ion Trap Mass Spectrometer

Purified peptide samples were reconstituted in 0.1% FA 5% ACN and were subject to LC-MS/MS analysis on an Agilent 1200 nanoflow system equipped with a dual column nanoelectrospray system [118], coupled to a Thermo Fisher Scientific LTQ linear ion trap mass spectrometer (Burlington, ON). This system allowed rapid analysis of peptide samples by decreasing the time spent equilibrating the column between runs. It consisted of an Agilent 1050 "loading pump" running isocratically at 5% ACN, 0.1% FA used to load and equilibrate the non-running column. A second "gradient pump" was used to run the other column and perform MS analysis (Agilent 1200 nanocapillary pump). A 10 µL sample was introduced to the system by an 1100 Agilent auto sampler. A voltage switching valve and solvent switching valve control which column is in use or being loaded. The columns (30 cm × 75 µm i.d.) were packed with C12 Jupiter beads (4 µm, 90 A pore size) from Phenomenex (Torrance, USA) and operated at 250 nL/min. The gradient of solvent A (0.1% FA in water) and solvent B (0.1% FA in acetonitrile) was as follows: 0 min, 5% B; 0.1 min, 7.5% B; 90 min, 20% B; 115 min, 25% B; 120 min, 35% B; 121 min, 80% B; 125 min, 80% B; 125.1 min, 5% B. The LTQ was set to data dependent mode (MS followed by MS/MS of top 3 peaks). The BSA digests were each subjected to 4 replicate LC-MS/MS injections (250 fmol/injection). The GELFrEE samples were subject to 6 LC-MS/MS injections per independently prepared sample (*i.e.* 6×6 injections), corresponding to approximately 1 µg per injection. The whole *E. coli* samples were injected in duplicate corresponding to approximately 1 µg per injection. The plasma samples were injected and analyzed in singlet.

2.7.2 Kidney Sample Analysis

Kidney samples were analyzed on an Orbitrap Velos Pro (Thermo Fisher Scientific, Burlington, ON) coupled to a Dionex Ultimate 3000 Rapid Separation LC nanosystem (Bannockburn, IL), operating in MS mode at a resolution of 30 000 FWHM, scanning in rapid mode for MS^2 (66,666 Da/s, at <0.6 Da FWHM). The column was a Phenomenex Onyx monolithic C18 (150 × 0.1 mm, Torrance, CA), coupled to a 10 μm New Objective PicoTip non-coated Emitter Tip (Woburn, MA). The gradient from solvent A to solvent B was as follows: 0 min, 3% B; 3 min, 3% B; 5 min, 5% B; 69 min, 35% B; 72 min, 95% B; 77 min, 95 % B; 80 min, 3% B. The Orbitrap was set to data dependent mode (MS followed by MS/MS of the top 10 peaks). Each of the six kidney extracts was subjected to a single injection for LC-MS/MS analysis.

2.7.3 Peptide Database Searching

Peptide identification was carried out by database searching using the Thermo Proteome Discoverer software package (v 1.3 or 1.4) and the SEQUEST searching algorithm [119]. MS spectra were searched against the species-specific UniProt databases bovine, human, *Escherichia coli* (strain K12), or *Rattus norvegicus*, with a precursor mass tolerance of 1 Da (for LTQ data) or 10 ppm (for Orbitrap). A fragment tolerance of 1.0 Da was used for both instruments. Allowable modifications included static carbamidomethylation (+57.0215 Da) of cysteine residues, and dynamic oxidation (+15.9949 Da) of methionine, with up to two missed trypsin cleavages unless otherwise specified. In chapter 4, a peptide false discovery rate of 1% was set by decoy database searching, with a minimum of two peptides per protein to filter the data. In chapter 5, a peptide false discovery rate of 5% was set by decoy database searching, with a minimum of one peptide per protein to

filter the data. Label-free quantitative analysis of relative protein abundance was accomplished by spectral counting which uses the number of peptide spectral hits as a measure of quantitation [120].

2.8 Data Analysis

Protein spectral counts (PSM) [120] were normalized by dividing the individual PSM by the sum total PSMs from a particular run, and multiplying by the largest total sum of PSMs across the 6 biological samples. Normalized spectral count data were subjected to data analysis using a modified R script [121] based on QuasiTel [36]. All proteins with a quasi-Poisson p-value < 0.05 were considered to be significantly altered in abundance. Hierarchal clustering and generation of a heat map of all significantly altered proteins was accomplished using the R heatmap2 function (gplots library) and displaying a $\log_2$ fold change in PSMs across the 6 biological samples. Proteins with a PSM of zero were mapped to the lowest fold change across all statistically altered proteins. The altered protein list was subject to the online functional enrichment database tool DAVID, or Database for Annotation, Visualization and Integrated Discovery, version 6.7 in order to identify patterns in functional enrichment of gene ontology terms [122,123]. This free online tool was used according to manufactures instructions. The master list of all proteins identified across the runs was used as a background, and all filters were set to default

3. Chapter 3: Ion-Pairing In Organic Solvents[1]

3.1 Introduction

Precipitation is a classic approach to purifying proteins, being first described through the application of high salt concentrations (salting out) [81], and later through the addition of organic solvents [124]. Currently, a number of protein precipitation techniques have been developed for use in protein purification. These include precipitation through the addition of salts [125], organic solvents [72], acids [126], and high molecular weight polymers [80]. Historically, protein precipitation has been a vital technique for the study and isolation of proteins [90]. The large scale fractionation of plasma proteins is still heavily reliant on the use of ethanol precipitation [127]. Sample purification ahead of LC-MS/MS using protein precipitation has never gained widespread popularity. With the development of more automated approaches, such as solid phase extraction [128,129] and ultrafiltration [64], protein purification by precipitation has seen a decrease in use. However these techniques suffer from non-quantitative protein yield in the removal of sodium dodecyl sulfate at the intact protein level (see Section 1.3.6).

Solvent precipitation using the organic solvent acetone has been shown to be highly effective at removing SDS from protein samples ahead of LC-MS/MS analysis [1]. However the use of this technique is not widespread. The reported variability in protein recovery (as described in Section 1.4.4) may have hindered the widespread application of this technique. Recent work by Dr. Mark Wall[2] and Dr. Alan Doucette[2] revealed near quantitative recoveries (>95%) from acetone precipitation when

[1]Portions of this work are published in the manuscript: A.M. Crowell, M. J. Wall, A.A. Doucette. Maximizing recovery of water-soluble proteins through acetone precipitation. Anal Chim Acta. 2013, 796, 48-54. Results presented in this thesis were carried out by thesis author, unless otherwise noted.
[2] Department of Chemistry, Dalhousie Univesity, Halifax, N.S.

precipitating BSA in varying concentrations of SDS (Figure 1.7A). Protein recoveries under 10% are noted at low concentrations of SDS. The complete lack of protein precipitation was unexpected, and was not fully understood. A great amount of time and effort was put into gathering data to explain this trend [108,130]. This improvement in precipitation efficiency was later determined to be caused by the ionic properties of SDS; an identical trend is observable through the addition of sodium chloride (Figure 1.7B). However, the current theories of organic solvent precipitation cannot explain the requirement of salt to be present for protein precipitation of BSA to occur.

In aqueous solution, proteins adopt a structure which exposes the hydrophilic regions to the surrounding solvent. This causes the formation of a hydration layer that shields protein-protein interactions. Disruption of this hydration layer, through the addition of a precipitating agent, is the general cause of protein precipitation [40]. The actual protein-protein forces which cause protein aggregation are a highly debated subject. It is agreed that the addition of organic solvents to an aqueous system causes a disruption of the hydration layer in two ways. The first is that water binds more favorably to the organic solvent molecules than the proteins; this reduces the protein-protein shielding effect of the hydration sphere [93]. Secondly, the addition of an organic solvent to an aqueous system causes a reduction of the dielectric strength. This causes an increase in electrostatic interactions between proteins. The most popular theory of solvent precipitation relies on this increase of attractive electrostatic forces between proteins, as described by Coulomb's law [40,86,90,92]. This equation relates the magnitude of the electrostatic force to the dielectric constant of the medium. For a heterogeneously charged protein surface, the positively charged region of one protein can

combine with the negative charged region of another, leading to aggregation of proteins. However, by a similar logic, repulsive forces between like charges on opposing proteins would also increase in organic solvents. Thus, this simple model of precipitation does not fully describe the cause of protein aggregation in acetone.

The use of low concentrations of organic solvent for selective protein precipitation provides further evidence that this model is highly limited. One such example of this is Cohn fractionation of blood plasma. In this process the major plasma proteins such as serum albumin, gamma globulin, fibrinogen, thrombin, and others are isolated from plasma using ethanol precipitation [97]. This methodology relies on changes in ethanol concentration and pH to induce selective protein precipitation. It is known that proteins have reduced solubility at their intrinsic isoelectric point (pI) due to the decrease of electrostatic charge on the protein's surface. This decrease in charge reduces the protein-solvent interactions causing the proteins to be less soluble in the solution. The addition of organic solvents (~50%) can further reduce the solubility [86,93–97]. This observation is clearly in conflict with the theory that el ectrostatic attractions cause protein aggregation in organic solvents. Therefore other forces must be the cause of protein aggregation. It has been suggested that this process is mainly due to van der Waals forces and not hydrophobic interactions. This is due to the stabilizing nature of organic solvents, which should favorably interact with the hydrophobic regions of the protein [93]. It should be noted that protein precipitation at high organic concentrations has not been found previously to be dependent on the pI of the protein, only the molecular weight.

No matter the cause of protein precipitation, it is clear that a reduction of electrostatic charge on the protein is favorable to assist in the precipitation of proteins from solution. Here, this observation is extended to explain the phenomenon of increased protein recovery from acetone precipitation upon the addition of salts to the system. In this work it was verified that the solubility of a given protein in 80% acetone is highly dependent on the pH of the solution. The addition of trace levels of salts, allows for the effective precipitation of proteins removing the effect of pI. This is explained through an ion-pairing mechanism which is compatible with all previous theory. The combined influence of salt and organic solvent results in consistent and quantitative recovery for all water soluble proteins including complex proteome mixtures.

3.2 Experimental in Brief

Organic solvent precipitation was carried out as described in Section 2.3.1 and 2.3.2. In brief, 50 µL of protein solution and 50 µL of sample additive were combined to provide the final concentrations described in the results. Organic solvent was added to the sample at a 4:1 ratio to induce protein precipitation. However, for the variable acetone concentration experiment, sample and acetone volumes were adjusted as needed to provide 500 µL as a final volume. The protein pellet was collected through the use of centrifugation with an additional solvent wash following overnight incubation. The pellet was suspended in 1% SDS. Protein recovery was assessed through the use of either a BCA assay as described in Section 2.6.1 or SDS-PAGE for visualization as described in Section 2.5.3. Protein recovery was calculated through the comparison of the protein detected in the pellet following precipitation against a non-precipitated control.

3.3 Results and Discussion

3.3.1 Protein Recovery in the Presence of NaCl

Previous work has revealed a link in the protein precipitation ability of the organic solvent acetone and the amount of salt present in the sample (for BSA). To verify the inability of acetone to precipitate BSA (Figure 1.7), an increased concentration of both BSA and acetone was examined. Protein yield did not improve as the concentration of BSA was increased above 0.1 g/L (up to 20 g/L), nor as the level of acetone in the precipitating solvent was increased from 80% (up to 95%). Clearly, from these results it can be said that BSA is soluble in high concentrations of acetone (<95%). This trend would not have been predicted by the current model of solvent-based protein precipitation, wherein aggregation is induced by the lower dielectric strength of the solvent. It is further noted that the level of salt required to induce precipitation of BSA in 80% acetone is nowhere near that employed through the conventional method of salting out (typically 3 M ammonium sulfate). The salt required to restore protein recovery in acetone is over 3 orders of magnitude lower in concentration. These previous (Figure 1.7) and new observations clearly demonstrate a synergistic relationship between salt and solvent to control the efficiency of protein precipitation in acetone.

3.3.2 Extending the Observation to Other Protein Standards

To obtain a further understanding of the synergistic properties of salt and acetone, other proteins were tested. The precipitation efficiency of ten standard proteins was determined in 80% acetone. Table 3.1 summarizes the recoveries obtained from 0.1 g/L protein, individually prepared in pure water. The proteins were also precipitated with inclusion of 10 mM NaCl in the initial sample (*i.e.* ten-fold higher than what was

Table 3.1: Protein recovery in 80% acetone, with or without inclusion of 10 mM NaCl in the solution

	% Recovery (0 mM NaCl)	% Recovery (10 mM NaCl)
Lysozyme	8± 1	104± 8 [(A)]
cytochrome C	8± 3	87± 5
α-casein	11± 20	98± 5
ubiquitin	12± 7	94± 9
BSA	12± 9	102± 2
Invertase	15± 26	79± 4
carbonic anhydrase	95± 10	99± 13
ribonuclease B	95± 14	95± 17
Myoglobin	97± 2	94± 6
β-lactoglobulin B	100± 14	96± 11

[(A)] Recovery after addition of 30 mM salt

required to restore recovery for BSA). In the absence of salt, the test proteins displayed highly variable recoveries. Including BSA, six of the ten proteins tested showed poor recovery (at or below 15%), while the remaining four proteins demonstrated near quantitative recoveries (≥95%). Variability of recoveries across protein samples was expected, as precipitation efficiency is thought to be protein dependent. However, such drastic difference (either <15% or >95%) was surprising. As seen in Table 3.1, upon addition of salt all ten proteins demonstrated high precipitation efficiency. Those proteins which displayed high recovery in water remained high upon the addition of salt; the recovery of the remaining six proteins improved considerably in the presence of 10 mM NaCl. The sole exception was lysozyme, which required a minimum 30 mM NaCl to facilitate quantitative recovery of the protein (61±7% recovery was observed in 10 mM NaCl). Proteins that did not precipitate in 80% acetone must share a similar

chemical or physical property which prevents precipitation. No obvious chemical or physical property exists between the proteins that fully precipitated without the addition of salt or those that require salt for quantitative yield. However, the requirement for a higher concentration of salt to precipitate lysozyme provides insight into the mechanism of synergistic protein precipitation with salt and acetone.

The full recovery trend for lysozyme as a function of NaCl concentration is shown in Figure 3.1. As expected the protein recovery following the addition of acetone is minimal in low concentrations of salt. Upon the addition of 5 mM NaCl protein recovery starts to increase (11±7%) reaching a maximum at 30 mM (104±9%) and continue to maintain near quantitative yield until 100 mM (maximum salt concentration tested). The amount of salt required to induce quantitative precipitation of lysozyme is higher than the other proteins tested. Lysozyme has been found previously, to precipitate in lower yields than other proteins. Yuan *et al.* found reduced recovery for lysozyme compared to other proteins in the same sample [102]. This reported observation may be explained by the additional ionic strength required to precipitate lysozyme compared to other proteins. The sample examined by Yuan *et al.* may have contained enough salt to induce protein precipitation of other proteins, but insufficient amounts to quantitatively precipitate lysozyme. Increasing the concentration of ionic species can improve protein recovery of all proteins in a sample. Looking at this protein compared to the other standards tested (Table 3.1) reveals no trend in MW or hydrophobicity. However lysozyme has the pI which is the furthest from the solutions pH (~6). This suggests that the charge of the protein may be an important factor in preventing precipitation.

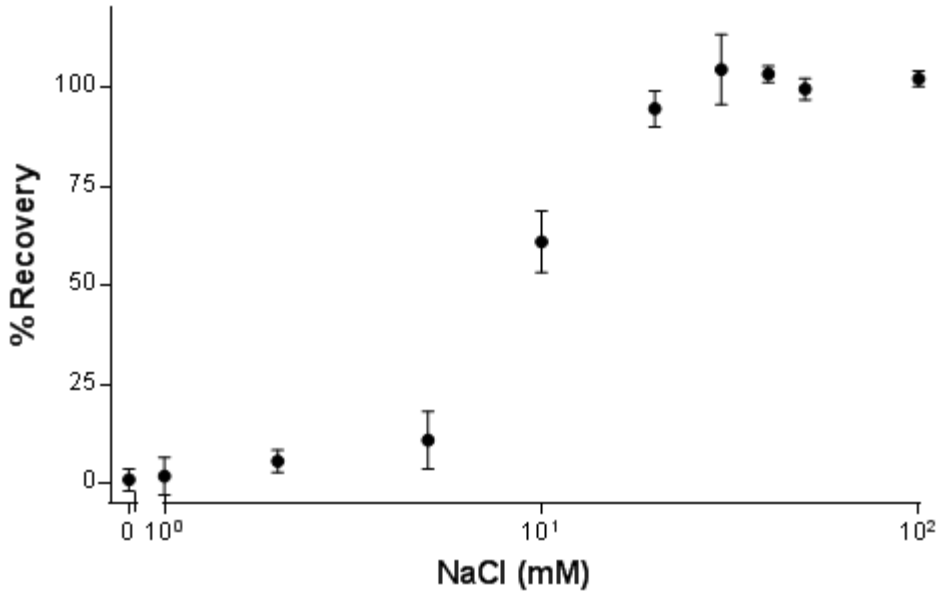

Figure 3.1: The recovery of 0.1 g/L Lysozyme (100 µl initial volume) following precipitation with 80% acetone, as a function of the NaCl concentration in the initial aqueous sample. Error bars represent the standard deviation from 3 measurements.

3.3.3 Effect of pH on Acetone Precipitation of Protein Standards

The effect of pH on protein solubility in an ethanol-water solvent system is well documented [97]. The dependence of pH to precipitate proteins is herein extended to an 80% acetone-water solvent system. As is seen in aqueous solutions, it is predicted that proteins will be least soluble in 80% acetone at or near their pI. Lysozyme (pI 11.4) yields precipitation efficiency below 10% in water (Table 3.1). However, in 25 mM NaOH (pH ~12) the recovery improves to 74±9%. Similarly, myoglobin, with a pI near 7, readily precipitates in 80% acetone/ water (see Table 3.1). However, in 25 mM NaOH, myoglobin remains fully soluble in acetone (recovery drops to 3±2%). Modifying the pH of the solution would be an impractical strategy to improve protein precipitation efficiency. Not only may the pI of the protein be unknown, but the precipitation of complex proteome mixtures would not be possible at a single pH, as the pI varies across the sample. As shown in Table 3.1, the inclusion of NaCl is a simple approach to maximize protein recovery through acetone precipitation.

3.3.4 Effect of Protein Concentration on Acetone Precipitation

The effect of protein concentration on the time required for precipitation to fully occur is well documented [100]. Here, the effect of protein concentration on the amount of salt required to induce protein precipitation of BSA using acetone precipitation is examined. Figure 3.2 provides the trends in precipitation efficiency as a function of salt at four concentrations of BSA (0.1, 0.5, 1.0, and 10 g/L). The recovery trend for 0.1 g/L BSA shown in Figure 3.3A is the same as that shown in Figure 1.7. All the protein recovery trends follow sigmoidal curves, with maximal recovery observed beyond a critical concentration of NaCl. It is noted, that near quantitative recovery of protein is

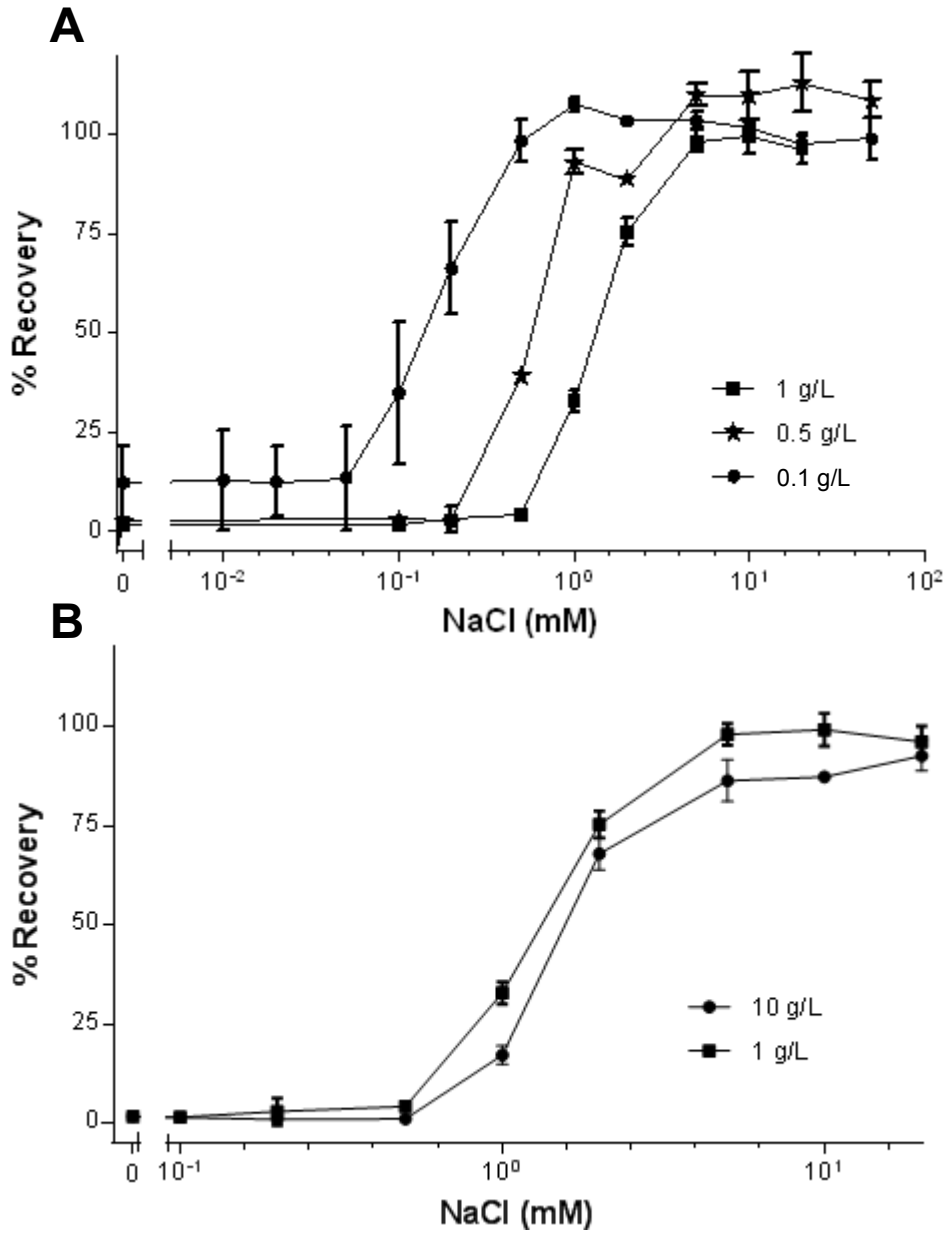

Figure 3.2: The recovery of BSA at varying concentrations (0.1 to 10 g/L as listed, 100 µl initial volume) following precipitation with 80% acetone, as a function of the NaCl concentration in the initial aqueous sample. To improve visualization of the data, points were connected with a line. Error bars represent the standard deviation from 3 measurements.

maintained at low protein concentrations. Precipitation of 10 g/L BSA (*i.e.* 1 mg total) generates an extremely large protein pellet, which is difficult to isolate from the supernatant through pipetting. As seen in Figure 3.2A, the concentration of salt required to induce protein precipitation is clearly influenced by the amount of protein in solution. Specifically, as the concentration of BSA is raised from 0.1 to 1.0 g/L BSA, the level of salt required for optimal protein recovery increases proportionally to the concentration of protein in the sample. The data was fitted to sigmoidal curves allowing for the calculation of EC_{50} values (effective salt concentration inducing 50% of maximal protein recovery) for each protein concentration. It was determined that the EC_{50} represents a constant mole ratio of approximately 100:1 (salt to protein). This ratio does not hold at extremely high protein concentration. Figure 3.2B reveals that similar concentrations of salt are required for both 1 g/L and 10 g/L. This suggests that the amount of salt required for precipitation is independent of concentration at this extreme.

Overall an observation of salt and protein concentration dependence on the recovery of protein from acetone precipitation is clearly present. Any model to explain the dependence of the trend of salt improving acetone precipitation must also account for this observation. It is also noted that the concentration of salt recovered in the pellet is far lower than the ratio in solution; an approximate 1:1 mole ratio was observed through precipitation of 5 g/L BSA in 1 M NaCl, translating into a 20,000 fold reduction of the salt content from the original solution (Na^+ assayed through flame atomic absorption). Thus, the requirement for salt in the solution to induce precipitation of the sample does not negatively influence the purity of the resulting pellet.

3.3.5 Effect of Acetone Concentration on Protein Precipitation

Coulomb's Law defines the relationship between the magnitudes of electrostatic attraction between two charged species to be inversely proportional to the dielectric strength of the solution. Therefore, it is hypothesized that a higher salt concentration may be required to induce maximum protein precipitation in an increasingly aqueous medium. To test this prediction the precipitation ability of various concentrations of acetone was examined as a function of salt (1 g/L BSA as a protein standard). Figure 3.3[1] shows the effect of salt on samples containing 40 to 80% acetone. As expected, lower amounts of acetone required a higher critical concentration of NaCl to maximize protein recovery. As the acetone was lowered from 80 to 50%, an approximate 10 fold increase in salt was required to maintain protein recovery. It is noted that BSA could not be effectively precipitated in 40% acetone as shown in Figure 3.3B, regardless of salt concentration used. From these results it can be concluded that lower concentrations of acetone require higher salt concentrations to induce protein precipitation.

3.3.6 Precipitation of Complex Proteome Mixtures

The systems examined thus far represent single proteins prepared in controlled solvent systems. The controlling influence of salt on precipitation efficiency in 80% acetone was visualized through SDS-PAGE images (Figure 3.4). Here, an *E. coli* proteome extract was subject to acetone precipitation with or without inclusion of 10 mM NaCl. The resulting protein pellet was isolated from the supernatant, and the proteins contained in each fraction were visualized. As shown in Figure 3.4B, the absence of salt results in poor precipitation efficiency, with the majority of the proteins observed in the supernatant. In sharp contrast, addition of 10 mM NaCl causes a complete reversal

[1] This experiment is based off of a previously published experiment [108].

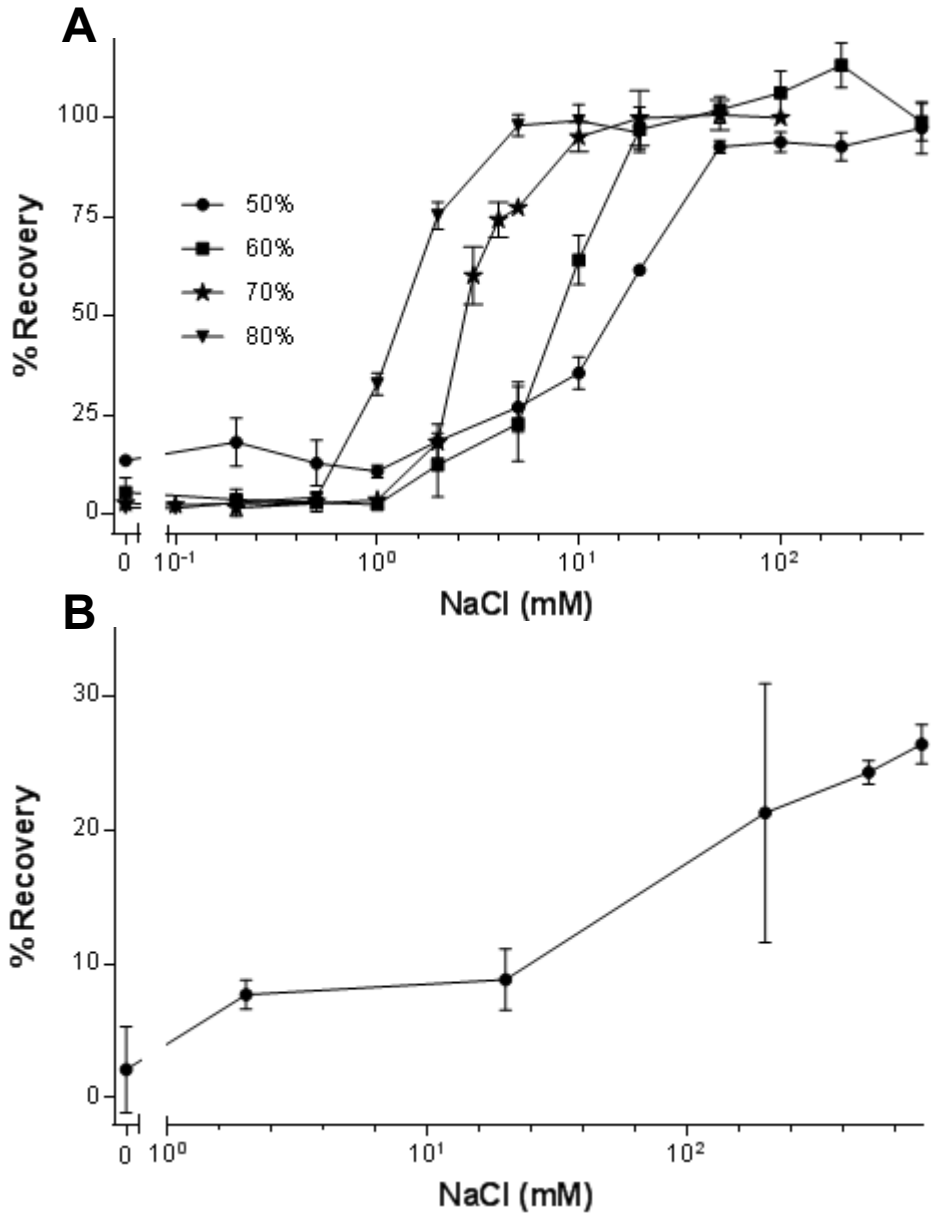

Figure 3.3: (A) The recovery of 1 g/L BSA following precipitation with varying compositions of acetone (50 to 80%) as a function of the NaCl concentration in the initial aqueous sample. Error bars represent the standard deviation from 3 measurements. (B) Plot of protein recovery following precipitation with 40% acetone (1 g/L)

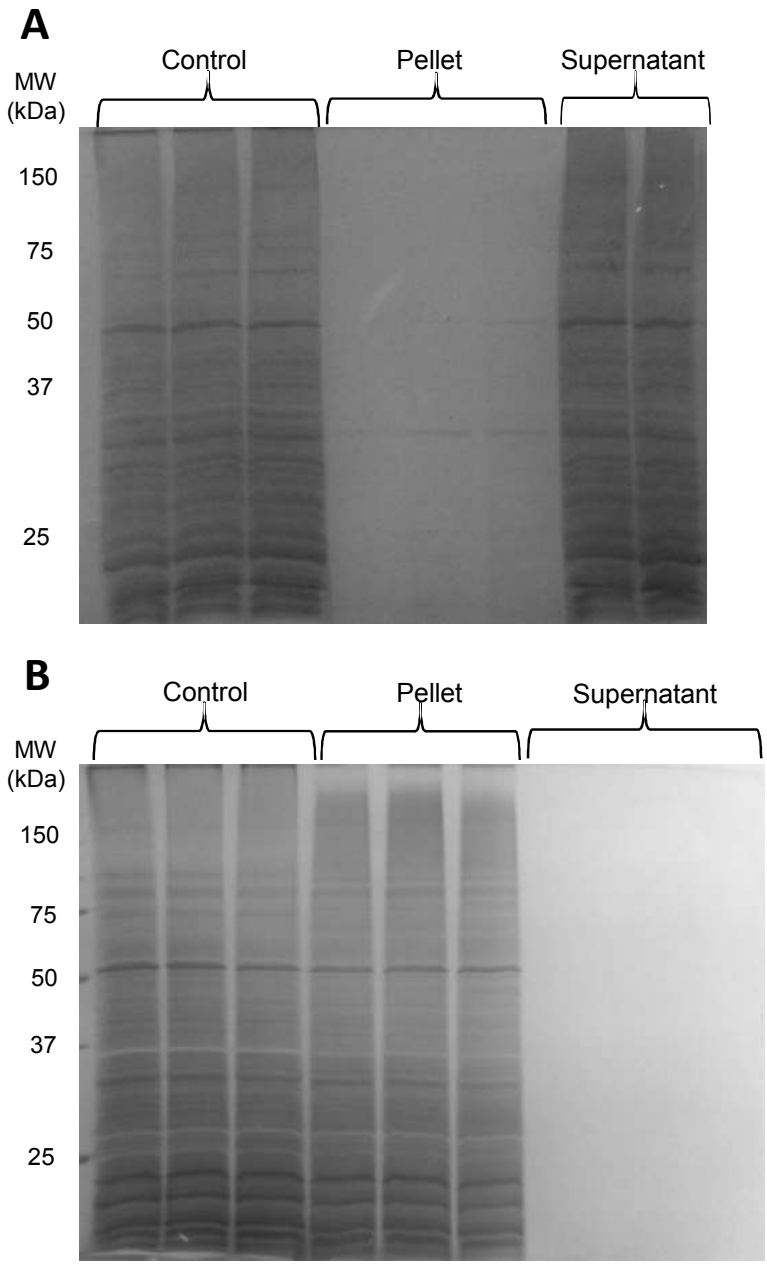

Figure 3.4: SDS-PAGE (Coomassie stain) reveals *E. coli* proteins (20 µg) recovered in the pellet or supernatant depending on the addition of NaCl to the sample (A) no NaCl and (B) 10 mM added to the initial protein sample. Control lanes consisted of 20 µg *E. coli* protein which did not undergo precipitation.

of the gel image, where the majority of proteins are observed in the lanes corresponding to the protein pellet.

3.3.7 Ion-Pairing in Organic Solvents

The addition of salt to improve precipitation efficiency for certain proteins in acetone may be explained through a model of ion pairing. As reviewed by Marcus and Hefter, it is known that ion-pairing is more prevalent in solvents of low bulk permittivity (dielectric) [131]. Though a 1:1 salt such as sodium chloride would be hydrated in water, preventing ion pairing in aqueous solutions, the reduced dielectric strength of 80% acetone (permittivity ~40 at 4°C, [132]) will facilitate ion pairing, including pairing between Na^+ or Cl^- and charges on the surface of proteins. It is hypothesized that such ion pairing will shield the repulsive electrostatic forces which are otherwise responsible for maintaining protein solubility in acetone. In water, electrostatic repulsion is a dominant force controlling protein solubility; these forces explain the poor solubility of protein in water at a pH equal to its isoelectric point.

The same argument can be extended to the solubility of proteins in organic solvents. Of the ten proteins examined (Table 3.1), lysozyme has the highest pI (11.4) and thus is highly charged in solution (pH ~6). The addition of salt to a sample of lysozyme can shield the protein charge through ion pairing in high concentrations of organic solvent. Once the charge is shielded, aggregation may occur as a consequence of van der Waals forces or hydrophobic interactions between proteins. This model of protein precipitation draws parallel to a method of DNA precipitation; a high concentration of ammonium acetate or similar salt is combined with 70% ethanol, such

that the salt will neutralize repulsive electrostatics arising from the negatively charged DNA backbone [133].

This model can provide explanation for the variable recoveries obtained for acetone precipitation without the addition of salt. If proteins are only mildly charged at the pH of solution where precipitation occurs, they will precipitate effectively from solution upon the addition of acetone. However, if they are highly charged (the pH is far from their pI) then they will not precipitate without the addition of an ion-pairing agent to shield the charge. The dependence of salt on protein concentration for the recovery of protein from acetone precipitation is consistent with the ion-pairing model. In such a model, one would expect the increased amount of protein to require more salt due the presence of more charged groups that require an ion-pairing "partner". Therefore as protein concentration increased more salt was required to be present in solution. Ion-pairing can also explain the requirement of higher salt concentrations at lower organic solvent compositions. As stated previously, Coulombs Law relates the electrostatic attraction between charges to the dielectric strength of solution. As dielectric is increased, the attractive forces between charges is decreased. Therefore, the ion-pairing ability of salt is decreased. For this reason a higher salt concentration is required to maintain salt-protein pairing in an increasingly aqueous medium.

3.3.8 Extending the Trend to Other Organic Solvents

The requirement of salt to induce near-quantitative precipitation of certain proteins in organic solvents has only been shown for the solvent acetone. Here, preliminary work which examines the recovery of 0.1 g/L BSA from both ethanol and acetonitrile precipitation as a function of salt, is shown in Figure 3.5. As can be seen the protein

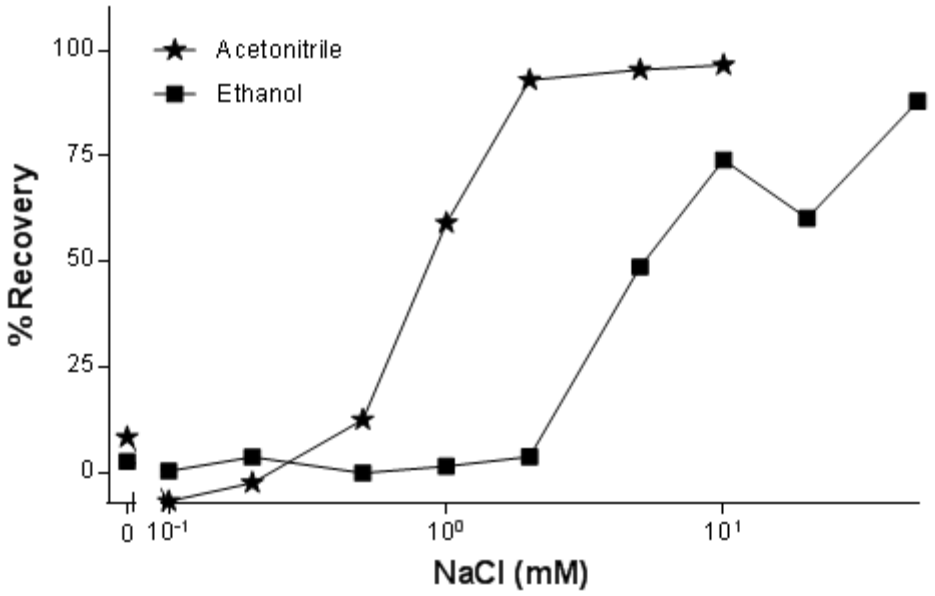

Figure 3.5: The recovery of 0.1 g/L BSA following precipitation with various organic solvents, as a function of the NaCl concentration in the initial aqueous sample. (n=1)

recovery in both these solvents is minimal at low concentrations of NaCl. However upon the addition of larger amounts of salt, precipitation occurs in high yield. For ethanol, maximal recovery was not observed until 50 mM (the highest concentration tested); however acetonitrile required significantly less salt (2 mM) to obtain maximal recovery. It is noted that both solvents required more salt than acetone (Figure 1.7B). This cannot be explained by differences in dielectric constant between solvents. Acetonitrile has the highest dielectric constant of all three solutions and therefore was expected to require the presence of more salt to induce optimal precipitation. Ethanol has only a slightly higher dielectric constant than acetone but requires significantly more salt to induce maximal precipitation. Further experimentation is needed to fully examine the ability of different organic solvents and different salts to induce protein precipitation. These results do suggest that the synergistic effect of salt on protein recovery is not limited to acetone and can be extended to other organic solvents

3.3.9 Practical Applications of an Ion Pairing Model

All the previous results reveal that the near quantitative recovery can be obtained from acetone precipitation upon the addition of minimal amounts of ionic species. This has been hypothesized to be due to an ionic pairing effect between the salt and the proteins. It is therefore critical to ensure that proper ionic buffers are present prior to acetone (or other organic solvent) precipitation. This thesis suggests the use of an ionic buffer (50 to 100 mM) should be present during acetone precipitation to ensure proper precipitation of all proteins. Figure 3.6 illustrates a practical application of acetone precipitation which emphasizes the need to control the ionic strength of the solution. Here, a cytosolic protein fraction was obtained through osmotic lysis of the mammalian cell NRK-52E. In

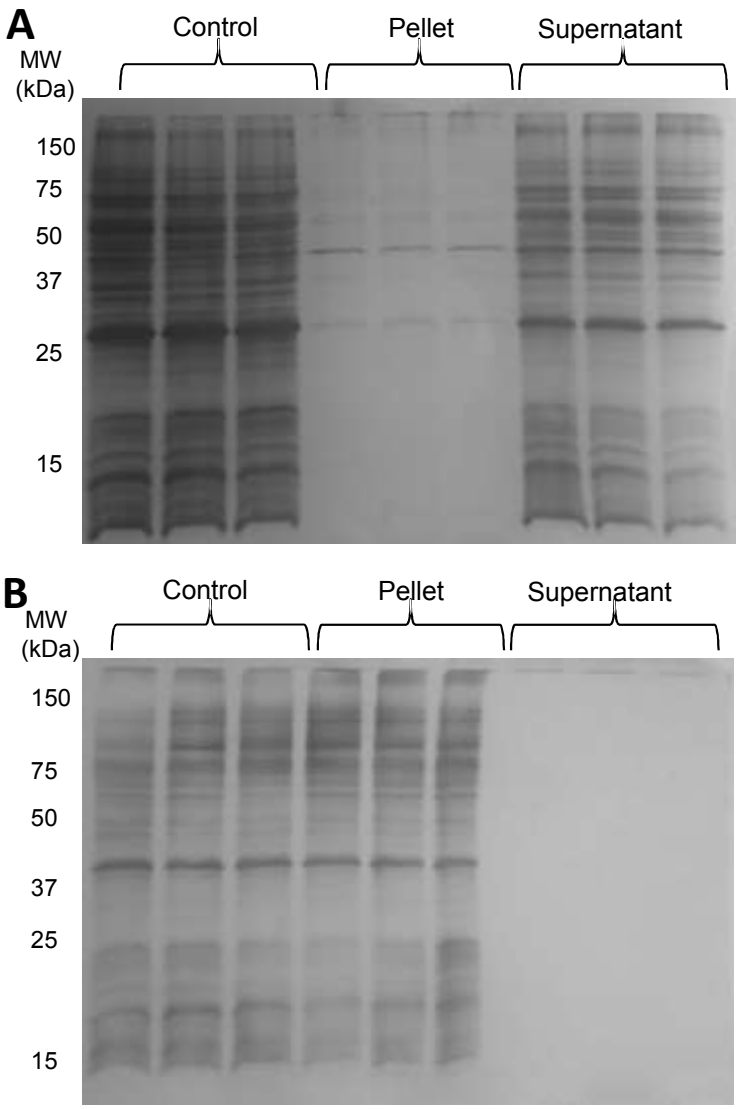

Figure 3.6: SDS-PAGE (silver stain) reveals protein from NRK-52E (20 µg) recovered in the pellet or supernatant fraction following precipitation with or without the addition of NaCl to the solution. (A) 0 mM, and (B) 10 mM. Control lanes consisted of 20 µg protein samples which did not undergo protein precipitation.

such a protocol, the salt concentration of the solution must be maintained at a low level to extract the protein fraction. Protein precipitation can be applied to concentrate the resulting dilute protein fraction following cell lysis. As shown in Figure 3.6A, acetone precipitation of the raw lysate results in relatively poor protein precipitation efficiency (11±7%). However, these proteins are readily precipitated in quantitative yield through the addition of 10 mM salt (Figure 3.6B) to the solution prior to acetone precipitation (86±9%). This ability to improve protein recovery is a direct extension of the improved fundamental understanding of variables controlling the precipitation efficiency.

3.4 Conclusion

Precipitation in organic solvent is a century-old technique to recover proteins in high purity. The conventional model of acetone precipitation presumes an increase in the attractive forces between proteins as the dielectric strength of the solution is decreased. However, in 80% acetone, the repulsive electrostatic forces dominate, leading to increased solubility at a pH away from the pI of the protein. By changing the pH one can induce near-quantitative recovery of a protein from solution. However, the practicality of this is limited in complex mixtures where a variety of proteins with varying pIs are present in solution. The addition of salt (NaCl) or other ionic species is shown to be essential for quantitative precipitation of proteins, including complex mixtures, as explained through a model of ion pairing. By ensuring a sufficient concentration of salt in the protein sample (typically between 1 and 100 mM), all water-soluble proteins can be precipitated in high yield. The method can therefore be applied to concentrate dilute protein solutions. Preliminary results for other organic solvents (alcohols, acetonitrile) demonstrate that this observation may extend to other solvent systems. It is

recommended for all solvent-induced precipitation protocols that researchers consider the addition of salt to improve the recovery of proteins.

4. Chapter 4: A Two-Stage Spin Cartridge for Protein Precipitation[1]

4.1 Introduction

Though top-down platforms for intact proteome analysis by mass spectrometry are gaining in popularity and capability [19], bottom-up approaches continue to be a dominant player in the field of proteomics. As such, proteins must be processed before presenting digested peptides for instrumental analysis. Protein purity is also critical to ensure maximal detection efficiency. Among the many sample additives encountered in a proteomics experiment, sodium dodecyl sulfate is a favored detergent to enhance the extraction [54] and solubilization [134] of proteins from biological sources. SDS is also useful for mass-based protein fractionation through SDS-PAGE [113] or GELFrEE [63]. However, SDS is notorious as an interfering contaminant, and above a critical concentration will cause signal suppression in MS [1], deterioration of LC separation [47,135], and loss of enzyme activity for protein digestion [49,136].

In Chapter 3 it was shown that near quantitative recovery for soluble proteins within complex mixtures is obtainable by acetone precipitation. Prior to this, the Doucette group and others have reported that solvent-based protein precipitation can achieve extremely high protein purity, with over a 2000-fold reduction of SDS [1,72]. Therefore acetone precipitation can be considered a viable method of SDS removal. The downside to protein precipitation is that the method is challenging to perform consistently, particularly at low analyte concentrations where the protein pellet may be invisible to the unaided eye. Although the protein may have precipitated from solution,

[1] Portions of this work are published in the manuscript: A.M. Crowell, D. L. MacLellan, A.A. Doucette. A two-stage spin cartridge for integrated protein precipitation, digestion and SDS removal in a comparative bottom-up proteomics workflow. J. Proteomics. 2014, *In Press*. doi:10.1016/j.jprot.2014.09.030. Results presented in this thesis were carried out by thesis author, unless otherwise noted.

the protein pellet is not readily visible to the individual and as such is difficult to manipulate. Removing too much of the supernatant runs a risk of accidental transfer of the protein pellet, resulting in inadvertent protein loss; leaving too much solvent behind will not deplete the SDS. Reduced protein recovery due to loss of the pellet is nearly unavoidable without significant care, making the use of protein precipitation in the proteomics workflow highly cumbersome and time consuming. The most common approach to avoid protein loss is in the application of a wash step which allows one to leave behind significant portions of acetone, thereby reducing the chance of accidental disruption of the protein pellet [1]. However improper formation of the protein pellet during the wash may also cause protein loss. The variable recovery of proteins attained through solvent-based protein precipitation has previously been attributed to the solvent manipulation steps [137].

Isolation of the protein pellet is normally carried out through the use of centrifugation. Filtration of the organic supernatant to isolate the insoluble proteins for further analysis is rare. Although a number of filtration devices have been commercialized for other uses, none fit the requirements needed to assist in the precipitation workflow. Protein precipitation requires long incubation periods to allow for precipitation to occur, and for resolubilization of the protein following precipitation. These two steps limit the use of most currently available devices. Organic solvent precipitation requires a long incubation period to ensure complete protein removal from solution. Solvent must then be allowed to flow through the filter for removal. Following precipitation, the protein pellet must be brought back into solution, requiring another blockage of solvent flow. For these reasons, a filter cartridge compatible with protein

precipitation requires a detachable and re-attachable plug. A filtration device (termed the ProTrap XG) was designed by Dr. Mark Wall and Dr. Alan Doucette[1] to assist in protein precipitation. The overall device is shown in Figure 4.1A; it consists of an upper filtration cartridge, a removable plug, cap, and an optional solid-phase extraction cartridge. This device was designed to be used with a conventional 2 mL micro-centrifuge tube. The main advantage of this device is that it can allow for protein precipitation and subsequent sample manipulation in an "online" format.

The objective of this work is to facilitate the process of protein precipitation, together with the subsequent steps of protein resolubilization, digestion, and cleanup in a robust and reproducible format. Acetone precipitation can be used to purify SDS from proteins prior to subsequent digestion. Here, the complete process of protein precipitation, protein digestion, and peptide purification occurs within a disposable two-stage spin cartridge. Using this device, a comparative proteomic assessment of kidney samples excised from an animal model of antenatal hydronephrosis was assessed. In this model, a complete ureteral obstruction is introduced by surgical blockage of the ureter. This congenital disorder affects 1-5% of all pregnancies, and can require surgical intervention to avoid loss of renal function [138]. As shown here, the resulting proteome profiles indicate marked changes in the obstructed kidney, consistent with reduced metabolic activity and an up regulation of proteins involved in cytoskeletal organization, among other affected pathways. The novel two-stage spin cartridge is therefore a powerful tool to facilitate the protein sample processing ahead of MS analysis.

[1] US Patent D693467, issued Nov. 12, 2013, licensed to Bike Scientific.

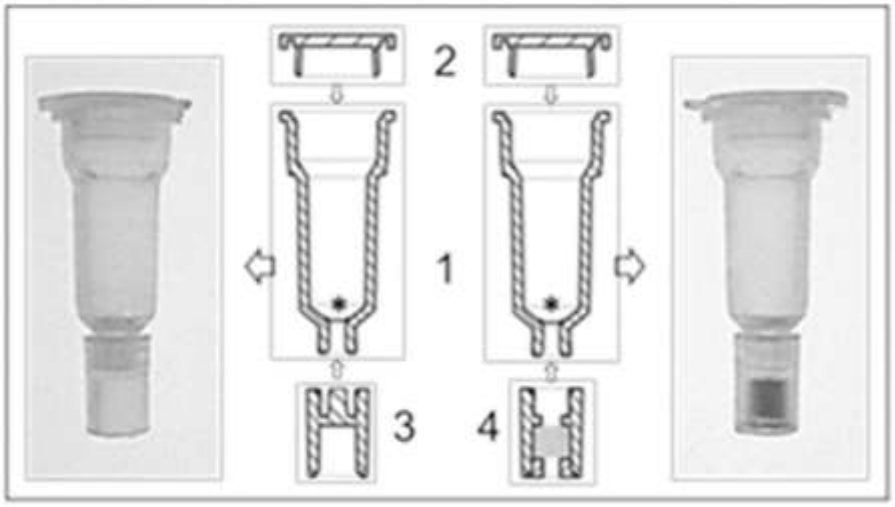

Figure 4.1: Photo and schematic of the two-stage spin cartridge. A PTFE membrane (*) is positioned at the base of the upper filtration cartridge, 1. The filtration cartridge is enclosed by a cap, 2, and removable plug, 3, at the base. A solid phase extraction cartridge, 4, can also be positioned at the base of the filtration cartridge. The assembled device is contained within a conventional 2 mL microcentrifuge tube.

4.2 Methods in Brief:

4.2.1 Sample preparation

All samples were made up in 100 mM Tris-HCl with varying concentrations of SDS or GELFrEE running buffer. *E. coli* was grown according to standard protocols. Protein isolation occurred through the use of a French press or use of 1% SDS. Membrane and cytosolic proteins were enriched using ultracentrifugation. Kidney samples were harvested from rats which underwent complete ligation of the left ureter or control rats. Proteins were extracted in a modified RIPA buffer. Protein separation of *E. coli* extracts by GELFrEE was carried out as described in Section 2.5.2.

4.2.2 ProTrap XG: The Two-stage Spin Cartridge

The two-stage spin cartridge was constructed in-house prior to use. Acetone precipitation was carried out in the filtration cartridge though the addition of acetone to 100 µL sample at a 4:1 ratio. The cartridge was sealed and incubated overnight at -20°C. Chloroform/methanol/water precipitation was carried out through the addition of chloroform, methanol, and then water to a 50 µL sample (1:3:3:1 ratio of chloroform, methanol, and water to sample). For both methods, the protein was first pelleted in the cartridge (1 min at 400 ×*g*). The solvent was then removed through centrifugation at low speeds with the plug removed. An acetone wash was used to remove any trace solvents. The protein pellet was resuspended in SDS (for BCA assay), formic acid, or urea (for tryptic digestion). Following reconstituted urea proteins were diluted, reduced, and alkylated prior to tryptic digestion. Tryptic digestion buffer was removed using the attachable SPE cartridge prior to LC-UV (peptide quantitation) or LC-MS/MS. Samples

were compared to non-precipitated controls and/or conventional vial based acetone precipitation (Section 2.3).

4.2.3 Sample Analysis

Protein recovery assessment following precipitation and capture of the protein pellet was carried out using the Pierce BCA assay. Protein visualization was carried out using SDS-PAGE [113]. SDS removal efficiency was assessed using a methylene blue active substance assay [116]. Peptide recovery assessment was carried out using a LC-UV assay [117]. Peptide identification of samples occurred through the use of an LTQ or Orbitrap MS coupled with reversed phase LC. Identification was carried out using the SEQUEST algorithm [119]. Spectral counting was used as a MS quantitation method [120].

4.2.4 Ethical Considerations

All animal based experimental procedures were carried out in compliance with the Canadian Council on Animal Care (Dalhousie University Animal Care and Use Committee, protocol number 09-068) by Dr. Dawn MacLellan[1].

4.3 Results and Discussion

4.3.1 Overview

The overall device was built in-house using pre-produced plastic pieces. Referring to Figure 4.1, the upper filtration cartridge of the two-stage device contains a PTFE membrane filter, which allows capture of protein aggregates. To avoid possible contaminants the filters were melted into place using a heat stake. A removable plug at the base of the upper filtration cartridge prevents solution from passing through the membrane while protein aggregation is slowly induced in the acetone solvent system. With plug detached, solvent and SDS are removed to waste through centrifugation of the

[1] Department of Pathology, Dalhousie University, Halifax, NS, Canada

device. The plug is reattached, allowing the captured protein to be resolubilized, derivatized, and digested with trypsin. A final desalting step is enabled through attachment of a solid phase extraction (SPE) cartridge to the base of the filter. The SPE cartridge is designed to contain reversed phase media between two membrane filters. The flow-through can be isolated and subject to solvent evaporation prior to LC-MSMS analysis.

It should be noted that this approach is fundamentally different from one using spin cartridges containing molecular weight cut-off filters [64,68]. With a MWCO filter, proteins are retained at a molecular level, while smaller molecules filter through. Protein-bound SDS is depleted with the addition of 8 M urea or other suitable additives [71]. The approach developed in this thesis incorporates protein precipitation, which eliminates bound and unbound SDS. The supernatant is separated from the aggregated protein by passing the sample through a PTFE membrane. The flow characteristics of the chemically inert PTFE membrane allow rapid and reproducible separation of the protein pellet without bias in protein size or type. MWCO filters have poor flow characteristics, easily becoming clogged by particulate matter in the sample [68]. This is not the case when using large (0.45 μm) pored filtration membranes.

4.3.2 Evaluation of the Filtration Cartridge

The effectiveness of the membrane in the upper filtration cartridge to retain protein aggregates while allowing SDS to flush through was assessed. A 0.45 μm PTFE membrane was employed to trap protein aggregates from acetone precipitation. The residual SDS associated with the pellet in the upper filtration cartridge is shown in Figure 4.2A. The initial sample (100 μL with 2% SDS) corresponds to a 2000 μg load of the

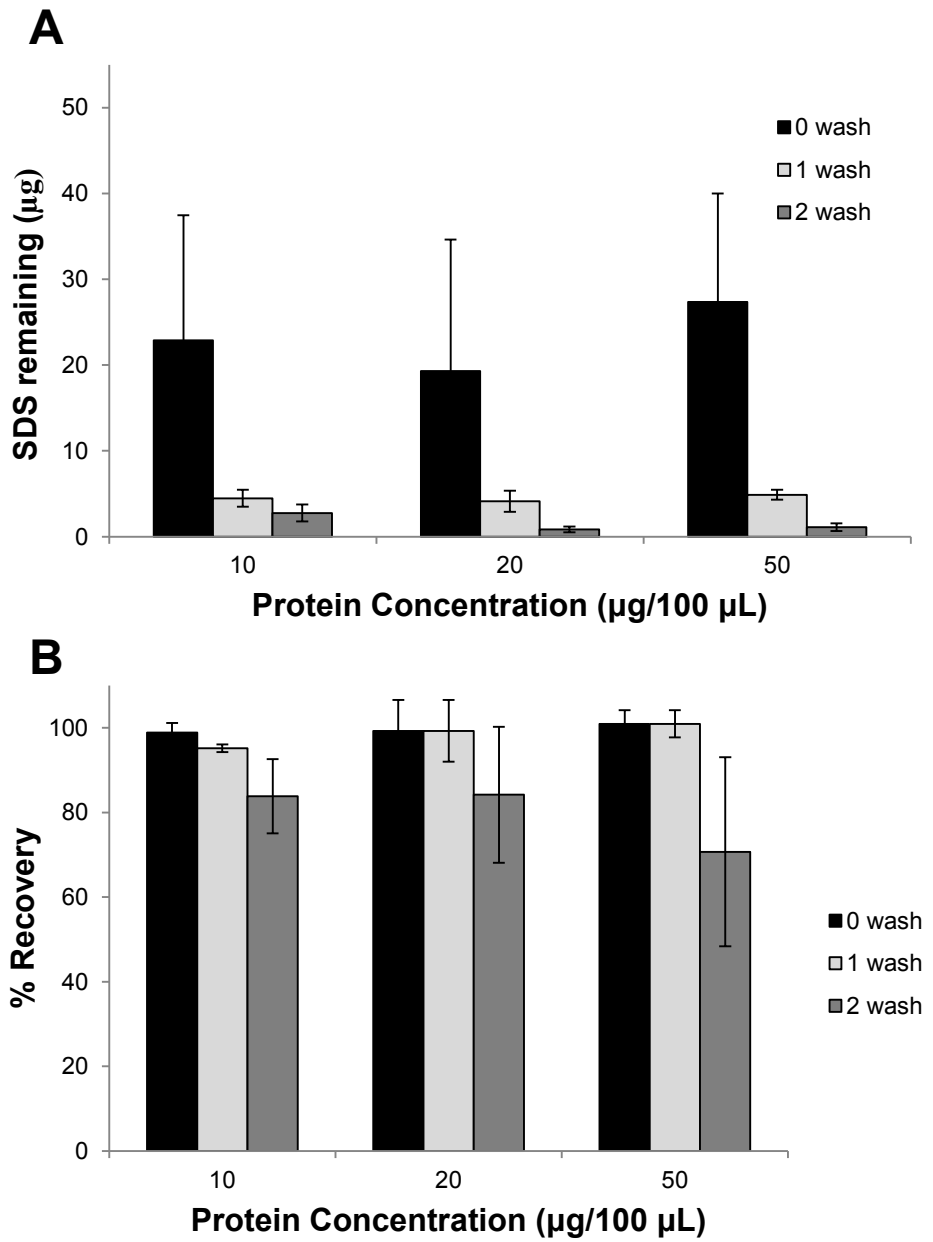

Figure 4.2: (A) The efficiency of SDS removal is displayed following acetone precipitation in the upper filtration cartridge. The initial sample (100 µL of BSA) contained 2% SDS (i.e. 2000 µg) and 100 mM Tris-HCl (pH 8). Inclusion of one or two washes (300 µL acetone per wash) further reduced the remained SDS while also improving precision. (B) The recovery of BSA on the PTFE membrane of the upper filtration cartridge is also shown as a function of the number of wash steps. Error bars represent the standard deviation from measurements using three independent spin cartridges.

detergent in the device. Assuming the protein is resolubilized to the same volume, the critical level of residual SDS must fall below 100 µg, to enable LC-MS/MS analysis of a 1 µg protein sample [1]. As seen from Figure 4.2, in the absence of a washing step, approximately 20 to 30 µg SDS remains, which already corresponds to ~99% depletion of the detergent. However, without a wash, the repeatability of the method suffered, possessing an absolute standard deviation on the order of 15 µg SDS across independent spin cartridges. The residual SDS does not correlate with the amount of protein in the cartridge; it is attributed to the high variation in residual SDS associated with a low volume of supernatant (~5-10 µL) which wets the filter, even after low speed centrifugation of the cartridge. Removal of this residual solvent is most easily achieved through dilution and subsequent centrifugation. As seen in Figure 4.2, the addition of a single wash brought the remaining SDS below 5 µg, which is below the critical threshold for SDS depletion. The wash step also greatly improves the repeatability of the method, as applied across multiple cartridges (standard deviation of 1 µg SDS for 1 wash). This now represents a 99.75% efficiency of SDS depletion. Given the amount of protein in the sample, such a high efficiency of SDS depletion would also permit sample preconcentration, for example, by solubilizing the protein or peptides to a lower final volume. Referring to Figure 4.2B, the filter retained the majority of the proteins (<95%) with a single wash. Inclusion of a second wash step, although further depleting the SDS, had a negative effect on protein recovery. It is recommend to use a single wash following acetone precipitation to deplete SDS, while maintain protein recovery above 95%. These results demonstrate that the use of a membrane filter to facilitate protein

precipitation achieves comparable results to conventional (vial-based) protein precipitation [1], with minimal manipulation of sample and at reduced spin times.

The spin cartridge also facilitates capture of aggregated protein at low analyte concentration. Protein recovery was assessed down to 200 ng initial protein (in 100 µL with 1% SDS), as shown in Figure 4.3. Although the pellet is no longer visible at this concentration, proteins will continue to aggregate in 80% acetone. The challenge in maintaining high recovery with conventional precipitation is in the removal of the supernatant without disturbing the loosely formed pellet. This is made extremely difficult at low concentrations where the pellet is not visible. Chapter 3 reported the recovery of soluble proteins through acetone precipitation ranging from 80 to 100%, beginning with 10 µg protein or greater. As seen in Figure 4.3, beginning with 2 or 0.5 µg BSA, the protein is quantitatively and consistently retained on the membrane of the spin cartridge. At 200 ng, recovery on the membrane exceeds 50%, as the intensity of the protein bands in the membrane fraction greatly exceed that of the flow through. Though it may be possible to achieve high recovery at low protein concentration through conventional precipitation, the reproducibility of the method comes into question as the pellet size is decreased. Isolation of the pellet from the supernatant is no longer a concern with the spin cartridge, being fully automated through a brief centrifugation protocol.

It can clearly be seen that the recovery of precipitated BSA from acetone precipitation can be facilitated with high yield using the filtration cartridge; however the recovery of other protein standards and complex protein samples has not been shown. Figure 4.4A represents the protein yield of other "standard" protein samples obtained

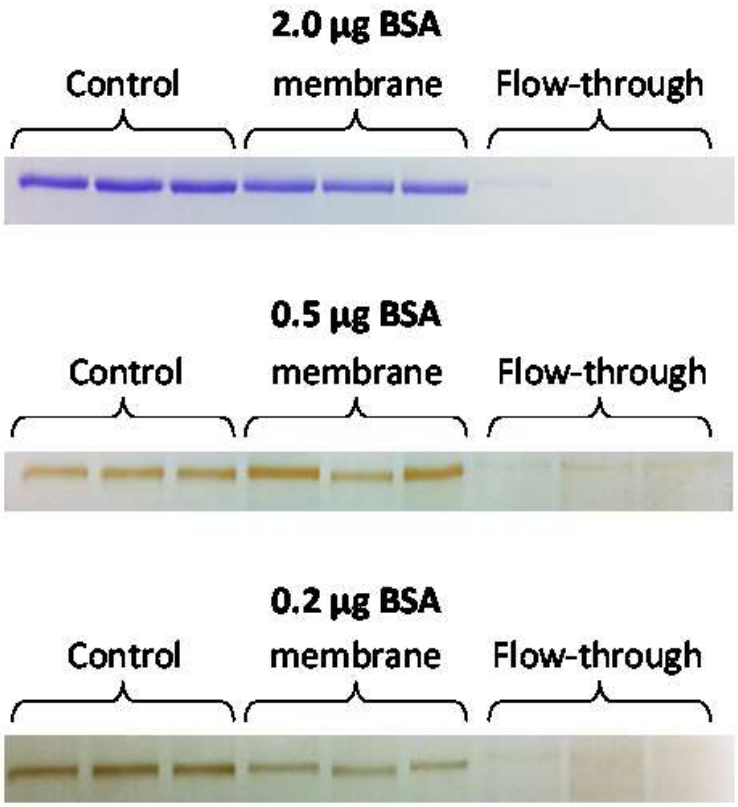

Figure 4.3: SDS-PAGE is used to follow the recovery of BSA protein following acetone precipitation and capture of the protein pellet on the membrane of the upper filtration cartridge. The total protein specified in the Figure was originally prepared in 100 µL of 1% SDS and 100 mM Tris-HCl (pH 8). The control represents the equivalent mass of protein loaded directly on the SDS-PAGE gel. The flow through of the upper filtration cartridge was fully evaporated and reconstituted in gel loading buffer, demonstrating that the bulk of the protein is captured on the membrane filter.

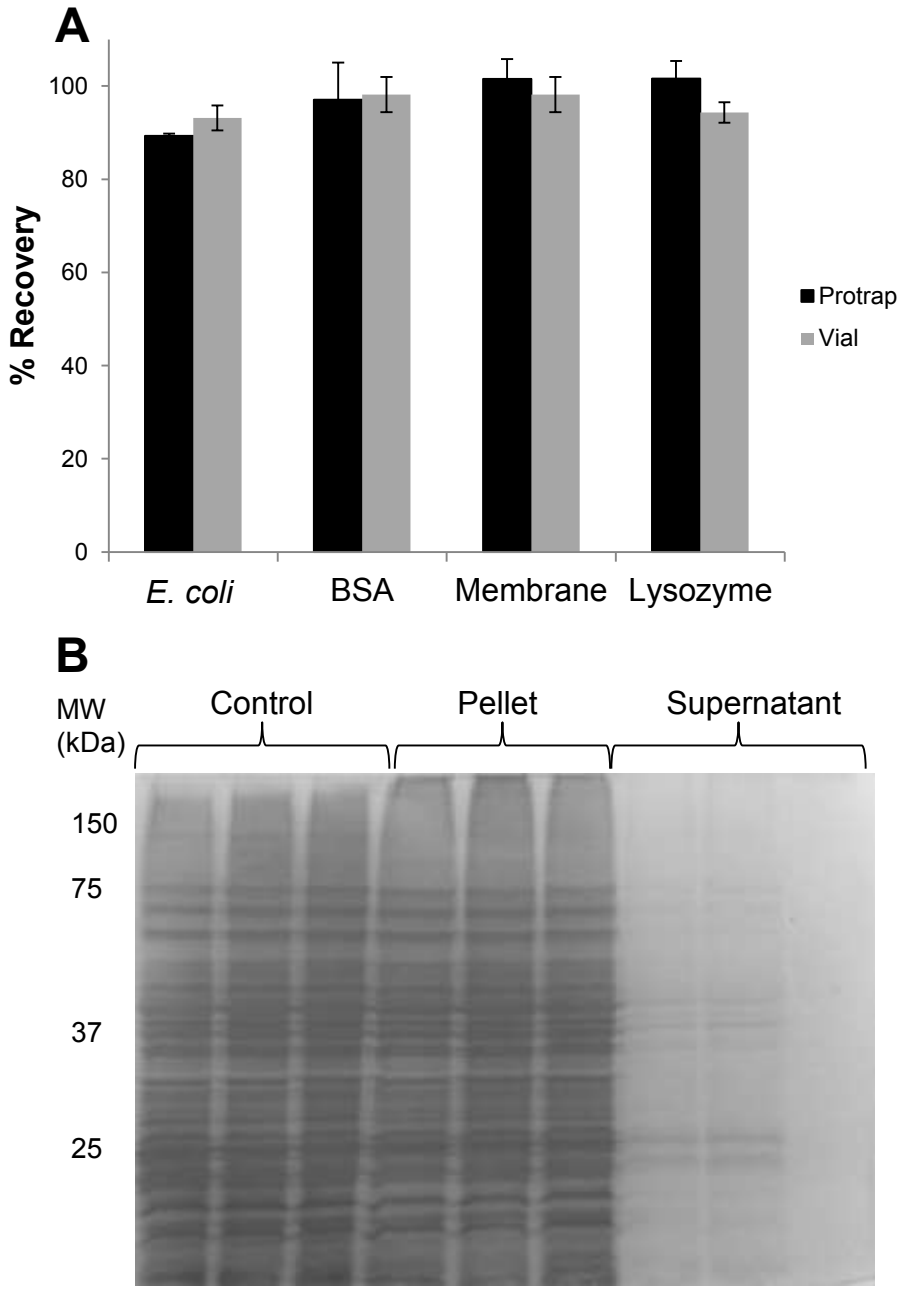

Figure 4.4: (**A**) Protein recovery following acetone precipitation with capture of the protein pellet on the PTFE membrane of the filtration cartridge. Samples contained 0.1 g/L of various proteins in 1% SDS and 100 mM Tris-HCl (pH 8). Conventional protein precipitation was also performed (vial ppt). (n=3). (**B**) SDS-PAGE was used to visualize the protein pellet recovered on the membrane following precipitation in the spin cartridge of 20 µg proteins extracted from *E. coli* in 1% SDS. The flow through of the upper filtration cartridge was also collected, and fully evaporated with reconstitution in gel loading buffer, demonstrating that the bulk of the protein is captured on the membrane filter.

from both vial-based precipitation and precipitation within the filtration cartridge. The recovery of the protein standards, BSA and lysozyme, was found to be similar for both the vial based and filtration assisted precipitation. To examine the recovery of complex protein mixtures, proteins extracted from the bacteria *E. coli* were both assayed (a 0.1 g/L sample) and visualized through SDS-PAGE (a 0.2 g/L sample). From Figure 4.4A, it can be seen that protein recoveries of near 85% were obtained on the filter. A similar recovery was found in the vial based method precipitation (~90%). To observe the loss which occurs through during the precipitation of the *E. coli* mixture on the filter, both the acetone supernatant and the pellet were subjected to SDS-PAGE visualization as shown in Figure 4.4B. As can be seen the protein loss occurs over the entire mass range visualized by SDS-PAGE, suggesting a non-biased protein loss. Low concentrations of protein can be observed in the supernatant. To observe the effect of acetone precipitation on more hydrophobic proteins, a membrane enriched protein sample from *E. coli* was subjected to acetone precipitation in the vial and in the filter cartridge. As can be seen from Figure 4.4A, equally high recovery was obtained for the two methods. From these observations it can be stated that the spin cartridge was successful at retaining proteins in high yield over a range of molecular weights and hydrophobicity's.

All the data presented at this time have been for acetone precipitation of protein samples, however the filtration device can be used to assist in the isolation of the protein pellet from any method of protein precipitation. The protein recovery from another common precipitation method using chloroform, methanol, and water was examined (as described in Section 2.4.3). As can be seen from the gel images in Figure 4.5, protein precipitated by CMW in the filtration cartridge can be retained in high yield. Minimal

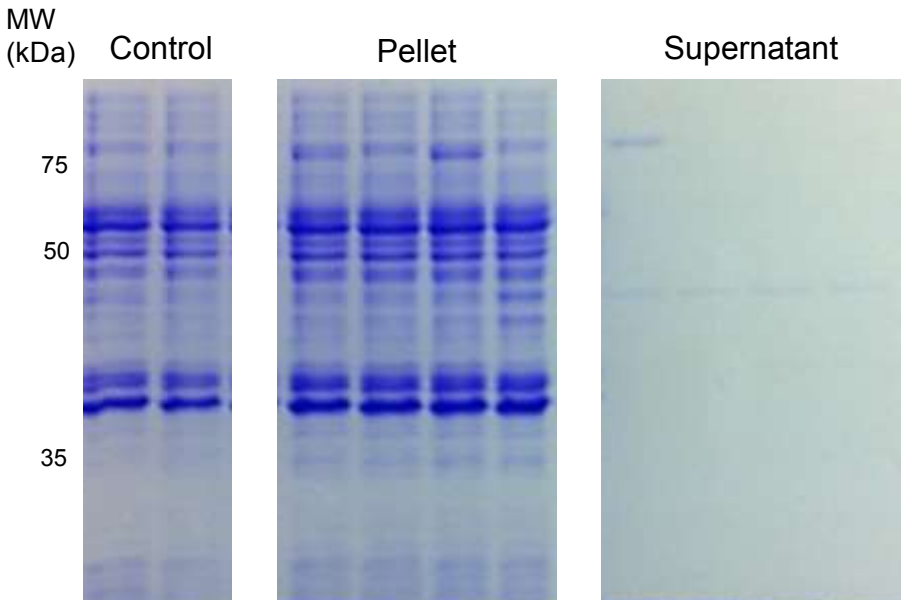

Figure 4.5: SDS-PAGE is used to visualize the protein pellet recovered on the membrane following chloroform/methanol/water precipitation in the spin cartridge of 10 µg proteins extracted from *E. coli*. The flow through of the upper filtration cartridge was also collected, and fully evaporated with reconstitution in gel loading buffer, demonstrating that the bulk of the protein is captured on the membrane filter.

amounts of protein are detected in the flow through. Other precipitation techniques such as trichloroacetic acid, ethanol, and methanol can be used within this device to facilitate protein precipitation ahead of LC-MS/MS analysis.

4.3.3 Evaluation of the SPE Column

By attaching the plug to the base of the upper filtration cartridge, the device essentially acts as a conventional microfuge tube, allowing for extended protein workup, including resolubilization, derivatization, and digestion. Following precipitation, proteins are resolubilized through sonication in 8 M urea, together with overnight tryptic digestion. The plug is replaced with the SPE cartridge to facilitate peptide cleanup. Performing the cleanup in an on-line format not only streamlines the sample preparation process but also avoids the need for subsequent off-line manipulations, which would increase the risk of sample loss [139]. To fully utilize the SPE cartridge the solvent conditions required to elute the peptides were examined. Acetonitrile (ACN) acidified with trifluroacetic acid (TFA) is a common HPLC solvent for the separation of peptides. The benefit of this solvent system is its easy removal through evaporation. For these reasons this solvent system was investigated for peptide retrieval from the clean-up cartridge.

Using a test system of tryptically digested BSA the SPE clean-up column was examined using a combination of 1 wash and 2 washes to identify the most efficient method for peptide recovery. The overall trend for peptide recovery following one wash of various concentrations of ACN (30-60%, acidified with 0.1% TFA) is shown in Figure 4.6. As can be seen from the Figure, maximal peptide recovery is obtained using one wash of 50 or 60% ACN/0.1% TFA. It was found that 80% ACN caused a reduction in recovery from the SPE cartridge. To improve peptide recovery, a second wash was

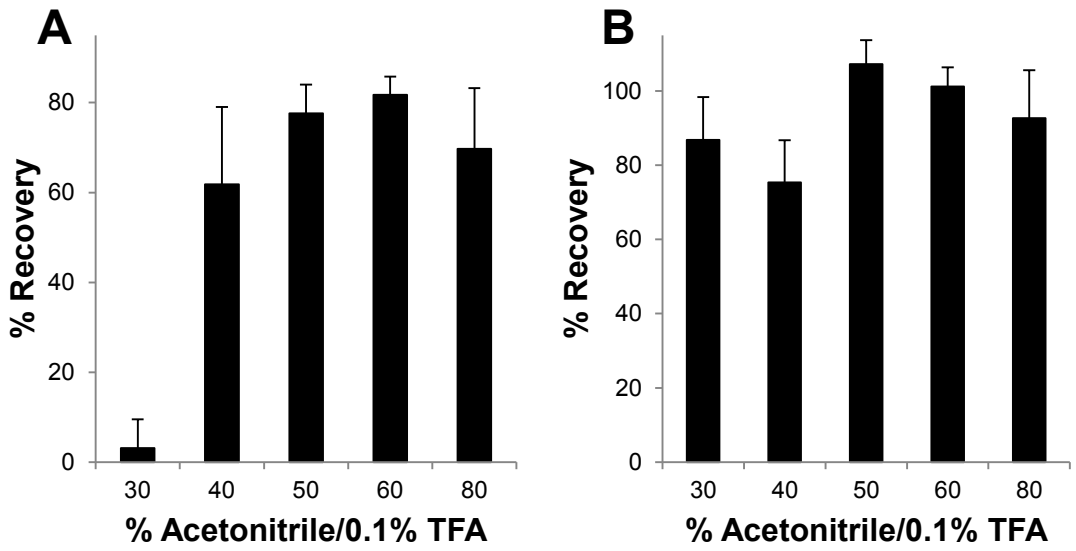

Figure 4.6: (A) The peptide recovery obtained from solid phase extraction desalting (250 µL of acidified BSA protein digest, 0.04 g/L) in the ProTrap XG as a function of eluting solvent (1 wash). Varying concentrations of acetonitrile (acidified with 0.1%) were tested (300 µL) (B) Peptide recovery obtained following two washes of eluting solvent. The first wash was maintained at 50% ACN/0.1% TFA, while the second wash consisted of varying ACN concentrations (0.1% TFA). Samples were dried under vacuum and redissolved in 1% SDS. Peptide quantitation was carried out through use of the BCA assay. Error bars represent the standard deviation from measurements using three independent SPE cartridges.

incorporated. The peptide recovery obtained following one wash of 50% ACN/0.1% TFA coupled with a second wash of varying ACN concentrations (30-60%, acidified with 0.1% TFA) is plotted in Figure 4.6B. As can be seen from the figure, maximal peptide recovery is obtained following two washes of 50% ACN/0.1% TFA. It is therefore suggested that two washes (300 μL) of 50% ACN should be used to facilitate peptide clean-up in the ProTrap XG. All peptide purification using the SPE cartridge is carried out using this protocol.

The purpose of the ProTrap XG is to facilitate the removal of SDS from a protein sample. Although the removal process is carried out by precipitation of the protein, it is important to examine the effect of SDS on peptide purification using the SPE cartridge. The protein recovery and SDS removal efficiency of the clean-up column is shown in Figure 4.7 for samples spiked with varying concentrations of SDS. Referring to Figure 4.7A, the recovery of the peptide is not inversely affected by the concentration of SDS until the sample contains 1% SDS. Peptide recovery is noted to increase as SDS concentration is increased. However, when samples contain 1% SDS, recovery was significantly decreased. Figure 4.7B, shows the percentage of SDS remaining following purification in the SPE cartridge. It can be clearly seen that SDS was not removed until SDS concentrations greater than 0.1% were present. The level of SDS remaining at both 0.1% and 1% SDS was far greater than the concentration tolerated by LC-MS/MS. From these results it can be seen that SDS does not negatively affect peptide recovery from the SPE at low concentrations (<1%), but it does not facilitate SDS depletion. Therefore it can be concluded that SDS removal must occur prior to SPE clean-up in the precipitation step of the protocol.

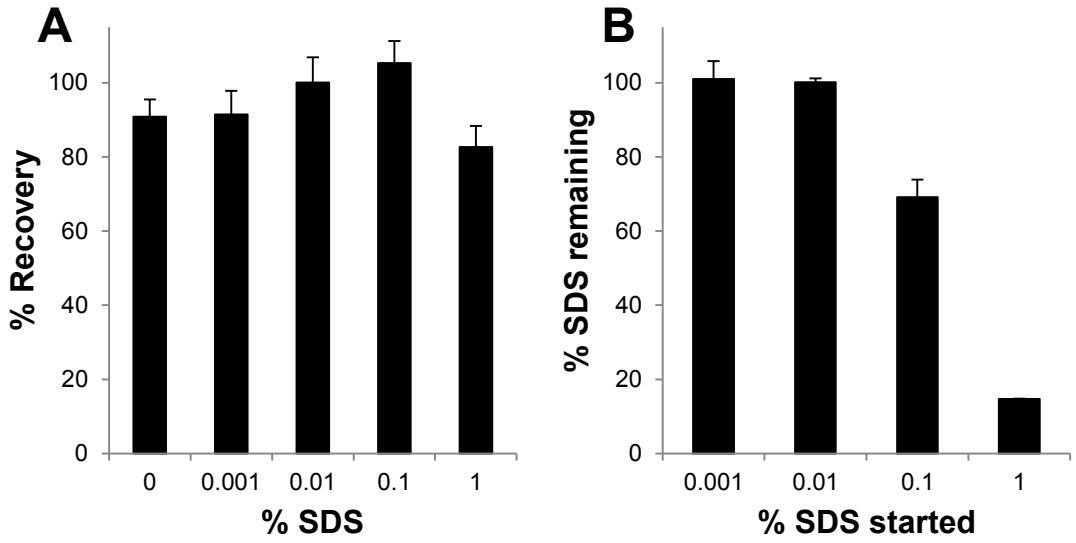

Figure 4.7: The effect of SDS on peptide recovery obtained from SPE purification of 250 µL, 0.04 g/L BSA digest using 2×300 µL, 50% ACN/0.1%TFA wash. (A) The peptide recovery as a function of SDS following SPE clean-up. (B) Percent of SDS remaining following SPE purification. Error bars represent the standard deviation from measurements using three independent SPE cartridges.

To examine the capacity of the cartridge for peptide clean-up, digested BSA peptides were subject to SPE clean-up at a variety of concentrations. As can be seen from Figure 4.8, the SPE cartridge has capacity to bind up to 100 µg of peptides. However, at this concentration peptide recovery has dropped to 68 ± 3%. It was found that 21 ± 2% of the BSA peptides were contained in the flow-through and wash steps of the SPE purification. This is demonstrating that the capacity of the cartridge is being approached. Samples with lower concentrations of peptide provide higher recovery. At 5 µg of peptide, 91±8% of the peptides are obtained, which decreases to 80% at 10 and 25 µg. This suggests that samples below 25 µg of proteins are ideal for sample preparation through the ProTrap XG.

4.3.4 Two-Stage Spin Cartridge for Bottom-up Protein Analysis

Previous experiments have examined the efficiency of both the upper filtration cartridge in capturing the protein pellet following precipitation and the bottom SPE cartridge in purifying the peptides. The overall sample recovery from precipitation, digestion, and peptide cleanup has not been investigated. To fully investigate the ability of the ProTrap XG to facilitate bottom-up protein preparation (shown in Figure 4.1B) a number of test systems were examined (0.1 g/L of protein in 1% SDS, 100 mM Tris-HCl pH 8). Figure 4.9A, plots the final peptide recovery obtained from a number of protein test systems ranging in protein complexity from a single protein to a complex protein mixture. As can be seen from the data, all protein samples provided overall peptide recoveries greater than 80%. However, as the complexity of the system increased, recovery was shown to decrease. The reduced recovery of the *E. coli* extract can be attributed to the inefficient precipitation of low molecular weight components (<5 kDa)

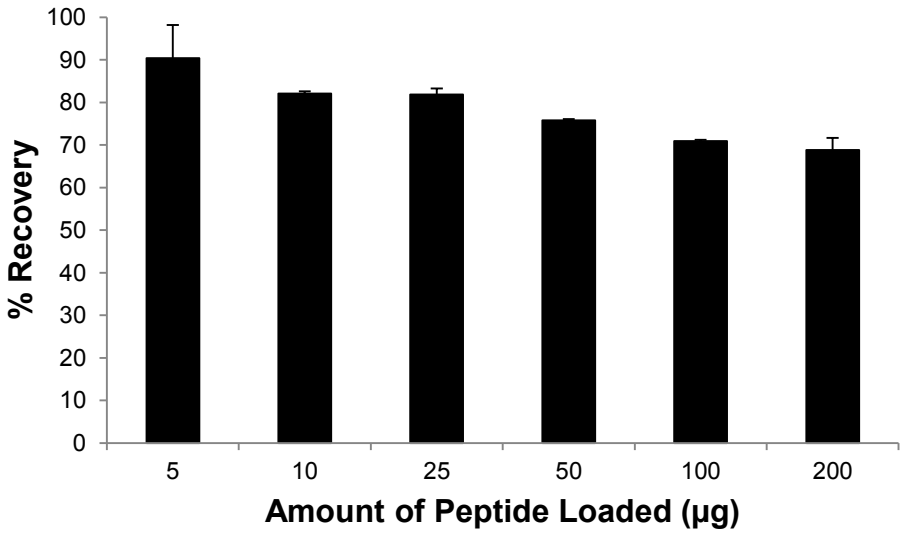

Figure 4.8: The peptide recovery obtained from solid phase clean-up of tryptically digested BSA (volume of 250 µL, acidified with TFA) protein as a function of the amount of peptide purified. Elution occurred with two 300 µL, 50% ACN/0.1%TFA washes. Peptide quantitation was carried out through use of LC-UV peptide assay. Error bars represent the standard deviation from measurements using three independent SPE cartridges.

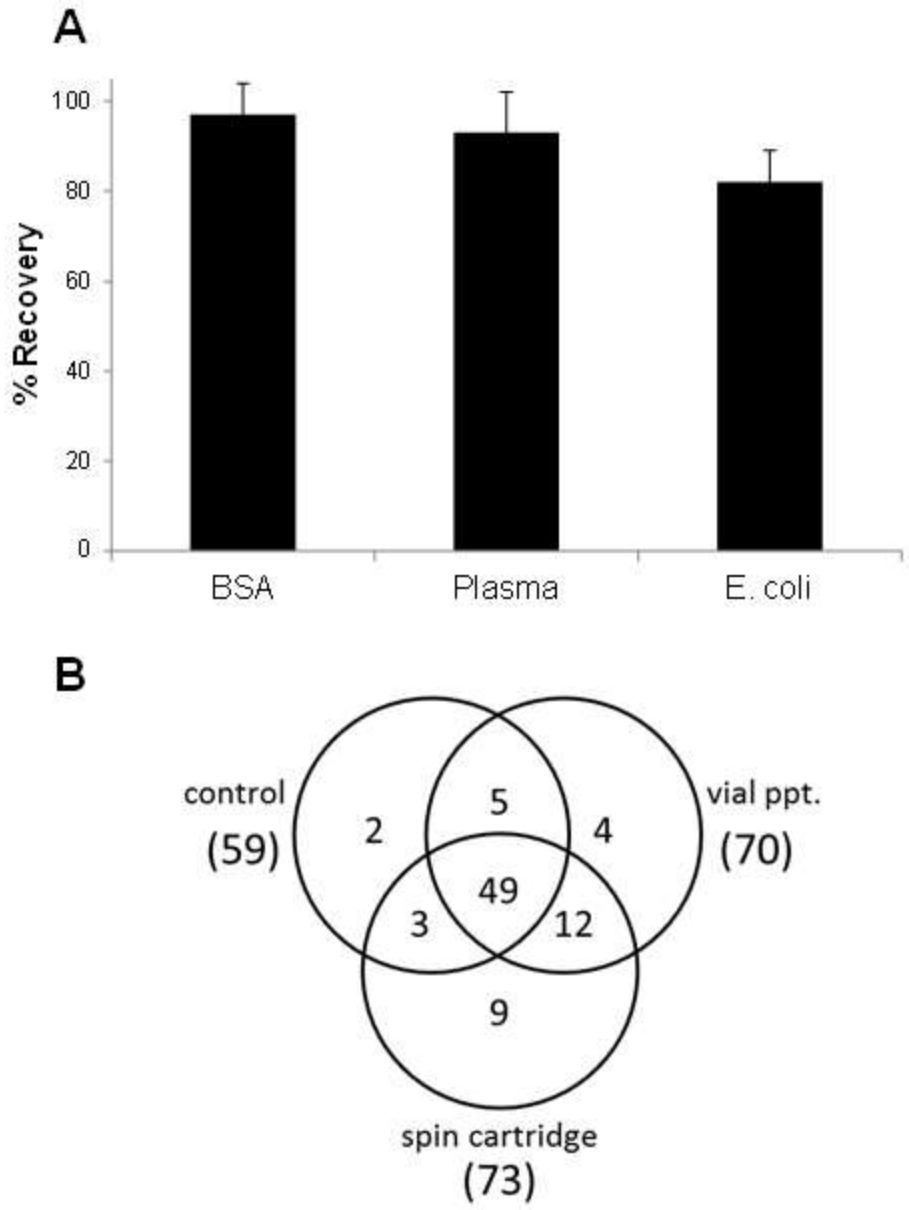

Figure 4.9: (A) Shown is the recovery of peptides following SDS removal through precipitation, protein digestion and column cleanup in the ProTrap XG. The initial protein loading was 10 µg/sample with 1% SDS (n=3). (B) Venn diagram summarizes bottom-up MS analysis of BSA (10 µg) following SDS removal and digestion in the spin cartridge relative to a conventional (vial-based) acetone precipitation protocol. Numbers represent the number of both redundant and unique peptides identified for each system. The control sample represents an equivalent concentration of BSA prepared in the absence of SDS, and digested without precipitation.

in acetone or losses in the SPE clean-up step, as opposed to the resolubilization step. From these recoveries, it can be said that the ProTrap XG is a reliable semi-automated platform to perform complex protein sample manipulations, including SDS depletion, resolubilization, digestion, and subsequent cleanup. Once cleaned, the solvent can be evaporated and the peptides stored in the freezer until such time as they are ready to be analyzed by MS [62]. The compatibility of the ProTrap XG to process SDS-containing samples ahead of MS analysis was examined. As shown in Figure 4.9B, a similar number of BSA peptides were identified, with considerable overlap in detected peptides relative to a conventional (vial-based) precipitation and digestion workflow with HPLC for final sample cleanup. The number of peptides identified from the SDS-depleted samples exceeded that of a control sample prepared in the absence of SDS. This increase may be attributed to differences in the digestion efficiency of precipitated proteins, wherein the proteins are fully denatured prior to precipitation [46].

4.3.5 Evaluation of the ProTrap XG Using a Complex Sample

To investigate the ability of the ProTrap XG to purify samples prior to LC-MS/MS, a complex protein mixture was examined. A sample of protein extracted from *E. coli* and spiked with SDS was subject to sample preparation for LC-MS/MS analysis using the ProTrap XG. A full list of identified proteins is provided as supplementary files. Table 4.1 summarizes the identified proteins/peptides processed through five independent spin cartridges, vial based precipitations, and non-SDS containing controls. Vialed based precipitation samples involved conventional acetone precipitation and tryptic digestion in a vial. Both SDS containing protocols provided higher protein and peptide identifications

than the control. However, the vial-based protocol provided higher identifications than that of the ProTrap XG.

Table 4.1: Proteins and peptides identified from aliquots of an *E. coli* protein extract, following SDS removal and protein digestion. VB ppt represents conventional vial base precipitation workflow. ProTrap XG represents two-stage filtration cartridge workflow.

Sample	Total Protein ID	Total peptide ID	Unique Peptide ID
Control 1	169	1372	823
Control 2	167	1311	783
Control 3	163	1445	829
Control 4	193	1671	996
Control 5	199	1651	972
Average	178±17	1490±163	881±97
VB Ppt 1	212	1806	1137
VB Ppt 2	242	1917	1233
VB Ppt 3	247	1880	1286
VB Ppt 4	266	2101	1389
VB Ppt 5	227	2180	1326
Average	239±20	1977±157	1274±96
ProTrap XG 1	170	1198	773
ProTrap XG 2	266	2216	1410
ProTrap XG 3	176	1417	879
ProTrap XG 4	198	1605	1010
ProTrap XG 5	200	1535	933
Average	202±38	1594±380	1001±244

To provide a more detailed examination of the samples, spectral counting was used as a semi-quantitative method to compare proteins between the workflows. Figure 4.10A plots the spectral hits for the top 10 protein found in the three workflows. As can be seen from the figure, similar spectral counts were found between the three workflows for the top 10 most abundant proteins. The overlap of the proteins identified in all three

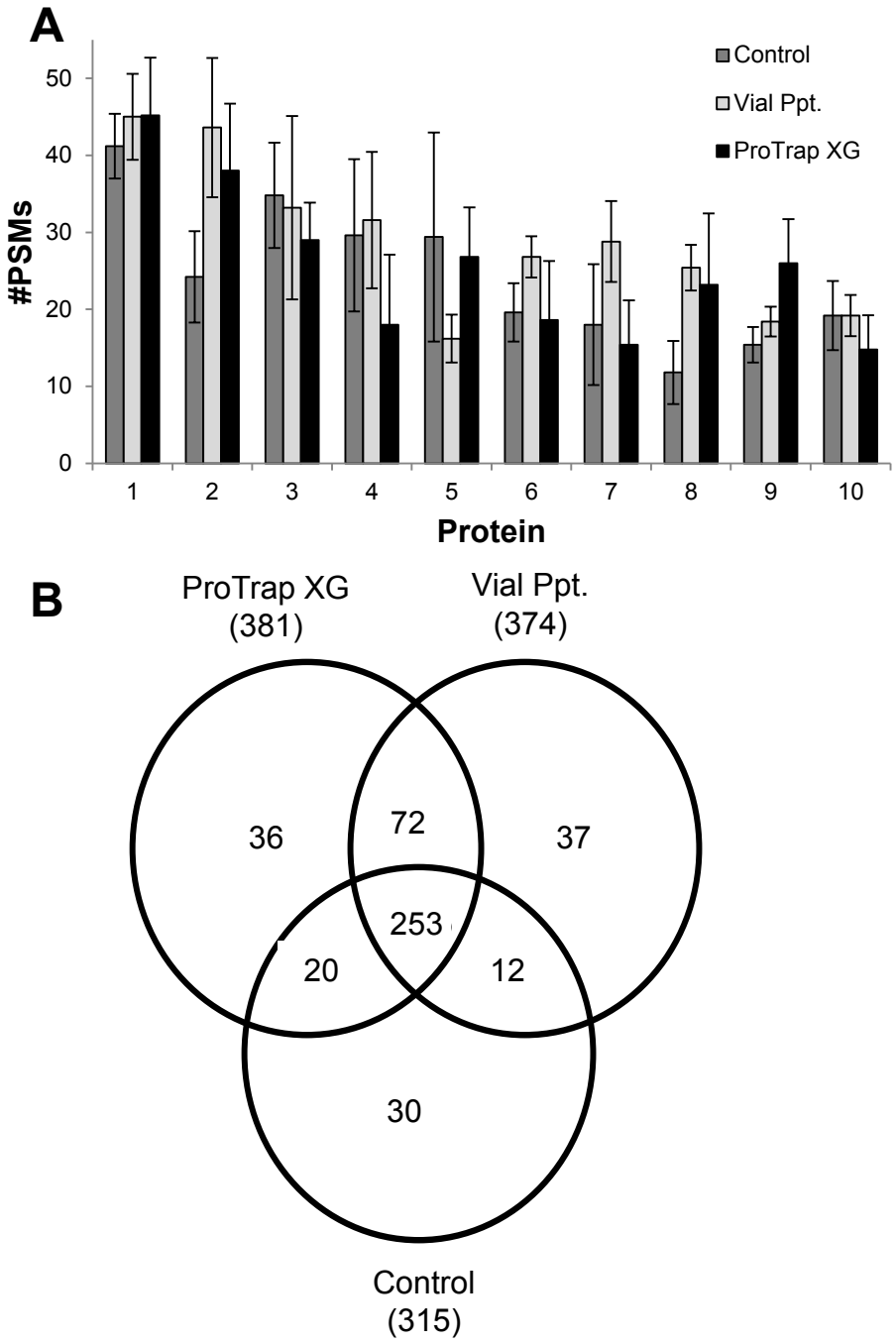

Figure 4.10: (A) Peptide spectral matches for the top 10 most abundant proteins identified in a *E. coli* extraction, following SDS removal and digestion using conventional acetone precipitation in a vial *vs*. the two-stage spin cartridge compared to a non-SDS containing control. The error bars represent the standard deviation of independently processed aliquots (n=5). (B) Venn diagram showing the protein overlaps of all the proteins identified in all three workflows. Each sample consists of two LC-MS/MS injections of $1/10^{th}$ of the purified peptide sample.

workflows was compared in Figure 4.10B. As can be seen from this data, a high protein overlap was observed between all three methods. Referring to Figure 4.10B, 70% of proteins were common between the vial and cartridge precipitation workflows. It was found that both the vial and the cartridge precipitation workflows provide 60% overlap to the control. The cartridge precipitation workflow was found to provide a 13% increase in unique peptides and proteins identified when compared to the control. However, the vial based precipitation protocol found a 20% increase in unique peptide identification and 15% increase in protein identification compared to the cartridge precipitation workflow. This is explained by the observed (~80%) recovery obtained previously for *E. coli* through the cartridge precipitation workflow (Figure 4.9A).

4.3.6 In-depth Examination of the LC-MS/MS Data

4.3.6.1 Trypsin Digestion Efficiency

The increase in identification over the control is attributed to differences in the digestion efficiency of precipitated proteins. Through the detailed examination of the list of peptides identified in the *E. coli* experiment, the overall digestion efficiency can be examined. To do this the raw MS/MS data was searched again, allowing for peptides with up to four missed-cleavages to be found. There was an increase in the number of missed cleavages in the identified peptides for both precipitation protocols (~14-16% of the total peptides identified) compared to the control (~6%). A summary of the results can be found in Table 4.2. This evidence suggests that either the introduction of the precipitation or urea hinders trypsin digestion. An alternative explanation is that the use of urea as a resolubilisation agent does not provide complete solubilisation of the proteins prior to trypsin digestion.

Table 4.2: Percentage of total peptides which contain a missed cleavage following sample preparation using a vial-based precipitation, the ProTrap XG, and a non-precipitated control.

	1 missed cleavage	2 missed cleavage	3 missed cleavage	4 missed cleavage	Total
Control	6.16	0.30	0.00	0.00	6.45
VB Ppt	14.01	0.85	0.00	0.01	15.56
ProTrap XG	14.48	1.06	0.03	0.00	14.87

4.3.6.2 Examination for Unwanted Modifications

Urea solution is a commonly employed agent for tryptic digestion in proteomic studies. However, the use of urea is problematic due to the possible carbamylation reaction that can occur at the N-terminal, and at the amino acids lysine and arginine. This unwanted modification can reduce protein identification and interfere with protein analysis. Unfortunately, the proposed ProTrap XG workflow makes use of this reagent to assist in protein digestion. To examine the samples from Figure 4.10 for the presence of this modification, the raw MS/MS data was researched allowing for carbamylation of possible amino acids and the N-terminal as a dynamic modification. The inclusion of urea into the two precipitation protocols was found to increase the carbamylation modifications in the detected peptides (~3% from 0.25% in the urea-negative control). The increase in missed cleavages and the increased modifications suggests that another solubilisation strategy over urea should be developed/examined for both bottom-up and top-down protein analysis. However, a 3% modification rate will not prevent protein analysis of a biological sample. The use of urea, although imperfect, will still provide high quality LC-MS/MS data for bottom-up analysis.

Urea is not the only source of possible unwanted modifications within this workflow. Simpson *et al*. described a +40 Da peptide modification that is introduced to the sample upon the addition of acetone to the peptide mixture [107]. Incomplete removal of acetone prior to digestion may cause this modification to occur. Searching the MS/MS data for any +40 Da dynamic modifications reveals a significant amount of modified peptides in the conventional vial approach (4.7±0.6% of all peptides identified). A significantly lower amount was identified when using the ProTrap XG; only 1.7±0.7% of all peptides identified contained the +40 Da modifications, which is similar to the amount found in the non-acetone exposed control (1.1±1%). Therefore the use of a filter to separate the protein from the organic solvent following protein precipitation, is more effective than centrifugation (and air drying) for removal of residual solvent.

4.3.7 Kidney Sample Analysis

The ProTrap XG was applied to process kidney proteome extracts constituting an animal model of ureteral tract obstruction (UTO). An SDS-containing buffer was used to facilitate extraction of the proteome mixture from the kidney tissues of 3 control and 3 surgically obstructed rat kidneys. Figure 4.11A provides a 1D gel image of the resulting proteome extracts. A full list of identified proteins is provided as supplementary tables. A total of 402 proteins were identified from the control samples, while 378 proteins were observed from the obstructed kidneys (42% in common, Figure 4.11B). From these lists, and examining the protein spectral counts, a total of 219 proteins were found to have a statistical difference in MS spectral counts between the two groups (p value <0.05). A heat map of these altered proteins is shown in Figure 4.11C, plotting the $\log_2$ fold change in protein spectral counts and revealing the differences across the sample types.

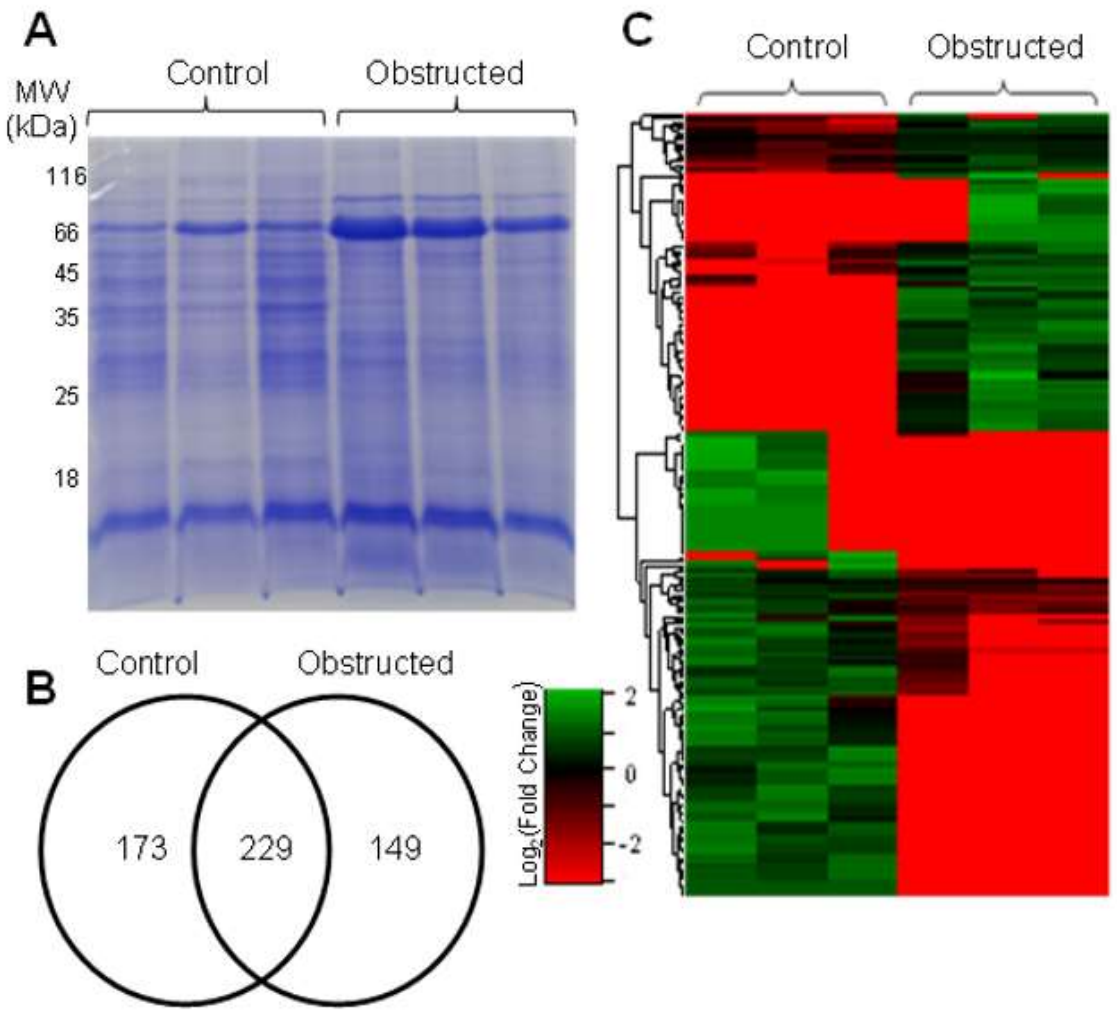

Figure 4.11: (A) SDS-PAGE is used to visualize the proteins extracted from rat kidneys obtained from three independent biological replicates (controls) relative to those from rats undergoing surgically obstruction of the kidney. (B) Bottom-up LC-MS/MS analysis was used to characterize the protein extract, resulting in the total number of protein identifications summarized in the Venn diagram as shown. (C) Protein spectral counts were used to generate a heat map through hierarchical clustering of individual protein expression levels between the control vs obstructed kidney samples. Proteins with higher protein expression in the obstructed samples are shown in green, while those with lower relative expression are shown in red.

The Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 was used to extract trends in the differentially expressed proteins. Of the 219 statistically significant proteins with altered expression, 89 were identified having a greater number of MS spectral counts in the obstructed kidneys relative to the control This implies a higher relative concentration of these particular proteins in the obstructed kidneys. The remaining 130 of 219 proteins were found to have lower MS spectral counts in the obstructed kidneys relative to the control. The proteins primarily map to cytoskeletal organization, including actin, calreticulin and profilin among several others. Antenatal hydronephrosis manifests in the enlargement of the kidney as it becomes filled with fluid resulting from blockage of urine outflow. The volumes of the obstructed kidneys were over 10 times that of the controls; though only approximately double the mass. Cytoskeletal components would therefore be expected to be altered in response to this stress. DAVID analysis also revealed an increase in inflammatory response, with proteins including kininogen, being involved in vasodilation [140], and haptoglobin, which prevents kidney damage from free hemoglobin [141]. An increase in blood circulatory proteins was observed in the kidneys, including increased expression of albumin (observed as the strong band in Figure 4.11A) as well as apolipoprotein E.

The proteins with decreased spectral counts in the obstructed kidneys relative to the control map to all levels of cellular metabolism. Functional annotation clustering links to decreased cellular respiration, organic acid and carbohydrate catabolism, amino acid and nucleotide metabolism, as well as decreased protein complex assembly. This comparative analysis of control *vs* completely obstructed kidneys provides insight into the proteomic changes manifested in the kidney. Further work in the Doucette Lab will

examine how such changes manifest in the urinary proteome, particularly in response to partially obstructed kidneys.

4.3.8 Application to Top-down Proteomics

Currently the ProTrap XG has only been applied to a bottom-up LC-MS/MS methodology. However, the upper filtration cartridge can be applied to assist in sample preparation prior to top-down analysis. Purified proteins can be collected post-precipitation and subject to LC-MS/MS analysis. The use of urea as a solubilization technique is known to be partially compatible with LC-MS/MS analysis, as the urea can be removed through the use of SPE using a trap or desalting column. However, the solubilization efficiency of urea with the addition of trypsin is poor. The use of formic acid to dissolve proteins post-precipitation has been found to be an effective means to analyze proteins post-precipitation [142]. Formic acid solubilized samples can be injected onto a trap column and subject to top-down LC-MS/MS analysis. To examine the efficiency of formic acid to solubilize the protein pellet following protein precipitation in the upper filtration unit, *E. coli* extract was subject to C/M/W precipitation and the protein pellet was dissolved using either SDS or 80% FA. Figure 4.12 shows a SDS-PAGE gel of 20 µg *E. coli* protein extract solubilized using 1% SDS, 80% formic acid, and 5% ACN/0.1% FA following CMW precipitation on the filtration cartridge (as described in Section 2.4.3). The protein is not subject to SPE purification. As can be seen the FA resolubilization is an effective method to solubilize the proteins prior to top-down LC-MS/MS analysis. It should be noted that the use of the SPE cleanup column should be avoided as proteins were found to irreversibly bind to the solid phase support (protein loss of 30-50%). Currently, SPE purification is not

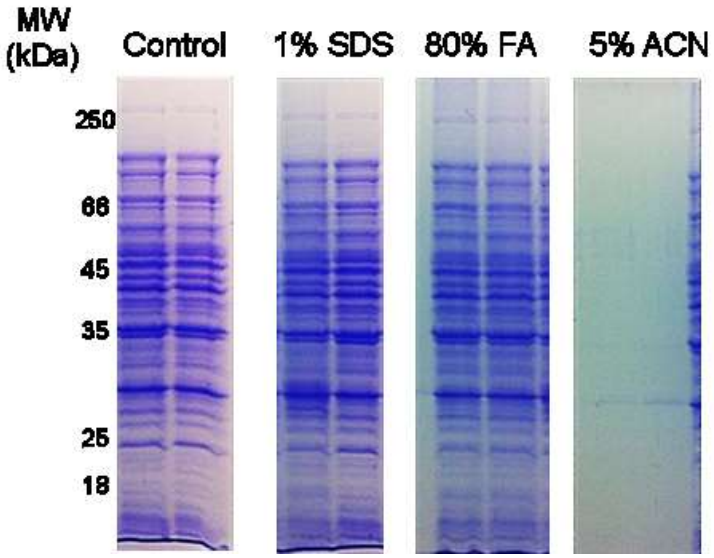

Figure 4.12: SDS-PAGE is used to visualize the proteins extracted from *E coli*, following chloroform/methanol/water precipitation (20 µg, in 1% SDS and 50 mM Tris-HCl pH 8) and resolubilization in various solvent systems.

recommended as a method of protein purification, due to high sample loss. It may be possible to reduce sample loss through the use of another SPE material such as size exclusion, or a monolithic material but the current SPE cartridge should be avoided for protein purification.

4.3.9 Coupling GELFrEE to the two-stage spin cartridge

Another area of SDS contamination is from the intact protein separation using GELFrEE. As stated previously, GELFrEE relies on the presence of SDS to separate proteins by molecular weight. The resulting protein fractions are highly contaminated with SDS which must be removed. Therefore SDS removal, subsequent digestion, and clean-up using the ProTrap XG, would provide a streamlined approach for bottom-up sample preparation following GELFrEE fractionation. Acetone precipitation of GELFrEE samples is made complicated by the presence of glycine in the running buffer. Glycine precipitates out of solution during acetone precipitation. However, glycine does not interfere with trypsin digestion and can be removed by a reversed phase desalting column (*i.e.* the SPE cartridge) following protein digestion.

To verify the ability of the complete ProTrap XG cartridge protocol to purify proteins following GELFrEE separation, an *E. coli* extracted protein samples of varying concentration were spiked with running buffer and subject to both the complete filtration cartridge and the vial based workflows. Figure 4.13 shows an increased recovery for the ProTrap XG (101±6%) compared to the vial workflow (86±0.6%) for samples containing 5 µg. However as protein concentration increases the recovery of the vial based strategy increases to near quantitative levels. The filtration based strategy recovery decreases to 75±0.6% at 25 µg expected to be caused by incomplete binding during SPE clean-up.

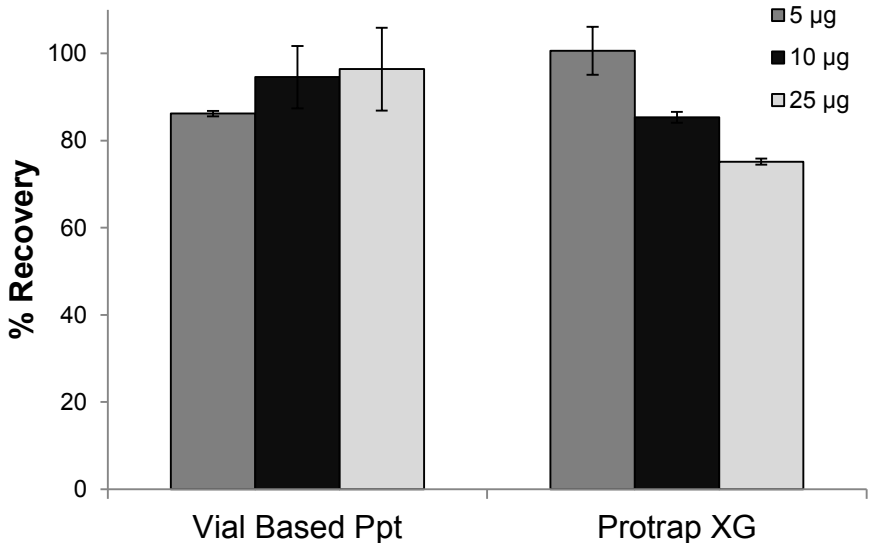

Figure 4.13: Shown is the recovery of peptides following SDS removal from a pseudo-GELFrEE fraction through protein precipitation, protein digestion and column cleanup in the ProTrap XG or through a vial based workflow. The initial protein loading was 10 µg of *E. coli* protein extract /sample spiked with GELFrEE running buffer. A non-spiked control was used for quantitation. (n=3).

These results suggest that the filtration based approach is more effective when working with dilute protein samples (<10 µg) compared to the conventional vial based approach. Fortunately, a normal GELFrEE fraction typically contains 5 to 10 µg of protein. Therefore, the ProTrap XG should be ideal for SDS removal following GELFrEE fractionation. Indeed an overall peptide recovery of 85±16% was achieved from the GELFrEE separation of 100 µg of *E. coli* followed by SDS removal and peptide digestion using the two-stage spin cartridge.

To further examine the compatibility of GELFrEE to the ProTrap XG, a detailed LC-MS/MS examination of a single fraction was employed. The ProTrap XG was employed to process a GELFrEE-fractionated *E. coli* extract (MW range ~48-55 kDa). A full list of identified proteins is provided as supplementary files. Table 4.3 summarizes the identified proteins /peptides from the GELFrEE fraction processed through 3 independent spin cartridges. Control samples involved conventional acetone precipitation and tryptic digestion in a vial. Recovery numbers were found to be identical for both workflows, and were highly repeatable for independent replicate.

Table 4.3: Proteins and peptides identified from aliquots of an *E. coli* fraction, following SDS removal and protein digestion.

		Proteins				**Peptides**			
	relative recovery*	run 1	run 2	run 3	total unique	run 1	run 2	run 3	total unique
vial ppt.	100 +/- 3	181	182	163	216	1072	1060	929	1554
spin cartridge	100 +/- 4	184	187	190	226	1221	1117	1194	1753

* recovery normalized to average peptide yield obtained across the two sample preparation workflows

The overlap in identified proteins from the three spin cartridges was found to be 69%, which is similar to the 66% overlap from three independent vial precipitations. Comparing the proteins identified across each method, 79% were common between the vial and cartridge precipitation workflows. A marginal 5% increase in the total identified proteins (p-value = 0.2) and a 13% increase in unique peptides (p value = 0.05, unpaired t-test) is observed when processed in the spin cartridge. The MS spectral counts per identified protein from the GELFrEE fraction are compared in Figure 4.14. The regressed slope of 1.06±0.02 whole, these results demonstrate the capacity of the ProTrap XG to deplete SDS from GELFrEE separated protein samples, with subsequent digestion and cleanup ahead of MS in a highly streamlined, efficient, and reproducible format.

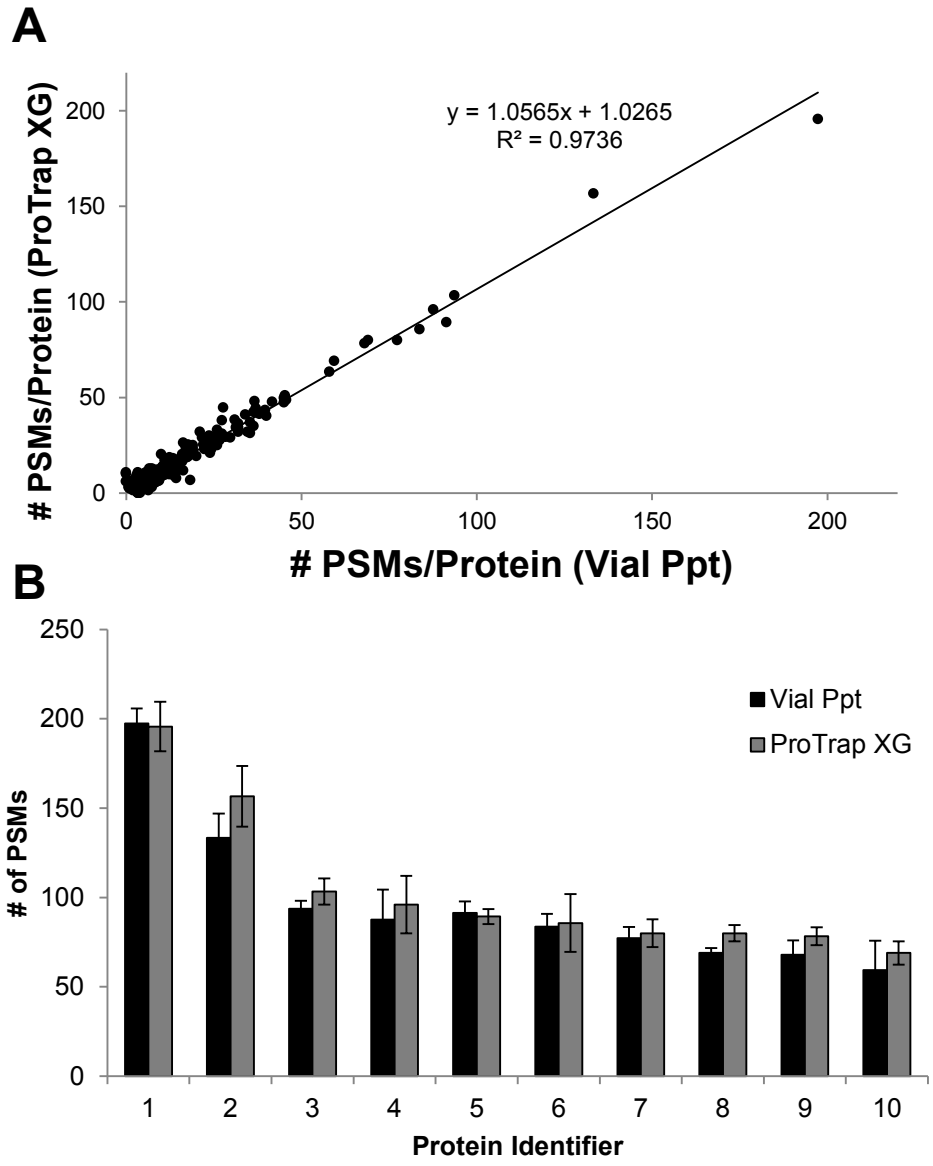

Figure 4.14: (A) Semi-quantitative analysis of proteins identified in a GELFrEE fraction of *E. coli*, following SDS removal and digestion using conventional acetone precipitation in a vial vs. the two-stage spin cartridge. The 8 proteins with the highest PSM (indicated) are also plotted in (B) with error bars representing the standard deviation of independently processed aliquots of the GELFrEE fraction (n=3).

4.4 Conclusion

The two-stage spin cartridge, termed the "ProTrap XG", was designed to automate the process of protein precipitation, which is difficult to perform in a reproducible fashion while maintaining high protein recovery and analyte purity. As seen here, use of the spin cartridge yields similar protein recovery, purity, and MS identifications compared to conventional vial-based precipitation. The results attainable with the two-stage spin cartridge should not be anticipated to be superior to a conventional approach, as great care was taken to perform the manual manipulations. The primary advantage of the device over a conventional workflow is that it ensures reliable and consistent recovery and purity of digested peptides from SDS-containing protein samples. All sample processing steps can be performed within a single disposable device. Multiple samples can be simultaneously processed through independent cartridges, resulting in highly repeatable digests, and facilitating label-free comparative proteome studies, as shown through spectral counting [120].

5. Chapter 5: High Molecular Weight Protein Depletion by GELFrEE

5.1 Introduction

One major focus of proteomics research has been in the identification and validation of protein biomarkers. Biomarkers are molecular components which can be used in the diagnosis or prognosis of diseases such as cancer, Alzheimer's, and cardiovascular disease [3,8,143–146]. In recent years, plasma has seen a great deal of interest as a potential source of protein biomarkers [3,6]. Blood contains a proteome of enormous complexity. The main plasma proteins include: albumin, immunoglobulins, fibrinogen, transferrin, haptoglobin, and lipoproteins [147]. However, it is also believed that proteins secreted by other tissues and cells throughout the body are present in blood [148]. Blood circulates throughout the circulatory system carrying oxygen, nutrients, and waste products to and from cells within the body. It is believed that blood interacts directly or indirectly with all cells in the human body [149]. Indeed, glycoproteins derived from tissues have been detected in the blood through MS analysis [150]. This suggests that the examination of the proteins in plasma will allow for the identification of biomarkers released from affected cells and tissues within the body.

Given the proteomic information contained within human plasma samples, it is clearly an ideal medium for the study of diseases within the human body. However the identification of proteins from human plasma has been severely limited. In 2004 a non-redundant list of 1175 unique proteins from plasma was compiled from 4 sources [151]. However, only 4% overlap was found between all four sources. This has been attributed to differences in plasma depletion and sample preparation. The difference in the storage of the plasma samples may have also led to the low protein overlap [152,153]. The first

large scale collaborative study in characterizing the plasma proteome only identified 889 proteins (95% confidence) [154]. Currently over 10 000 proteins are estimated to exist in plasma, most at low concentrations [155]. Newer in-depth methodologies do not provide many more identifications than these studies (~1 700 [156]).

A major issue limiting protein identification in plasma is the large dynamic range in concentration for different plasma proteins. Although the protein content in plasma is high (50 to 80 g/L), 99% of that concentration is made up of 22 proteins [147]. The majority of plasma proteins (~10 000) constitutes the remaining 1% of protein concentration in the plasma [147]. The presence of the overly abundant proteins in plasma makes the characterization of the lower abundant proteins incredibly difficult. For this reason the analysis of the plasma proteome cannot occur until either depletion of the overly abundant proteins or enrichment of the low abundant proteins is carried out. A number of protein depletion techniques have been proposed to allow for the examination of low abundant plasma proteins. These include affinity purification [157,158], intensive fractionation [159], selective protein precipitation [84,160], and ultrafiltration [161]. Affinity purification relies on the use of either an antibody or a chemical agent that specifically binds to the protein of interest and allows for either enrichment or depletion of the protein. The drawback of affinity based techniques is that albumin is a known carrier protein for low molecular weight proteins/peptides that may be of interest [158,162]. Therefore depletion of albumin using this method may also deplete these bound proteins. Protein fractionation relies on the separation of the plasma proteins into fractions with fewer proteins in each, allowing for more proteins to be identified by LC-MS/MS. This extends the dynamic range of the experiment. This method does not

normally remove any of the higher abundant proteins from the system, but it increases the LC-MS/MS time required to analyze the sample.

Selective protein precipitation and ultracentrifugation rely on the depletion of the more abundant high molecular weight proteins (>45 kDa) to isolate the low abundant, low molecular weight proteins. Protein precipitation makes use of the organic solvent (acetonitrile) [84] or ammonium sulfate [160] to selectively precipitate the higher molecular weight proteins from solution. The lower mass proteins remain soluble in the system. The benefit of this technique is that the addition of the organic solvent causes protein denaturation which disrupts protein-albumin binding. The alternative technique, ultrafiltration uses a molecular weight cut-off filter to retain the high molecular weight proteins on the filter while the LMW proteins pass through [163]. This is normally done in the presence of a small concentration of detergent and/or organic solvent, to prevent protein binding to albumin. Neither ultracentrifugation nor protein precipitation are highly effective at removing the high molecular weight protein without the loss of the low molecular weight proteins [164,165].

The main plasma proteins are secreted by the liver and intestines and have a larger molecular weight than the kidney filtration limit (>45 kDa) [6]. The large size of plasma proteins means that depletion by molecular weight is an effective method for enrichment of the less abundant proteins. These smaller proteins have been termed the low molecular weight proteome [161]. The high tumor and vascular permeability of the LMW proteome makes them interesting targets for biomarker discovery [163,166–168]. This means that this subset of proteins should contain the majority of the proteins secreted into the plasma by the tissues within the body. For these reasons it is of no

surprise that the proteins within the LMW proteome has been associated with a number of pathological conditions including cancer, diabetes, cardiovascular diseases, and infectious diseases [161]. It is clear that the LMW proteome can provide a true snap-shot of what is occurring within the body, making it an ideal system for human biomarker discovery.

The inclusion of SDS into the plasma sample prior to any sample manipulations steps could have a large benefit for the analysis of plasma proteins. Currently, plasma samples are required to be stored at -80ºC or in liquid nitrogen to prevent sample degradation [169]. This limits the ability of researchers to easily store and study plasma samples. The inclusion of SDS into the plasma samples may reduce sample degradation through the inactivation of enzymes which cause protein degradation or modification [170,171]. The addition of SDS would also be beneficial in the disaggregation of proteins [45]. SDS would denature all proteins with the sample. This would prevent albumin from binding any proteins, preventing protein loss through this mechanism.

Currently, isolation of the LMW proteins from plasma is limited to ultrafiltration and protein precipitation. Mass-based protein fractionation using gel-eluted liquid fraction entrapment electrophoresis coupled with acetone precipitation has been shown in Chapter 4 to be a viable method for the separation and analysis of protein samples containing SDS. GELFrEE fractionation allows both the fractionation and enrichment of the LMW proteins in one step. Both depletion and fractionation have been shown to be required for the identification of more than 200 proteins of the LMW proteome in a single experiment [156,172]. The pore size of the gel column can be modified to allow

for protein separation in a specific mass window (5-50 kDa). The pore size of a polyacrylamide gel is controlled by two factors: (1) %T and (2) %C. The %T represents the total amount of acrylamide present in the gel. As the amount of acrylamide increases the pore size decreases. The %C describes the amount of cross-linker present in the gel. Higher %C causes more crosslinking to be present thereby causing pore sizes to decrease. Here, the %T of the resolving gel column was changed to allow the fractionation of a lower molecular weight region of proteins (Figure 5.1).

GELFrEE has previously been used to isolate and prefractionate the low molecular weight proteins obtained from HeLa S3 cells for top-down proteomics [173]. Although this system is much simpler than plasma, the same principles of separation should still apply. The difficulty of applying the GELFrEE to the plasma proteome is that the majority of the proteome's mass is found in a specific MW range (*i.e.* >66 kDa). All previous works utilizing GELFrEE for separating protein samples by MW have used samples with an evenly distributed proteome. The application of GELFrEE to plasma samples has not been previously published. Here, a modified GELFrEE protocol was employed to isolate the LMW proteins from plasma; the unwanted high molecular weight proteins are left in the gel column to be discarded. This methodology has the advantage of allowing for the use of SDS to denature all proteins prior to depletion, preventing protein loss through protein-protein aggregation [45].

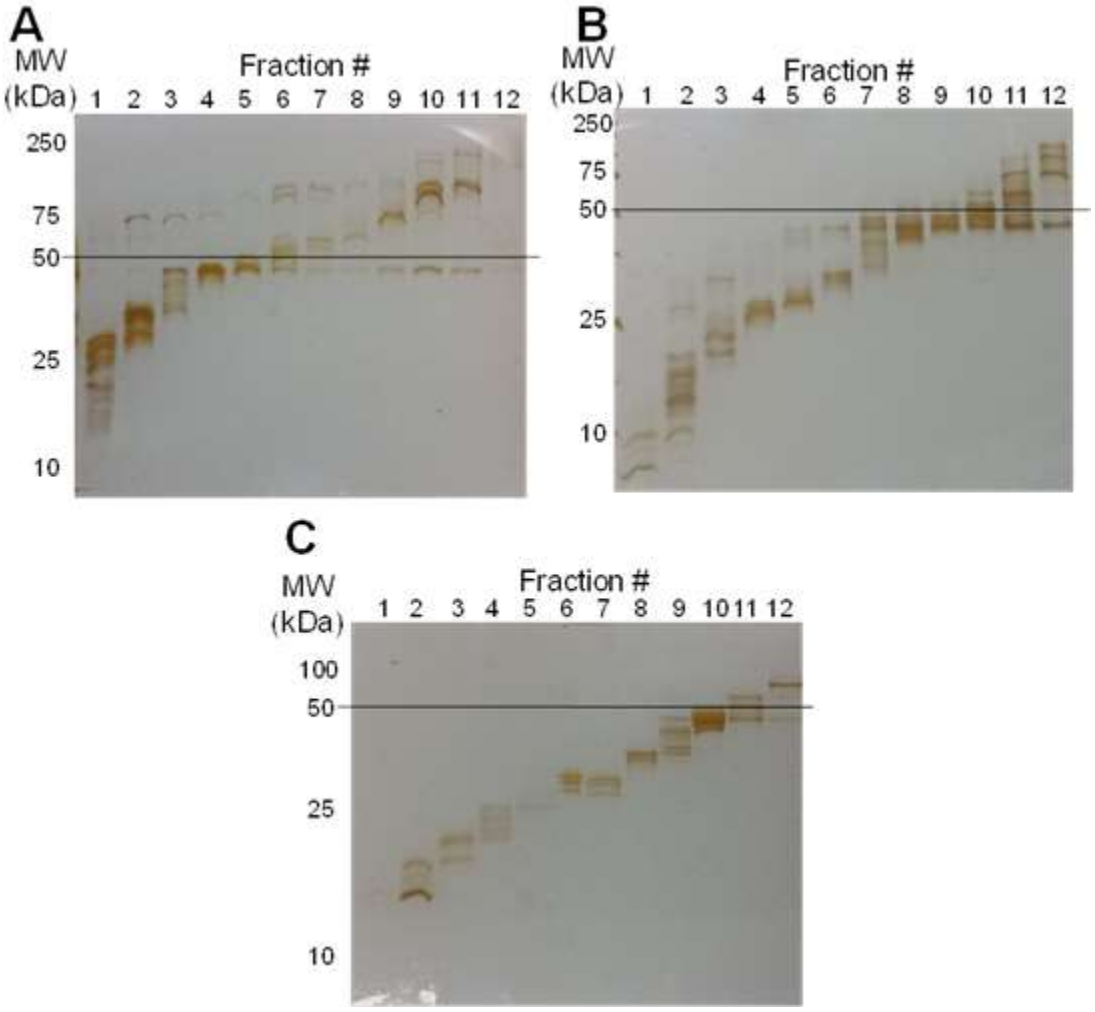

Figure 5.1: SDS visualization of 100 µg *E. coli* protein extract fractionated by GELFrEE with varying %T resolving columns: (A) 12 %T, (B) 14 %T, and (C) 16 %T. Fractionation was carried out through use of the standard protocol (as described in Section 2.5.1). One tenth of the first twelve fractions collected, where subject to SDS-PAGE visualization. The solid line represents the 50 kDa mass range.

5.2 Methods in Brief

Lyophilized plasma standards were dissolved in either 1% SDS (for GELFrEE depletion) or water (for direct peptide analysis as described previously) at the same volume as original samples (*i.e.* 1 mL). GELFrEE depletion at the 45-50 kDa was carried out using a 17% resolving gel column of length 1 cm, coupled with a 2.2 cm 4%T stacking column, in a Laemmli format [63,113]. A modified Laemmli sample buffer containing a 5 fold decrease in bromophenol blue was used for sample loading. In the final experiment, 2-mercaptoethanol was not present in the sample buffer. The samples were loaded into the polyacrylamide gels using a loading voltage of 200 V, which was maintained until the tracking dye fully migrated off the gel column. Separation occurred at 240 V. Following separation and depletion samples were subject to acetone precipitation with a 1.5 mL acetone wash. The pellet was resolubilized in 8 M urea (tryptic digestion and LC-MS/MS analysis) or Laemmli buffer (SDS-PAGE visualization on a 17%T gel). Protein searching was done using SEQUEST as part of the Proteome Discover 1.4 package. Peptide false discovery rates of 5%, with 1 unique peptide per protein were used as filters. LC-MS/MS controls consisted of tryptically digested plasma standard (1 µg per injection).

5.3 Results and Discussion

To isolate the LMW proteome of plasma, a fractionation protocol was developed using a bovine plasma standard. As shown in Figure 5.2A, a 17%T resolving gel column was used to provide efficient fractionation (11 fractions) under 50 kDa MW. The collection timing was designed to allow for the collection of serum albumin in the final fraction. It is noted that visualization of the proteins within the plasma fractions required loading of

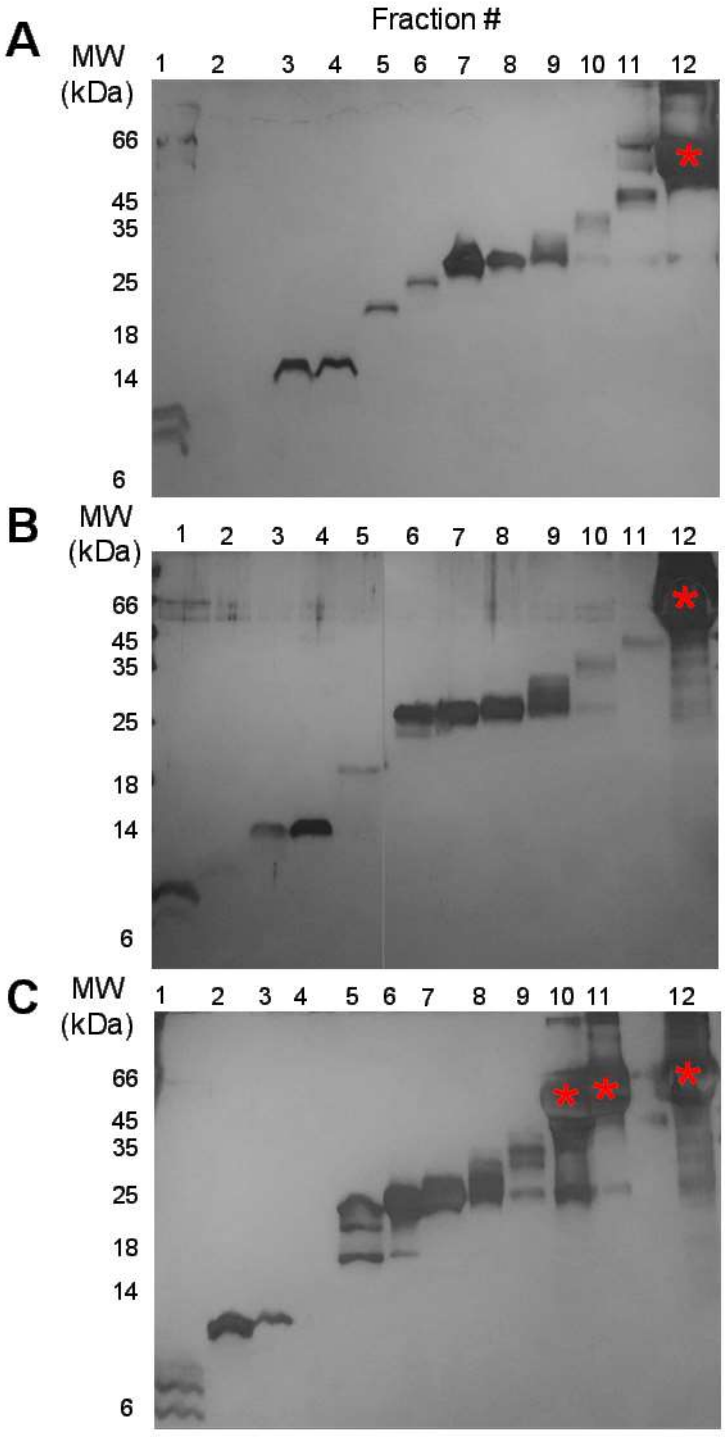

Figure 5.2: SDS-PAGE visualization of fractionated and high molecular weight depleted plasma standard through the use of GELFrEE on a 17%T column. Variable amounts of bovine plasma were loaded into the system for separation: (A) 55 µg, (B) 110 µg, and (C) 220 µg. Proteins fractions were subject to acetone precipitation and resolved on a 17 #T gel. Silver stain was used for visualization. The presence of serum albumin is denoted by *.

the entire fraction following acetone precipitation. Peptide quantitation of the fractions was carried out following acetone precipitation and tryptic digestion. The LC-UV assay revealed peptide amounts below the detection limit of the assay (<0.5 μg) for the majority of fractions. This result is not entirely surprising as only 55 μg of total plasma protein was loaded into the system. To isolate the bottom 1% (by mass) of proteins a large amount of plasma will be required.

5.3.1 Determining the Loading Capacity of the System

One advantage of GELFrEE is its ability to load a large amount of protein. Sample loadings up to 1 mg have been shown to be acceptable for this unit [63]. However, the loading capacity for plasma has not been tested previously. Different amounts of plasma protein were subject to the proposed GELFrEE separation and shown in Figures 5.2A to 5.2C. As can be seen the presence of albumin is normally visible in the final protein fraction (#12) for all loadings. However, after the protein loading increased to 220 μg, albumin was clearly observed in early fractions (Figure 5.2C). This suggests that the system is not capable of loading more than 110 μg of protein. The limited loading capacity of plasma proteins is explained by the presence an uneven distribution of proteins by mass within plasma. Proteins must occupy physical space even when dissolved in solution. Electrophoresis through a porous medium separates protein by molecular weight, creating distinct "bands" of proteins. However, when one or more bands possess an overabundance in mass, they must occupy more space. This has two effects which could potentially cause the proteins to increase in velocity. The first effect is that physical crowding of the proteins may push the lower weight and lower abundant proteins ahead. The second effect is electrostatic in nature; the large amount of

negatively charged proteins in one space may cause the LMW (also negatively charged) proteins to increase in velocity due to the electrostatic repulsion.

Regardless of the cause of interference, it is clear that overloading the gel column with plasma proteins causes a reduction in the separation ability of the system. Therefore, protein loadings above 110 µg (of plasma proteins) are not possible with the current setup. However, a 110 µg separation was unable to provide adequate peptide amounts for LC-MS/MS analysis. Therefore it is critical to increase sample loading. The easiest method to increase sample loading is to increase the amount of space the proteins can occupy. To do this, the 4 mm gel columns were replaced with 6 mm columns. Figure 5.3 shows the SDS-PAGE visualization of the protein fractions following depletion and separation using a 6 mm column. Increasing amounts of protein were tested to determine the loading capacity of the new system. As can be seen, protein loadings of up to 550 µg can occur prior to overloading the column (Figure 5.3B). This is a fivefold increase over the smaller diameter tubes.

5.3.2 Application of the System to LC-MS/MS Analysis

To extend the developed GELFrEE methodology for high molecular weight plasma depletion to LC-MS/MS protein identification, a human plasma standard was examined. The standard was spiked with 1% SDS, to prevent protein degradation prior to sample manipulation. The sample loading was kept at 550 µg (capacity of the system) to allow for adequate sample collection. To verify the success of the depletion and fractionation, the fractions were concentrated using acetone precipitation and subjected to SDS-PAGE visualization. Referring to Figure 5.4A, MW fractionation is clearly visible. The presence of protein bands in the LMW fractions suggest that LMW protein enrichment is

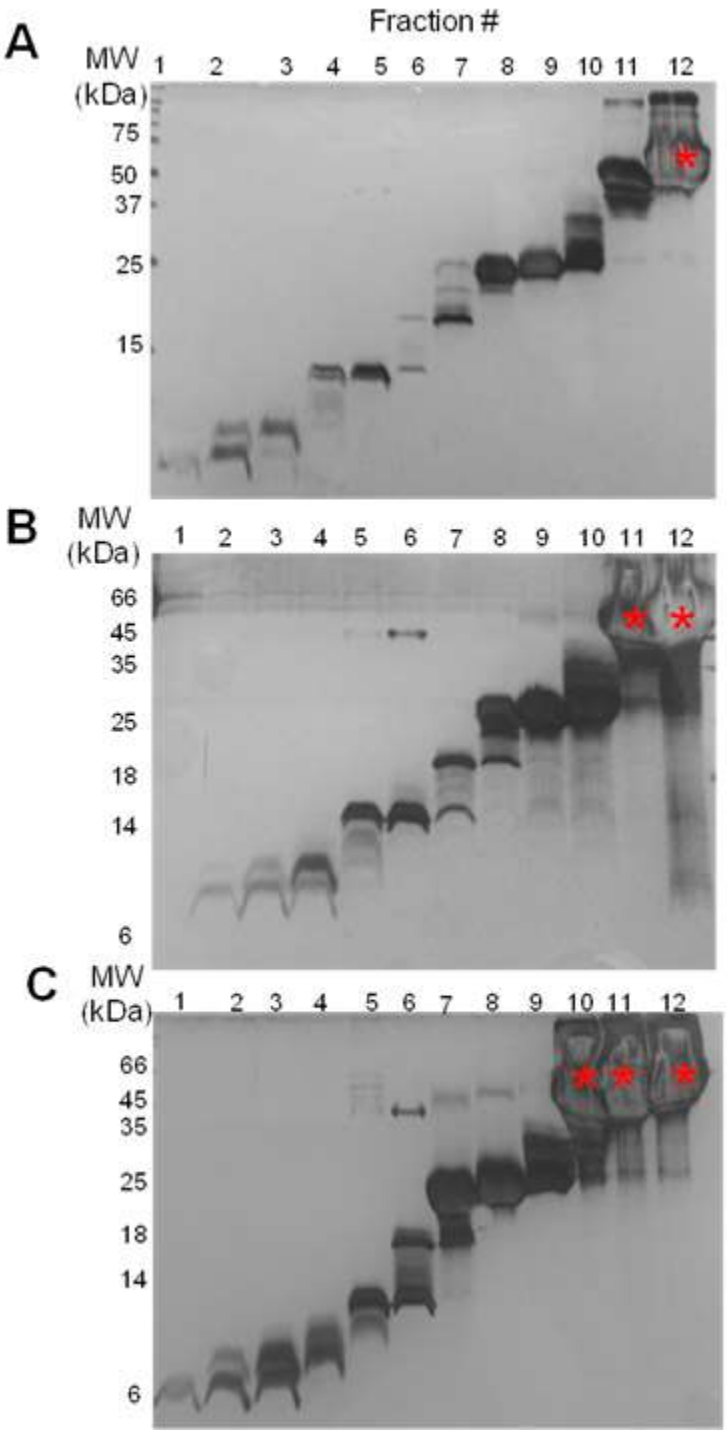

Figure 5.3: SDS-PAGE visualization following fractionation and depletion of bovine plasma standard using GELFrEE on a 6 mm, 17%T column. Variable amounts of bovine plasma protein was loaded into the system for separation: (A) 550 µg, (B) 825 µg, and (C) 1100 µg. Proteins fractions were subject to acetone precipitation and one half of each fraction was resolved on a 17%T gel. Silver stain was used for visualization. The presence of serum albumin is denoted by *.

occurring. Protein bands corresponding to as low as 10 kDa and as high as 66 kDa are clearly visible throughout the gel image. However, SDS-PAGE does not have the ability to identify proteins within the bands.

To fully examine the ability of this technique to enrich the LMW proteome, protein characterization of the resulting fractions is required. The LMW protein fractions were precipitated using acetone, resolubilized in 8 M urea, and subject to tryptic digestion and LC-MS/MS analysis. A non-SDS containing and non-precipitated plasma sample was digested and subjected to LC-MS/MS analysis as a comparison. The overlap of protein identifications between the two samples can be seen in Figure 5.5B. The GELFrEE processed sample allowed for the identification of 163 proteins (1 MS replicate per fraction, for 12 fractions) versus 111 proteins in the control (4 MS replicates). A total of 29% of the proteins identified in the GELFrEE were also found in the control plasma. Of these overlapping proteins, 61% were found to have a MW of <50 kDa. Looking at the proteins identified in the two systems, an increased proportion of low molecular weight proteins (<50 kDa) are found in the GELFrEE processed sample (67%) compared to the control (52%). From these results, it can be said that an enrichment of the low molecular weight proteins did occur in the GELFrEE processed samples. However, protein identifications are not quantitative, and therefore do not reveal information about the relative concentrations of each protein in the samples.

To provide quantitative information about protein enrichment, a semi-quantitative technique known as spectral counting [120] was applied to the LC-MS/MS data. To examine the list of proteins identified for changes in MW composition, the spectral hits for specific mass ranges of proteins were calculated and plotted in Figure 5.4C. To

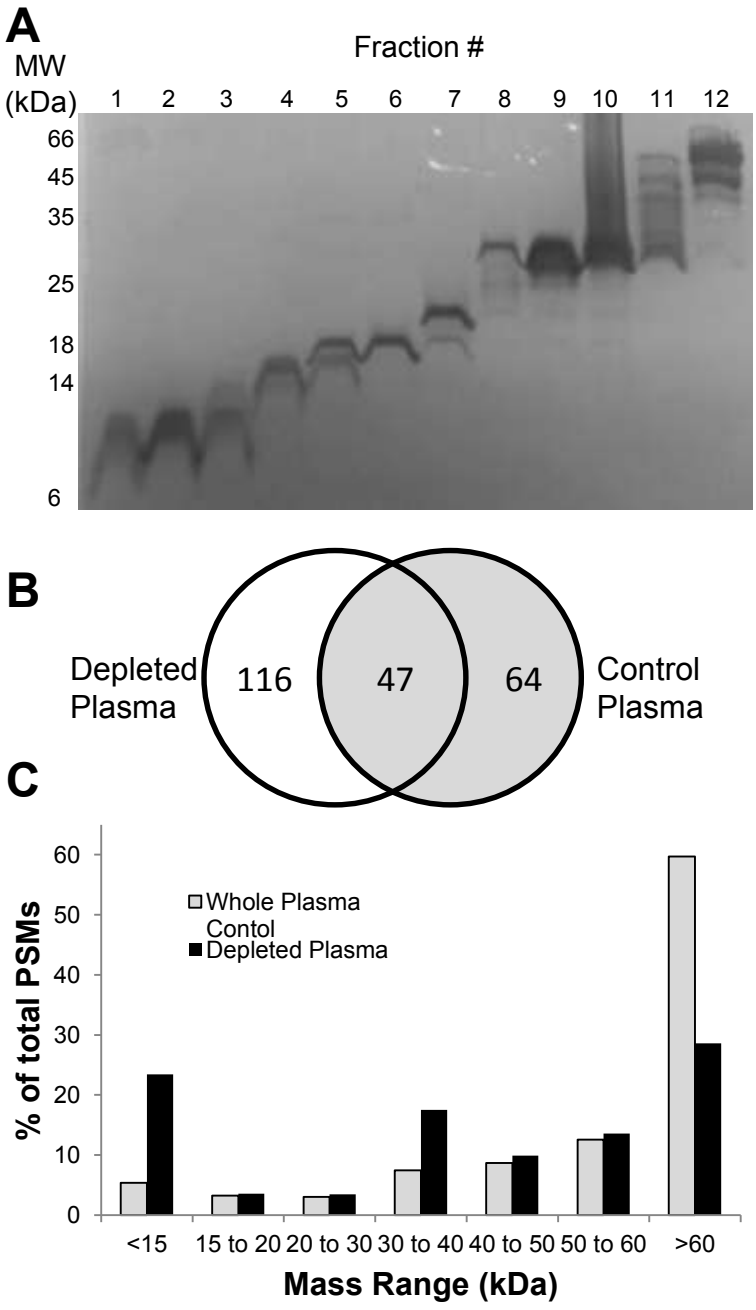

Figure 5.4: (A) Visualization of the high molecular weight depletion and fractionation of human plasma (550 µg) by GELFrEE. (B) Venn diagram of proteins identified in the fractions (1 technical replicate for each fraction) compared to a non-depleted control (4 replicated of 1 µg plasma protein). (C) Semi-quantitative study of mass distribution of proteins in the non-depleted and depleted plasma samples. The proteins were grouped within specific mass ranges, and the normalized peptide hits for each range were plotted.

accurately compare spectral hits between both systems, the number of spectral hits for each protein was normalized to the total spectral hits found in each system (expressed as a percentage). Referring to Figure 5.4C, depletion of spectral hits in the >60 kDa range is clearly visible for the depleted sample, compared to the control. This suggests that high molecular weight protein depletion did occur. It is noted that a decrease in the number of spectral hits for albumin was found be decreased in the depleted plasma. Enrichment in the <15 kDa and 30-40 kDa regions are noted. The other protein ranges, 15 to 30 kDa, and 40 to 60 kDa were not found to be enriched. These results clearly demonstrate an enrichment of LMW proteins using this technique. However, it was found that a large number of peptide hits are the result of immunoglobulin proteins. In total, 24% of the spectral hits of the depleted sample are from immunoglobulin proteins versus 7% in the whole plasma. This explains the enrichment seen in the < 15 kDa range, as light chain immunoglobulins (~10 to 15 kDa range) fall into this range.

5.3.3 Removing the Reducing Agent in the Sample Loading Buffer

The enrichment of immunoglobulins is unwanted as they interfere with the identification of other proteins and do not provide information about potential biomarkers. The enrichment previously observed in Figure 5.4C is believed to be caused by the presence of the reducing agent, 2-mercaptoethanol, in the sample loading buffer. Reduction of the immunoglobulins would disrupt the disulfide bond connecting the light and heavy chains of the protein. The resulting light chain immunoglobulins would then be enriched during GELFrEE fractionation. By removing the reducing agent, the disulfide bond would not be disrupted. The light chain immunoglobulins would be removed with the heavy chain immunoglobulins. To test this hypothesis, human plasma was subjected to the GELFrEE

depletion protocol without the presence of 2-mercaptoethanol in the sample loading buffer. The resulting protein fractions were precipitated and subjected to SDS-PAGE and LC-MS/MS analysis. Referring to Figure 5.5, isolation and fractionation of the low molecular weight protein did occur. Bands are clearly visible in the LMW fractions, and increase in MW as the fraction number increases. However, it is noted that albumin is present in fraction 11 (visualized in SDS-PAGE), suggesting that the loading capacity of the system was decreased when reduction of the sample was not carried out. The LC-MS/MS analysis of the protein fractions was unsuccessful as only a handful of proteins were identified (~10 proteins, mainly located in the later fractions). It was found that the low mass fractions contained protein amounts under the LoD of a LC-UV peptide assay (0.5 μg). Therefore it is concluded that not enough protein material was present to perform proper protein identification by LC-MS/MS on an LTQ instrument.

To overcome the limited quantity of protein obtained from the GELFrEE depletion of plasma, both the combination of fractions and replicate pooling was carried out. Fractions 1-2, 3-4, 5-6, and 7-8 were combined to increase total protein quantity. To further increase the protein amount obtained, pooling of technical replicates was performed. The GELFrEE depletion was performed with six replicates of the same plasma sample. The pooling of the 6 replicates occurred during SPE purification. The resulting fractions were estimated to have peptide amounts of 3-4 μg by an LC-UV peptide assay. This correlates to peptide amounts of 0.5 μg to 0.66 μg per fraction prior to replicate pooling. The final fractions were estimated to contain >10 μg of protein, this is due to the presence of albumin protein. The resulting fractions were expected to contain enough peptide material to allow for protein identification by LC-MS/MS.

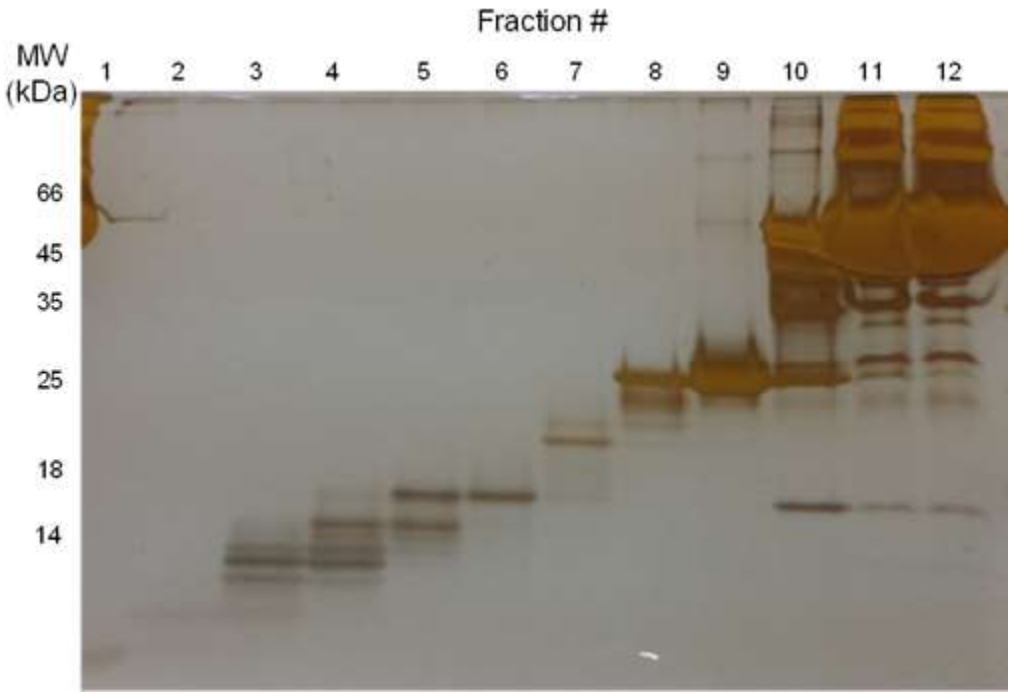

Figure 5.5: SDS-PAGE visualization of the fractions obtained through the depletion of 550 µg of human plasma standard using GELFrEE without the presence of a reducing agent in the sample buffer. Proteins fractions were subjected to acetone precipitation and one half of each fraction was resolved on a 17%T gel. Silver stain was used for visualization. The presence of multiple protein bands in the low molecular weight fractions should be noted.

Subjecting the GELFrEE depleted fractions to LC-MS/MS analysis allowed for the identification of 104 proteins (1 MS replicate per fraction), compared to 88 proteins in the non-depleted control sample (8 MS replicates of non-depleted plasma). In this experiment, only 20% of the proteins found in the GELFrEE processed sample was also identified in the control (Figure 5.6A). The low overlap suggests that protein enrichment or depletion did occur. To verify LMW enrichment, the proteins identified in each sample were examined. A slight enrichment of LMW proteins was noted in the GELFrEE depleted sample (58% of proteins below 50 kDa vs. 48% in the control). The normalized spectral hits for each mass range were plotted in Figure 5.6B. The previously observed enrichment in the >15 kDa range (Figure 5.4C) is no longer present, suggesting that light chain immunoglobulins are no longer being enriched. Referring to Figure 5.6B, an overall enrichment in the 15-40 kDa region is clearly visible compared to the control. Overall, 29% of the total peptide hits were found in this region compared to 6% in the control. This significant increase suggests that enrichment in this area did occur. A decrease in proteins in the >60 kDa range is clearly visible (46% of the total PSMs *vs.* 75% in the control) as well as a decrease in immunoglobulins (2% of the total PSMs *vs.* 10% in the control). A significant decrease in the identifications of serum albumin is also noted. Overall it is clear that by removing the reducing agent in the sample buffer, LMW enrichment can occur with minimal amounts of contaminating immunoglobulins.

The low number of proteins that were identified was attributed to the residual SDS in the fractions. Acetone precipitation is not 100% effective at removing SDS from the protein samples, and residual SDS (<0.01%) can be left behind. By pooling 6 replicate fractions, the residual SDS in each sample may cause issues with LC-MS/MS

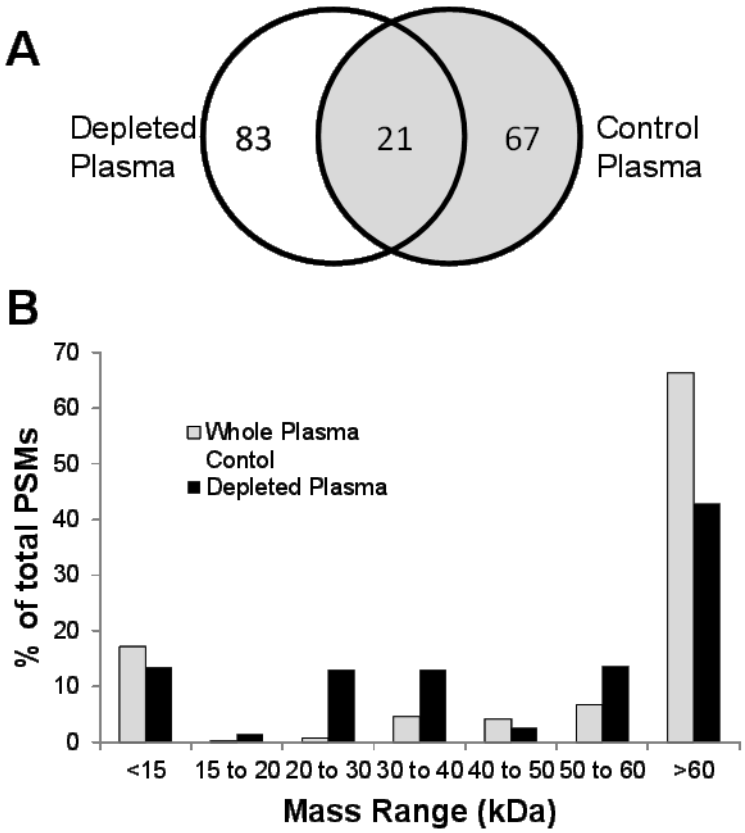

Figure 5.6: (A) Venn diagram of proteins identified in the fractions (1 technical replicate for each fraction) compared to a non-depleted control (4 replicated of 1 µg plasma protein). (C) Semi-quantitative study of mass distribution of proteins in the non-depleted and depleted plasma samples. The proteins were grouped within specific mass ranges, and the normalized peptide hits for each range was plotted.

analysis. This is certainly the case for the lower abundant fractions (fractions 1-3) where protein identification was minimal (3 to 22 proteins per fraction). To allow for greater protein identification through pooling of replicate GELFrEE fractions, a second acetone wash step may be required to ensure proper SDS removal. Even with this limitation, this method shows promise in the isolation and fractionation of low molecular weight proteins from plasma.

5.4 Conclusion

The low molecular weight plasma proteome is a highly untapped source of potential biomarkers [161]. Current LMW isolation techniques are both limited in their tolerance for SDS and in their effectiveness in isolating the LMW proteome. Here, a novel GELFrEE depletion strategy, coupled to acetone precipitation is presented for the effective isolation and fractionation of low molecular weight plasma proteins. This method is highly compatible with SDS and can be modified to provide fractionation of any mass range. A modified protocol was used to isolate proteins with a MW below 35 kDa, as seen in Figure 5.7. The enrichment of the LMW proteome was carried out, allowing for the identification of 104 proteins, with the majority of identifications occurring in the low molecular weight range (~58%). In total, 54% of the peptides identified were matched to proteins in the less than 60 kDa mass range. Significant LMW enrichment was achieved compared to a non-enriched control. These preliminary results show promise for the use of GELFrEE as a LMW protein enrichment strategy ahead of LC-MS/MS analysis.

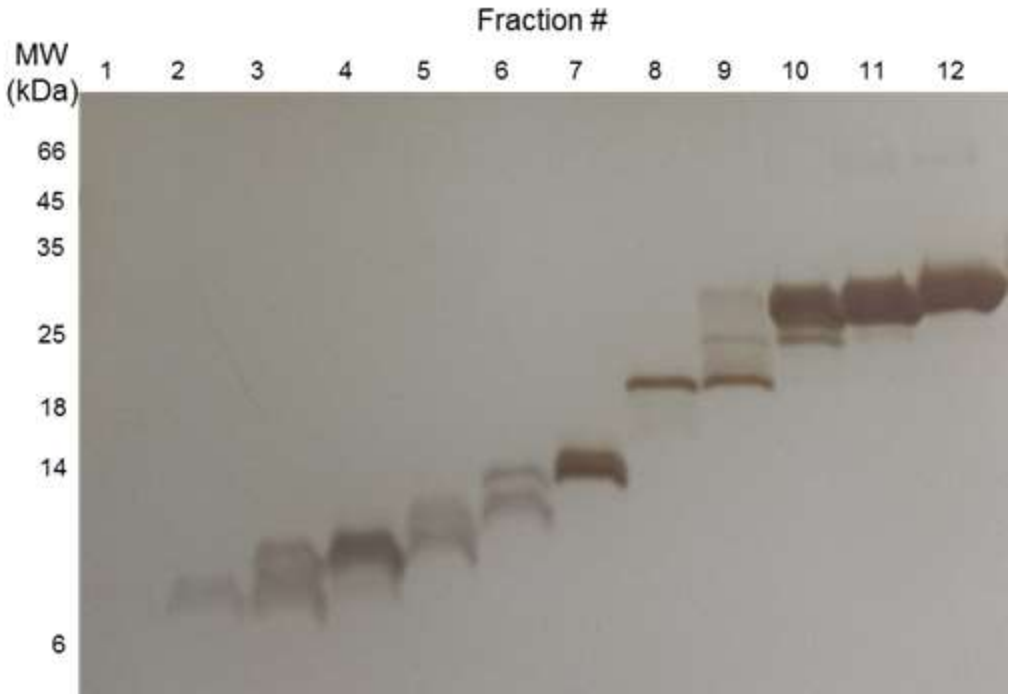

Figure 5.7: GELFrEE fractionation and depletion of blood plasma (550 µg) in the <35 kDa range. Fractionation was carried out through the use of a 17%T, 1.2 cm long resolving gel column (6 mm in diameter). Collection timing was as follows: 6×1 min, 5×2min, and 1×5 min.

6. Chapter 6: Conclusion

The use of sodium dodecyl sulfate in the proteomics workflow can be accommodated through complete removal prior to LC-MS/MS analysis. SDS was shown to be highly beneficial in the extraction and solubilization of proteins from a number of different sample types, such as tissues and cell cultures. The mass based separation platform GELFrEE is also dependent on the presence of SDS to ensure proper protein separation. To overcome the limited tolerance of LC-MS/MS with SDS, an efficient and reliable technique for SDS removal is required.

Here, acetone precipitation is presented as a highly effective protein purification strategy which can efficiently remove SDS from a protein sample. In Chapter 3, the improved recovery obtained from acetone precipitation upon the addition of ionic additives is explained through a model of ion-pairing. Overall, this chapter reveals acetone precipitation can provide near quantitative protein yields through the addition of an ionic buffer. Therefore, by ensuring proper ionic strength in samples, acetone precipitation can be used as a highly effective and reproducible protein purification strategy.

In Chapter 4, a semi-automated workflow for bottom-up protein analysis is presented. In this workflow, a two-stage filtration unit with an SPE de-salting column is used to effectively remove SDS from protein samples ahead of LC-MS/MS analysis. Highly reproducible protein yields are obtained through acetone precipitation in the two-stage unit. This technique was coupled with detergent lysis using an SDS containing extraction buffer to allow for the examination of proteins from a rat kidney from a congenital model of ureteral tract obstruction (UTO) [138]. This workflow was also

shown to be compatible with GELFrEE for SDS removal, protein digestion, and peptide purification ahead of LC-MS/MS analysis.

In Chapter 5, the protein separation ability of GELFrEE was used to isolate the low molecular weight proteins from human plasma. This methodology is compatible with high concentration of SDS, allowing for the use of SDS as a preservation agent ahead of sample manipulation. Following separation, SDS is removed from the GELFrEE fractions using acetone precipitation. Preliminary results using this method, shows low molecular weight protein enrichment compared to a non-depleted sample.

7. Chapter 7: Future Work

7.1.1 The Effect of Salt on Acetone Precipitation

The addition of simple salts to samples prior to acetone precipitation was shown to provide near quantitative protein recovery for water-soluble proteins. This trend was extended to other organic solvent systems. However, the requirement of salt to provide maximal recovery was not found to be dependent on dielectric strength of the solution, but on another unknown variable. Further investigation of the effect of salt concentration on other water miscible organic solvent for protein precipitation requires investigation. To do this various organic solvents should be tested with the system 0.1 g/L, to look for possible chemical and/or physical properties of the solvents to explain why different concentrations of sodium chloride are required to induce maximal protein precipitation. Not only is the type of solvent used a variable worth investigating but also the type of salt. The addition of different salt to the precipitation may cause different protein salts to be formed. The main disadvantage of protein precipitation is in the difficulty in solubilizing the protein pellet following protein precipitation. The use of various salts may make resolubilization easier or more difficult. The examination of protein resolubilization following protein precipitation using various salts and organic solvents would be beneficial in improving the ability to solubilize the protein pellet.

7.1.2 The ProTrap XG

One of the main goals of this thesis was to provide an effective acetone precipitation strategy using the two-stage spin cartridge (ProTrap XG). In Chapter 4, the application of the ProTrap XG, to SDS removal using acetone precipitation was validated using a number of protein samples. However, the validation process focused on the use of a bottom-up workflow ahead of protein identification by LC-MS/MS. The ProTrap XG is

not limited to the protocols shown in this thesis. The versatility of the device is highly understated. Any protein precipitation can be accomplished in the upper filtration cartridge. The comparison of protein recovery and SDS removal following protein precipitation using a variety of methods coupled to the filtration unit is required. Different precipitation methods may have advantages in speed, ease of protein resolubilization, and even effectiveness with different sample types. An in-depth analysis of the SDS removal and effectiveness of all possible protein precipitation techniques in the ProTrap XG ahead of LC-MS/MS is required.

The ProTrap XG was shown to be an easy and semi-automated method to remove SDS from GELFrEE fractionated protein samples ahead of LC-MS/MS analysis. However, only one fraction was examined in detail. The ability of the ProTrap XG to purify proteins from GELFrEE for LC-MS/MS analysis should be carried out. This device should be tested with both the in-house built system described previously [60] and the commercial system currently on the market. The two systems use different gel columns and different buffers. Overall, a more rigorous validation of the GELFrEE coupled to the ProTrap XG is required. The system would then be required to profile the proteome of a health related sample.

It was speculated that sample loss which occurred when working with samples >20 µg was caused by the limited capacity of the SPE clean-up method. The R2 resin was chosen for use due to its low price and availability. Other resins have not been tested for improved loading capacity. It is possible to use any reverse phase resin in the SPE cartridge for peptide or protein de-salting. The R2 resin was shown to cause protein loss when working with intact-proteins. To overcome this limitation, either a size exclusion

or monolithic column should be examined for protein purification. The SPE cartridge is not limited to the use of reverse phase resins. It is possible to use other types of SPE material. The use of weak anion exchange, strong cation exchange, titanium dioxide, and size exclusion will allow for a number of experiments following protein purification by protein precipitation. These include, but are not limited to, peptide fractionation, phosphopeptide enrichment, and peptide level SDS removal. The coupling of SPE to protein precipitation is easily carried out through the use of this attachable cartridge.

The biggest limitation in the proposed ProTrap XG workflow is in the use of urea or formic acid as a resolubilization agent. The use of either reagent causes a significant amount of modifications to the proteins, making both top-down and bottom-up analysis limited. Urea is also limited in its ability to dissolve membrane proteins. Other reagents are required to assist in protein resolubilization. These should either be compatible with LC-MS/MS or can be removed using the SPE cartridge. One possibility is the use of minimal amounts of SDS, followed by dilution (to under 0.1%), and tryptic digestion. The SDS can be removed using a SCX SPE cartridge following tryptic digestion. Another possible method lies within the use of sodium deoxycholate, a detergent which is highly compatible with tryptic digestion (<5%) and easily removed from solution [174,175]. However this detergent has been found to have limited success when being used to disrupt cell membranes [176]. Overall, it is clear an alternative method of dissolving the protein pellet following precipitation is required for both bottom-up and top-down proteomic analysis.

7.1.3 Low Molecular Weight Protein Isolation using GELFrEE

In Chapter 5, a GELFrEE protocol was presented which allowed for the low molecular weight enrichment of proteins from plasma. Overall, protein enrichment was observed but total protein identification was poor. This is attributed to the incomplete removal of SDS from the 6 pooled replicates. To overcome this issue, a significant number of acetone washes should be applied during protein precipitation. Secondly, only minimal amounts of peptide material were recovered in the protein fractions. For this methodology to become a viable technique for the examination of the LMW proteome of plasma, the limited loading capacity of the system (~500 mg) must be increased. Through the manipulation of the column dimensions it may be possible to increase sample loading. However, the in-house built system should be replaced with the commercial system. The commercial system allows for the use of 6 mm tubes. The collection volume of this system is significantly lower (100-150 µL). This would decrease the amount of resolubilization agent required post precipitation by reducing the amount of glycine present in the protein pellet. Glycine precipitates during acetone precipitation, increasing the difficulty of resolubilization. Another benefit of the system is that glycine is actually not present in the running buffer. A tris-acetate buffer is used to perform the electrophoretic separation.

References

[1] Botelho D., Wall M.J., Vieira D.B., Fitzsimmons S., Liu F., Doucette A., Top-down and bottom-up proteomics of SDS-containing solutions following mass-based separation. *J. Proteome Res.* 2010, 9, 2863–2870.

[2] Atkinson A.J., Colburn W.A., DeGruttola V.G., DeMets D.L., Downing G.J., Hoth D.F., Oates J.A., Peck C.C., Schooley R.T., Spilker B.A., Woodcock J., Zeger S.L., Biomarkers and surrogate endpoints: preferred definitions and conceptual framework. *Clin. Pharmacol. Ther.* 2001, 69, 89–95.

[3] Orton D., Doucette A., Proteomic workflows for biomarker identification using mass spectrometry — technical and statistical considerations during initial discovery. *Proteomes* 2013, 1, 109–127.

[4] Rifai N., Gillette M.A., Carr S.A., Protein biomarker discovery and validation: the long and uncertain path to clinical utility. *Nat. Biotechnol.* 2006, 24, 971–983.

[5] Parker C.E., Borchers C.H., Mass spectrometry based biomarker discovery, verification, and validation--quality assurance and control of protein biomarker assays. *Mol. Oncol.* 2014, 8, 840–858.

[6] Anderson N.L., Anderson N.G., The human plasma proteome: history, character, and diagnostic prospects. *Mol. Cell. Proteomics* 2002, 1, 845–867.

[7] Mitchell P., Proteomics retrenches. *Nat. Biotechnol.* 2010, 28, 665–670.

[8] Roche S., Gabelle A., Lehmann S., Clinical proteomics of the cerebrospinal fluid: Towards the discovery of new biomarkers. *Proteomics. Clin. Appl.* 2008, 2, 428–436.

[9] Zubarev R.A., The challenge of the proteome dynamic range and its implications for in-depth proteomics. *Proteomics* 2013, 13, 723–726.

[10] Meyer B., Papasotiriou D.G., Karas M., 100% protein sequence coverage: a modern form of surrealism in proteomics. *Amino Acids* 2011, 41, 291–310.

[11] Stosova T., Havlis J., Lenobel R., Sebela M., Proteolytic enzymes: Significance for proteomics. *Chem. List.* 2005, 99, 896–905.

[12] Rodriguez J., Gupta N., Smith R.D., Pevzner P.A., Does trypsin cut before proline? *J. Proteome Res.* 2008, 7, 300–305.

[13] Paizs B., Suhai S., Fragmentation pathways of protonated peptides. *Mass Spectrom. Rev.* 2005, 24, 508–548.

[14] Boyd R., Somogyi A., The mobile proton hypothesis in fragmentation of protonated peptides: a perspective. *J. Am. Soc. Mass Spectrom.* 2010, 21, 1275–1278.

[15] Hunt D.F., Yates J.R., Shabanowitz J., Winston S., Hauer C.R., Protein sequencing by tandem mass spectrometry. *Proc. Natl. Acad. Sci.* 1986, 83, 6233–6237.

[16] Webb K.J., Xu T., Park S.K., Yates J.R., Modified MuDPIT separation identified 4488 proteins in a system-wide analysis of quiescence in yeast. *J. Proteome Res.* 2013, 12, 2177–2184.

[17] Hebert A.S., Richards A.L., Bailey D.J., Ulbrich A., Coughlin E.E., Westphall M.S., Coon J.J., The one hour yeast proteome. *Mol. Cell. Proteomics* 2014, 13, 339–347.

[18] Chait B.T., Mass spectrometry: bottom-up or top-down? *Science.* 2006, 314, 65–66.

[19] Tran J.C., Zamdborg L., Ahlf D.R., Lee J.E., Catherman A.D., Durbin K.R., Tipton J.D., Vellaichamy A., Kellie J.F., Li M., Wu C., Sweet S.M.M., Early B.P., Siuti N., LeDuc R.D., Compton P.D., Thomas P.M., Kelleher N.L., Mapping intact protein isoforms in discovery mode using top-down proteomics. *Nature* 2011, 480, 254–258.

[20] Smith L.M., Kelleher N.L., Proteoform: a single term describing protein complexity. *Nat. Methods* 2013, 10, 186–187.

[21] Karas M., Hillenkamp F., Laser desorption ionization of proteins with molecular masses exceeding 10,000 daltons. *Anal. Chem.* 1988, 60, 2299–2301.

[22] Tanaka K., Waki H., Ido Y., Akita S., Yoshida Y., Yoshida T., Matsuo T., Protein and polymer analyses up to *m/z* 100 000 by laser ionization time-of-flight mass spectrometry. *Rapid Commun. Mass Spectrom.* 1988, 2, 151–153.

[23] Yamashita M., Fenn J.B., Electrospray ion source. Another variation on the free-jet theme. *J. Phys. Chem.* 1984, 88, 4451–4459.

[24] Wilm M., Principles of electrospray ionization. *Mol. Cell. Proteomics* 2011, 10, M111.009407.

[25] Iribarne J. V., On the evaporation of small ions from charged droplets. *J. Chem. Phys.* 1976, 64, 2287–2294.

[26] Thomson B.A., Iribarne J. V., Field induced ion evaporation from liquid surfaces at atmospheric pressure. *J. Chem. Phys.* 1979, 71, 4451–4463.

[27] Nguyen S., Fenn J.B., Gas-phase ions of solute species from charged droplets of solutions. *Proc. Natl. Acad. Sci. U. S. A.* 2007, 104, 1111–1117.

[28] Wilm M., Mann M., Analytical properties of the nanoelectrospray ion source. *Anal. Chem.* 1996, 68, 1–8.

[29] Wilm M.S., Mann M., Electrospray and Taylor-Cone theory, Dole's beam of macromolecules at last? *Int. J. Mass Spectrom. Ion Process.* 1994, 136, 167–180.

[30] Oss M., Kruve A., Herodes K., Leito I., Electrospray ionization efficiency scale of organic compounds. *Anal. Chem.* 2010, 82, 2865–2872.

[31] El-Faramawy A., Siu K.W.M., Thomson B.A., Efficiency of nano-electrospray ionization. *J. Am. Soc. Mass Spectrom.* 2005, 16, 1702–1707.

[32] Gygi S.P., Rist B., Gerber S.A., Turecek F., Gelb M.H., Aebersold R., Quantitative analysis of complex protein mixtures using isotope-coded affinity tags. *Nat. Biotechnol.* 1999, 17, 994–999.

[33] Ross P.L., Huang Y.L.N., Marchese J.N., Williamson B., Parker K., Hattan S., Khainovski N., Pillai S., Dey S., Daniels S., Purkayastha S., Juhasz P., Martin S., Bartlet-Jones M., He F., Jacobson A., Pappin D.J., Multiplexed protein quantitation in *Saccharomyces cerevisiae* using amine-reactive isobaric tagging reagents. *Mol. Cell. Proteomics* 2004, 3, 1154–1169.

[34] Ong S.E., Blagoev B., Kratchmarova I., Kristensen D.B., Steen H., Pandey A., Mann M., Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics. *Mol. Cell. Proteomics* 2002, 1, 376–386.

[35] Melanson J.E., Chisholm K.A., Pinto D.M., Targeted comparative proteomics by liquid chromatography/matrix-assisted laser desorption. *Rapid Commun. Mass Spectrom.* 2006, 20, 904–910.

[36] Li M., Gray W., Zhang H., Chung C.H., Billheimer D., Yarbrough W.G., Liebler D.C., Shyr Y., Slebos R.J.C., Comparative shotgun proteomics using spectral count data and quasi-likelihood modeling. *J. Proteome Res.* 2010, 9, 4295–4305.

[37] Zhang N., Chen R., Young N., Wishart D., Winter P., Weiner J.H., Li L., Comparison of SDS- and methanol-assisted protein solubilization and digestion methods for *Escherichia coli* membrane proteome analysis by 2-D LC-MS/MS. *Proteomics* 2007, 7, 484–493.

[38] Klapper M.H., On the nature of the protein interior. *Biochim. Biophys. Acta - Protein Struct.* 1971, 229, 557–566.

[39] Liang J., Dill K.A., Are proteins well-packed? *Biophys. J.* 2001, 81, 751–766.

[40] Arakawa T., Timasheff S.N., Theory of protein solubility. *Methods Enzymol.* 1985, 114, 49–77.

[41] Ruckenstein E., Shulgin I.L., Effect of salts and organic additives on the solubility of proteins in aqueous solutions. *Adv. Colloid Interface Sci.* 2006, 123-126, 97–103.

[42] Fuguet E., Ràfols C., Rosés M., Bosch E., Rafols C., Roses M., Critical micelle concentration of surfactants in aqueous buffered and unbuffered systems. *Anal. Chim. Acta* 2005, 548, 95–100.

[43] Turro N.J., Yekta A., Luminescent probes for detergent solutions. A simple procedure for determination of the mean aggregation number of micelles. *J. Am. Chem. Soc.* 1978, 100, 5951–5952.

[44] Reynolds J.A., Tanford C., Binding of dodecyl sulfate to proteins at high binding ratios. Possible implications for the state of proteins in biological membranes. *Proc. Natl. Acad. Sci. U. S. A.* 1970, 66, 1002–1007.

[45] Bhuyan A.K., On the mechanism of SDS-induced protein denaturation. *Biopolymers* 2010, 93, 186–199.

[46] Zhang N., Li L., Effects of common surfactants on protein digestion and matrix-assisted laser desorption/ionization mass spectrometric analysis of the digested peptides using two-layer sample preparation. *Rapid Commun. Mass Spectrom.* 2004, 18, 889–896.

[47] Vieira D.B., Crowell A.M.J., Doucette A.A., Perfluorooctanoic acid and ammonium perfluorooctanoate: volatile surfactants for proteome analysis? *Rapid Commun. Mass Spectrom.* 2012, 26, 523–531.

[48] Asanuma M., Fukuzawa S., Matsuda T., Hirota H., The effect of sodium dodecyl sulfate and anion-exchange silica gel on matrix-assisted laser desorption/ionization mass spectrometric analysis of proteins. *Rapid Commun. Mass Spectrom.* 2009, 23, 1647–1653.

[49] Kadiyala C.S.R., Tomechko S.E., Miyagi M., Perfluorooctanoic acid for shotgun proteomics. *PLoS One* 2010, 5, e15332.

[50] Rundlett K.L., Armstrong D.W., Mechanism of signal suppression by an ionic surfactants in capillary electrophoresis electrospray ionization mass spectrometry. *Anal. Chem.* 1996, 68, 3493–3497.

[51] De Duve C., Beaufay H., A short history of tissue fractionation. *J. Cell Biol.* 1981, 91, 293s–299s.

[52] Cañas B., Piñeiro C., Calvo E., López-Ferrer D., Gallardo J.M., Trends in sample preparation for classical and second generation proteomics. *J. Chromatogr. A* 2007, 1153, 235–258.

[53] Ericsson C., Nistér M., Protein extraction from solid tissue. *Methods Mol. Biol.* 2011, 675, 307–312.

[54] Kushnirov V.V., Rapid and reliable protein extraction from yeast. *Yeast* 2000, 16, 857–860.

[55] Venter J.C., Adams M.D., Myers E.W., Li P.W., et al., The sequence of the human genome. *Science* 2001, 291, 1304–1351.

[56] Almén M.S., Nordström K.J.V., Fredriksson R., Schiöth H.B., Mapping the human membrane proteome: a majority of the human membrane proteins can be classified according to function and evolutionary origin. *BMC Biol.* 2009, 7, 50.

[57] Ahram M., Litou Z.I., Fang R., Al-Tawallbeh G., Estimation of membrane proteins in the human proteome. *In Silico Biol.* 2006, 6, 379–386.

[58] Kohnke P.L., Mulligan S.P., Christopherson R.I., Membrane proteomics for leukemia classification and drug target identification. *Curr. Opin. Mol. Ther.* 2009, 11, 603–610.

[59] Le Maire M., Champeil P., Møller J. V, Interaction of membrane proteins and lipids with solubilizing detergents. *Biochim. Biophys. Acta - Biomembr.* 2000, 1508, 86–111.

[60] Lin Y., Liu H., Liu Z., Wang X., Liang S., Shotgun analysis of membrane proteomes using a novel combinative strategy of solution-based sample preparation coupled with liquid chromatography-tandem mass spectrometry. *J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci.* 2012, 901, 18–24.

[61] Yu Y.Q., Gilar M., Kaska J., Gebler J.C., A rapid sample preparation method for mass spectrometric characterization of N-linked glycans. *Rapid Commun. Mass Spectrom.* 2005, 19, 2331–2336.

[62] Shevchenko A., Tomas H., Havlis J., Olsen J. V, Mann M., In-gel digestion for mass spectrometric characterization of proteins and proteomes. *Nat. Protoc.* 2006, 1, 2856–2860.

[63] Tran J.C., Doucette A.A., Gel-eluted liquid fraction entrapment electrophoresis: An electrophoretic method for broad molecular weight range proteome separation. *Anal. Chem.* 2008, 80, 1568–1573.

[64] Wiśniewski J.R., Zougman A., Nagaraj N., Mann M., Universal sample preparation method for proteome analysis. *Nat. Methods* 2009, 6, 359–362.

[65] Sun D., Wang N., Li L., Integrated SDS removal and peptide separation by strong-cation exchange liquid chromatography for SDS-assisted shotgun proteome analysis. *J. Proteome Res.* 2012, 11, 818–828.

[66] Wu L.-L., Gao H.-W., Gao N.-Y., Chen F.-F., Chen L., Interaction of perfluorooctanoic acid with human serum albumin. *BMC Struct. Biol.* 2009, 9, 31.

[67] Sickmann A., Dormeyer W., Wortelkamp S., Woitalla D., Kuhn W., Meyer H.E., Identification of proteins from human cerebrospinal fluid, separated by two-dimensional polyacrylamide gel electrophoresis. *Electrophoresis* 2000, 21, 2721–2728.

[68] Manza L.L., Stamer S.L., Ham A.-J.L., Codreanu S.G., Liebler D.C., Sample preparation and digestion for proteomic analyses using spin filters. *Proteomics* 2005, 5, 1742–1745.

[69] Wiśniewski J.R., Zielinska D.F., Mann M., Comparison of ultrafiltration units for proteomic and N-glycoproteomic analysis by the filter-aided sample preparation method. *Anal. Biochem.* 2011, 410, 307–9.

[70] Wiśniewski J.R., Rakus D., Multi-enzyme digestion FASP and the "Total Protein Approach"-based absolute quantification of the *Escherichia coli* proteome. *J. Proteomics* 2014, 109C, 322–331.

[71] Erde J., Loo R.R.O., Loo J.A., Enhanced FASP (eFASP) to increase proteome coverage and sample recovery for quantitative proteomic experiments. *J. Proteome Res.* 2014, 13, 1885–1895.

[72] Puchades M., Westman A., Blennow K., Davidsson P., Removal of sodium dodecyl sulfate from protein samples prior to matrix-assisted laser desorption/ionization mass spectrometry. *Rapid Commun. Mass Spectrom.* 1999, 13, 344–349.

[73] Vissers J.P.C., Chervet J.P., Salzmann J.P., Sodium dodecyl sulphate removal from tryptic digest samples for on-line capillary liquid chromatography electrospray mass spectrometry. *J. Mass Spectrom.* 1996, 31, 1021–1027.

[74] Zhou J.-Y., Dann G.P., Shi T., Wang L., Gao X., Su D., Nicora C.D., Shukla A.K., Moore R.J., Liu T., Camp II D.G., Smith R.D., Qian W.-J., Simple Sodium Dodecyl Sulfate-Assisted Sample Preparation Method for LC-MS-Based Proteomics Applications. *Anal. Chem.* 2012, 84, 2862–2867.

[75] Jenö P., Scherer P.E., Manning-Krieg U., Horst M., Desalting electroeluted proteins with hydrophilic interaction chromatography. *Anal. Biochem.* 1993, 215, 292–298.

[76] Konigsberg W.H., Henderson L., Removal of sodium dodecyl sulfate from proteins by ion-pair extraction. *Methods Enzymol.* 1983, 91, 254–259.

[77] Andersen P., Heron I., Simultaneous electroelution of whole SDS-polyacrylamide gels for the direct cellular analysis of complex protein mixtures. *J. Immunol. Methods* 1993, 161, 29–39.

[78] Chick H., Martin C.J., The precipitation of egg-albumin by ammonium sulphate. A contribution to the theory of the "salting-out" of proteins. *Biochem. J.* 1913, 7, 380–398.

[79] Sivaraman T., Kumar T.K., Jayaraman G., Yu C., The mechanism of 2,2,2-trichloroacetic acid-induced protein precipitation. *J. Protein Chem.* 1997, 16, 291–297.

[80] Zeppezauer M., Brishammar S., Protein precipitation by uncharged water-soluble polymers. *Biochim. Biophys. Acta - Biophys. Incl. Photosynth.* 1965, 94, 581–583.

[81] Kunz W., Henle J., Ninham B.W.W., "Zur Lehre von der Wirkung der Salze" (about the science of the effect of salts): Franz Hofmeister's historical papers RID D-2847-2009. *Curr. Opin. Colloid Interface Sci.* 2004, 9, 19–37.

[82] Jiang L., He L., Fountoulakis M., Comparison of protein precipitation methods for sample preparation prior to proteomic analysis. *J. Chromatogr. A.* 2004, 1023, 317–320.

[83] Ma J.G., Stoter G., Verweij J., Schellens J.H.M., Comparison of ethanol plasma protein precipitation with plasma ultrafiltration and trichloroacetic acid protein precipitation for the measurement of unbound platinum concentrations. *Cancer Chemother. Pharmacol.* 1996, 38, 391–394.

[84] Kay R., Barton C., Ratcliffe L., Matharoo-Ball B., Brown P., Roberts J., Teale P., Creaser C., Enrichment of low molecular weight serum proteins using acetonitrile precipitation for mass spectrometry based proteomic analysis. *Rapid Commun. Mass Spectrom.* 2008, 22, 3255–3260.

[85] Polson C., Sarkar P., Incledon B., Raguvaran V., Grant R., Optimization of protein precipitation based upon effectiveness of protein removal and ionization effect in liquid chromatography-tandem mass spectrometry. *J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci.* 2003, 785, 263–275.

[86] Scopes R.K., *Protein Purification*, Springer New York, New York, NY 1994.

[87] Piettre, Vila A., On the separation of the proteins of the serum. *Comptes Rendus Hebd. Des Seances L Acad. Des Sci.* 1920, 170, 1466–1468.

[88] Jiang L., He L., Fountoulakis M., Comparison of protein precipitation methods for sample preparation prior to proteomic analysis. *J. Chromatogr. A* 2004, 1023, 317–320.

[89] Biddlecombe R.A., Pleasance S., Automated protein precipitation by filtration in the 96-well format. *J. Chromatogr. B* 1999, 734, 257–265.

[90] Englard S., Seifter S., Precipitation techniques. *Methods Enzymol.* 1990, 182, 285–300.

[91] Zayas J.F., *Functionality of Proteins in Food*, Springer Berlin Heidelberg, Berlin, Heidelberg 1997.

[92] Green A.A., Hughes W.L., Protein fractionation on the basis of solubility in aqueous solutions of salts and organic solvents. *Methods Enzymol.* 1955, 1, 67–90.

[93] Van Oss C.J., On the mechanism of the cold ethanol precipitation method of plasma protein fractionation. *J. Protein Chem.* 1989, 8, 661–668.

[94] Choudhary C., Kumar C., Gnad F., Nielsen M.L., Rehman M., Walther T.C., Olsen J.V., Mann M., Lysine acetylation targets protein complexes and co-regulates major cellular functions. *Science* 2009, 325, 834–840.

[95] Fu Q., Garnham C.P., Elliott S.T., Bovenkamp D.E., Van Eyk J.E., A robust, streamlined, and reproducible method for proteomic analysis of serum by delipidation, albumin and IgG depletion, and two-dimensional gel electrophoresis. *Proteomics* 2005, 5, 2656–2664.

[96] Cohn E.J., Strong L.E., Hughes W.L., Mulford D.J., Ashworth J.N., Melin M., Taylor H.L., Preparation and Properties of Serum and Plasma Proteins. IV. A System for the Separation into Fractions of the Protein and Lipoprotein Components of Biological Tissues and Fluids. *J. Am. Chem. Soc.* 1946, 68, 459–475.

[97] Cohn E.J., Gurd F.R.N., Surgenor D.M., Barnes B.A., Brown R.K., Derouaux G., Gillespie J.M., Kahnt F.W., Lever W.F., Liu C.H., Mittelman D., Mouton R.F., Schmid K., Uroma E., A System for the Separation of the Components of Human Blood: Quantitative Procedures for the Separation of the Protein Components of Human Plasma. *J. Am. Chem. Soc.* 1950, 72, 465–474.

[98] Thongboonkerd V., McLeish K.R., Arthur J.M., Klein J.B., Proteomic analysis of normal human urinary proteins isolated by acetone precipitation or ultracentrifugation. *Kidney Int.* 2002, 62, 1461–1469.

[99] Merrell K., Southwick K., Graves S.W., Esplin M.S., Lewis N.E., Thulin C.D., Analysis of low-abundance, low-molecular-weight serum proteins using mass spectrometry. *J. Biomol. Tech.* 2004, 15, 238–248.

[100] Barritault D., Expert-Bezancon A., Guérin M.F., Hayes D., The use of acetone precipitation in the isolation of ribosomal proteins. *Eur. J. Biochem.* 1976, 63, 131–135.

[101] Hurkman W.J., Tanaka C.K., Solubilization of plant membrane proteins for analysis by two-dimensional gel electrophoresis. *Plant Physiol.* 1986, 81, 802–806.

[102] Yuan X., Russell T., Wood G., Desiderio D.M., Analysis of the human lumbar cerebrospinal fluid proteome. *Electrophoresis* 2002, 23, 1185–1196.

[103] Hansson S.F., Puchades M., Blennow K., Sjögren M., Davidsson P., Validation of a prefractionation method followed by two-dimensional electrophoresis - Applied to cerebrospinal fluid proteins from frontotemporal dementia patients. *Proteome Sci.* 2004, 2, 7.

[104] Thongboonkerd V., Chutipongtanate S., Kanlaya R., Systematic evaluation of sample preparation methods for gel-based human urinary proteomics: quantity, quality, and variability. *J. Proteome Res.* 2006, 5, 183–191.

[105] Srivastava O.P., Srivastava K., Purification of α-crystallin from human lenses by acetone precipitation method. *Curr. Eye Res.* 1998, 17, 1074–1081.

[106] Ashri N.Y., Abdel-Rehim M., Sample treatment based on extraction techniques in biological matrices. *Bioanalysis* 2011, 3, 2003–2018.

[107] Simpson D.M., Beynon R.J., Acetone precipitation of proteins and the modification of peptides. *J. Proteome Res.* 2010, 9, 444–450.

[108] Crowell A.M.J., Proteome Purification: Studying the Synergistic Effects of Salt and Acetone upon Protein Precipitation. Honours Thesis, Dalhousie University, 2012.

[109] *The QIAGEN Guide to Good Microbiological Practice. Part III: Growth of E. coli cultures*, QIAGEN News, 1999.

[110] Wu F., Sun D., Wang N., Gong Y., Li L., Comparison of surfactant-assisted shotgun methods using acid-labile surfactants and sodium dodecyl sulfate for membrane proteome analysis. *Anal. Chim. Acta* 2011, 698, 36–43.

[111] Ulm A.H., Miller F., An operation to produce experimental reversible hydronephrosis in dogs. *J. Urol.* 1962, 88, 337–341.

[112] Harlow E., Lane D., *Antibodies: A Laboratory Manual.*, Cold Spring Harbor Lab Press. New York, 1988

[113] Laemmli U.K., Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970, 227, 680–685.

[114] Shevchenko A., Wilm M., Vorm O., Mann M., Mass spectrometric sequencing of proteins from silver stained polyacrylamide gels. *Anal. Chem.* 1996, 68, 850–858.

[115] Neuhoff V., Arold N., Taube D., Ehrhardt W., Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250. *Electrophoresis* 1988, 9, 255–262.

[116] Hayashi K., A rapid determination of sodium dodecyl sulfate with methylene blue. *Anal. Biochem.* 1975, 67, 503–506.

[117] Wang N., Xie C., Young J.B., Li L., Off-line two-dimensional liquid chromatography with maximized sample loading to reversed-phase liquid chromatography-electrospray ionization tandem mass spectrometry for shotgun proteome analysis. *Anal. Chem.* 2009, 81, 1049–1060.

[118] Orton D.J., Wall M.J., Doucette A.A., Dual LC-MS platform for high-throughput proteome analysis. *J. Proteome Res.* 2013, 12, 5963–5970.

[119] Yates J.R., Eng J.K., McCormack A.L., Schieltz D., Method to correlate tandem mass spectra of modified peptides to amino acid sequences in the protein database. *Anal. Chem.* 1995, 67, 1426–1436.

[120] Liu H.B., Sadygov R.G., Yates J.R., A model for random sampling and estimation of relative protein abundance in shotgun proteomics. *Anal. Chem.* 2004, 76, 4193–4201.

[121] Orton D.J., Doucette A.A., Maksym G.N., MacLellan D.L., Proteomic analysis of rat proximal tubule cells following stretch-induced apoptosis in an in vitro model of kidney obstruction. *Spec. Issue Can Proteomics Fill Gap Between Genomics Phenotypes?* 2014, 100, 125–135.

[122] Huang D.W., Sherman B.T., Lempicki R.A., Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat.Protocols* 2008, 4, 44–57.

[123] Huang D.W., Sherman B.T., Lempicki R.A., Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. *Nucleic Acids Res.* 2009, 37, 1–13.

[124] Piettre, M., Vila A., Sur la separation des proteines du serum. *C. R. Hebd. Seances Acad. Sci.* 1920, 170, 1466–1468.

[125] Saha S., Fractional precipitation of plasma proteome by ammonium sulphate:case studies in leukemia and thalassemia. *J. Proteomics Bioinform.* 2012, 05, 163–171.

[126] Natarajan S., Xu C., Caperna T.J., Garrett W.M., Comparison of protein solubilization methods suitable for proteomic analysis of soybean seed proteins. *Anal. Biochem.* 2005, 342, 214–220.

[127] Burnouf T., Modern plasma fractionation. *Transfus. Med. Rev.* 2007, 21, 101–117.

[128] Subden R.E., Brown R.G., Noble A.C., Determination of histamines in wines and musts by reversed-phase high-performance liquid chromatography. *J. Chromatogr.* 1978, 166, 310–312.

[129] Hennion M.-C., Solid-phase extraction: method development, sorbents, and coupling with liquid chromatography. *J. Chromatogr. A* 1999, 856, 3–54.

[130] Spear J., Maximizing Proteome Recovery during SDS Removal through Solvent Precipitation. Honours Thesis, Dalhousie Univeristy, 2011.

[131] Marcus Y., Hefter G., Ion pairing. *Chem. Rev.* 2006, 106, 4585–4621.

[132] Shakhparonov M., Akhadov Y., Dielectric properties and molecular structure of water-acetone solutions. *J. Struct. Chem.* 1965, 6, 15–19.

[133] Piskur J., Rupprecht A., Aggregated DNA in ethanol solution. *FEBS Lett.* 1995, 375, 174–178.

[134] Nagaraj N., Lu A., Mann M., Wiśniewski J.R., Detergent-based but gel-free method allows identification of several hundred membrane proteins in single LC-MS runs. *J. Proteome Res.* 2008, 7, 5028–5032.

[135] Kawasaki H., Suzuki K., Separation of peptides dissolved in a sodium dodecyl sulfate solution by reversed-phase liquid chromatography: removal of sodium dodecyl sulfate from peptides using an ion-exchange precolumn. *Anal. Biochem.* 1990, 186, 264–268.

[136] Choi N.S., Hahm J.H., Maeng P.J., Kim S.H., Comparative study of enzyme activity and stability of bovine and human plasmins in electrophoretic reagents, beta-mercaptoethanol, DTT, SDS, triton X-100, and urea. *J. Biochem. Mol. Biol.* 2005, 38, 177–181.

[137] Sharma R., Dill B.D., Chourey K., Shah M., VerBerkmoes N.C., Hettich R.L., Coupling a detergent lysis/cleanup methodology with intact protein fractionation for enhanced proteome characterization. *J. Proteome Res.* 2012, 11, 6008–6018.

[138] Nguyen H.T., Herndon C.D.A., Cooper C., Gatti J., Kirsch A., Kokorowski P., Lee R., Perez-Brayf-Ield M., Metcalfe P., Yerkes E., Cendron M., Campbell J.B., Perez-Brayfield M., The Society for Fetal Urology consensus statement on the evaluation and management of antenatal hydronephrosis. *J. Pediatr. Urol.* 2010, 6, 212–231.

[139] Magdeldin S., Moresco J.J., Yamamoto T., Yates J.R., Off-Line Multidimensional Liquid Chromatography and Auto Sampling Result in Sample Loss in LC/LC-MS/MS. *J. Proteome Res.* 2014, 13, 3826–3836.

[140] el-Dahr S.S., Dipp S., Molecular aspects of kallikrein and kininogen in the maturing kidney. *Pediatr. Nephrol.* 1993, 7, 646–651.

[141] Zager R.A., Vijayan A., Johnson A.C.M., Proximal tubule haptoglobin gene activation is an integral component of the acute kidney injury "stress response". *Am. J. Physiol. Renal Physiol.* 2012, 303, F139–F148.

[142] Ma J., Hou C., Sun L., Tao D., Zhang Y., Shan Y., Liang Z., Zhang L., Yang L., Zhang Y., Coupling formic acid assisted solubilization and online immobilized pepsin digestion with strong cation exchange and microflow reversed-phase liquid chromatography with electrospray ionization tandem mass spectrometry for integral membrane proteome analysis. *Anal. Chem.* 2010, 82, 9622–9625.

[143] Parikh C.R., Mishra J., Thiessen-Philbrook H., Dursun B., Ma Q., Kelly C., Dent C., Devarajan P., Edelstein C.L., Urinary IL-18 is an early predictive biomarker of acute kidney injury after cardiac surgery. *Kidney Int.* 2006, 70, 199–203.

[144] Portilla D., Dent C., Sugaya T., Nagothu K.K., Kundi I., Moore P., Noiri E., Devarajan P., Liver fatty acid-binding protein as a biomarker of acute kidney injury after cardiac surgery. *Kidney Int.* 2008, 73, 465–472.

[145] Diamandis E.P., Mass spectrometry as a diagnostic and a cancer biomarker discovery tool: opportunities and potential limitations. *Mol. Cell. Proteomics* 2004, 3, 367–378.

[146] Blennow K., Cerebrospinal fluid protein biomarkers for Alzheimer's disease. *NeuroRx* 2004, 1, 213–225.

[147] Veenstra T.D., Conrads T.P., Hood B.L., Avellino A.M., Ellenbogen R.G., Morrison R.S., Biomarkers: mining the biofluid proteome. *Mol. Cell. Proteomics* 2005, 4, 409–418.

[148] Thadikkaran L., Siegenthaler M.A., Crettaz D., Queloz P.-A., Schneider P., Tissot J.-D., Recent advances in blood-related proteomics. *Proteomics* 2005, 5, 3019–3034.

[149] Issaq H.J., Xiao Z., Veenstra T.D., Serum and plasma proteomics. *Chem. Rev.* 2007, 107, 3601–3620.

[150] Zhang H., Liu A.Y., Loriaux P., Wollscheid B., Zhou Y., Watts J.D., Aebersold R., Mass spectrometric detection of tissue proteins in plasma. *Mol. Cell. Proteomics* 2007, 6, 64–71.

[151] Anderson N.L., Polanski M., Pieper R., Gatlin T., Tirumalai R.S., Conrads T.P., Veenstra T.D., Adkins J.N., Pounds J.G., Fagan R., Lobley A., The human plasma proteome: a nonredundant list developed by combination of four separate sources. *Mol. Cell. Proteomics* 2004, 3, 311–326.

[152] Lee D.H., Kim J.W., Jeon S.Y., Park B.K., Han B.G., Proteomic analysis of the effect of storage temperature on human serum. *Ann. Clin. Lab. Sci.* 2010, 40, 61–70.

[153] Insenser M., Martínez-García M.Á., Nieto R.M., San-Millán J.L., Escobar-Morreale H.F., Impact of the storage temperature on human plasma proteomic analysis: implications for the use of human plasma collections in research. *Proteomics. Clin. Appl.* 2010, 4, 739–744.

[154] States D.J., Omenn G.S., Blackwell T.W., Fermin D., Eng J., Speicher D.W., Hanash S.M., Challenges in deriving high-confidence protein identifications from data gathered by a HUPO plasma proteome collaborative study. *Nat. Biotechnol.* 2006, 24, 333–338.

[155] Adkins J.N., Varnum S.M., Auberry K.J., Moore R.J., Angell N.H., Smith R.D., Springer D.L., Pounds J.G., Toward a human blood serum proteome - Analysis by multidimensional separation coupled with mass spectrometry. *Mol. Cell. Proteomics* 2002, 1, 947–955.

[156] Luczak M., Marczak L., Stobiecki M., Optimization of plasma sample pretreatment for quantitative analysis using iTRAQ labeling and LC-MALDI-TOF/TOF. *PLoS One* 2014, 9, e101694.

[157] Wang Y.Y., Cheng P., Chan D.W., A simple affinity spin tube filter method for removing high-abundant common proteins or enriching low-abundant biomarkers for serum proteomic analysis. *Proteomics* 2003, 3, 243–248.

[158] Björhall K., Miliotis T., Davidsson P., Comparison of different depletion strategies for improved resolution in proteomic analysis of human serum samples. *Proteomics* 2005, 5, 307–317.

[159] Fujii K., Nakano T., Kawamura T., Usui F., Bando Y., Wang R., Nishimura T., Multidimensional protein profiling technology and its application to human plasma proteome. *J. Proteome Res.* 2004, 3, 712–718.

[160] Mahn A., Ismail M., Depletion of highly abundant proteins in blood plasma by ammonium sulfate precipitation for 2D-PAGE analysis. *J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci.* 2011, 879, 3645–3648.

[161] Tirumalai R.S., Chan K.C., Prieto D.A., Issaq H.J., Conrads T.P., Veenstra T.D., Characterization of the low molecular weight human serum proteome. *Mol. Cell. Proteomics* 2003, 2, 1096–1103.

[162] Burtis, C. A., Ashwood E.R., *Tietz Fundamentals of Clinical Chemistry*, W. B. Saunders Company, Philadelphia, PA 2001.

[163] Greening D.W., Simpson R.J., A centrifugal ultrafiltration strategy for isolating the low-molecular weight (<or=25K) component of human plasma proteome. *J. Proteomics* 2010, 73, 637–648.

[164] Mostovenko E., Protein fractionation for quantitative plasma proteomics by semi-selective precipitation. *J. Proteomics Bioinform.* 2012, 5, 217–221.

[165] Georgiou H.M., Rice G.E., Baker M.S., Proteomic analysis of human plasma: failure of centrifugal ultrafiltration to remove albumin and other high molecular weight proteins. *Proteomics* 2001, 1, 1503–1506.

[166] Jain R.K., Transport of molecules across tumor vasculature. *Cancer Metastasis Rev.* 1987, 6, 559–593.

[167] Yuan F., Dellian M., Fukumura D., Leunig M., Berk D.A., Torchilin V.P., Jain R.K., Vascular permeability in a human tumor xenograft: molecular size dependence and cutoff size. *Cancer Res.* 1995, 55, 3752–3756.

[168] Dellian M., Yuan F., Trubetskoy V.S., Torchilin V.P., Jain R.K., Vascular permeability in a human tumour xenograft: molecular charge dependence. *Br. J. Cancer* 2000, 82, 1513–1518.

[169] Rai A.J., Gelfand C.A., Haywood B.C., Warunek D.J., Yi J., Schuchard M.D., Mehigh R.J., Cockrill S.L., Scott G.B.I., Tammen H., Schulz-Knappe P., Speicher D.W., Vitzthum F., Haab B.B., Siest G., Chan D.W., HUPO Plasma Proteome Project specimen collection and handling: towards the standardization of parameters for plasma proteome samples. *Proteomics* 2005, 5, 3262–3277.

[170] Otzen D., Protein-surfactant interactions: A tale of many states. *Biochim. Biophys. Acta-Proteins Proteomics* 2011, 1814, 562–591.

[171] Proc J.L., Kuzyk M.A., Hardie D.B., Yang J., Smith D.S., Jackson A.M., Parker C.E., Borchers C.H., A quantitative study of the effects of chaotropic agents, surfactants, and solvents on the digestion efficiency of human plasma proteins by trypsin. *J. Proteome Res.* 2010, 9, 5422–5437.

[172] Hoffman S.A., Joo W.-A., Echan L.A., Speicher D.W., Higher dimensional (Hi-D) separation strategies dramatically improve the potential for cancer biomarker detection in serum and plasma. *J. Chromatogr. B. Analyt. Technol. Biomed. Life Sci.* 2007, 849, 43–52.

[173] Lee J.E., Kellie J.F., Tran J.C., Tipton J.D., Catherman A.D., Thomas H.M., Ahlf D.R., Durbin K.R., Vellaichamy A., Ntai I., Marshall A.G., Kelleher N.L., A robust two-dimensional separation for top-down tandem mass spectrometry of the low-mass proteome. *J. Am. Soc. Mass Spectrom.* 2009, 20, 2183–2191.

[174] Masuda T., Tomita M., Ishihama Y., Phase transfer surfactant-aided trypsin digestion for membrane proteome analysis. *J. Proteome Res.* 2008, 7, 731–740.

[175] Lin Y., Zhou J., Bi D., Chen P., Wang X., Liang S., Sodium-deoxycholate-assisted tryptic digestion and identification of proteolytically resistant proteins. *Anal. Biochem.* 2008, 377, 259–266.

[176] Lin Y., Huo L., Liu Z., Li J., Liu Y., He Q., Wang X., Liang S., Sodium laurate, a novel protease- and mass spectrometry-compatible detergent for mass spectrometry-based membrane proteomics. *PLoS One* 2013, 8, e59779.

Appendix A:

Supplementary Tables

Table A.1: Proteins identified in the rat kidneys which were found to be significantly up regulated in obstructed kidneys compared to the control

UniProt Accession	Protein Name	PSMs Control	PSMs Obstructed
P47875	Cysteine and glycine-rich protein 1	0±0	3±0
P68511	14-3-3 protein eta	0±0	6±1
Q5XI73	Rho GDP-dissociation inhibitor 1	0±0	5±1
P62329	Thymosin beta-4	0±0	3±1
P04897	Guanine nucleotide-binding protein G(i) subunit alpha-2	0±0	3±1
P29457	Serpin H1	0±0	3±1
P68035	Actin, alpha cardiac muscle 1	0±0	46±9
P61589	Transforming protein RhoA	0±0	3±1
Q91ZN1	Coronin-1A	0±0	3±1
F1M983	Protein Cfh	0±0	3±1
Q07439	Heat shock 70 kDa protein 1A/1B	0±0	7±2
E2RUH2	Protein LOC100360501	0±0	2±1
G3V8L3	Lamin A, isoform CRA_b	0±0	2±1
Q5XI38	Lymphocyte cytosolic protein 1	0±0	5±1
Q4V7C7	Actin-related protein 3	0±0	4±1
Q5RKI0	WD repeat-containing protein 1	0±0	4±1
G3V6P7	Myosin, heavy polypeptide 9, non-muscle	3±1	14±1
P31232	Transgelin	0±0	19±5
Q91Y81	Septin-2	0±0	3±1
Q3MIE4	Synaptic vesicle membrane protein VAT-1 homolog	0±0	3±1
Q10758	Keratin, type II cytoskeletal 8	0±0	3±1
P06866	Haptoglobin	0±0	3±1
Q5BJY9	Keratin, type I cytoskeletal 18	0±0	3±1
P08649	Complement C4	0±0	3±1
G3V7Q7	IQ motif containing GTPase activating protein 1 (Predicted), isoform CRA_b	0±0	3±1
Q5M860	Protein Arhgdib	0±0	4±2
G3V7K3	Ceruloplasmin	0±0	22±8
G3V8C3	Vimentin	0±0	11±5
Q03626	Murinoglobulin-1	0±0	16±7
Q08163	Adenylyl cyclase-associated protein 1	0±0	4±2
G3V852	Protein Tln1	2±2	18±3

UniProt Accession	Protein Name	PSMs Control	PSMs Obstructed
M0R9D5	Protein Ahnak	0±0	7±3
P10959	Carboxylesterase 1C	0±0	3±1
Q62952	Dihydropyrimidinase-related protein 3	0±0	3±2
P85972	Vinculin	0±0	12±6
M0RBF1	Complement C3	4±1	16±4
G3V712	Keratin complex 2, basic, gene 7, isoform CRA_a	0±0	4±2
Q9QX79	Fetuin-B	0±0	5±2
D3ZB30	Polypyrimidine tract binding protein 1, isoform CRA_c	0±0	4±2
C0JPT7	Filamin alpha	0±0	7±4
Q63279	Keratin, type I cytoskeletal 19	0±0	5±3
Q5XFX0	Transgelin-2	5±2	18±4
Q9JKB7	Guanine deaminase	0±0	6±3
M0RD14	Pyruvate kinase	6±3	18±2
P60711	Actin, cytoplasmic 1	45±13	92±4
P68255	14-3-3 protein theta	4±1	8±1
P01048	T-kininogen 1	1±1	11±5
P08932	T-kininogen 2	1±1	11±5
P07335	Creatine kinase B-type	3±1	13±5
P18418	Calreticulin	5±2	12±2
P11598	Protein disulfide-isomerase A3	3±1	8±2
Q63081	Protein disulfide-isomerase A6	1±1	7±1
P17475	Alpha-1-antiproteinase	1±1	8±3
Q63610	Tropomyosin alpha-3 chain	0±0	7±5
D4ACV3	Histone H2A	10±1	13±1
P05545	Serine protease inhibitor A3K	2±2	10±3
Q63041	Alpha-1-macroglobulin	2±2	10±2
P61980	Heterogeneous nuclear ribonucleoprotein K	2±2	7±1
P09495	Tropomyosin alpha-4 chain	1±1	14±8
P45592	Cofilin-1	5±2	9±0
P24268	Cathepsin D	1±1	6±1
P20059	Hemopexin	2±3	16±6
I6L9G6	Protein Tardbp	0±0	2±2
Q3T1J1	Eukaryotic translation initiation factor 5A-1	0±0	2±2
Q62636	Ras-related protein Rap-1b	0±0	2±2
Q5XI19	Fermitin family homolog 2 (Drosophila)	0±0	2±2
P10960	Sulfated glycoprotein 1	0±0	2±2
Q9Z1P2	Alpha-actinin-1	0±0	4±4
P13832	Myosin regulatory light chain RLC-A	0±0	4±3

UniProt Accession	Protein Name	PSMs Control	PSMs Obstructed
P02651	Apolipoprotein A-IV	0±0	2±2
B5DFD8	SH3 domain-binding glutamic acid-rich-like protein	0±0	2±2
P36972	Adenine phosphoribosyltransferase	0±0	2±2
P62963	Profilin-1	4±2	9±1
Q5U300	Ubiquitin-like modifier-activating enzyme 1	1±2	7±1
D3ZWE0	Histone H2A	0±0	4±4
P47942	Dihydropyrimidinase-related protein 2	1±1	8±4
Q64119	Myosin light polypeptide 6	3±1	6±2
Q07936	Annexin A2	0±0	8±7
G3V8L9	Polymerase I and transcript release factor	0±0	3±3
P02770	Serum albumin	137±44	297±93
P51886	Lumican	0±0	4±4
D3ZZT9	Protein Col14a1	0±0	3±3
D4A111	Protein Col6a3	0±0	3±3
P47853	Biglycan	0±0	2±2
O35567	Bifunctional purine biosynthesis protein PURH	0±0	2±2
D3ZQ25	Fibulin 1 (Predicted)	0±0	2±2
D3Z9F8	Collagen alpha-1(XII) chain	0±0	2±2
P02650	Apolipoprotein E	2±2	7±3
P34058	Heat shock protein HSP 90-beta	8±2	14±3

Table A.2: Proteins identified in the rat kidneys which were found to be significantly down regulated in obstructed kidneys compared to the control

UniProt Accession	Protein Name	PSMs Control	PSMs Obstructed
P26772	10 kDa heat shock protein, mitochondrial	4±0	0±0
Q9JJ19	Na(+)/H(+) exchange regulatory cofactor NHE-RF1	3±0	0±0
P80254	D-dopachrome decarboxylase	2±0	0±0
P13221	Aspartate aminotransferase, cytoplasmic	2±0	0±0
P14604	Enoyl-CoA hydratase, mitochondrial	7±1	0±0
P07171	Calbindin	3±1	0±0
Q9WUS0	GTP:AMP phosphotransferase AK4, mitochondrial	3±1	0±0
Q811X6	Lambda-crystallin homolog	9±2	0±0
D4A5L9	Protein LOC690675	13±3	0±0
Q64573	Liver carboxylesterase 4	11±2	0±0
D3ZWT8	Uncharacterized protein	5±1	0±0
P07340	Sodium/potassium-transporting ATPase subunit beta-1	7±2	0±0
P19468	Glutamate--cysteine ligase catalytic subunit	15±3	0±0
UniProt Accession	Protein Name	PSMs Control	PSMs Obstructed
Q5I0P2	Glycine cleavage system H protein, mitochondrial	3±1	0±0
Q7TP48	Adipocyte plasma membrane-associated protein	3±1	0±0
P00502	Glutathione S-transferase alpha-1	5±1	0±0
P07861	Neprilysin	7±2	0±0
P29410	Adenylate kinase 2, mitochondrial	2±1	0±0
D4A1J4	3-hydroxybutyrate dehydrogenase type 2	2±1	0±0
D4A2K1	Protein Hoga1	5±1	0±0
O08557	N(G),N(G)-dimethylarginine dimethylaminohydrolase 1	4±1	0±0
Q64319	Neutral and basic amino acid transport protein rBAT	4±1	0±0
O35077	Glycerol-3-phosphate dehydrogenase [NAD(+)], cytoplasmic	6±2	0±0
Q68FY0	Cytochrome b-c1 complex subunit 1, mitochondrial	6±2	0±0
Q5U2Q3	Ester hydrolase C11orf54 homolog	9±3	0±0
Q4KLZ6	Bifunctional ATP-dependent dihydroxyacetone kinase/FAD-AMP lyase (cyclizing)	4±1	0±0
Q5M9H2	Acyl-Coenzyme A dehydrogenase, very long chain	4±1	0±0

UniProt Accession	Protein Name	PSMs Control	PSMs Obstructed
Q711G3	Isoamyl acetate-hydrolyzing esterase 1 homolog	4±1	0±0
F7ESM5	Nitrilase 1, isoform CRA_a	4±1	0±0
P00786	Pro-cathepsin H	10±2	3±1
P09034	Argininosuccinate synthase	11±4	0±0
P25093	Fumarylacetoacetase	12±4	0±0
Q6AYQ8	Acylpyruvase FAHD1, mitochondrial	3±1	0±0
P07895	Superoxide dismutase [Mn], mitochondrial	3±1	0±0
Q6PDU7	ATP synthase subunit g, mitochondrial	3±1	0±0
Q5M884	Protein Eci3	3±1	0±0
P70473	Alpha-methylacyl-CoA racemase	3±1	0±0
Q68FU3	Electron transfer flavoprotein subunit beta	3±1	0±0
Q9EQS4	Cystathionase (Cystathionine gamma-lyase)	14±5	0±0
P14740	Dipeptidyl peptidase 4	5±2	0±0
Q4KLP0	Probable 2-oxoglutarate dehydrogenase E1 component DHKTD1, mitochondrial	3±1	0±0
Q9WVK7	Hydroxyacyl-coenzyme A dehydrogenase, mitochondrial	4±2	0±0
P50554	4-aminobutyrate aminotransferase, mitochondrial	5±2	0±0
G3V6C2	Protein Hgd	5±2	0±0
P04176	Phenylalanine-4-hydroxylase	5±2	0±0
P07154	Cathepsin L1	5±2	0±0
O70490	Acyl-coenzyme A synthetase ACSM2, mitochondrial	6±2	0±0
P04636	Malate dehydrogenase, mitochondrial	25±5	9±1
P46413	Glutathione synthetase	4±2	0±0
O88989	Malate dehydrogenase, cytoplasmic	14±2	5±1
Q9QX71	Napsin	4±2	0±0
Q6AYT0	Quinone oxidoreductase	4±2	0±0
Q63530	Phosphotriesterase-related protein	4±2	0±0
Q5PQT3	Glycine N-acyltransferase	3±2	0±0
Q5M7T9	Threonine synthase-like 2	3±2	0±0
P04182	Ornithine aminotransferase, mitochondrial	31±9	3±2
Q64428	Trifunctional enzyme subunit alpha, mitochondrial	4±2	0±0
Q07523	Hydroxyacid oxidase 2	52±4	3±5
P27867	Sorbitol dehydrogenase	5±3	0±0
Q6P6R2	Dihydrolipoyl dehydrogenase, mitochondrial	10±2	1±1
Q06647	ATP synthase subunit O, mitochondrial	8±2	1±1
P32755	4-hydroxyphenylpyruvate dioxygenase	5±3	0±0

UniProt Accession	Protein Name	PSMs Control	PSMs Obstructed
P24329	Thiosulfate sulfurtransferase	9±1	1±1
P56574	Isocitrate dehydrogenase [NADP], mitochondrial	19±6	3±2
P48508	Glutamate--cysteine ligase regulatory subunit	9±2	1±1
Q497B0	Omega-amidase NIT2	9±3	1±1
Q02974	Ketohexokinase	9±2	1±1
B0BMW2	3-hydroxyacyl-CoA dehydrogenase type-2	7±5	0±0
P63039	60 kDa heat shock protein, mitochondrial	22±5	6±3
Q9QYU4	Thiomorpholine-carboxylate dehydrogenase	4±3	0±0
G3V9U2	3-ketoacyl-CoA thiolase, mitochondrial	13±3	1±2
Q64565	Alanine--glyoxylate aminotransferase 2, mitochondrial	12±5	1±1
P98158	Low-density lipoprotein receptor-related protein 2	32±3	3±5
P00507	Aspartate aminotransferase, mitochondrial	9±2	2±2
Q9WUW9	Sulfotransferase 1C2A	8±2	1±1
G3V7W7	Alanyl (Membrane) aminopeptidase	15±3	1±2
Q6JE36	Protein NDRG1	4±3	0±0
Q64602	Kynurenine/alpha-aminoadipate aminotransferase, mitochondrial	9±4	1±1
F8WFI0	Delta-1-pyrroline-5-carboxylate dehydrogenase, mitochondrial	10±5	1±1
P19112	Fructose-1,6-bisphosphatase 1	20±1	2±4
P15999	ATP synthase subunit alpha, mitochondrial	24±8	9±3
P07314	Gamma-glutamyltranspeptidase 1	13±4	1±2
Q9ER34	Aconitate hydratase, mitochondrial	20±10	5±1
B2GV06	Succinyl-CoA:3-ketoacid coenzyme A transferase 1, mitochondrial	11±7	1±1
P38918	Aflatoxin B1 aldehyde reductase member 3	5±4	0±0
Q68FZ8	Propionyl coenzyme A carboxylase, beta polypeptide	3±3	0±0
P15651	Short-chain specific acyl-CoA dehydrogenase, mitochondrial	2±2	0±0
P35435	ATP synthase subunit gamma, mitochondrial	2±2	0±0
P14173	Aromatic-L-amino-acid decarboxylase	2±2	0±0
G3V827	Cysteine conjugate-beta lyase 1, isoform CRA_a	2±2	0±0
M0RAP9	Uncharacterized protein	2±2	0±0
G3V709	Nicotinate phosphoribosyltransferase	2±2	0±0
P13086	Succinyl-CoA ligase [ADP/GDP-forming] subunit alpha, mitochondrial	2±2	0±0

UniProt Accession	Protein Name	PSMs Control	PSMs Obstructed
O55171	Acyl-coenzyme A thioesterase 2, mitochondrial	2±2	0±0
Q9JM53	Apoptosis-inducing factor 1, mitochondrial	2±2	0±0
Q6TXG7	Serine hydroxymethyltransferase	2±2	0±0
M0RDI1	Glutathione S-transferase	2±2	0±0
Q920L2	Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial	8±7	0±0
Q63010	Liver carboxylesterase B-1	4±4	0±0
P52759	Ribonuclease UK114	17±2	2±4
P16086	Spectrin alpha chain, non-erythrocytic 1	11±7	1±1
O89035	Mitochondrial dicarboxylate carrier	3±3	0±0
P49432	Pyruvate dehydrogenase E1 component subunit beta, mitochondrial	3±3	0±0
Q5XI95	Alcohol dehydrogenase 6	3±3	0±0
Q5XIH3	NADH dehydrogenase (Ubiquinone) flavoprotein 1	2±2	0±0
P24470	Cytochrome P450 2C23	2±2	0±0
P20817	Cytochrome P450 4A14	2±2	0±0
O35078	D-amino-acid oxidase	5±4	0±0
F1M8E9	Putative lysozyme C-2	11±3	1±2
P51635	Alcohol dehydrogenase [NADP(+)]	13±6	4±1
P28037	Cytosolic 10-formyltetrahydrofolate dehydrogenase	4±4	0±0
P52873	Pyruvate carboxylase, mitochondrial	15±5	2±3
Q561S0	NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 10, mitochondrial	3±3	0±0
F1LZW6	Protein Slc25a13 (Fragment)	3±3	0±0
P97852	Peroxisomal multifunctional enzyme type 2	3±3	0±0
P28826	Meprin A subunit beta	3±3	0±0
F1LNU2	Cubilin	3±3	0±0
P07632	Superoxide dismutase [Cu-Zn]	9±3	2±2
Q5XI78	2-oxoglutarate dehydrogenase, mitochondrial	4±4	0±0
P07379	Phosphoenolpyruvate carboxykinase, cytosolic [GTP]	11±4	1±2
P42123	L-lactate dehydrogenase B chain	16±8	5±1
D3ZXY4	Protein Aldh8a1	11±5	1±2
Q63965	Sideroflexin-1	3±3	0±0
F1LQC1	Acyl-coenzyme A oxidase	3±3	0±0
P06757	Alcohol dehydrogenase 1	8±8	0±0
G3V796	Acetyl-Coenzyme A dehydrogenase, medium chain	2±2	0±0

UniProt Accession	Protein Name	PSMs Control	PSMs Obstructed
Q6PDW8	Glutathione peroxidase	4±1	2±0
Q63716	Peroxiredoxin-1	10±3	6±0
Q9JJ40	Na(+)/H(+) exchange regulatory cofactor NHE-RF3	8±5	1±1

Supplementary Files

S1.xlsx Raw peptide list for BSA digest samples

S2.xlsx Raw peptide list for *E. coli* digest samples

S3.xlsx Raw protein list for *E. coli* digest samples

S4.xlsx Peptide list for *E. coli* digest searched for up to 4 missed cleavages

S5.xlsx peptide list for *E. coli* digest searched for carbamylation as a modification

S6.xlsx peptide list for *E. coli* digest searched for any +40 Da modification

S7.xlsx Full protein list for rat kidney control vs obstructed

S8.xlsx Raw peptide list for GELFrEE fraction digest samples

S9.xlsx Protein list for GELFrEE-fractionated E. coli proteome

S10.xlsx Protein list for plasma samples following LMW enrichment and control.

S11.xlsx Protein list for plasma samples without the use of 2-mercaptaethanol.

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