

DEVELOPMENT OF TECHNIQUES FOR REMOVAL OF SODIUM DODECYL
SULFATE FOR ENHANCED PROTEIN IDENTIFICATION AND
CHARACTERIZATION

by

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Submitted in partial fulfilment of the requirements
for the degree of Master of Science

at

Dalhousie University
Halifax, Nova Scotia
October 2011

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DALHOUSIE UNIVERSITY
DEPARTMENT OF CHEMISTRY

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Dated: October 31, 2011

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DALHOUSIE UNIVERSITY

DATE: October 31, 2011

AUTHOR: Shayla Melissa Fitzsimmons

TITLE: DEVELOPMENT OF TECHNIQUES FOR REMOVAL OF SODIUM
DODECYL SULFATE FOR ENHANCED PROTEIN IDENTIFICATION
AND CHARACTERIZATION

DEPARTMENT OR SCHOOL: Department of Chemistry

DEGREE: MSc CONVOCATION: May YEAR: 2012

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Abstract

Mass spectrometry is a powerful tool employed in proteomics; however, sodium dodecyl sulfate (SDS), a surfactant used for protein solubilization, is known to cause severe interference at concentrations greater than 0.01%. Thus, methods for SDS removal are paramount. This thesis presents the development of techniques for efficient SDS removal while maintaining high protein recoveries.

Due to the lack of sensitivity and selectivity demonstrated by current high-throughput SDS quantitation methods, a negative-mode LC-ESI-MS technique was optimized (LOQ 0.5 ng, LOD 0.15 ng SDS).

The Pierce Detergent Spin Removal Columns are a commercial product which efficiently removes SDS, but offers poor protein recovery. An alternate protocol is developed which maintains effective SDS removal while providing protein yields of >65%.

Proteomic experiments often involve numerous samples, thus necessitating high-throughput methods for SDS removal. A fully automated strong cation exchange-reversed phase technique was therefore developed, which efficiently removes SDS while providing >75% protein recovery.

List of Abbreviations and Symbols Used

α	Selectivity factor
γ	Surface tension
Γ	Surface excess
μ	Chemical potential
μ_e	Electrophoretic mobility
μg	Microgram
v	Velocity
$\times g$	Times gravity
2D	Two dimensional
A	Surface area
ACN	Acetonitrile
APFO	Ammonium perfluorooctanoate
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate
CID	Collision induced dissociation
CMC	Critical micelle concentration
CMW	Chloroform/methanol/water
CNBr	Cyanogen bromide
CTA	Cetyl trimethylammonium
CTAB	Cetyl trimethylammonium bromide
Da	Dalton
DR-beads	Detergent removal beads
DNA	Deoxyribonucleic acid
DS	Dodecyl sulfate
E	Electric field
ESI	Electrospray ionization
FASP	Filter aided sample preparation
G	Gibbs energy
GC	Gas chromatography
GeLC-MS	Gel electrophoresis liquid chromatography mass spectrometry
GELFrEE	Gel-eluted liquid fraction entrapment electrophoresis
HILIC	Hydrophilic interaction chromatography
HPLC	High-performance liquid chromatography
IEC	Ion exchange chromatography
k'	Capacity factor
kDa	Kilodalton
KDS	Potassium dodecyl sulfate
K_{sp}	Solubility product constant
LC	Liquid chromatography
LOD	Limit of detection
LOQ	Limit of quantitation

M	Molar (moles per liter)
MALDI	Matrix-assisted laser desorption/ionization
<i>m/z</i>	Mass to charge ratio
MS	Mass spectrometry
MW	Molecular Weight
N	Column efficiency
nanoESI	Nano-electrospray ionization
PAGE	Polyacrylamide gel electrophoresis
PMF	Peptide mass fingerprinting
ppm	Parts per million
PTM	Post-translational modification
RNA	Ribonucleic acid
RPLC	Reversed phase liquid chromatography
rpm	Revolutions per minute
R_s	Chromatographic resolution
RSD	Relative standard deviation
RP	Reversed-phase
SCX	Strong cation exchange
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SPE	Solid phase extraction
TCA	Trichloroacetic acid
TFA	Trifluoroacetic acid
t_m	Retention time of an unretained compound
t_r	Retention time
Tris	Tris(hydroxymethyl)aminomethane
TPCK	Tosyl phenylalanyl chloromethyl ketone
UV	Ultraviolet
Vis	Visible
w_b	Base peak width

Acknowledgements

I would first like to thank my supervisor, Dr. Alan Doucette, for his help and support during my time at Dalhousie. His enthusiasm, ideas and constructive criticisms have taught me a great deal.

I thank my past and present committee members, Dr. Robert Guy, Dr. Michael Quilliam and Dr. Louis Ramaley, for reviewing my thesis, and also for their advice.

Thank you to the office staff in the Department of Chemistry, particularly Giselle Andrews, for their help over the years. I also extend my thanks to the lab instructors who I worked with during my time as a teaching assistant.

I would especially like to thank my current group members, Dr. Mark Wall, Fang Liu, Dennis Orton, Douglas Vieira and Andrew Crowell. Thank you also to my former group members, Dr. Diane Botelho, Leanne Lucas, Jacqueline Spears and Bjorn Weilens.

Finally, I would like to thank my entire family; particularly my mother, Lesley, who has always given me unwavering love and support. Thank you so much.

Chapter 1

Introduction

1.1 The Necessity of Studying Proteins

Genomics, the study of an organism's hereditary information contained within the genome, is a well-established discipline wherein much effort has been expended in determining the entire DNA sequence of several organisms. The most famous of these, the Human Genome Project, is an international scientific research project focused on elucidation of the human genome through DNA sequencing and through mapping of the 20,000 to 25,000 genes from which it is comprised [1]. To date, approximately 92.3% of the human genome has been completed, and further analyses are ongoing [2, 3].

Information gathered from genomic studies provides a great deal of information about the function and physiology of a studied organism. However, determination of the mechanisms involved in cellular function generally requires knowledge of proteins. Given that the DNA sequence has been reported for many organisms, and that DNA is transcribed into RNA, which is in turn translated into proteins, early studies attempted RNA analysis for the determination of protein content in cells. Though an excellent starting point, it was found that correlation between RNA level and protein content is poor; identical RNA concentrations may produce proteins which vary in concentration by more than 20-fold [4, 5]. This discrepancy occurs for a number of reasons. An abundantly available RNA may be translated ineffectively or degraded rapidly [6], therefore producing significantly smaller amounts of protein than expected. Forms of a protein which differ slightly in their amino acid sequence (isoforms) may arise from a single

RNA sequence due to alternative splicing (a post-transcriptional process in which portions of the RNA sequence are spliced and reconnected in various combinations), a process which is, at best, difficult to predict [7]. Additionally, many proteins function only after formation of complexes with other molecules [8], or after having undergone a post-translational modification (PTM, a chemical modification) [9], both of which are impossible to determine using RNA analysis. From the above, it is clear that the set of proteins expressed in an organism is much larger and more complex than its genome. Thus, it is necessary to directly study the protein content of cells.

1.2 The Importance of Studying Proteins

Proteins are essential components of all living organisms, vital in metabolic pathways and often acting as catalysts for biochemical reactions. Protein PTMs in particular are known to be especially important in the regulation of protein function and cell metabolism. Proteins are also critical components in cell signalling (a form of communication which governs cellular actions and activities), cellular adhesion (binding of a cell to another surface, essential in maintaining multicellular structure), immune responses, and the cell cycle. The study of proteins is therefore fundamental, as knowledge of their structure and function is crucial for providing insights into cellular processes.

Proteomics is the large-scale study of proteins, wherein the entire complement of proteins produced within an organism (*i.e.*, the proteome) is subject to study. The relatively simplistic model of yeast contains approximately 6,000 proteins [10], while the much larger human proteome is estimated to contain upwards of 100,000 unique protein

forms (including protein isoforms) [11]. Beyond the number of components, any given proteome exhibits additional complexity in terms of protein expression level and protein properties. Not all proteins are expressed concurrently since a cell's exact protein content depends on its physiological state, which differs over time. The implications of such knowledge allows for the comparative analysis of different cell states for the determination of differential protein expression, such as in biomarker discovery experiments, wherein a diseased cell may express proteins with different relative abundances relative to those in a healthy cell.

1.3 Biomarker Discovery

A biomarker is a substance which is used as an indicator for a specific biological or cellular state. While the exact definition varies according to the scientific field in which the term is used, a proteomic biomarker is a protein whose presence, absence, or relative abundance is indicative of a disease. Knowledge of such a protein may also provide insights into the mechanism of said disease, allowing for the targeted development of drugs or therapeutics as treatment [12].

The process for discovery of biomarkers follows one of two pathways: focused or comprehensive. In focused proteomic studies, the molecule(s) of interest are known, and may be a single protein or a collection of proteins. To prevent interferences from other compounds, separation techniques are often incorporated for the purification of the desired protein(s), and methods for detection and quantitation are tailored to obtain maximum selectivity; antibody-based (*i.e.*, Western blot, enzyme-linked immunosorbent assay) and mass spectrometry-based (*i.e.*, multiple reaction monitoring) are commonly

used techniques. For comprehensive proteomic studies, the entire proteome is characterized with the hopes of identifying a protein (or proteins) whose expression is differentiated between two unique cell states (*i.e.*, healthy versus diseased). The inherent complexity of a proteome necessitates that the first step in such an experiment be protein separation.

1.4 Protein Separation

Protein separation platforms work by using a given characteristic of the protein (*i.e.*, isoelectric point, mass, hydrophobicity, charge, selected affinity) to divide the sample components into several fractions. The ability to detect a given protein is often influenced by the presence of other proteins or peptides in the fraction. It therefore follows that samples containing fewer components would present less interference; indeed, improved fractionation is generally reflected in a higher percentage of detected proteins. Separations are also effective at removing non-protein interferences, which would otherwise cause signal suppression in the detector (*e.g.*, mass spectrometer).

Thus, it is clear that separation is an essential step in proteomics experiments. The protein separation platforms available for proteome processing may be divided amongst two categories: solution-based platforms which encompass chromatography (*e.g.*, size-exclusion, affinity, reversed-phase and ion exchange), and gel-based platforms (*e.g.*, sodium dodecyl sulfate polyacrylamide gel electrophoresis, SDS-PAGE, or gel-eluted liquid fraction entrapment electrophoresis, GELFrEE). A thorough exploration of each technique can be found in numerous texts. A brief overview is provided below.

1.4.1 Solution-Based Separation Platforms

Liquid chromatography is a separation technique often applied to proteomic samples, wherein a mixture may be fractionated based on chemical or physical properties such as size, charge, hydrophobicity, or affinity to a ligand. Analytes are loaded onto a column containing a solid stationary phase, and separation occurs through differential partitioning between the stationary and mobile (solvent) phases; choice of stationary phase is dependent on the type of separation desired. The efficiency of separation is defined by the chromatographic resolution (R_s) obtained between individual sample components and is dependent on three parameters: column performance, selectivity factor and capacity factor.

Column efficiency (N) is a measure of the efficiency of a column, and its effect on resolution is influenced by the following: (i) longitudinal diffusion, wherein solute diffusion is driven by a concentration gradient, (ii) mass transfer kinetics of analyte between the mobile and stationary phases, and (iii) the multiple pathways in which an analyte may travel down a column of porous packing material. The relative importance of each factor is dependent on mobile phase velocity.

The capacity factor (k') is a measure of analyte retention as defined by

$$k' = \frac{t_r - t_m}{t_m}, \quad (1.1)$$

where t_m is the time required for elution of an unretained compound, and t_r is the retention time of a specific analyte. The selectivity factor is related and describes the separation of two species on the column. It is defined as,

$$\alpha = \frac{k'_2}{k'_1}, \quad (1.2)$$

where k'_1 and k'_2 are the capacity factors for two different analytes. Put together, the three terms define the general resolution equation,

$$R_s = \left(\frac{\sqrt{N}}{4}\right) \left(\frac{\alpha-1}{\alpha}\right) \left(\frac{k'_{t_2}}{k'_{t_2}+1}\right). \quad (1.3)$$

For maximum resolution, these three key parameters must be optimized; however, even at optimal conditions, the complexity of proteomic samples is such that baseline resolution is not possible. Nonetheless, chromatographic separation is a useful and powerful tool for decreasing sample complexity and under the right conditions may be employed for protein- or peptide-level separations.

1.4.1.1 Ion exchange Chromatography

Ion exchange chromatography (IEC) employs a polymeric or crystalline solid support matrix (*e.g.*, polystyrene, silica) wherein the matrix beads are derivatized with a charged functional group [13], and used to separate proteins according to their net charge (see Figure 1.1). There are four general forms: cation, anion, strong, and weak. The first two refer to functional group charge; cation exchange utilizes negatively-charged functional groups (*i.e.*, propyl sulfonic acid, carboxyl [13]) for separation of positively-charged molecules, while anion exchange employs positively-charged functional groups (*e.g.*, quaternary amines, diethylaminoethyl, polyethylenimine [13]) for separation of negatively-charged molecules. The latter two refer to functional group strength; strong ion exchangers (*i.e.*, propyl sulfonic acid, quaternary amines) are ionized over a wide range of pH levels (*i.e.*, pH 0 – 14), whereas weak ion exchangers (*i.e.*, carboxyl, polyethylenimine) are ionized over a narrower pH range (*i.e.*, pH 0 – 8 for weak anion exchange, or 6 – 14 for weak cation exchange).

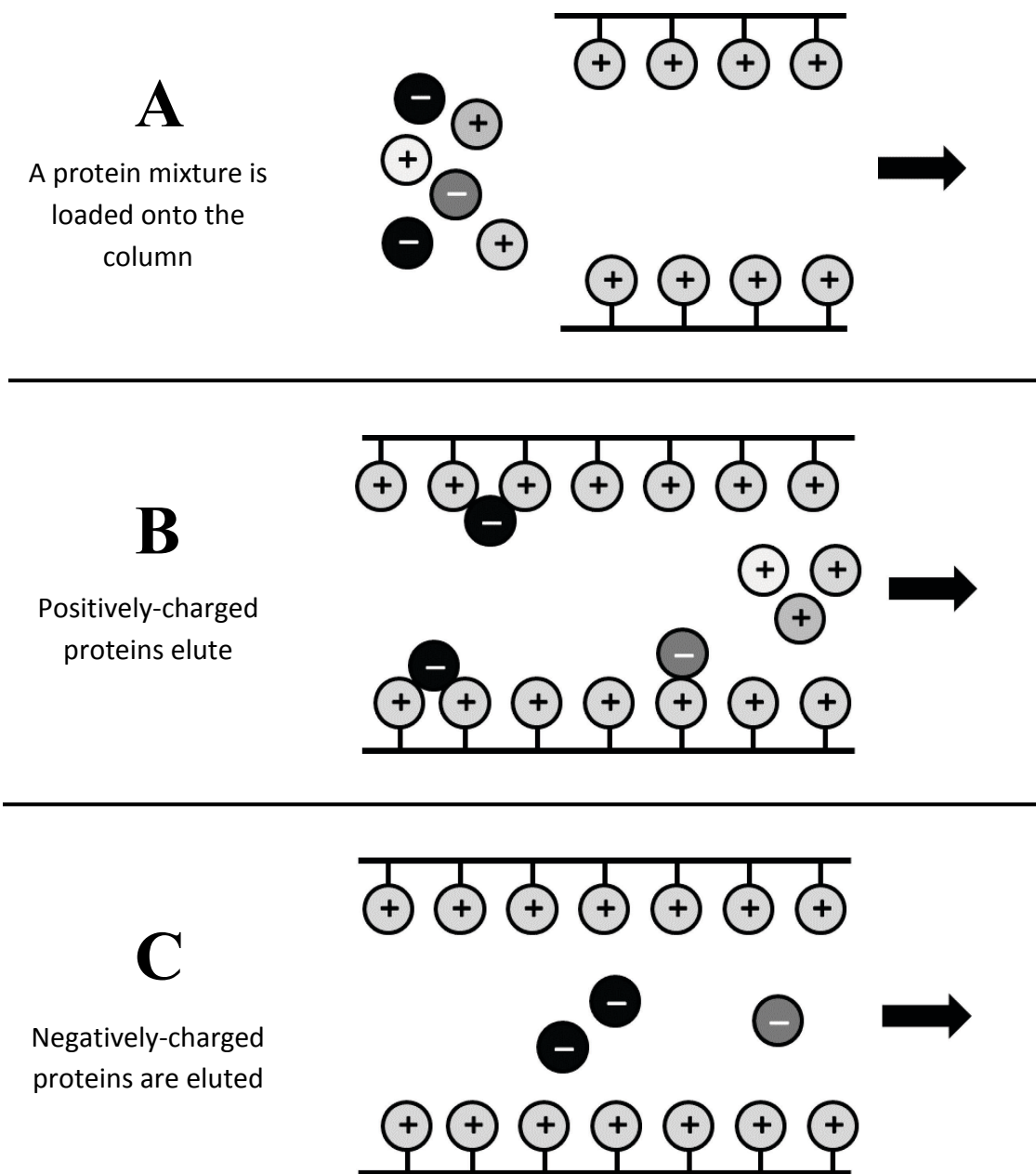


Figure 1.1: The basis of anion exchange chromatography; cation exchange employs the same mechanism, but with opposing charges. (A) A protein mixture is injected onto a column containing positively-charged packing material. (B) Proteins containing a net negative charge are retained, while all other molecules are eluted. (C) Retained proteins are eluted by a change in eluting solvent pH, or through application of a solution with high ionic strength.

For all ion exchange types, molecules are retained on the column based on ionic interactions. Elution may occur by increasing the ionic strength of a similarly charged species, causing said species to preferentially bind and thereby displacing the analyte. It may also occur by adjusting the mobile phase pH to alter the charge of (i) analyte functional groups or (ii) the ion exchange resin (weak ion exchange only), such that analyte-resin ionic interactions are no longer able to form [14].

Both anion and cation exchange have been used extensively for protein and peptide separation [15-20]; a judicious selection of mobile phase [21] has been shown to allow for separation of protein isoforms which differ by a single charge. Such resolution from a chromatographic method is exceeded only by affinity chromatography [14]. However, elution of proteins from ion exchange often requires buffers whose components are incompatible with downstream analysis, necessitating further sample preparation. Nonetheless, ion exchange remains a powerful tool for protein fractionation.

1.4.1.2 Reversed-Phase Chromatography

Reversed-phase liquid chromatography (RPLC) is a well-characterized chromatographic technique for separation of proteins and peptides [22], and has the ability to bind and select for proteins containing a wide range of polar and non-polar side chains [23]. It utilizes a column packing based on spherical silica particles, to which alkyl chains are covalently bonded. Proteins are retained based on hydrophobic interactions, and eluted (in order of increasing hydrophobicity) by application of a moderately polar mobile phase (*i.e.*, methanol, acetonitrile). While this technique allows for high resolution separations of the complex mixtures commonly used in proteomics [24], greater

resolution may be obtained by employing it as a second dimension of separation prior to MS analysis [25-27].

1.4.1.3 Multidimensional Separations

Orthogonal chromatographic techniques may be combined for enhanced protein separation. Many of the above-mentioned separation methods may be coupled [28, 29], although employing ion exchange ahead of reversed-phase is arguably the most popular. This is due to the high loading capacity of ion exchange and the high resolution obtainable from reversed-phase. Many combinations of IEC-RP-HPLC have been reported [18, 30, 31].

1.4.1.4 Coupling to MS

Coupling of liquid-based separation platforms to mass spectrometry for MS-based proteomics initially faced two major problems: production of gas phase ions from solubilized proteins, and removal of MS-incompatible contaminants (*i.e.*, salts, detergents). The first was overcome upon the advent of electrospray ionization (ESI), wherein proteins are ionized in-solution and subsequently transferred into the gas phase. The second is most commonly solved by the online coupling of reversed-phase chromatography to mass spectrometry [32], wherein proteins are retained and contaminants eluted in the void volume. As such, reversed-phase chromatography is commonly employed as the final dimension of separation prior to MS analysis [18, 29, 33].

1.4.2 Electrophoretic Separation Platforms

Electrophoretic separation occurs according to electrophoretic mobility; that is, according to the motion of solubilized particulates when under a uniform electric field. The movement of particles may be described by equation 1.4,

$$\mu_e = \left(\frac{v}{E}\right), \quad (1.4)$$

where μ_e is the electrophoretic mobility of the molecule, v is the velocity of the molecule (dependent on solution viscosity and net molecular charge), and E is the applied electric field. Assuming no barriers are encountered, compounds with a greater electrophoretic mobility will migrate through solution faster.

By far, the most common electrophoretic separation platform in proteome applications is polyacrylamide gel electrophoresis (PAGE), which may be operated under denaturing (*i.e.*, sodium dodecyl sulfate PAGE [34]) or non-denaturing (*i.e.*, Blue-Native PAGE [35]) conditions. The latter is beyond the scope of this thesis, and will not be discussed further. As protein denaturation aids in solubilisation, it is under such conditions that the overwhelming majority of PAGE separations are performed. The most common denaturation agent is sodium dodecyl sulfate (SDS); gel electrophoresis separations employing SDS are known as SDS-PAGE.

1.4.2.1 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

The most popular buffering system employed in SDS-PAGE experiments is the Tris-Glycine Laemmli system [36], which allows separation in the 10 kDa – 250 kDa range. Separation of lower molecular weight proteins, including peptides, may be achieved by use of a different buffering system (*i.e.*, Tris-Tricine, < 20 kDa [37]). For both, SDS is introduced to the sample through addition of SDS-containing sample buffer.

The mechanism of separation in SDS-PAGE begins with the uniform binding of SDS to proteins (1.4 grams SDS per gram protein [38]), which causes denaturation (Figure 1.2a) of the protein, creating rod-like molecules with identical charge per unit mass. This ensures identical electrophoretic mobility for all sample components, and allows protein separation as a function of molecular weight (MW). The medium in which separation occurs, a polyacrylamide gel, is a polymer consisting of acrylamide and cross-linking bis-acrylamide. Adjusting the concentration of each prior to polymerization allows for tailoring of the pore sizes, thus changing the effective range of molecular weights which may be separated [39]. Gels which allow for separation in the 10 kDa – 250 kDa mass range are typically cast at 10 to 15% acrylamide, of which approximately 3% is bis-acrylamide.

Resolution in SDS-PAGE is increased through use of discontinuous PAGE [36], wherein the gel is divided into two discrete sections. Samples are loaded onto the upper portion, referred to as the stacking gel, which is cast with a low concentration of acrylamide (typically 4%). The lower section, the resolving gel, is where protein separation occurs. Both parts are buffered using Tris-HCl; the stacking gel to pH 6.8, and the resolving gel to pH 8.8. Upon application of an electric field, a technique known as isotachopheresis occurs. This allows for improved resolution by focusing the proteins into a tight band prior to separation. Such focusing is the result of differing glycine mobility due to pH differences in the stacking and resolving gels.

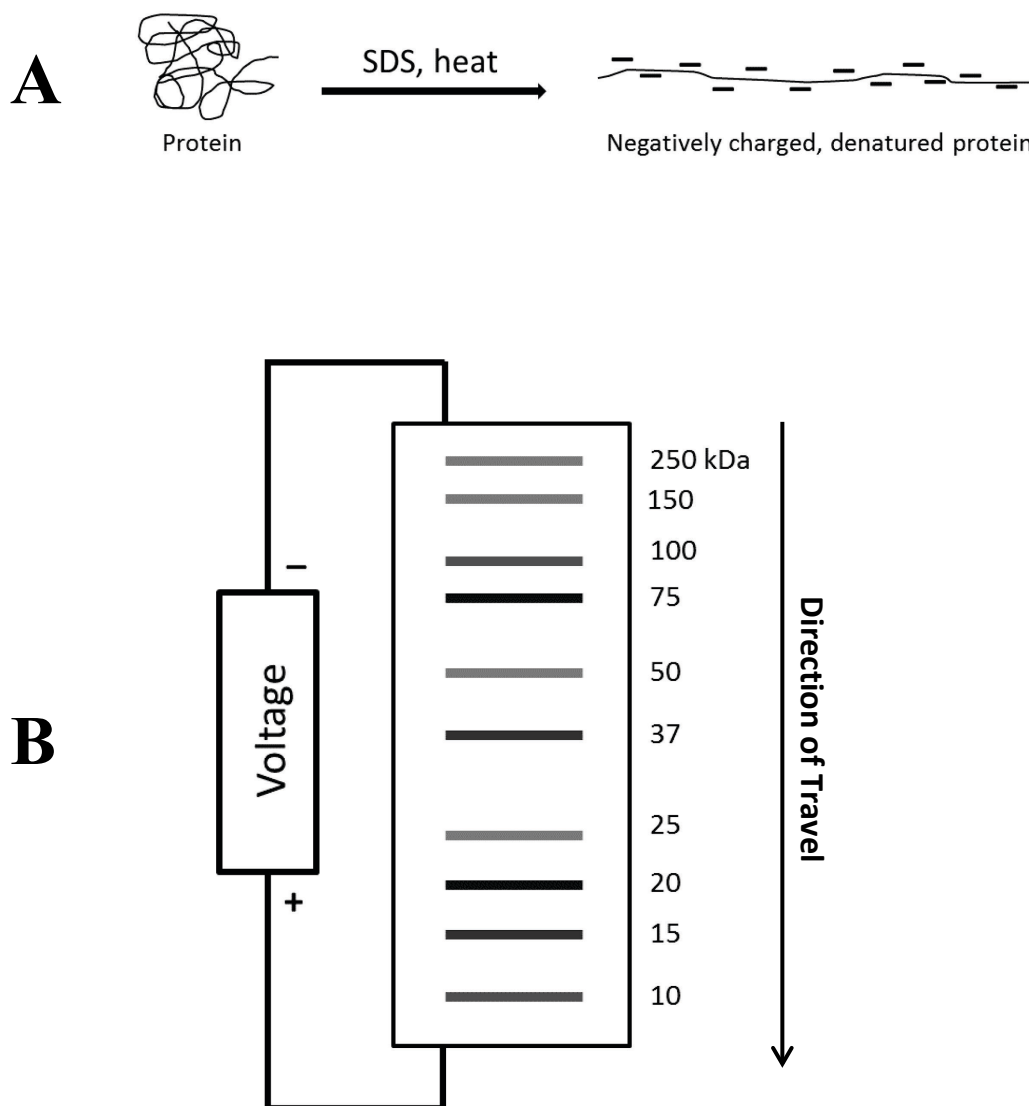


Figure 1.2: Diagram illustrating SDS-PAGE. (A) Proteins are denatured through the application of SDS and heat, resulting in a rod-like molecule with a net negative charge. (B) The sample is loaded into a polyacrylamide gel and separated by application of an electric field, wherein the velocity of protein migration is logarithmically related to protein molecular weight. The migration of small proteins is faster than that of large proteins. A standard protein molecular weight ladder, used for determining the mass of unknown proteins, is shown.

In the resolving gel, proteins of lower molecular weight sieve through the pores more easily, thus migrating through the gel at a faster rate relative to proteins of higher molecular weight. The end result is a separation wherein the logarithm of molecular weight for a given protein is related to its mobility, which in turn is related to the distance it traveled down the gel (Figure 1.2b) [34]. Post-separation, proteins may be visualized by staining, most often with Coomassie Brilliant Blue [40] or silver stain [41].

1.4.2.2 Coupling SDS-PAGE to MS

SDS-PAGE is not capable of direct (online) coupling to MS for analysis, although offline coupling is possible. First, proteins must be extracted from the polyacrylamide gel. Overnight solvent extraction allows for the recovery of proteins up to 70 kDa [42-44]. However, because recovery decreases as a function of mass, recovery of larger proteins is still challenging, thus resulting in the loss of a large portion of the proteome. Electroelution, wherein electrophoresis is continued to allow migration of proteins off the gel, is generally a time- and labour-intensive effort often accompanied by poor protein yields and unacceptably large sample dilution [45]. Exceptions have been reported [46-47], but require specialty apparatuses which are not readily available. It is therefore common to facilitate extraction through in-gel enzymatic digestion, whereby proteins are cleaved into smaller fragments [48]. Subsequent wash and extraction steps remove contaminants and extract peptides into solution, at which point they may be subjected to liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) [49].

When protein bands from an SDS-PAGE are excised and treated as described above, the approach is termed GeLC-MS, and it has a number of benefits. It requires relatively little total sample material (30 – 50 μ g), and thus is applicable to analysis of

proteomes with limited availability. It also allows for separation and analysis of proteins with varying biochemical characteristics [49]. It is currently unsurpassed as a method for protein separation and peptide analysis, and has achieved wide-spread use as a powerful and simple approach for protein analysis, capable of identifying thousands of proteins from a complex sample.

1.4.2.3 Gel-Eluted Liquid Fraction Entrapment Electrophoresis

Gel-eluted liquid fraction entrapment electrophoresis (GELFrEE) [50] is an electrophoretic separation platform which utilizes polyacrylamide gels and the Laemmli buffer system for separation; however, it discards the traditional slab-gel format of SDS-PAGE, instead employing a tube-gel. Separation occurs via the same mechanism as previously described. However, proteins are allowed to elute from the gel into a solution-based collection chamber and are subsequently collected. Protein separation is maintained through collection of sample at multiple time points, where the end result is a proteome fractionated into individual mass ranges in the solution phase (Figure 1.3). An advantage of the GELFrEE method is that it allows for the collection of intact proteins at high recovery, therefore facilitating study and characterization of a proteome at the intact level. However, the buffer in which samples are retained contains SDS. It is therefore incompatible with mass spectrometry [51], necessitating a clean-up step prior to MS analysis.

1.5 Mass Spectrometry

As previously mentioned, mass spectrometry is the principal method employed in proteomics experiments for the identification and characterization of proteins. Though

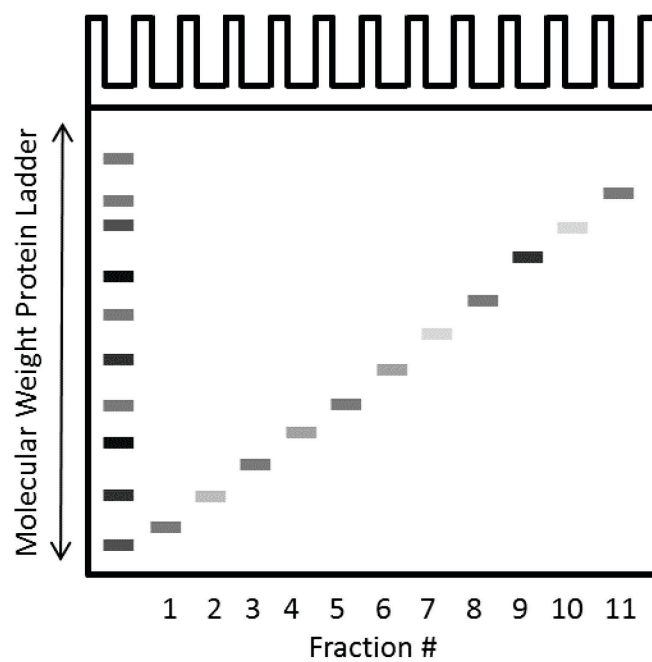


Figure 1.3: Proteins fractionated by GELFrEE are collected in-solution. Employing SDS-PAGE for subsequent visualization allows for determination of the mass ranges contained in individual fractions.

several methods exist currently, difficulties in transforming large biomolecules into gas phase ions frustrated early studies; this was owed largely to extensive decomposition during vaporization. However, the advent of ‘soft’ ionization techniques allowed, with varying success, the production of intact ions from proteins; the most successful and widely used methods to emerge were electrospray ionization (ESI) [52] and matrix-assisted laser desorption ionization (MALDI) [53]. The choice of ionization method is dependent on the experiment, as both have advantages and disadvantages; ESI permits online LC coupling while MALDI is more suited to offline coupling. ESI was the only ionization method utilized in the present study and hence it will be the only method discussed.

1.5.1 Electrospray Ionization

ESI is an ionization method capable of producing either positively- or negatively-charged gas phase ions (positive ion mode or negative ion mode, respectively); for simplicity’s sake, and because it is the most commonly used mode in proteomics experiments, only positive ion mode will be discussed herein. Gas phase ions are produced in a three step process: (i) formation of charged droplets, (ii) solvent evaporation resulting in the creation of smaller, charged daughter droplets, and (iii) production of gas phase ions (Figure 1.4). First, high voltage (1-5 kV) is applied to a capillary located opposite a counter electrode connected to the entrance of a mass spectrometer. Analyte-containing solution is directed through the capillary, and upon reaching the tip an electrochemical reaction occurs creating an excess of positive ions which gather at the surface of the meniscus, thus forming a Taylor cone. It is from the tip

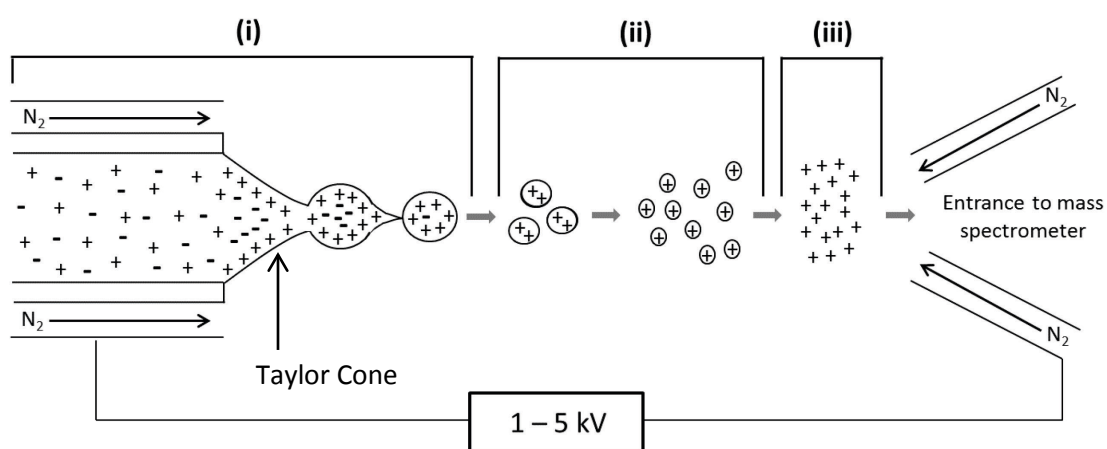


Figure 1.4: Electrospray ionization allows for the production of charged gas phase protein molecules: (i) formation of charged droplets through application of a voltage to the capillary tip; (ii) solvent evaporation, resulting in the creation of smaller charged droplets; (iii) production of gas phase ions through the ion evaporation model or charged residue model.

of the Taylor cone that Coulomb forces disperse the liquid into a fine aerosol spray of charged droplets. The initial droplet size is dependent on capillary dimensions, solvent composition, and the flow rate at which the system is operated. Using capillaries with smaller internal diameter, volatile solvents, and nanoESI (flow rates of nL/min) produces smaller droplets [54].

As the charged droplets are accelerated towards the counter electrode, nebulization occurs, sometimes aided by the addition of heat and/or a flow of inert gas; solvent evaporation generates an increased charge density on the surface of a droplet. Once the Rayleigh limit is reached (where surface tension equals Coulomb repulsion), the resulting instability causes disintegration of the droplet into smaller, highly charged daughter droplets, whereupon the process is again repeated [54].

The method for production of the final gas phase ions is unclear; however, there are two major theories. The Ion Evaporation Model [55] proposes that, as the droplet reaches a critical radius, the strength of charge at the surface becomes large enough to eject ions from the charged droplet into the gas phase. The Charged Residue Model [56] suggests that solvent evaporation and droplet disintegration continues, eventually leading to droplets containing only one analyte molecule; subsequent evaporation of all remaining solvent molecules then results in a gas phase ion. Produced ions are then fed into one of many existing mass analyzers, a discussion of which is beyond the scope of this thesis.

1.6 Proteomic Workflows

Used in combination with one or multiple separation platforms, mass spectrometry forms the basis for the proteomic experiments commonly performed. Currently, there are two primary workflows – top-down (analysis of intact proteins) and bottom-up (analysis of enzymatically-generated peptides). Reversed-phase nano-scale LC-ESI-MS provides high resolution of peptides while also allowing full automation, and is almost universally coupled to MS in bottom-up proteomic experiments [57-59]. An overview of each is provided.

1.6.1 Bottom-Up Workflow

In bottom-up proteomics, proteins are enzymatically digested to form peptides; this may occur pre- or post-separation. Generated peptides may also be subject to separation. Most commonly, digestion is performed using trypsin, which generally cleaves at the C-terminus of lysine and arginine residues [48]. Once a peptide separation step is complete, peptides are subject to MALDI-MS or ESI-MS. Finally, identification is performed using either peptide mass fingerprinting (PMF) or peptide sequencing.

PMF is a technique which identifies proteins by comparing their theoretical peptide masses, as generated by a protein database, to the masses of peptides obtained and identified by MS following enzymatic digestion [60]. However, the method is applicable only to mixtures of low complexity; even highly-fractionated proteomes are often too complex for analysis by PMF.

Peptide sequencing experiments commonly couple liquid chromatography with mass spectrometry; such experiments are known as shotgun proteomics and are the method of choice for many researchers (and consequently, the method most amenable to

high-throughput analysis) [61]. Peptides are dissociated by collision-induced dissociation (CID), wherein energy is applied to ions in the mass analyzer to promote collision with an inert gas (*e.g.*, nitrogen), thus producing a predictable fragmentation pattern. Through calculation of the mass difference between fragments, the amino acid sequence of a protein may be elucidated. Based on such knowledge, the protein from which the fragments originated may be identified, even when the peptide exists in a complex mixture.

The bottom-up strategy utilizing shotgun proteomics is the most widely-used workflow for protein analysis, and has been extremely successful in identifying proteins in digests originating from complex proteomes (*e.g.*, cell lysates, yeast) [33, 62]. The identification of peptides is eased by their ready solubilisation and separation. However, the bottom-up workflow employing peptide sequencing is most advantageous due to the large number of proteins which may be identified in a single run. When coupled with multidimensional separation techniques, the identification of greater than 10,000 proteins has been reported [63]. However, this approach is not without limitations. Spectra dominated by ions from highly abundant peptides can cause loss of information about low-abundant peptides, which is of particular concern when employing data dependent scanning (wherein only the ions with highest intensity are sequenced by MS/MS). Additionally, a large portion of total peptide population is not identified; and, as a consequence, much information about PTMs is lost [64]. Despite these impediments, bottom-up proteomics is a mature field which provides valuable information for protein identification and characterization. Currently, application of the bottom-up workflow to complex proteomes is focused almost exclusively on the characterization of ‘real’

samples (*e.g.*, human plasma) [65]. The information it provides is complimentary to that obtained from top-down proteomics [64].

1.6.2 Top-Down Workflow

In top-down proteomics, intact proteins are separated and then subject to ionization; the charged molecules are introduced into a mass spectrometer, followed by gas phase fragmentation [66]. To avoid ambiguity in determination of masses from multiply-charged ions, such experiments are most commonly performed on mass spectrometers with high mass measurement accuracy (*e.g.*, a Fourier Transform ion cyclotron resonance mass spectrometer, mass accuracy < 2 ppm [39]). The elimination of the time-consuming digestion required for bottom-up strategies is an advantage to top-down proteomics. However, the major advantage of this strategy is the potential access to the entirety of a given protein's sequence, coupled with the ability to examine PTMs; proteins differing only by post-translational modifications may be identified [67]. The top-down proteomic workflow is an extremely powerful tool for MS-based proteomics. However, compared to bottom-up, it is still a relatively young field. Limitations include the inability to sequence proteins larger than approximately 50 kDa due to stable tertiary structures interfering with efficient fragmentation [68], and the expense of the necessary instrumentation [69]. However, with continuing advances in the field of mass spectrometry instrumentation, the latter limitation may become a non-issue. Additionally, the complex spectra generated by even a single multiply-charged protein limits analysis to simple protein mixtures, at best [64]. Regardless, top-down proteomic experiments yield valuable data, often complementary to that obtained from the bottom-up approach.

Though mass spectrometry is a powerful technique used in both top-down and bottom-up proteomics, the ionization mechanisms (ESI and MALDI) common in both workflows are sensitive to matrix effects. Signal suppression due to ions and surfactants are of great concern, because they greatly reduce the ability to confidently identify peptides or proteins from the obtained mass spectra. Of greatest concern is the commonly-used SDS; it has been reported that the critical threshold level for SDS tolerance in an LC-ESI-MS experiment is 0.01% [51]. However, it must be noted that SDS threshold tolerance is likely dependent on experimental conditions, and thus may differ between experiments.

1.7 Sodium Dodecyl Sulfate

SDS is the most-used anionic surfactant in proteomics experiments, and is often utilized in proteomics experiments for disruption of lipid cell membranes in protein extraction, for protein solubilisation [70], enhancement of digestion efficiency [71], protein separation by molecular weight (*e.g.*, SDS-PAGE and GELFrEE), or as a disaggregating agent [72]. It exhibits a number of properties which affect its function in proteomics experiments.

1.7.1 Behaviour of SDS in Aqueous Solutions

Surfactants are a class of (usually) amphiphilic organic compounds which work to lower the surface tension of a liquid, and it is to this class that SDS belongs. In aqueous solutions, H₂O forms hydrogen bonds between adjacent molecules; however, the number of available bonds at the surface is less than in bulk solution, producing a net inward attraction [73]. Due to the resultant high surface energy, it is energetically favourable for

a solution to obtain minimized surface area. The tension required to maintain such minimal surface area is defined as surface tension,

$$\gamma = \left(\frac{dG}{dA} \right), \quad (1.5)$$

where γ is surface tension, G is the Gibbs energy, and A is the surface area.

When added to aqueous solution, surfactant molecules adsorb at the liquid-vapour interface; the hydrophobic tails extend out of solution into the vapour phase, while the hydrophilic head groups remain in solution (Figure 1.5a). Such partitioning at the interface interferes with hydrogen-bonding and results in decreased surface tension; the strength of the effect is dependent on chemical potential and surface excess (difference in concentration between bulk and surface solution) of all components (*i.e.*, SDS and H₂O). However, the values of both are related to detergent concentration [74] and are defined by the Gibbs adsorption isotherm,

$$-d\gamma = \Gamma_1 d\mu_1 + \Gamma_2 d\mu_2, \quad (1.6)$$

where γ is surface tension, Γ is the surface excess, and μ is the chemical potential of components 1 and 2, respectively. The hydrocarbon chains of surfactant monomers which remain in the bulk solution are surrounded by a cage-like structure of hydrogen-bonded water molecules [75].

As with many surfactants, SDS monomers existing in bulk solution are capable of aggregation (Figure 1.5b); the creation of such clusters is driven by entropic considerations. As the concentration of hydrophobic SDS tails increases, there comes a point at which the entropy decrease caused by aggregation is less than that of the entropy decrease caused by caging the hydrophobic tails, thus resulting in spontaneous micelle formation [75]. The concentration at which this occurs is the critical micelle

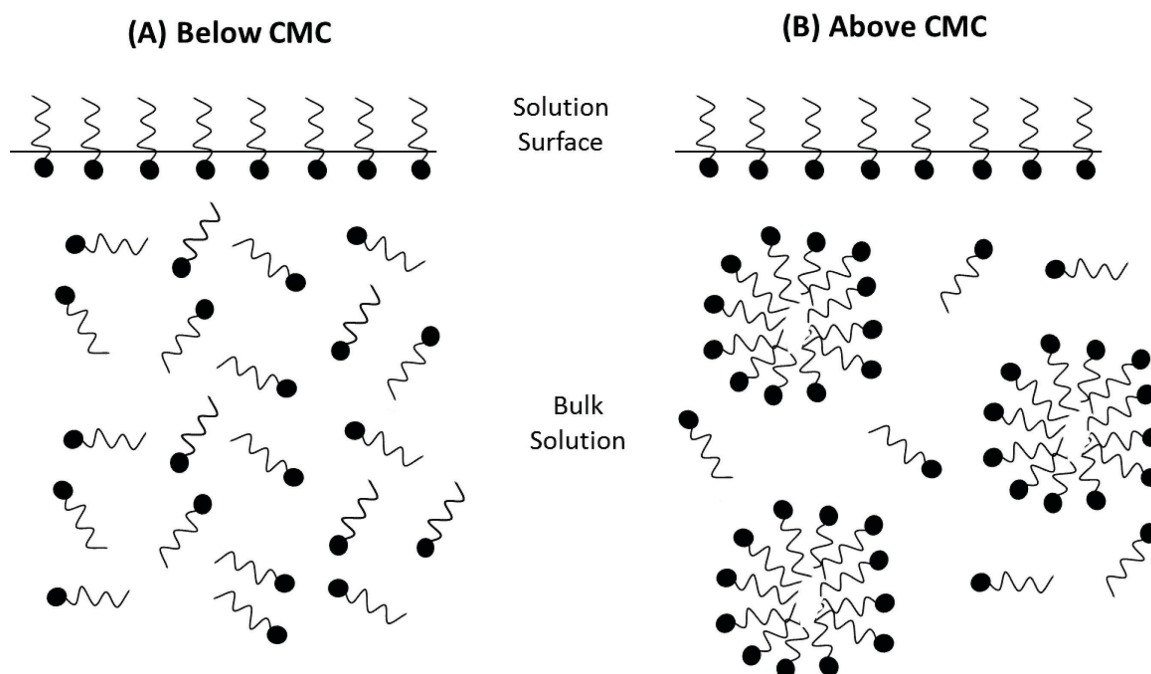


Figure 1.5: The behaviour of SDS in an aqueous system. Below the critical micelle concentration (A), SDS monomers adsorb to the liquid-vapour interface; the hydrophilic head (●) remains in solution, while the hydrophobic tail (∩∩∩) extends out into the vapour phase; SDS remains as monomers in the bulk solution. Above the critical micelle concentration (B), most SDS not adsorbed at the liquid-vapour interface aggregates to form micelles.

concentration (CMC), a value that is dependent on temperature, pressure, and on the presence of other surface active agents and electrolytes. Clusters of ionic surfactants generally contain 10 – 100 molecules; larger clusters are unstable, since the charged head groups tend to break them apart [76]. The CMC for SDS, in pure water at 25°C, is 0.0082 M [77], with micelles containing 62 molecules [78].

1.7.2 SDS-Protein Interactions

When in their native state, proteins fold into structures which have four distinct aspects. Primary structure refers to the amino acid sequence; secondary structure to the localized structures within the protein (*i.e.*, α -helix, β -sheet); tertiary structure to the three-dimensional structure of a single protein; and quaternary structure to the structure formed by several polypeptides.

Upon addition of SDS, proteins are dissociated and denatured, losing their quaternary and tertiary structures; this is due to disruption of intra- and inter-protein binding, caused by hydrophobic interactions between protein apolar sites and the alkyl chain of SDS monomers. At concentrations above the CMC, SDS micelles nucleate on the protein chain, with inter-micellar and protein-micellar charge-charge repulsions, causing loss of much of the existing secondary structures. The nucleation of micelles on the hydrophobic patches of a protein chain explains the mechanism with which SDS aids in protein solubilisation. Insoluble portions are incorporated into the core of a micelle, which is itself dissolved in bulk solution due to favourable ionic-dipole interactions between water and the anionic head groups of SDS [72]. The process of SDS-mediated protein unfolding is endothermic, [79] and is controlled by entropic considerations,

wherein protein denaturation increases system entropy [80]; the absolute values observed are dependent on protein concentration [81].

The structure of the protein-SDS complex has been described by a “necklace” model [82], wherein SDS micelles of constant size are formed along the hydrophobic portions, interspersed with sections wherein the protein retained its secondary structure (Figure 1.6) [83]. Studies have suggested that perturbation in protein structure is independent of protein structure, conformation, and ionization state [72]. As previously mentioned, the saturation point at which SDS no longer binds to protein occurs at an SDS to protein ratio of 1.4 to 1 [38]. However, that ratio holds only when cysteine-cysteine disulfide bonds do not restrict denaturation; if such bonds are not reduced, SDS cannot access the entirety of a protein’s structure and the ratio is therefore lower [84].

1.7.3 SDS and MS

SDS is an excellent tool for preparation and separation of protein samples. Unfortunately, SDS interferes with electrospray ionization-mass spectrometry analysis [85, 86]. It has been reported that complete removal of detergent is necessary in order to obtain reliable mass spectra [87]. Further investigations into the effect of background ions on the ESI process have shown that ion concentrations above 10^{-5} M caused severe signal suppression [88]; from this, it may be inferred that SDS concentrations above $\sim 3 \times 10^{-4}\%$ cannot be tolerated by ESI-MS. However, the values above refer to signal suppression observed *via* direct infusion MS, while proteome analysis is primarily through LC-ESI-MS. Referring to a bottom-up LC-ESI-MS experiment, SDS was well-tolerated at levels up to 0.01% [51].

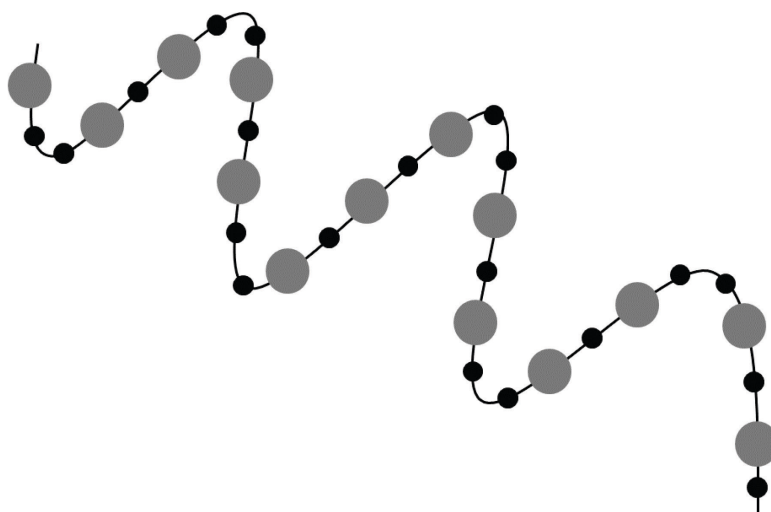


Figure 1.6: A schematic representation of the protein-SDS “necklace” model. SDS micelles (●) form along the hydrophobic portions of the protein, and are interspersed with sections of the protein which retained its secondary structure (●).

Signal suppression occurs both above and below the CMC for SDS, thus signifying that SDS monomers, and not micelles, are the cause. Studies indicate that the large number of anionic surfactant molecules existing at the liquid-vapour interface are the source of the two effects which interfere with ionization. The decrease in surface tension produced by the addition of a surfactant causes destabilization of the Taylor cone and results in reduced spray efficiency; this effect dominates at high SDS concentrations. Furthermore, Coulombic attractions occur between the produced cationic ions and the negative surfactant, reducing the charge at the surface of a droplet and thus interfering with the transfer of such ions into the gas phase; this effect dominates at low SDS concentrations [89].

Additionally, SDS has a negative effect on reversed-phase liquid chromatography separations. The alkyl chain of SDS is highly non-polar and adsorbs strongly onto the reversed-phase column, where the anionic head group works as an ion exchanger, attracting proteins/peptides and thereby retarding their elution and decreasing resolution [90]. Furthermore, elution of SDS does not occur until the end of a gradient, when organic solvent concentration is high (>80% acetonitrile); thus, proteins retained due to hydrophobic interactions with SDS will elute in a large, poorly resolved peak, significantly impacting the number of proteins which may be analyzed. As such, it is necessary to either substitute another detergent for SDS, or remove SDS prior to MS analysis.

1.7.4 Alternative Detergents

While SDS is the most popular detergent for use in proteomics experiments, it is not the only one which has been shown effective for protein extraction, solubilisation and

disaggregation. Indeed, there exist a number of alternatives, including chaotropic agents and detergents with properties which differ from SDS. However, not all are 'MS-friendly', and thus may require removal prior to MS analysis. For chaotropes and detergents which form low molecular weight micelles, application of the sample to a dialysis membrane is an effective method for removal. Chaotropes may be combined into one category, but detergents fall into several: ionic, non-ionic, zwitterionic, and acid cleavable. The benefits and drawbacks of each are discussed below.

1.7.4.1 Ionic Detergents

Ionic detergents may be anionic (*i.e.*, ammonium perfluorooctanoate (APFO), SDS) or cationic (*i.e.*, cetyl trimethylammonium bromide (CTAB)). Solutions containing such compounds exhibit a high ionic strength, and thus denature proteins, a useful trait for protein extraction and solubilisation, and also molecular weight separations [91]. However, they bind to ion exchange resins (*i.e.*, IEC), and move in an electric field (*i.e.*, isoelectric focusing), thus limiting their use in other separation platforms [92]. Additionally, ionic detergents tend to interfere with MS analysis and thus are not ideal alternatives for SDS in a proteome analysis experiment. While there is no one method suitable for depletion of ionic detergents, CTAB is dialyzable [93] and APFO is volatile [57], allowing for their removal with relative ease.

1.7.4.2 Non-Ionic Detergents

Non-ionic detergents (*i.e.*, Triton X-100, n-octyl- β -D-glucopyranoside) are uncharged compounds which work to decrease hydrophobic interactions, and are best suited for the breaking of lipid-protein and lipid-lipid interactions. An interesting property of non-ionic detergent micelles is their temperature dependence; micellar size

increases substantially with higher temperatures. At the cloud point (a compound-specific temperature point) micelles separate out of solution and into a non-aqueous phase. This property is particularly advantageous for membrane protein extraction; however, care must be taken not to use such detergents in separation techniques in which heat is generated (*i.e.*, electrophoresis) [91].

The lack of charge-charge repulsions experienced by non-ionic detergents renders such compounds unsuitable for molecular weight separations, however it does allow solubilized proteins to retain a large portion of their native structure and/or activity [91]. As such, non-ionic detergents are preferred in experiments for determination of protein activities. Additionally, their lack of charge precludes binding to ion exchange resins, thus easily allowing detergent removal by ion exchange chromatography [92] prior to MS analysis.

1.7.4.3 Zwitterionic Detergents

Zwitterionic detergents (*i.e.*, CHAPS) are molecules which have no net charge, offering properties of both ionic and non-ionic detergents. The compounds lack electrical mobility, conductivity, and the ability to bind to ion exchange resins, while still disrupting protein-protein interactions for protein denaturation. Such molecules may be used in both charge-related and electrophoretic separation techniques. Most importantly, they do not have a significant impact on mass spectrometric signal, and thus removal before MS analysis is not necessary [91, 93]. However, their solubilisation efficiency is less than that of SDS, and they are therefore not a suitable substitute for SDS in electrophoretic separation platforms (*i.e.*, SDS-PAGE, GELFrEE).

1.7.4.4 Acid Cleavable Detergents

Acid cleavable detergents (*i.e.*, RapiGest [94]), as applied to sample preparation for mass spectrometry, are a fairly recent class of ‘MS-friendly’ surfactants. They are molecules which aid in protein solubilisation and digestion, but are degraded by cleavage (wherein cleavage usually occurs under acidic conditions). Thus, samples may be applied directly to mass spectrometry without further work-up. Although such detergents are not suitable for gel electrophoresis and are available only at relatively high monetary cost, they are of great interest as a promising alternative to SDS.

1.7.4.5 Chaotropic Agents

Chaotropic agents interfere with non-covalent interactions (*i.e.*, hydrogen-bonding) and thus disrupt the structure of water. The resultant decrease in the number of hydrogen bonds allows for additional water-protein interactions and consequently increases protein solubility. Most suitable for use in proteomics experiments are urea, thiourea and guanidine [95]. Although the most potent, guanidine is ionic and thus suitable only for non-electrophoretic applications; the chaotrope of choice in PAGE is often a combination of urea and thiourea. Advantageous because it does not alter protein molecular weight or isoelectric point, it is nonetheless imperfect. Urea may undergo carbamylation with amino acid side groups, and the combination is not capable of overcoming protein aggregation at high protein concentrations. Additionally, all chaotropes are active only at multimolar concentrations, and thus must be removed by dialysis or solid phase extraction prior to MS analysis [96].

1.7.5 Techniques for SDS Removal

Despite a number of alternative detergents available for use in proteomics experiments, SDS remains among the most powerful and well-characterized, and is still the top choice for many researchers. It is most commonly employed to aid in protein solubilisation, or to allow for molecular weight separation in SDS-PAGE applications. This includes the traditional slab-gel format, but also GELFrEE, wherein protein fractions are collected in Laemmli running buffer, a solution which contains approximately 0.1% SDS. As such, there is considerable interest in the development of high-throughput methods for efficient detergent depletion. A significant number of techniques have previously been published, some as recently as 2010 [97].

The techniques for detergent depletion may be broken into several categories: precipitation, solid phase extraction, ion-pair extraction, electrophoresis, 'other', and techniques which are commercially available. A review of these existing SDS removal methods is provided below.

1.7.5.1 Precipitation

SDS depletion methods which rely on precipitation fall into two groupings: protein precipitation and dodecyl sulfate precipitation. The latter exploits the insolubility of dodecyl sulfate (DS) potassium and divalent salts [98] to precipitate free dodecyl sulfate. Two separate studies found that potassium phosphate, added to SDS-containing solutions at a final concentration of 20 mM (pH 7.4), removed sufficient detergent for subsequent trypsin digestion [99], analysis by Lowry protein assay [98], or amino acid sequencing [100]. The protocol was later expanded for removal of salts and other undesirable compounds, whereby the supernatant from the DS-precipitate was retained

for subsequent precipitation of KDS-bound proteins [101, 102]. The highest yields were found to occur at low pH (10% trichloroacetic acid) and low temperature (2 to 4°C) conditions.

When performed together, KDS and subsequent KDS-protein precipitation were effective at SDS removal, even at high SDS concentrations (up to 2.3% SDS), with less than 0.01% remaining after treatment. There was slight loss of hydrophilic proteins during free-DS precipitation; however, the majority of proteins precipitated out during KDS-protein precipitation. Recoveries for hydrophilic proteins were determined to be greater than 75%, and hydrophobic proteins greater than 90% [102]. However, protein precipitation suffers because these proteins which become insoluble are difficult to handle after removal of detergent. Additionally, while the described methods are inexpensive, they are also labour-intensive – a number of steps are required for each precipitation, and these methods are therefore not suitable for high-throughput analysis.

Organic solvent protein precipitation is a popular technique for removal of many contaminants, since it is relatively quick, inexpensive, and amenable to high-throughput analysis. The mechanism of precipitation occurs as such: addition of organic solvent progressively displaces water from the protein hydration layer, where the resultant decrease in hydration layer allows for protein aggregation and precipitation by attractive electrostatic and dipole forces.

Two methods available for organic solvent precipitation are (i) cold acetone, whereby incubation in cold acetone causes precipitation [51, 103-105], and (ii) chloroform/methanol/water (CMW), whereby addition of the aforementioned reagents at a ratio of 1:4:3 (to one part sample) results in a two-phase system where protein

precipitates at the interface [51, 103]. The originally reported method for CMW precipitation provided sufficient SDS removal for subsequent MS analysis (1000-fold reduction), though it is also possible to obtain further SDS reduction through washing of the protein pellet. It was also demonstrated that protein pellets obtained from acetone precipitation of samples initially containing >0.1% SDS require at least one wash in order to obtain adequate detergent depletion (*i.e.*, to a level below 0.01% SDS). Additionally, while both techniques exhibited moderate to high protein recoveries (>80% for acetone, >50% for CMW) [51], recovery dropped significantly for proteins and peptides smaller than 10 kDa¹.

Additional methods for protein precipitation which allow for SDS removal include acid precipitation and salting-out. Acid precipitation employs trichloroacetic acid (TCA), in the presence of a carrier molecule (*i.e.*, deoxycholate), to achieve recoveries of approximately 90% from even highly dilute solutions [106-108]. Salting-out precipitates proteins based on their decreased solubility at high salt concentrations. The mechanism occurs as such: salt ions interact with water molecules, thus decreasing the number available for protein solvation. At a given salt concentration (dependent on salt and protein properties), the strength of protein-protein interaction becomes greater than protein-water interaction, resulting in precipitation. This is commonly performed using ammonium sulfate [109, 110].

As with KDS-protein precipitation, many of the precipitated proteins become difficult to re-solubilize after removal of detergent. As such, despite simplicity and ease of use, the applicability of organic solvent precipitation, TCA precipitation, and salting-out are dependent on the type of sample to be treated.

¹ Determined from unpublished work in the Doucette laboratory.

1.7.5.2 Solid Phase Extraction

The term ‘solid phase extraction’ encompasses several techniques; a number of these have been applied to remove SDS from protein samples. These include size exclusion, anion exchange, cation exchange, ion-retardation resin, ceramic hydroxyapatite, reversed-phase, and hydrophilic interaction liquid chromatography. Although size exclusion is not truly a solid phase extraction (SPE) technique, its application to SDS removal is similar to that of true SPE methods and it is therefore included here.

Relative to the size of proteins, SDS is a small molecule (MW 288.38 g/mol), and 100-fold depletion of SDS in samples with proteins of molecular weight as low as 2000 Da may be achieved by size exclusion chromatography, whereby smaller molecules take longer to elute [103, 111]. However, it is important to account for SDS micelles, which have significantly higher molecular weight (approximately 18 kDa [112]). It is thus necessary to work under conditions which promote the dissociation of micelles. Reported methods for such involve protein solubilisation and column equilibration with solutions containing organic solvents [113, 114] or chaotropic agents (*i.e.*, urea) [115]. Under these conditions, SDS removal is simple and effective, with protein recoveries typically greater than 75%. However, the presence of urea necessitates an additional clean-up step, and the presence of organic solvents may cause protein aggregation and subsequent precipitation, limiting the applicability of this method.

There are several chromatographic techniques which fall under the purview of ion exchange chromatography; the first is anion exchange, whereby positively-charged molecules are retained on the negatively-charged column. In such experiments for SDS

removal, SDS is retained and proteins eluted. Although reported depletion of SDS was consistently sufficient for subsequent MS analysis [90, 116, 117], protein recovery was component dependent and varied from 50% to 100% [116]. Losses were likely due to SDS-bound, hydrophobic or basic proteins binding irreversibly to the column [90]. However, incubation of sample in 8 M urea resulted in overall higher recoveries [117], likely due to a weakening of SDS-protein interactions in the presence of a chaotropic agent. Despite the effectiveness of anion exchange methods for removal of SDS, care should be taken when analyzing hydrophobic (*i.e.*, membrane) proteins so as to avoid significant sample loss, particularly when only limited analyte is available.

Cation exchange works in the opposite manner, wherein positively-charged molecules are bound to the negatively-charged resin. In such experiments, proteins are retained and SDS eluted. However, this area has not been the subject of significant research, with detergent depletion by cation exchange occurring as a secondary consideration to the main goal of the experiment [118, 119]. Although it does seem effective, with detergent-depleted proteins being successfully analyzed by mass spectrometry, absolute values for protein recovery and SDS reduction have not been published.

Ion retardation resins are similar to mixed-bed ion exchange, containing both positively- and negatively-charged ion exchangers; however, the makeup of ion retardation chromatography columns differs from mixed-bed in that there is not an equivalent mixture. Instead, negatively-charged molecules are held within a 'cage' of positively-charged resin. This type of ion exchange chromatography is capable of adsorbing both anions and cations, and is best buffered using a compound with a neutral

buffering range. It was shown to be capable of removing most free SDS, although it appears that much of the protein-bound SDS remains. Protein recoveries were component dependent, ranging from 58% to 98% (average 83%) [120, 121]. However, only a select few standard proteins were tested, which may not provide a complete picture for protein recovery. Care should be taken if using ion retardation resin for SDS removal from a complex proteome, as it is possible that many proteins will bind to the resin.

Ceramic hydroxyapatite chromatography utilizes ceramic hydroxyapatite for SDS reduction and reactivation of proteins. SDS-protein complexes are bound to the column, upon which they are washed with phosphate buffer containing a mild detergent. This detergent exchanges with SDS, and an increase in phosphate buffer elutes the retained protein at a recovery of 90% to 100% [122, 123]. Though an efficient and effective method, SDS is removed only at the cost of introducing an alternative detergent which may also interfere with MS analysis. It is therefore best to use this method only when protein reactivation is desired.

Traditionally, both SDS and proteins are retained on a reversed-phase column. However, when loading at high concentration of n-propanol, certain column packings (*i.e.*, Zorbax) retain proteins but elute SDS and other contaminants. All free SDS is removed, and the high concentration of organic solvent weakens SDS-protein binding, allowing for removal of some protein-bound SDS. Standard proteins were eluted with a decreasing gradient of n-propanol, and were recovered with 90% yield through addition of an ion-pairing reagent to the mobile phase [124]. Additionally, utilization of a high flow rate allows for sample collection in a small volume (< 100 μ L). However, despite the effectiveness of this method, its application to membrane proteins is limited – the

presence of n-propanol often leads to protein aggregation, resulting in significant sample loss.

HILIC, a variation of normal phase chromatography, was employed for SDS removal [125]. Protein recovery varied between proteins, but was never less than 50%. SDS removal was high, sufficient for subsequent N-terminal amino acid sequencing. However, the mobile phase contained a high concentration of n-propanol, and thus this method suffers from the same disadvantages as detergent depletion using reversed-phase chromatography.

1.7.5.3 Ion-Pair Extraction

Ion-pair extraction utilizes ion-pairing of dodecyl sulfate with triethyl- or tributylammonium ions; upon pairing with such organic compounds, the anionic detergent forms an organic solvent-soluble oil, and the protein is precipitated. SDS removal by ion-pairing works both in-solution [126-128] and in-gel [129], although protein precipitation does not occur under the latter condition. Observed protein recoveries were greater than 70%, and samples did not require further clean-up prior to MS analysis. However, in-solution removal necessitates that the sample be free of salts and buffers. If such contaminants are present, the sample must be dialysed prior to SDS extraction. Dialysis is a time-consuming process, and thus affects the application of this technique to high-throughput analysis. Additionally, in-gel removal of SDS requires that proteins be subsequently digested, and many protocols for peptide extraction already incorporate an effective SDS removal strategy. This same consideration also applies to substitution of SDS with an MS-compatible non-ionic detergent, such as octyl- β -glucopyranoside, wherein protein recovery was 80% and SDS reduced 10,000-fold [130].

1.7.5.4 Electrophoresis

There are a number of SDS removal techniques which rely on the application of an electric field, many of which are preceded by electroelution of protein from a polyacrylamide gel [45, 128, 131]. One electrophoretic method for SDS removal is electrodialysis, whereby proteins are eluted from an excised gel plug and retained on a dialysis membrane. SDS selectively migrates through this membrane, and the resulting sample has significantly reduced SDS content (< 0.001% SDS). Proteins are desorbed from the membrane by a ten second reversal of current; total recovery was high, as evidenced by the lack of signal change observed in treated versus un-treated samples. However, the sheer number of gel slices excised from a typical polyacrylamide gel (50+) limits the applicability of this method in high-throughput analysis.

An interesting electrophoretic technique for SDS removal utilizes two troughs in a polyacrylamide gel; the furthest upstream contains the SDS-protein sample, immediately followed by a trough containing Triton X-100. On application of an electric field, SDS-protein complexes migrate through the Triton and the anionic and non-ionic detergents interact to form micelles. The resultant micelles separate from the protein, which is then electroeluted and analyzed [132]. However, the necessity of electroelution adds significant time and labour to the method, rendering it unsuitable for high-throughput analysis.

Additionally, there exists a method applicable only to MALDI analysis, wherein proteins are electroblotted directly onto a surface support suitable for MALDI-MS analysis, followed by thorough washing to remove all contaminants. However, this

requires a large amount of sample, as greater than 98% is lost during the process, severely limiting its applicability [128].

In addition to methods requiring electrophoresis, it has been shown that overnight incubation of an SDS-containing polyacrylamide gel in methanol/acetic acid/water removes SDS by diffusion [133]. However, the extremely long incubation time renders this method less favourable as compared to others. Another method employs approximately two hours of washes for SDS removal, followed by overnight in-gel trypsin-digestion and subsequent peptide extraction [49]. Although the time required is significant, the direct applicability of obtained peptides to MS is beneficial.

1.7.5.5 'Other' Approaches to SDS Removal

Other techniques for removal of SDS include a specialized electrospray ionization apparatus. CTAB is added to the SDS-containing sample, forming a dodecyl sulfate-cetyl trimethylammonium (DS-CTA) ion-pair; both sample and acidic methanol aerosols are generated by separate tips, and the droplets fused. Due to its low solubility in methanol, the DS-CTA pair is excluded from the droplet and thus does not interfere with MS analysis. With such a technique, proteins were observed at up to 0.3% SDS, however the spectra did show more interference as compared to a non-SDS containing control sample [85]. This method is not applicable in most laboratories, since it requires building of a specialized ionization source, a cost not required by the majority of other SDS removal techniques.

Alternatively, absorption of a high concentration SDS sample (4% SDS) into a vacuum-dried polyacrylamide gel is effective for both hydrophobic and hydrophilic proteins [97]. Subsequent in-gel washing steps removed $80 \pm 6\%$ SDS with minimal

protein loss. The proteins identified following in-gel digestion, peptide extraction and LC-MS/MS analysis exhibited varying hydrophobicity and molecular weight, indicating the lack of bias in this method, a concern in many other SDS removal techniques. However, the method is not suitable for experiments where proteins must be kept intact, such as in top-down proteomics, since digestion is necessary to extract peptides from the polyacrylamide gel.

1.7.5.6 Commercial Strategies

There are a plethora of commercially available detergent depletion methods, the majority of which come packaged as a spin column or as an online HPLC guard cartridge (wherein the contaminant is retained on the guard column, and sample is eluted directly onto an additional column for separation). With the exception of SDS-Out [134], wherein free SDS is precipitated, all products bind to either SDS or protein. The Nest Group offers a reversed-phase guard column [135], and both The Nest Group and Glygen Corp offer HILIC spin columns [136, 137]. Michrom Bioresources, Inc. offers a combined anion exchange-HILIC trap cartridge [138], and Biotech Support Group an aqueous suspension of non-ionic adsorbent for binding to SDS (SurfactAway™ Non-Ionic) [139]. Pierce Detergent Spin Removal Columns [140], Detergent-OUT™ [141], QuickSpin Detergent Removal Kit [142], and the Filter Aided Sample Preparation (FASP™) Protein Digestion Kit [143] all utilize a spin-column format for detergent depletion. While the first three allow for collection of intact protein in solution, the mechanism of action for the last is different. It employs a molecular weight cut-off filter, wherein SDS and other contaminants are eluted, and proteins are retained. Proteins are then subject to trypsin digestion and the resultant peptides eluted through the membrane.

The aforementioned commercially available methods for SDS reduction do not make by any means a complete list, however they are representative of the techniques available for purchase.

1.7.5.7 Challenges of SDS Removal

There exist a large number of SDS removal techniques, and many are applicable to SDS removal in protein or peptide samples. However, none are universally applicable to all proteomics experiments (*i.e.*, MALDI *vs* ESI, sequencing *vs* activity characterization, or top-down *vs* bottom-up). Additionally, available methods often suffer from poor or biased protein yields; biased yields occur when different sample components are recovered with varying yields, thus potentially resulting in the loss of important sample component(s). This is of particular concern in biomarker experiments, whereas poor protein yield is of greatest concern when treating samples containing limited analyte.

The poor protein yields often observed are due to the depletion of SDS to which proteins are bound. Achievement of high recovery is therefore often accompanied by the presence of residual SDS. As a specific example, dodecyl sulfate precipitation is limited by the K_{sp} of the system; when performed without subsequent KDS-protein precipitation, it succeeds at removing approximately 90% SDS, which is not sufficient for MS analysis. Addition of a chaotropic agent (*i.e.*, urea) has been shown to be effective in the dissociation of SDS-protein complexes [144]. However, as urea interferes with non-covalent interactions, it also causes protein denaturation. It is therefore not suitable for experiments in which the recovery of protein activity is desired. Furthermore, addition of urea in elution buffers [115, 117] necessitates that dialysis be performed for its removal.

As an additional clean-up step, dialysis introduces further time, labour, and the potential for protein loss, and is therefore not ideal.

Urea has been successfully incorporated for SDS removal from intact proteins *via* the FASPTM method [144]; however, it requires protein digestion and is thus suitable only for bottom-up experiments. Furthermore, the FASP method is unsuitable for SDS removal from peptide samples, as many peptides are too small to be retained by the dialysis membrane. The limitations suffered by the currently available methods ensure that the development of new techniques for SDS removal remains an area of great interest.

1.8 SDS Quantification

To determine the efficiency of the previously discussed SDS removal techniques, it is necessary to quantify the amount of SDS remaining. There are many methods available for such, a number of which rely on colorimetric approaches. However, as a whole, colorimetric methods which depend on ion-pair extractions into organic solvents suffer from insufficiently low limits of detection and tend to lack specificity for dodecyl sulfate [145-148]. The methylene blue spectrometric assay [145] is an example of such, wherein a chloroform-extractable ion-pair is formed between methylene blue and dodecyl sulfate, inducing a quantifiable colorimetric change in the chloroform layer. However, it is prone to interferences from any number of common anions.

Titrimetry techniques employ titration for quantification of SDS (*i.e.*, CTAB as a titrant) [149], while capillary electrophoresis separates SDS from other sample components by its size, charge, and frictional force, with quantification occurring by incorporation of an internal standard and indirect UV detection [150]. Both techniques

encounter obstacles similar to those observed for spectroscopic techniques. Titrimetry protocols in particular are often time- and labor-intensive, prohibiting high-throughput analysis. While sufficient selectivity and sensitivity was demonstrated using gas chromatography [151] and gas chromatography-mass spectrometry [152], these techniques are either labour-intensive or are prone to interferences from protein. As such, they are not suitable methods for SDS quantification. Using LC-MS, sensitive techniques for the detection and quantification of multiple anionic surfactants in wastewater have been developed [153]. Though a sensitive and attractive option for SDS quantification, it is time-intensive and therefore not suitable for high-throughput analysis. The limits of detection for each technique are summarized in Table 1.1; capillary electrophoresis is least sensitive ($\text{LOD} = 1.38 \times 10^{-4} \text{ M}$) and LC-MS most sensitive ($\text{LOD} = 1.73 \times 10^{-9} \text{ M}$). However, the choice of method really depends on sample components and the levels of SDS to be quantified.

1.9 Research Proposal

Sodium dodecyl sulfate is a detergent commonly used in proteomics experiments. It aids in protein solubilisation and digestion efficiency, and also allows for electrophoretic molecular weight separation. However, the presence of SDS causes severe signal suppression in mass spectrometric analysis. Although commonly used at concentrations of 0.1% or greater, the threshold level to which SDS must be depleted for reliable mass spectra has been reported as 0.01% [51]. SDS removal to below this level is therefore necessary prior to MS analysis. There currently exist a number of methods for SDS removal, both commercial and non-commercial.

Table 1.1: Summary of the limits of detection for SDS quantification methods.

Technique	Limit of Detection (M)	Reference
Titration	1.0×10^{-5}	[149]
GC	1.7×10^{-6}	[151]
GC-MS	3.5×10^{-8}	[152]
LC-MS	1.7×10^{-9}	[153]
Spectrophotometric	3.0×10^{-6}	[145]
Capillary Electrophoresis	1.4×10^{-4}	[150]

Nonetheless, to determine the efficiency of a detergent depletion technique, it is necessary to quantify the SDS remaining in solution. Currently, methods available for high-throughput SDS quantification in proteome-containing samples lack sensitivity and selectivity. To overcome such limitations, a negative mode LC-ESI-MS technique was optimized.

Among the more recent commercial techniques for SDS removal are the Pierce Detergent Removal Spin Columns, wherein SDS-containing samples are applied to a spin column; the solid particulates within the column bind to SDS, but allow proteins to elute. The company advertises both efficient detergent depletion and high protein recovery; the latter is an important consideration, since protein loss may result in insufficient analyte remaining for subsequent analysis. However, Pierce reported recovery for only one protein, which is not sufficient data to justify the claim of “high protein recovery”. Therefore, the efficiency of protein recovery for a variety of samples will be investigated, including standard proteins and complex mixtures. Should their yields be poor, an alternate protocol will be developed for enhanced protein recovery.

Among the non-commercial techniques, cation exchange chromatography (wherein protein is retained and SDS eluted) has arguably received the least attention, particularly in regards to automation. Therefore, a fully automated SDS reduction technique employing cation exchange coupled to reversed-phase chromatography will be developed. Given the difficulty of removing SDS from low molecular weight proteins *via* protein precipitation methods, the system will be optimized with the relatively low molecular weight sample of cyanogen bromide-digested yeast.

Overall, the development of two techniques for SDS depletion will be presented, in which removal of SDS is efficient and protein recoveries are high. Both the developed negative mode LC-ESI-MS technique and the methylene blue spectrophotometric assay [51] will be employed for SDS quantification. Throughout the development of each protocol, protein recovery will be assessed through two quantitative strategies: the BCA assay [154] and peak areas as obtained from LC/UV absorbance.

Chapter 2

Sensitive and Specific Quantification of Sodium Dodecyl Sulfate by Negative Mode LC-ESI-MS

2.1 Introduction

One of the simplest methods available for SDS quantification is the methylene blue colorimetric assay [145], wherein a chloroform-extractable ion-pair is formed between SDS and methylene blue. It has a limit of detection of 0.2 μg SDS in 200 μL , or $1 \times 10^{-4}\%$ *w/v* SDS. Given the potential influence of SDS on MS signals, even at trace levels of the surfactant in a protein sample, an assay with improved sensitivity would be desirable. Additionally, the colorimetric protocol is prone to interference from any number of common anions, which further raises the limit of detection. Following a survey of alternative procedures for SDS quantification, it was concluded that no suitable published method is available (see Chapter 1). Thus, establishment of a technique with the required sensitivity and specificity to quantify trace levels of SDS in protein-containing samples is warranted. Having been shown effective for analysis of anionic surfactants in waste water, negative mode LC-ESI-MS [153] was chosen as a suitable instrument for the optimization of an SDS quantification method; however, the previously reported method was deemed unsuitable due to the requirement of a preconcentration step, thereby limiting the applicability of the method to high-throughput analysis. The potential for automation of this technique increases the appeal.

2.2 Materials and Methods

2.2.1 Reagents and Solutions

Protease inhibitor cocktail (cat. P8849) and *Saccharomyces cerevisiae*, Type II (cat. YSC2) were purchased from Sigma-Aldrich Canada Inc. (Oakville, Canada). Sodium dodecyl sulfate was obtained from Bio-Rad (Mississauga, Canada). Milli-Q grade water was purified to $18.2 \text{ M}\Omega\text{cm}^{-1}$. HPLC grade solvents were purchased from Fisher Scientific (Ottawa, Canada). The BCA protein assay was obtained from Pierce (Rockford, Illinois, USA). The Waters Spherisorb $5 \mu\text{m}$ C18 beads for HPLC were acquired from Waters Corporation (Milford, Massachusetts, USA).

2.2.2 Yeast Proteome Extraction

Five grams of lyophilized cells of *S. cerevisiae* were suspended in 40 mL of warm ($40 \text{ }^\circ\text{C}$) water until reconstituted (approximately 30 minutes), followed by centrifugation at $\sim 500 \times g$ and discarding of the supernatant. This process was repeated an additional ten times until the supernatant was colourless. The final volume of the cell pellet was 10 mL. Protease inhibitor cocktail was added to the remaining yeast-water mixture at a 1:20 volume ratio. The mixture was frozen by slowly pouring the suspension into liquid nitrogen, after which the frozen cells were lysed by grinding in a mortar with a pestle for ~ 10 min until the solid was in the form of a fine powder. Proteins were extracted by adding 5 mL of water with gentle shaking for 30 min, followed by centrifugation ($15,000 \times g$) on a Fisher-Scientific accuSpin Micro centrifuge for five minutes and collection of the supernatant. The protein concentration, as determined through BCA assay was 2.0 g/L (Section 2.2.5).

2.2.3 Organic Solvent Precipitation

The extracted yeast proteins were precipitated using a modified chloroform:methanol: water method, as described by Botelho *et al.* [51]. Briefly, 400 μL methanol, 100 μL chloroform and 300 μL water were added to 100 μL sample (100 μg protein), with gentle mixing after each addition, followed by centrifugation at $15,000 \times g$, and removal of the top layer (water/methanol). Methanol (400 μL) was added, and the solution was gently agitated to encourage mixing of the solvents. Further centrifugation at $15,000 \times g$ and decanting of the solution preceded addition of a final 400 μL of methanol, followed by centrifugation and decanting of the methanol. Any remaining traces of liquid were allowed to evaporate in the fume hood at room temperature.

2.2.4 Mass Spectral SDS Assay

An isocratic LC/MS method was employed to quantify the SDS, employing a self-packed C18 column (1 mm \times 10 cm, 5 μm , Waters Corp.) with an Agilent 1100 HPLC system. The solvent composition was held constant at 85% acetonitrile, 0.1% formic acid in water, with a flow rate of 50 $\mu\text{L}/\text{min}$. An injection volume of 5 μL was used. Effluent from the C18 column was directed to a Finnigan LCQ Duo ion trap mass spectrometer (Waltham, USA) with electrospray ionization operated in negative mode with spray voltage 3.5 kV and capillary temperature 200 $^{\circ}\text{C}$. The instrument was operated from m/z 260 to 270. The Xcalibur Qual Browser (Version 1.2) 'add peaks' function was used to determine the peak area from the total ion chromatogram. A 6-point calibration curve was created (0.5 ng to 100 ng) and original samples diluted to fit.

2.2.5 Pierce BCA Assay

The BCA assay was performed as described by Pierce [154]. Briefly, reagent A and reagent B were mixed in a 50:1 ratio to form working reagent, and 500 μL working reagent added to 25 μL sample followed by brief vortexing. Samples were incubated at 37 $^{\circ}\text{C}$ (standard protocol, 20 $\mu\text{L}/\text{mL}$ to 2000 $\mu\text{L}/\text{mL}$) or 60 $^{\circ}\text{C}$ (enhanced protocol, 5 $\mu\text{L}/\text{mL}$ to 250 $\mu\text{L}/\text{mL}$) for 30 minutes and absorbance measured at 562 nm, against a BSA calibration curve (0.125 μg to 6.25 μg for the enhanced protocol; 0.5 μg to 50 μg for the standard protocol).

2.3 Results and Discussion

Figure 2.1a shows an isocratic LC/MS method using negative mode ESI to create a calibration curve over the range of 0.5 ng to 100 ng SDS. Liquid chromatography was employed prior to MS as a means of eliminating interferences caused by buffers or salts. Injection occurred in twenty minute intervals, which allowed for a moderately high-throughput assay. However, Figure 2.1a clearly illustrates that peaks return to baseline after only eight minutes, and so it was concluded that the time between injections could be further shortened to ten minutes without affecting resolution, therefore increasing the throughput of the assay. The observed peak-tailing has two possible causes (i) hydrogen-bonding between SDS and the silanol backbone of the column packing material, or (ii) SDS binding and lingering in the MS source. Retention times (3.1 ± 0.2 min) remained constant and reproducible for all injections, while base peak widths increased as SDS concentration increased (*i.e.*, 5.0×10^{-4} μg SDS, $w_b = 4.32$ min; 0.10 μg SDS,

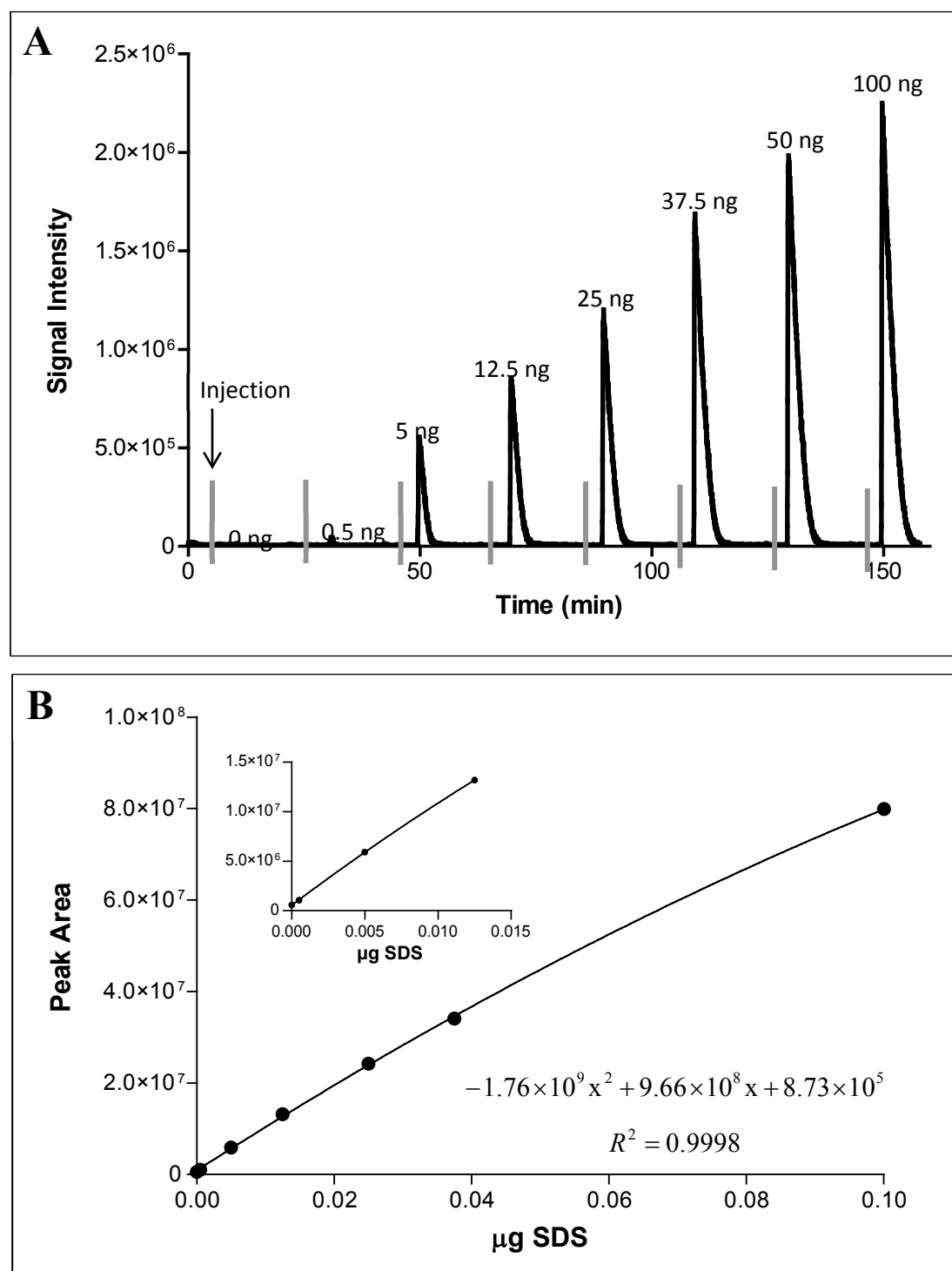


Figure 2.1: Employing isocratic LC-LCQ-MS in negative mode to quantify SDS. (A) SDS (0 – 0.1 μg) injected onto LC-LCQ-MS, negative mode, monitoring m/z 265.4 and (B) quadratic calibration curve generated by integration and plotting of peak areas.

$w_b = 8.01$ min). Due to the increase in peak width, the peak area was chosen to construct the calibration curve.

The resultant peak areas were obtained from the total ion chromatogram (m/z 260 – 270) and plotted to create a curve, as shown in Figure 2.1b. This data were fit to a quadratic equation since it provided the best correlation coefficient (0.9998). Additionally, analysis of quadratic versus linear curves by the F-test concluded that the quadratic fit was preferred. The shape of the calibration curve is likely due to detector saturation. However, the quadratic model does not in any way affect the validity of the results. Additionally, solutions of known SDS concentration were injected and the concentration determined using both a linear and quadratic model, as illustrated in Table 2.1. The quadratic fit produced values significantly closer to the true value, particularly at low values, proving the accuracy of this method. Although a linear region exists in the range of 0.5 ng to 12.5 ng, use of such would limit the quantitation range, thereby justifying the use of a non-linear trendline.

The standard curve was generated from SDS-containing samples which were free of protein. Addition of protein to the SDS-containing samples resulted in slight peak broadening, whereby base peak width increased by approximately 12%. This was likely due to protein-bound SDS eluting off the column with slightly different retention times, whereupon ionization caused separation of the SDS-protein complex. As demonstrated in Table 2.1, injection of a known standard containing 2.5 μg protein and 2×10^{-3} μg SDS shows that peak broadening had no significant effect on accuracy or sensitivity.

Table 2.1: Comparison of the accuracy of linear and quadratic fits from the calibration curve of mass spectrometric SDS assay. Calculated in the presence or absence of protein.

SDS Injected (μg)	Protein Injected (μg)	Linear Fit		Quadratic Fit	
		Calculated SDS Injected (μg)	Percent Error	Calculated SDS Injected (μg)	Percent Error
0.0020	-	0.00131	34.5	0.00205	2.5
0.0020	2.5	0.00135	32.5	0.00206	3.0
0.0050	-	0.00360	28.0	0.00499	0.2
0.0090	-	0.00797	11.4	0.00907	0.8

0.1% SDS in 100 μL (100 μg), the method would be sufficient for quantification of the detergent at a level of 99.99% removal.

Reproducibility of the system is an important consideration, as high reproducibility allows for more sample replicates and fewer technical replicates. Replicate injections at ten minute intervals for one hour (6 injections total) resulted in peak area variability with a relative standard deviation (RSD) of 1.5%. Peak area variability of 30 replicate injections over ten hours resulted in an RSD of 4.6%. Nevertheless, the experiment is valid only if the spray tip has not moved and if no source contamination has occurred. Since the instrument employed was a multi-user instrument, it was therefore necessary to calibrate at the beginning of each use.

Due to the interfering properties of SDS, one might expect a systematic decrease in intensity caused by a build-up of SDS inside the MS source. This was in fact observed when the system was operated at a flow rate of 100 $\mu\text{L}/\text{min}$. However, decreasing the flow rate to 50 $\mu\text{L}/\text{min}$ eliminated this effect. Figure 2.2 demonstrates that no trends in peak area variability were observed as a function of time, indicating that any variability is likely due only to variations in the instrument. These relatively low RSD values demonstrate the stability and reproducibility of this method. A summary of values developed for this assay are displayed in Table 2.2.

Sensitivity of this method is comparable to or better than the majority of SDS quantification methods published in the literature; quantification by GC-MS [152] is one of the most sensitive high-throughput methods published, with a reported LOD of <1 ng. However, the presence of protein renders this method unusable, while the SDS

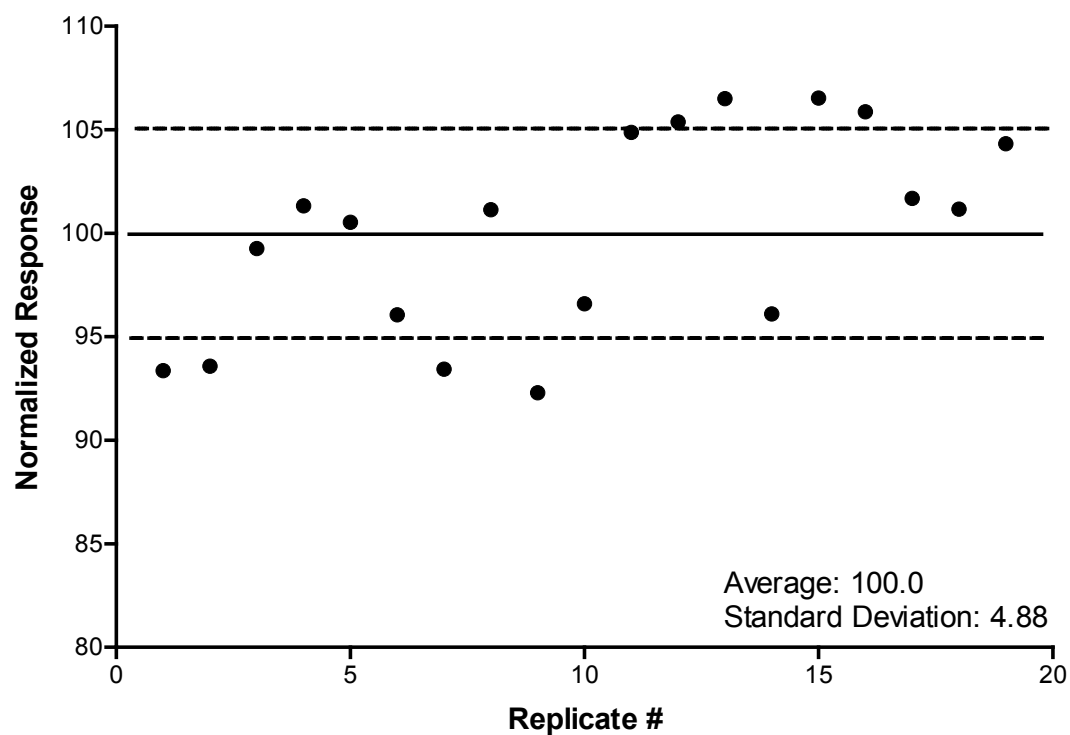


Figure 2.2: Peak areas obtained from repeated injections of 0.1 µg SDS onto LC-LCQ-MS, monitored at m/z 265.4. Injections occurred over ten hours. Data normalized to average TIC peak area.

Table 2.2: Summary of parameters determined for mass spectrometric SDS assay.

Parameter	Value
Quadratic Range	0.5 ng – 100 ng
Limit of Quantitation	0.5 ng
Limit of Detection	0.15 ng
Stability (RSD for 30 Minutes)	> 98.5%
Stability (RSD for 10 Hours)	> 95.0%
Coefficient of Variation (Quadratic Fit)	0.9998

quantification method chosen is not prone to interference from protein or other common contaminants. Indeed, LC-ESI-MS was chosen in part to ensure that interferences are minimized, since it separates both free SDS and protein-bound SDS from other sample components.

2.4 Conclusions

Herein, a study was presented which optimized an LC-MS-based protocol for SDS quantification in proteomics experiments. It is amenable to high-throughput analysis, and is both sensitive and selective. This method offers an attractive alternative to existing methods for SDS quantification in protein-containing samples.

Chapter 3

Development of an Alternate Protocol for Increased Protein Recovery with the Pierce Detergent Removal Spin Columns

3.1 Introduction

Sodium dodecyl sulfate (SDS) is the most widely used detergent in proteomics experiments, owing mainly to its use in SDS polyacrylamide gel electrophoresis (SDS-PAGE). SDS is typically used at concentrations between 0.1% and 2%. Unfortunately, SDS is known to cause severe signal suppression for proteins and peptides in electrospray mass spectrometry (ESI-MS) [87]. Thus, it is necessary to remove this ionic surfactant before ESI-MS analysis.

The specific tolerance level of SDS in an LC-ESI-MS experiment for proteome analysis was discussed in Chapter 1; the critical threshold value to which SDS must be depleted was found to be 0.01% [51]. Under the conditions used, this translated into an SDS to protein ratio of 4:1. This value is approximately three times higher than the maximal SDS to protein binding ratio of 1.4:1. The SDS threshold tolerance is likely dependent on experimental conditions, thus obtaining a technique which depletes SDS below 0.01% is important.

As outlined in Chapter 1, several methods are available for SDS depletion. Among them is the Pierce Detergent Removal Spin Columns, a commercial product which permits high-throughput and efficient detergent removal (upwards of 99% depletion) [140]. The specific mechanism of detergent depletion is unclear, though one notes that these columns are capable of removing ionic as well as non-ionic detergents, through binding of the surfactant to the column support.

For the Pierce Detergent Removal Spin Columns, Pierce advertises high protein recovery; 95% yield was recorded for a 100 μg sample of trypsin-digested BSA. Nevertheless, this reported recovery rate cannot sufficiently justify a claim of “high protein recovery” for two distinct reasons.

The first relates to analyte concentration; the sample used by Pierce had a protein concentration of 1 g/L, and an absolute amount of 100 μg . While a greater amount of protein is always desired, on the order of 100 μg starting material is a minimum recommended amount of protein for most proteome profiling experiments. However, it is noted that sample processing steps, including fractionation, often dilute or divide the sample to significantly lower amounts (1-10 μg). The study of low-abundant proteins is also common, but in such analyte-limited cases, SDS removal strategies must be optimized according to lower starting concentrations. When sample is limited, protein loss is of particular concern, as it may bring the final concentration of available analyte below instrument detection limits.

The second concern related to the reported protein yield is that it is based on a single, standard (and digested) protein. Complex proteome mixtures contain proteins exhibiting a wide range of characteristics (size, charge, hydrophobicity) which likely influence the respective recovery rate for individual proteins when subjected to an SDS removal strategy. Unbiased protein recovery, wherein recoveries are equal for all observed proteins, is an especially critical consideration in biomarker discovery experiments so as to avoid the loss of sample components which may be of importance. Intact (undigested) proteins are also expected to behave quite differently than peptides arising from trypsin digestion. Thus, it remains unclear if application of the Pierce

Detergent Removal Spin Columns to samples containing lower quantities of protein or to proteins with varying characteristics would yield similarly high recoveries as those reported with digested BSA.

Herein, protein yields were investigated after application of the detergent removal spin columns for analyte-limited samples of standard proteins and complex mixtures. It was determined that effective reduction of SDS was obtained with the standard protocol as recommended with the Pierce Detergent Removal Spin Columns. However, it was also found that protein yield varied according to protein type, and that yield suffered at low concentrations. An alternate protocol was therefore developed whereby the volume of the detergent depletion column was minimized in an attempt to improve protein recovery at low concentration, while maintaining the efficiency of SDS depletion.

3.2 Materials and Methods

3.2.1 Reagents and Solutions

Iodoacetamide, ammonium bicarbonate, myoglobin, lysozyme, ubiquitin, cytochrome c, β -lactoglobulin A, protease inhibitor cocktail (cat. P8849), trypsin (TPCK treated, cat. T1426) and *Saccharomyces cerevisiae*, Type II (cat. YSC2) were purchased from Sigma-Aldrich Canada Inc. (Oakville, Canada). Sodium dodecyl sulfate, urea and dithiothreitol were obtained from Bio-Rad (Mississauga, Canada). Sodium sulfate was from Caledon Laboratory Chemicals (Georgetown, Canada) and sulfuric acid from EMD Chemicals (Gibbstown, USA). Milli-Q grade water was purified to $18.2 \text{ M}\Omega\text{cm}^{-1}$. Chloroform, methanol, methylene blue, and HPLC grade solvents were purchased from Fisher Scientific (Ottawa, Canada). The BCA protein assay and Detergent Removal Spin

Columns were obtained from Pierce (Rockford, Illinois, USA). The Waters Spherisorb 5 μm C18 beads for HPLC were acquired from Waters Corporation (Milford, Massachusetts, USA), and the Jupiter 3 μm C18 beads were from Phenomenex (Torrance, USA).

3.2.2 Yeast Proteome Extraction

Yeast was prepared as described in Chapter 2, Section 2.2.2.

3.2.3 Organic Solvent Precipitation

Organic solvent precipitation was performed as described in Chapter 2, Section 2.2.3.

3.2.4 Trypsin Protein Digestion

Following organic solvent precipitation, proteins were re-suspended in 20 μL of 8 M urea, with a pipette used for repeated drawing and dispensing of the solution (50 times) to aid in solubilisation. Following this, the solution was diluted to 100 μL at a final concentration of 50 mM aqueous ammonium bicarbonate. Proteins were reduced through addition of 5 μL of 200 mM dithiothreitol (20 minutes at 60 $^{\circ}\text{C}$) and alkylated using 10 μL of 200 mM iodoacetamide (room temperature, 20 minutes in the dark). Digestion occurred following addition of 2 μg trypsin, with overnight incubation at 37 $^{\circ}\text{C}$. The digestion was terminated by acidification (pH \sim 3) with 10% trifluoroacetic acid (TFA).

3.2.5 Reversed-Phase HPLC Sample Clean-Up

Following trypsin digestion, samples were desalted on an Agilent 1200 HPLC system. Reversed-phase chromatography was performed using a self-packed C18 column (1 mm \times 10 cm, 5 μm beads) at a flow rate of 100 $\mu\text{L}/\text{min}$ with UV detection at 214 nm. Following sample injection, the gradient consisted of a five minute hold at

5% acetonitrile (ACN) and 0.1% TFA in water, then an increase from 5 to 95% ACN (0.1% TFA/water) over 22.85 minutes. The system was held at 95% ACN for five minutes before instantly dropping to initial conditions for 30 minutes to regenerate the column. Eluting peptides were collected as a single fraction over a 14 minute interval, beginning 19 minutes after injection. The samples were completely dried in a SpeedVac.

3.2.6 Pierce Detergent Removal Spin Column Standard Protocol

The standard protocol for detergent removal was carried out as described by Pierce [140]. Briefly, the spin column was placed in a 1.5 mL microcentrifuge tube and storage buffer removed by centrifugation at $1500 \times g$ for one minute on a Fisher-Scientific accuSpin Micro centrifuge at room temperature, followed by three consecutive washes in the same manner with 400 μ L of 50 mM ammonium bicarbonate (for standard proteins) or water (for yeast proteome). Detergent-containing samples (100 μ L) were added to the spin columns, with incubation at room temperature for two minutes. The detergent-depleted flow-through was collected after two minutes centrifugation at $1500 \times g$.

3.2.7 Pierce Detergent Removal Spin Column Alternate Protocol

The bead storage buffer was removed through repeated centrifugation and subsequent replacement of buffer with water. The process was repeated ten times to ensure complete removal of buffer. The resulting slurry (50% water, 50% beads v/v , total volume approximately 6 mL) was placed in a beaker and continuously agitated using a magnetic stir bar. The appropriate volume of bead slurry was dispensed with a pipette and added to detergent-containing samples in 1.5 mL vials. Samples were capped and manually agitated (continuous shaking and inversion of the sample vial) for a specified

period ranging from 10 seconds to 10 minutes, followed by centrifugation at 13,000 rpm on a benchtop centrifuge for five minutes. The supernatant was removed with a pipette for further analysis.

3.2.8 Spectrophotometric SDS Assay

A methylene blue spectrophotometric assay [145] was employed to quantify the SDS remaining in samples treated with the standard protocol (Section 3.2.7). Briefly, 200 μL of methylene blue reagent (per liter of reagent: 250 μg methylene blue, 50 g sodium sulfate, and 10 mL concentrated sulphuric acid in water) was added to 200 μL of sample. The sample was briefly mixed, and then 800 μL of chloroform was added with two minutes vortexing to extract the methylene blue-dodecyl sulfate ion-pair into the chloroform layer. The mixture was briefly centrifuged, and the upper (aqueous layer) was completely removed. A small quantity of sodium sulfate (not weighed, approximately 20 mg) was added to the organic layer, with brief vortexing to remove any remaining traces of water. The absorbance was measured at 651 nm with an Agilent G1103A UV/Vis spectrophotometer. An SDS stock was used to create a 7-point calibration curve (plus the blank) from 0.2 μg to 2 μg SDS, onto which the original samples were diluted to fit within the linear range of calibration.

3.2.9 Mass Spectral SDS Assay

A mass spectral SDS assay was performed as described in Chapter 2, Section 2.2.4.

3.2.10 Protein and Peptide Quantification by HPLC/UV

Standard proteins and trypsin-digested peptides were quantified using LC/UV at 280 nm and 214 nm, respectively, using reversed-phase chromatography (1 mm \times 10 cm,

5 μm C18 beads) on an Agilent 1200 HPLC system. The flow rate was 100 $\mu\text{L}/\text{min}$. For standard proteins, and the gradient used was as described in Section 3.2.6. For peptides, solvent composition was held at 5% ACN, 0.1% TFA, water for five minutes, followed by an instantaneous increase to 85% ACN, which was held for five minutes, then an instantaneous decrease to 5% ACN. The column was allowed to re-equilibrate at 5% ACN for 20 minutes. A 5-point calibration curve was created for myoglobin, lysozyme, ubiquitin, cytochrome C and β -lactoglobulin A (0.1 μg to 1 μg), and a 6-point curve for tryptically-digested yeast peptides (1 μg to 5 μg). Peak areas were determined by exporting the time and corresponding intensity values to a Microsoft Excel spreadsheet, wherein the baselines were normalized to zero, and the intensities at each time point (from 13 to 16.5 min) were summed.

3.2.11 Pierce BCA Assay

The Pierce BCA assay was performed as described in Chapter 2, Section 2.2.5.

3.2.12 Mass Spectrometry and Database Searching

Peptide LC-MS/MS analysis was performed using reversed-phase chromatography (capillary column, 75 μm \times 25 cm, 3 μm C18 beads) coupled to a Finnigan LTQ XL linear ion trap mass spectrometer (Waltham, USA). The flow rate used was 250 nL/min. Following the start of sample injection, the column was held at initial solvent conditions (5% ACN in 0.1% formic acid, water) for one hour, after which the gradient proceeded as follows: an instantaneous increase to 7.5% ACN, followed by an increase to 20% ACN over 90 minutes, then to 25% ACN over 25 minutes, with a subsequent increase to 35% ACN over five minutes and a final increase to 80% ACN over one minute. This was held for four minutes before an instantaneous decrease to 5%

ACN, with a ten minute post-time to regenerate the column. The MS instrument method used data dependent scanning, whereby the method cycles between full MS and zoom scans for determination of charge state, followed by MS/MS of the three ions with highest intensity. Charge state screening was employed to screen ions with charge state ≥ 4 , singly-charged ions, or ions for which a charge state could not be determined. MS/MS spectra were searched against the *Saccharomyces cerevisiae* yeast proteome database (downloaded November 1, 2007) using the SEQUEST algorithm in Bioworks v3.2. To maintain a false discovery rate of less than 1%, searches were also performed against a reversed *S. cerevisiae* database, adjusting the filter criteria according to the values employed by Botelho *et al.* [51].

3.3 Results and Discussion

3.3.1 Evaluation of the Pierce Detergent Removal Spin Columns

3.3.1.1 SDS Reduction using Standard Protocol

The data reported by Pierce for their Detergent Removal Spin Columns claims greater than 99% reduction of SDS. Given the SDS threshold value for MS experiments (0.01%), a hundred-fold reduction in detergent concentration would be sufficient, given that the initial sample is below 0.1%. The conventional running buffers for SDS-PAGE and GELFrEE separations contain 0.1% SDS; however, given the electrophoretic mobility of SDS, the final concentration of SDS in GELFrEE fractions can exceed this value². The efficiency of SDS reduction with the Detergent Removal Spin Columns was therefore evaluated using 100 μ L samples prepared in either 0.1% or 2% SDS. For

²Determined from unpublished work in the Doucette lab to be as high as 0.6% SDS in the collected fraction.

consistency with SDS removal in protein recovery experiments, each sample included 10 μg of a complex proteome mixture, namely intact (undigested) yeast extract. The results are reported in Table 3.1.

From Table 3.1, at each level of SDS, the claimed >99% SDS removal efficiency was easily confirmed. In fact, even beginning with 2% SDS, the detergent removal strategy brought the concentration of SDS an order of magnitude below the LC/MS tolerance threshold of 0.01%. The data reported by Pierce involved a sample containing 2.5% SDS, though it is noted that the concentration of SDS is rarely this high at the point of sample cleanup; 0.1% SDS is perhaps most common. As one might expect, the final concentration of SDS was lower when beginning from a more dilute detergent sample. However, the specific level to which SDS was reduced could not be determined with the methylene blue assay for all samples. Using the 0.1% SDS sample, the remaining concentration of SDS was below the detection limit of the assay (0.1 μg). Nonetheless, following SDS depletion, and without additional dilution, the concentration of SDS remaining from an initial 0.1% SDS would be at least two orders of magnitude lower than the threshold value permitting LC/MS analysis.

The first priority of any detergent depletion strategy is to permit effective removal of the SDS from the sample. In this regard, the Pierce column strategy is not only simple to use, but is among the most effective strategies for detergent depletion. Given successful SDS reduction, the next consideration must be towards maintaining high recovery for proteins. This is assessed below.

Table 3.1: Remaining SDS from samples containing 2% or 0.1% SDS, with 10 μg protein, following detergent depletion with the Pierce Detergent Removal Spin Column standard protocol.

Initial Mass SDS (μg)	Final Mass SDS (μg)	% SDS Removed
2000	0.7 ± 0.1	99.9 ± 0.005
100 ¹	< 0.1	> 99.9

¹Below limit of detection for methylene blue SDS assay

3.3.1.2 Protein Recovery using Standard Protocol

Complex mixtures of proteins are commonly used in proteomics experiments, and two common sample types are intact (undigested) and trypsin-digested. The average molecular weights for each sample type are estimated at approximately 1000 Da and >50 kDa for trypsin-digested and intact yeast, respectively. Additionally, the sample type to be used is dependent on the aims of the experiment (*i.e.*, intact proteins for top-down proteomics). Because the amount of analyte available in any given proteomic experiment is highly variable, protein recovery must be determined at both high and low concentrations.

Table 3.2 summarizes the protein recovery obtained for complex protein mixtures through the standard protocol for SDS removal. Protein concentration and sample type appear to have minimal effect on protein recovery. However, the recoveries are significantly lower than the 95% reported by Pierce. The highest recovery (52%) was exhibited by 5 μ g of intact yeast protein.

With any protein manipulation step, a degree of sample loss is expected to occur. Using the detergent removal beads (herein referred to as DR-beads), protein loss could occur through direct surface contact with the DR-beads, through interaction with SDS bound to the DR-beads, or even as a result of a decrease in protein solubility related to elimination of detergent from solution. The latter is unlikely to be an issue for digested protein samples, but may be an important factor when depleting SDS from hydrophobic protein samples. Given the large volume of DR-beads employed in the standard protocol (500 μ L column bed, recommended for 100 μ L samples), it is anticipated that the

Table 3.2: Percent recovery of complex proteomes at high (50 μg in 100 μL) and low (5 μg in 100 μL) concentrations, after treatment with the Pierce Detergent Removal Spin Column standard protocol.

μg Protein	% SDS	Intact Yeast Protein (%)	Trypsin-Digested Yeast Protein (%)
50	0.1	48 ± 2	42 ± 1
5	0.1	52 ± 8	37 ± 8

abundance of protein-bead interactions would result in large sample losses. This is of particular concern when sample concentration is low, as even small losses may bring the available analyte to levels below the limit of detection for subsequent analyses. Furthermore, the strength of protein binding to the beads is likely dependent on protein properties, and thus the degree of protein loss may vary in accordance with sample components.

A set of standard proteins (BSA, ubiquitin, cytochrome c, lysozyme, β -lactoglobulin A, and myoglobin) – were individually prepared at varying levels of SDS (0.1% or 2%) and at varying concentrations, down to 0.01 g/L (*i.e.*, 1 μ g total). The proteins were treated using the conventional protocol for SDS removal with the Pierce Detergent Removal Spin Columns, and quantified by LC/UV, as shown in Figure 3.1. This quantification protocol was chosen over the BCA assay, as it provides the necessary sensitivity for determination of recovery from low (1 μ g) starting quantities of material. Table 3.3 summarizes the results for protein recovery. Beginning with 100 μ g trypsin-digested BSA (2.5% SDS), as reported by Pierce, it was seen that 95% recovery can be obtained following detergent depletion. However, for all other samples tested, protein recovery was far below this value. The results reflect two important attributions of reporting recoveries: assessing yield over a range of sample types, and sample concentrations. These are discussed below.

First, with all other variables equal, the recovery of different proteins varies considerably. As shown in Table 3.3, recoveries were not near 95% as claimed by Pierce, and yields from one protein to another varied by almost an order of magnitude, or by as

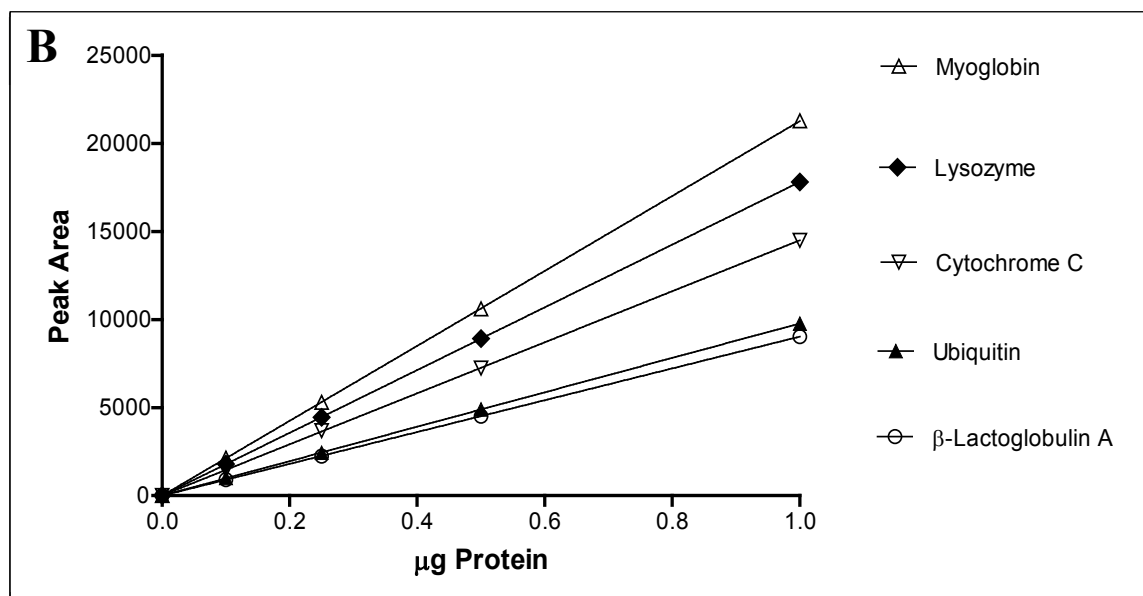
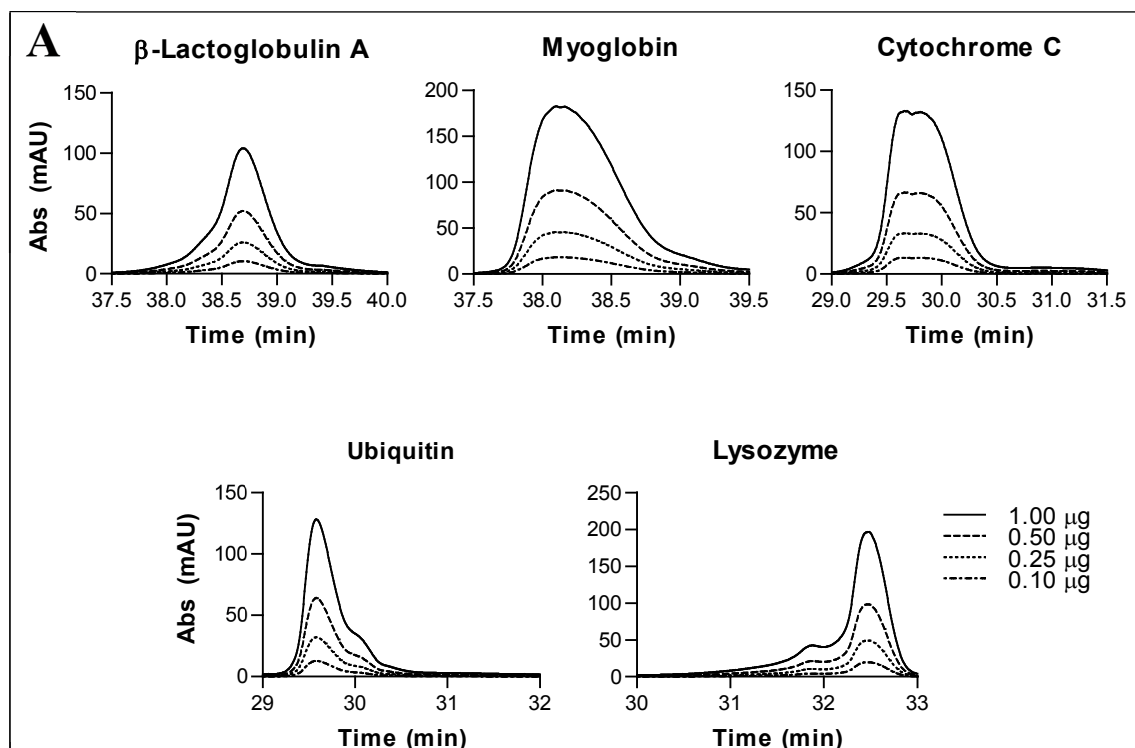


Figure 3.1: Protein quantitation by LC/UV. (A) Chromatograms of β -lactoglobulin A, myoglobin, cytochrome C, ubiquitin and lysozyme (0 – 1 μ g) injected onto an Agilent RP-HPLC and quantified using the peak area at 280 nm, and (B) plot of calibration curves.

Table 3.3: Percent recovery of trypsin-digested BSA and standard proteins, at varying masses of protein and of SDS, when treated with the Pierce Detergent Removal Spin Column standard protocol.

μg Protein	% SDS	BSA¹ (%)	Ubiq² (%)	Lyso³ (%)	Cyt C⁴ (%)	Myo⁵ (%)	β-Lac A⁶ (%)	Average
100	2.5	93	-	-	-	-	-	-
10	2	-	49	6	8	21	85	34 ± 33
10	0.1	-	54	12	62	47	70	49 ± 24
1	2	-	10	2	0	0	0	2 ± 4
1	0.1	-	22	4	10	0	0	7 ± 9

¹Trypsin-digested BSA

²Ubiquitin

³Lysozyme

⁴Cytochrome C

⁵Myoglobin

⁶β-Lactoglobulin A

much as 80%. Given that each protein exhibits different properties, it is likely that recovery is affected by some combination of protein properties such as size, charge, and hydrophobicity. The similarities between average standard protein and complex sample recoveries give further credence to the theory; both exhibit yields of ~50%.

Additionally, all proteins exhibited better recovery at higher concentrations, indicating that protein recovery is dependent on protein concentration. Given the reasonable yields previously reported in Table 3.2 for 5 μg of complex proteome and the extremely poor yields observed for 1 μg of standard protein, it seems that the effect of sample concentration may be most pronounced at sample amounts below 5 μg . It should, however, be noted that lower protein recoveries are better tolerated at higher concentrations. While the low (<50%) recoveries for 50 μg samples are not of concern, the similar recoveries for 5 μg samples may not leave sufficient analyte for subsequent analysis. Also, with the exception of β -lactoglobulin A, samples containing lower SDS concentrations (0.1% SDS) exhibited higher recoveries, giving credence to the theory that some protein loss is due to the removal of SDS-bound proteins. For standard proteins, the Pierce Detergent Removal Spin Column is therefore most suited when the SDS concentration is low (below ~0.1%) and protein concentration is high (above ~0.1 $\mu\text{g}/\mu\text{L}$).

As a general conclusion, application of the standard protocol should only be performed for samples containing a minimum of 10 μg protein. Treatment of samples at lower concentration may not provide sufficient protein for subsequent analysis. It is noted that the sample concentration cannot always be known ahead of detergent depletion. SDS is a known interference for several protein quantification assays, including LC/UV and

Bradford assays, although the Pierce BCA assay is tolerant to SDS. If sample quantity is low, or unknown, then an alternative SDS depletion strategy is recommended.

3.3.2 Development of an Alternate SDS Depletion Protocol

The standard protocol for the Pierce Detergent Removal Spin Columns is extremely effective at detergent depletion. However, the variable and often poor protein recoveries present a serious limitation of this cleanup strategy. Given that the level of SDS reduction is more than sufficient to permit LC/MS analysis, a logical strategy would sacrifice the efficiency of SDS removal for a gain in protein recovery. With a possible source of protein loss being to the surface of the DR-beads, a strategy was devised wherein fewer beads were applied to the sample.

The strategy of using fewer beads should still permit sufficient detergent depletion, while decreasing protein-bead interactions and therefore improving protein recovery. In the standard protocol, efficient surface contact is provided; no more than 100 μL of sample is applied to a 500 μL bed volume. Unfortunately, a significant reduction in the bed volume for a column-based protocol would result in poor contact between the solution and bead surface. Thus, an alternative approach to facilitate contact between sample and bead surface was developed. In this strategy, beads were directly dispensed into the detergent containing solution and manually agitated to ensure maximal contact of SDS with the bead surface. Without agitation, the bead particulates quickly settled to the bottom of the vial. Noting that an automated approach to agitation would be preferred, it was also found that the benchtop shakers / vortexers initially available in the Doucette laboratory were either too violent or too gentle for shaking. However, a suitable agitator was later found (Section 3.3.2.5). Optimal agitation was achieved in a tumbling format,

wherein the vials were rocked upside down and back, to allow beads to flow through the solution without causing detergent-containing samples to foam. Automated tumbler-style mixers are commercially available, and thus this strategy could be immediately automated.

Several variables are of concern to optimize this alternative protocol, an overview of which is supplied below:

- (1) Determination of the minimum volume of DR-beads required for reduction of SDS to the critical threshold concentration for obtaining reliable mass spectral data in LC-MS/MS experiments.
- (2) Determination of the effect of (i) volume of DR-beads, (ii) total volume, and (iii) agitation time in regards to protein recovery.
- (3) Determination of the compatibility of SDS-depleted samples with mass spectrometry proteome analysis.

3.3.2.1 Determination of Minimum DR-Bead Volume for SDS Removal

The Pierce Detergent Removal Spin Columns utilize 500 μL of DR-beads to quickly and efficiently remove SDS. However, the column capacity far exceeds what is required to deplete SDS below the threshold levels (Section 3.3.1.1). It may therefore be possible to deplete SDS to below the critical threshold level for MS while using fewer DR-beads, thus necessitating the study of the effect of DR-bead volume on SDS removal.

Figure 3.2 plots the mass of SDS remaining from a 100 μL solution of 0.1% SDS (100 μg SDS initial) after agitation with increasing volumes of DR-beads. Ten minutes of agitation was chosen. As expected, increasing the volume of DR-beads rapidly increased

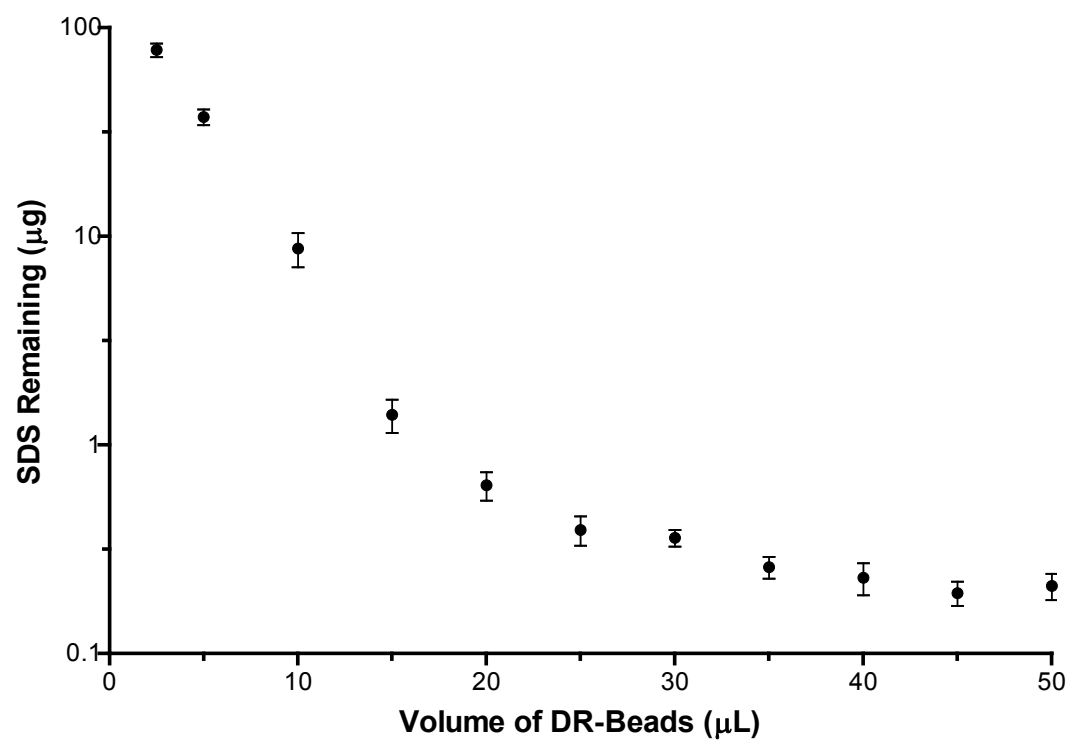


Figure 3.2: SDS remaining from an initial 100 µg after ten minutes of manual agitation with 0 – 50 µL of DR-beads.

the efficiency of SDS removal. Compared to the standard protocol for SDS depletion, which uses 500 μL of DR-beads, the final mass of SDS remaining in the alternative protocol was higher (0.2 μg using 60 μL of beads *vs* <0.1 μg). Nonetheless, relative to the threshold value of SDS (<0.01%) for bottom-up LC/MS, the values can be translated to show that 20 μL of DR-beads is sufficient to deplete an initial 0.1% SDS to below the threshold of 0.01% (*i.e.*, a 100-fold reduction in SDS). Doubling the volume of beads to 40 μL resulted in a 3-fold reduction in the level of SDS remaining, depleting SDS to a final mass of 0.23 μg . It was therefore determined that 40 μL of DR-beads be the recommended volume for SDS depletion under the conditions used (100 μL at 0.1% SDS).

3.3.2.2 Effect of Agitation Time and DR-Bead Volume

With greater than 20 μL of beads, agitation for ten minutes was sufficient to deplete SDS to below the critical threshold level for MS. Increasing agitation time may further reduce the level of SDS remaining in the sample. However, given the already sufficient reduction in SDS, it was determined that longer agitation times were not necessary. Likewise, shorter agitation times with more DR-beads could give rise to a higher throughput reduction protocol. The effects of shorter agitation times (ten seconds to ten minutes) were therefore explored, and are summarized in Figure 3.3.

Two points are evident from Figure 3.3. First, shorter agitation times resulted in less efficient SDS reduction, likely because there was less opportunity for DR-beads to physically contact and thus remove SDS molecules. For any given sample volume or volume of DR-beads, as agitation time was increased there was a near-exponential

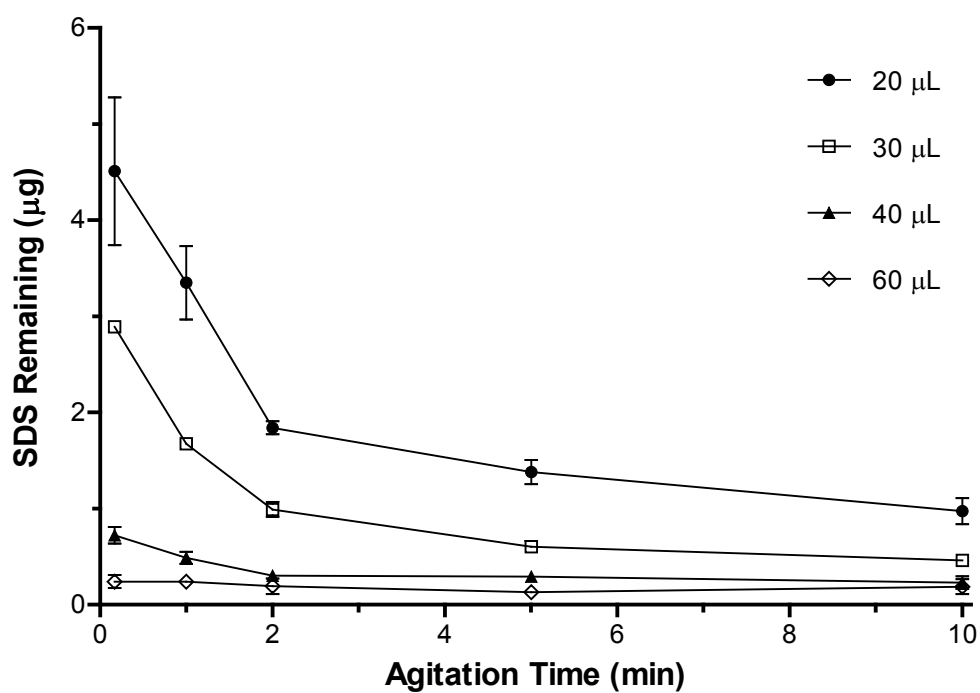


Figure 3.3: Use of alternate protocol for removal of 0.1% (100 µg) SDS as a function of agitation time and volume of DR-beads: (●) 20 µL; (□) 30 µL; (▲) 40 µL; (◇) 60 µL.

decrease in the level of SDS remaining. This is likely because, at greater agitation times, there was limited SDS remaining in the sample to interact with the DR-beads. While longer agitation times may prove beneficial, it is clear that manual agitation for ten minutes is sufficient for SDS removal from proteomic samples. Although 98% of the detergent was depleted after two minutes, an additional eight minutes of agitation still exhibited a further 1% decrease in SDS levels, for a total of 99% detergent removal. Given the ability to process multiple samples (20+) simultaneously, an extra eight minutes of agitation does not significantly affect the throughput of this method. Thus, to provide maximum SDS reduction, it is recommended that manual agitation be performed for ten minutes.

The second point illustrated from Figure 3.3 relates to the effect of larger DR-bead volume on agitation time. Figure 3.3 also plots the mass of SDS remaining after agitation with 20 μL – 60 μL of DR-beads. It was observed that a 50% increase in bead volume resulted in a 10-fold decrease in required agitation time to achieve the same level of SDS reduction. This observation is likely due to the increased concentration of DR-beads in the sample (*i.e.*, 20 μL of beads is 0.2 v/v , 60 μL of beads is 0.6 v/v). At any given time point, the probability of an SDS molecule contacting a bead is greater at higher DR-bead concentrations. Overall, at any given time, an increased volume of DR-beads corresponds to enhanced detergent depletion. However, due to concerns of larger DR-bead volumes resulting in increased sample losses, the minimum DR-bead volume of 40 μL is recommended.

3.3.2.2.1 Effect of Initial Sample Volume

Occasionally, the concentrations of non-SDS sample contaminants (*i.e.*, salts and buffers) are too high to permit subsequent analysis. One method for decreasing the effect of such impurities is to dilute the sample. However, SDS removal depends on physical contact between SDS molecules and DR-beads, and diluting the sample solution may decrease the efficiency of SDS removal. It is therefore unclear if SDS removal from a dilute solution would exhibit similarly high percent reductions as compared to SDS removal from an un-diluted solution.

Figure 3.4 plots the mass of SDS remaining following removal of 100 μg SDS when diluted into different volumes (100 μL vs 1000 μL) and treated with 40 μL of DR-beads. Comparison of the two plots demonstrates that SDS removal was significantly less effective in diluted solutions. One minute of agitation in 100 μL removed 99.5% of the SDS, while ten minutes of agitation in 1000 μL removed merely 76.5%. The observed effect is possibly due to equilibrium of free SDS and bead-bound SDS. It is possible that the thermodynamics of the system may be such that it is unfavourable for the DR-beads to bind further SDS, as the level of detergent depletion may lead to reduced protein solubility. Alternatively, the observed effect may be due to equilibrium between free DR-beads and DR-beads which have bound an SDS molecule.

Interestingly, after ten seconds of agitation, the rate of detergent depletion for dilute samples in Figure 3.4 was almost 100 times greater than in un-dilute samples. This is reminiscent of the rate of SDS depletion observed previously in Figure 3.3, wherein fewer beads resulted in accelerated depletion. It is thus likely that dilution of sample decreases the probability of contact between an SDS molecule and DR-bead, therefore

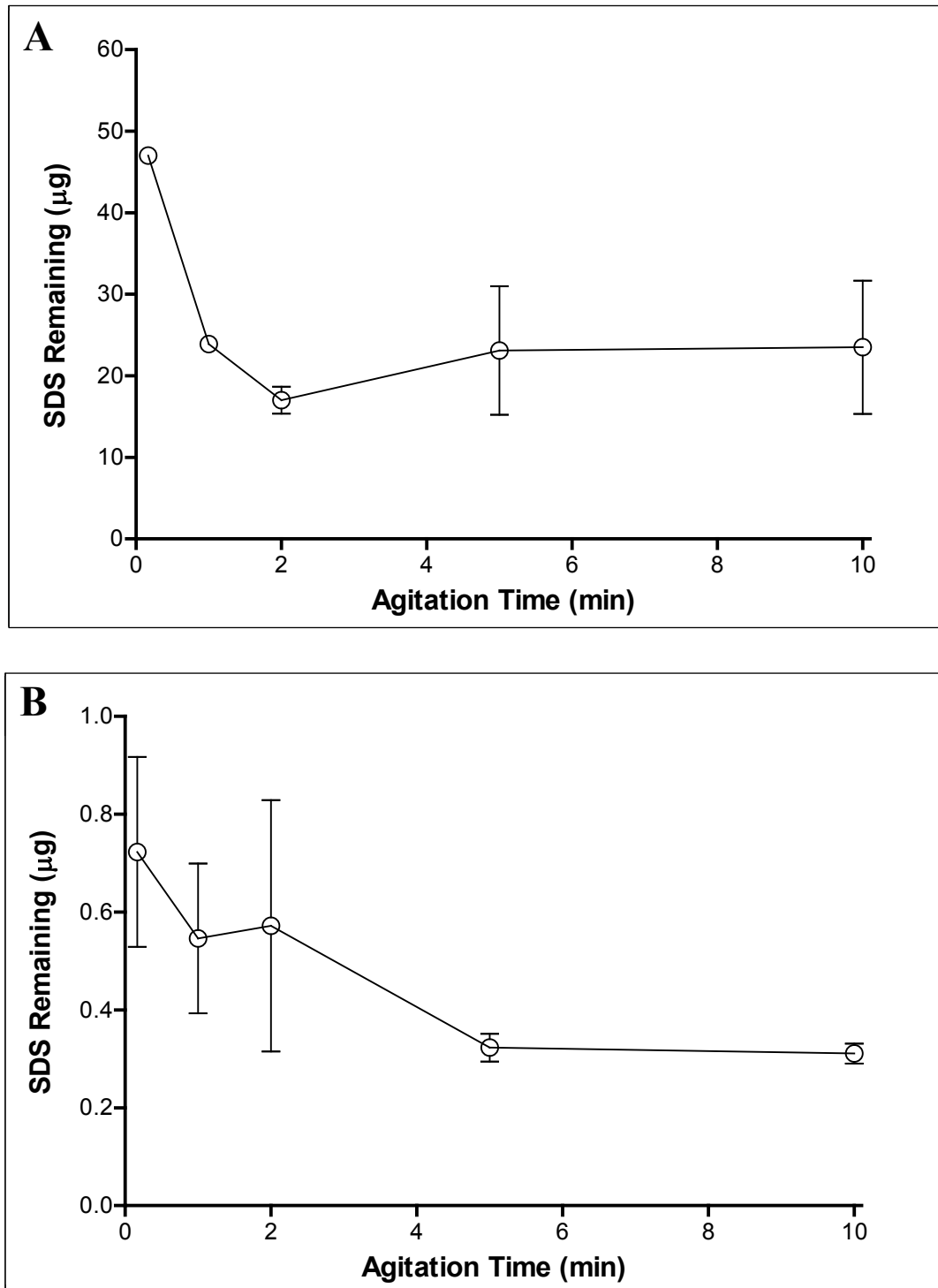


Figure 3.4: Use of alternate protocol for removal of 100 μg SDS as a function of agitation time and sample dilution. (A) 1000 μL , 0.01% SDS; (B) 100 μL , 0.1% SDS.

decreasing the effectiveness of SDS removal. It is however important to note that, despite the high mass of SDS remaining, the final concentration observed (0.003%) is still below the critical threshold value required for mass spectral analysis.

At higher initial volumes and shorter agitation times, the efficiency of SDS reduction was decreased. To obtain maximum SDS removal, an agitation time of ten minutes is recommended, and sample dilution is not recommended. However, as previously mentioned, it is anticipated that larger volumes of DR-beads would result in increased sample losses. Therefore, protein recovery experiments must be performed before optimal DR-bead volume can be determined.

3.3.2.3 Determination of Protein Recovery

3.3.2.3.1 Effect of DR-Bead Volume on Protein Recovery

It was shown in Section 3.3.2.2 that increased volume of DR-beads resulted in increased SDS removal. However, optimal bead volume is not only a function of SDS removal; it also depends on protein recovery. Due to an increased probability of protein-bead interaction, a greater volume of DR-beads may result in reduced protein yields. To determine whether this concern was warranted, it was necessary to study the effect of DR-bead volume on protein recovery in both analyte-abundant (50 μg) and analyte-limited (5 μg) samples.

The effect of DR-bead volume on protein recovery for intact yeast proteins (<1 kDa to >80 kDa) is plotted in Figure 3.5, while Figure 3.6 illustrates recovery for trypsin-digested proteins (<5 kDa). With the exception of the analyte-abundant samples for trypsin-digested yeast proteins (Figure 3.6a), results were as expected. SDS removal using 40 μL DR-beads resulted in an overall higher recovery as compared to the use of

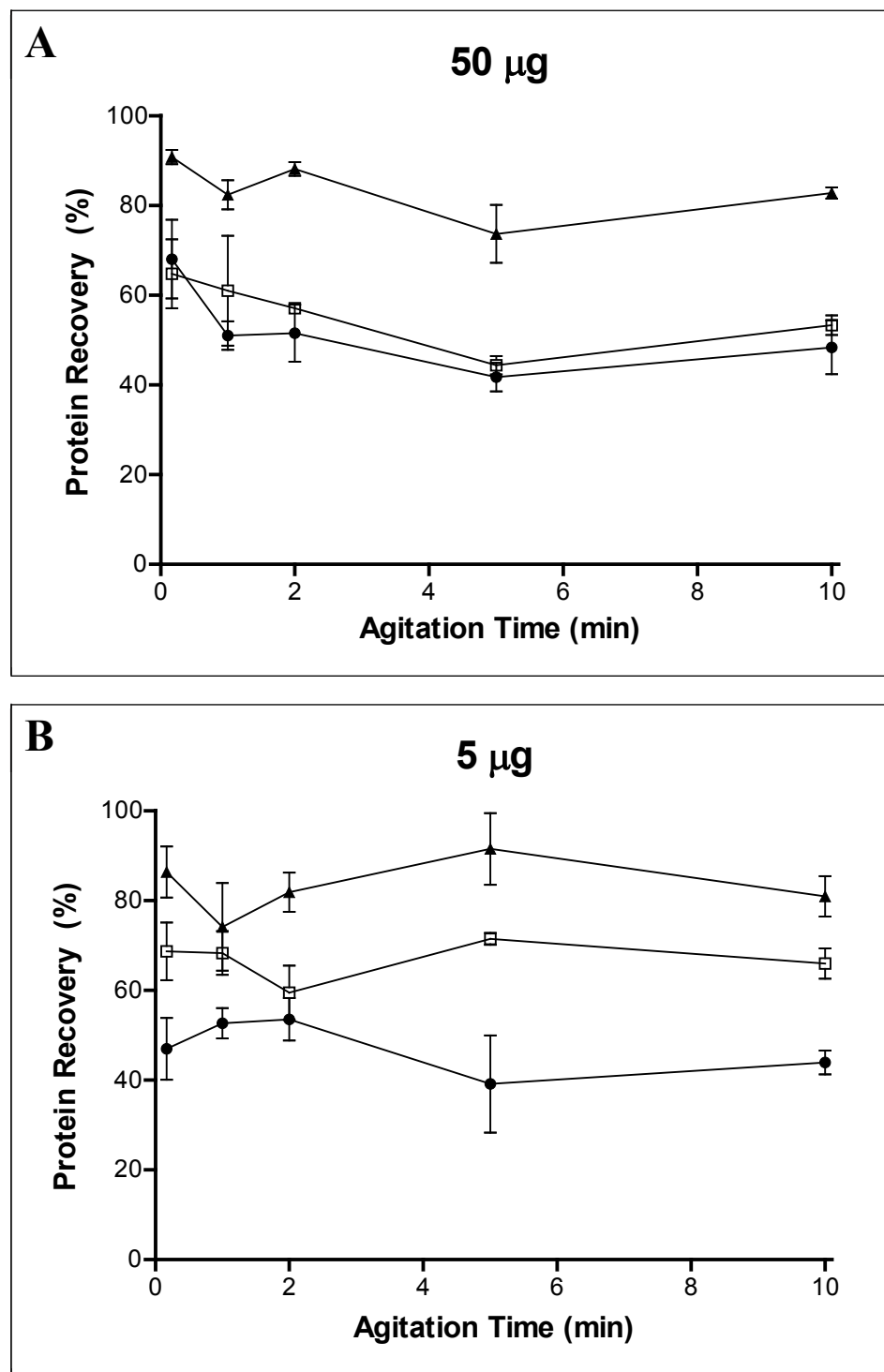


Figure 3.5: Protein recovery observed from alternate protocol when applied to (A) 50 µg and (B) 5 µg intact yeast proteins in 100 µg SDS, as a function of agitation time, sample dilution, and volume of DR-beads. (▲) 1000 µL total volume, 40 µL DR-beads; (□) 100 µL total volume, 40 µL DR-beads; (●) 170 µL total volume, 120 µL DR-beads.

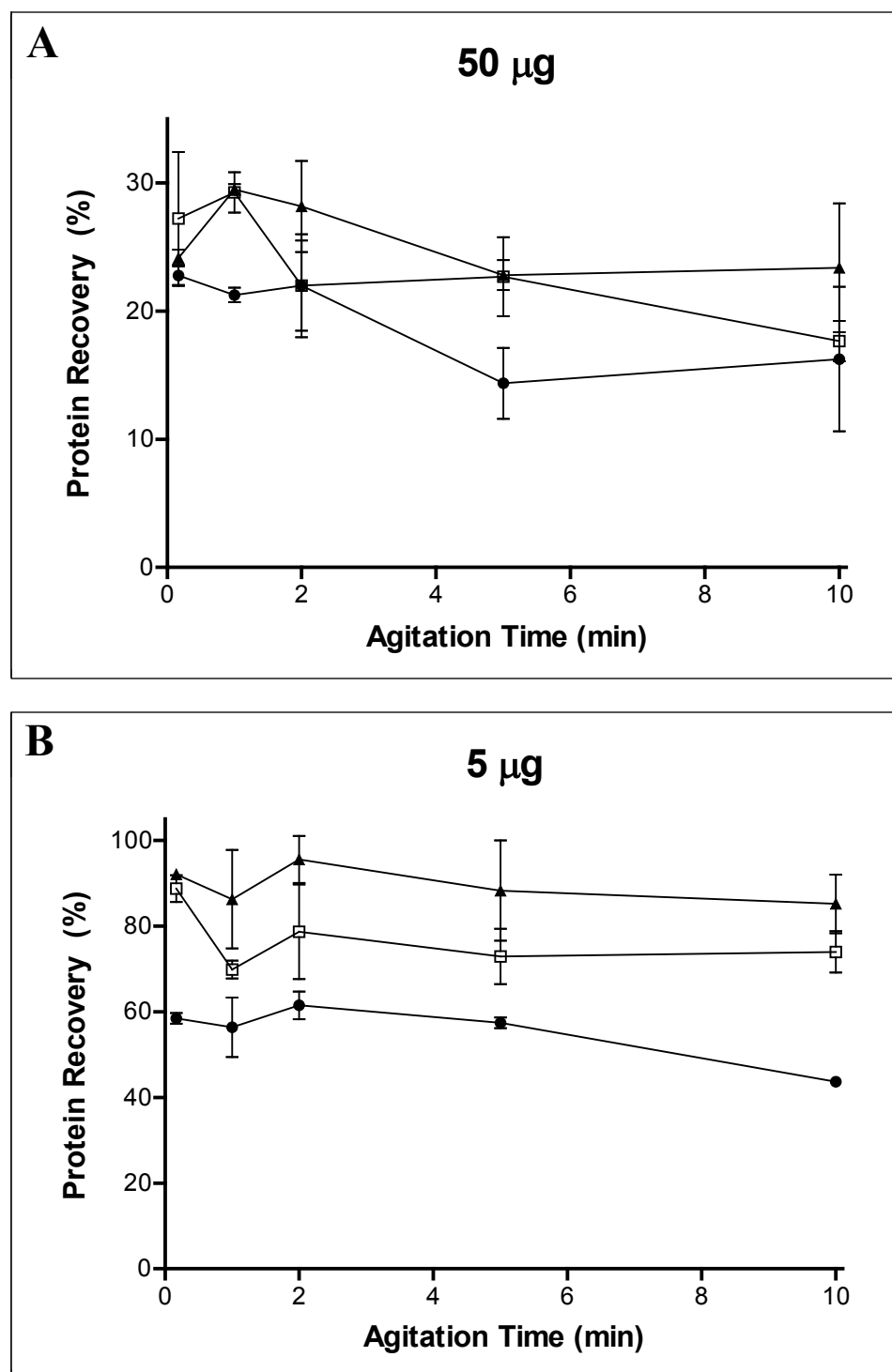


Figure 3.6: Protein recovery observed from alternate protocol when applied to (A) 50 μg and (B) 5 μg trypsin-digested yeast proteins in 100 μg SDS, as a function of agitation time, sample dilution, and volume of DR-beads. (▲) 1000 μL total volume, 40 μL DR-beads; (□) 100 μL total volume, 40 μL DR-beads; (●) 170 μL total volume, 120 μL DR-beads.

120 μ L DR-beads. The difference in recoveries between the two DR-bead volumes was 5% for intact proteins, and 30% for trypsin-digested proteins. However, other than the noted exception, all recoveries were greater than those observed using the standard protocol, as previously reported in Table 3.3.

Trends do not appear to exist for 50 μ g samples of trypsin-digested proteins (Figure 3.6a), and an overall lower yield was observed. This is possibly because of low sample pH due to the presence of residual TFA. Before addition and subsequent removal of SDS, trypsin-digested proteins were subject to reversed-phase HPLC clean-up, whereby TFA was removed by evaporation in a SpeedVac. However, evaporation may not have been sufficient to remove all traces of acid. The recommended operating range for the Pierce Detergent Removal Spin Columns is pH 4 – 10, and a more acidic pH may decrease the effectiveness of the method, therefore resulting in overall lower recovery. The higher recovery observed for such samples when employing the standard protocol (Table 3.2), wherein samples were not acidified prior to detergent depletion, gives further credence to the theory.

It is therefore clear that the use of fewer DR-beads in the alternate protocol does improve protein recovery. Overall, it is recommended to use the minimal volume of DR-beads required for sufficient SDS removal, which was previously defined as 40 μ L.

3.3.2.3.2 Effect of Agitation Time on Protein Recovery

For optimum SDS removal, the recommended agitation time was determined to be ten minutes. It is important to note, however, that sufficient SDS removal could be obtained with an agitation time as short as two minutes. Shorter agitation times decrease the probability of protein-particulate interactions and consequently may lead to improved

protein recovery. To determine the optimal agitation time for balanced SDS removal and protein recovery, yields of analyte-abundant (50 μg) and analyte-limited (5 μg) conditions were investigated as a function of agitation time.

3.3.2.3.2.1 Intact Yeast Proteins

Figure 3.5a and Figure 3.5b plot the relationship between agitation time and percent recovery for intact yeast proteins in 0.1% (100 μg) SDS. Two points are evident from the data. Firstly, percent loss was very similar for both analyte-limited and analyte-abundant samples, wherein recoveries differed by less than 10%. Secondly, there does not appear to be a significant decrease in protein recovery at increased agitation times, indicating that correlation between agitation time and protein recovery is low. Indeed, regardless of initial analyte mass, the majority of loss occurred within ten seconds of agitation. Further shaking demonstrated minimal decrease in protein yield (<10%). This is similar to the rate of SDS depletion previously observed in Figure 3.4b. SDS removal of 99.3% was observed within ten seconds, with a further decrease of 0.5% over an additional ten minutes of agitation. Given the rapidity of SDS and protein loss, the observed protein losses were likely due to the removal of SDS to which a protein is bound, in addition to removal of free SDS. However, loss may also be due to direct contact between protein and DR-beads.

In general, when working with intact proteins, longer agitation does not significantly decrease protein yields. An agitation time of ten minutes is therefore recommended, as it permits greater SDS depletion.

3.3.2.3.2.2 Trypsin-Digested Yeast Proteins

Protein recoveries for trypsin-digested yeast proteins in 0.1% (100 μg) SDS are illustrated in Figure 3.6a and Figure 3.6b, respectively. The analyte-limited (5 μg protein) samples show approximately the same trends as intact (Section 3.3.2.4.2.1) yeast protein, with the majority of protein loss occurring within ten seconds of agitation. There is also no obvious correlation between protein recovery and increased agitation times. However, it is difficult to discern trends for the analyte-abundant (50 μg protein) data, which may be due to the presence of residual TFA decreasing the effectiveness of the DR-beads. Due to the low correlation between protein recovery and agitation times, the recommended agitation time for balanced SDS removal and protein recovery is ten minutes.

3.3.2.3.3 Effect of Dilution on Protein Recovery

A further consideration is sample dilution. Although detergent depletion in diluted samples was still sufficient, it was less effective. This was likely due to a decreased number of SDS-bead interactions. However, sample dilution should also have reduced the number of protein-bead interactions. Consequently, it was probable that sample dilution would result in increased protein recovery, and it was therefore necessary to examine its effect.

Protein recoveries for each sample type in 100 μg of SDS, diluted to different volumes (100 μL and 1000 μL), are demonstrated by the hollow triangles and solid squares in Figure 3.5 (intact yeast proteins) and Figure 3.6 (trypsin-digested yeast proteins). Excluding the analyte-abundant (50 μg protein) data for trypsin-digested proteins (Figure 3.6), likely the result of residual TFA, results were as expected. Protein recoveries were significantly higher in dilute solutions (by approximately 30%). However,

given that SDS reduction in dilute samples was less effective, sample dilution to 1000 μL should be used with caution. It is an effective method for increased protein recovery only if the samples need not be concentrated after SDS removal. Concentrating samples through solvent evaporation would also concentrate SDS, thus bringing the detergent level to above the 0.01% required for MS analysis. Therefore, SDS removal from dilute solutions is recommended only if protein concentration is high enough to allow for subsequent analysis without sample concentration.

3.3.2.4 Standard Protocol versus Alternate Protocol

The optimal conditions for removal of 100 μg of SDS using the alternate protocol were determined to be ten minutes of agitation in 100 μL initial volume. Protein recoveries for standard versus original protocol, recorded in Table 3.4 and Table 3.5, illustrate that protein recoveries using the alternate protocol matched or exceeded those obtained by the standard protocol. The different recoveries which were observed for standard proteins (Table 3.2) demonstrated a bias in the recovery of individual protein components, and this is therefore expected to be a contributing factor in the clean-up of complex protein mixtures. Different sample components exhibited different recoveries, with the values for complex proteomes referring to the average recovery for all elements existing within the sample.

As proof of the ability of the alternate protocol to remove SDS ahead of MS, both the standard and alternate protocols were applied to 100 μL samples containing (i) 5 μg of trypsin-digested BSA and (ii) 5 μg of trypsin-digested yeast proteins in 0.1% SDS. Samples were subsequently subjected to mass spectral analysis on an ion trap mass spectrometer. MS analysis of 500 fmol of BSA (assuming 100% recovery from

Table 3.4: Comparison of protein recoveries obtained from 50 μg samples of complex proteome using the standard protocol and alternate protocol.

Sample Type	μg Protein	SP¹ Recovery (%)	AP² Recovery (%)	AP (Dilute)³ Recovery (%)
Intact Yeast Protein	50	48.4	53.3	82.8
Trypsin-Digested Yeast Protein	50	42.2	17.7	23.4

¹Standard protocol

²Alternate protocol

³Alternate protocol on sample diluted to 1000 μL

Table 3.5: Comparison of protein recoveries obtained from 5 μg samples of complex proteome using the standard protocol and alternate protocol.

Sample Type	μg Protein	SP¹ Recovery (%)	AP² Recovery (%)	AP (Dilute)³ Recovery (%)
Intact Yeast Protein	5	52.2	66.0	80.9
Trypsin-Digested Yeast Protein	5	37.2	74.0	85.2

¹Standard protocol

²Alternate protocol

³Alternate protocol on sample diluted to 1000 μL

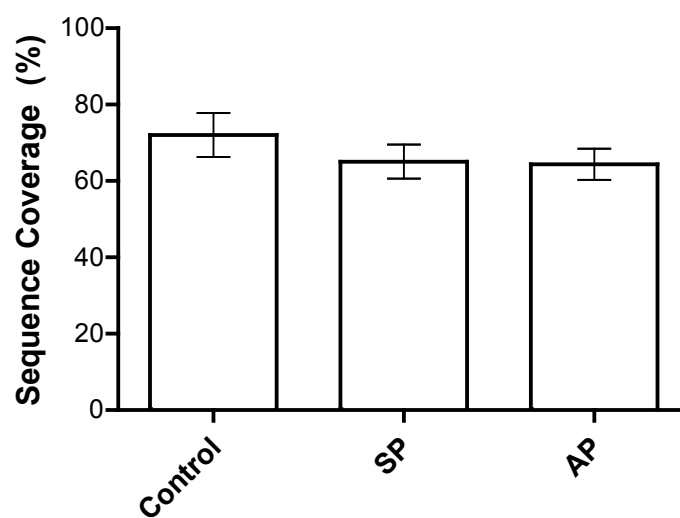


Figure 3.7: Comparison of BSA sequence coverage obtained from 500 fmol trypsin-digested BSA injected onto a nanoLC-ESI-MS. Prior to analysis, Control had no SDS present, while Standard Protocol (SP) and Alternate Protocol (AP) samples were treated for SDS removal by the standard and alternate protocols, respectively.

DR-beads) yielded the sequence coverages illustrated in Figure 3.7. In this manner, there seems to be very little variation between the two protocols. However, both provided sequence coverage that was slightly lower than a non-SDS containing control sample. Though such an effect may also be observed due to insufficient removal of SDS, this was likely due to protein recovery of less than 100%.

Additionally, proteome coverage was obtained for trypsin-digested yeast protein samples. Assuming 100% recovery, 1 μg of material was injected, and the number of proteins identified for each sample illustrated in Figure 3.8. The largest number of proteins, 669, was identified for the non-SDS containing control sample. Of those, 450 were identified by the alternate protocol-treated sample, plus an additional 114 for a total of 564 proteins. The number of proteins identified by the standard protocol-treated sample was the least, at 479. Previous experience from the Doucette laboratory is that run-to-run variability in MS analysis may contribute up to a 20% difference in the total number of identified proteins. The difference in identified proteins between the alternate and standard protocols therefore clearly indicated increased effectiveness for the alternate protocol. As with the BSA sequence coverage results, the fewer number of proteins identified in the treated samples as compared to the control sample was likely due to less than 100% protein recovery.

3.3.2.5 Application of Alternate Protocol to GELFrEE Fractions

To increase the applicability of the alternate protocol to high-throughput analysis, an automatic agitator was identified and employed for the subsequent experiment. For all previous considerations, samples treated with the alternate protocol were in aqueous 0.1% SDS solution. However, samples requiring detergent depletion often contain other

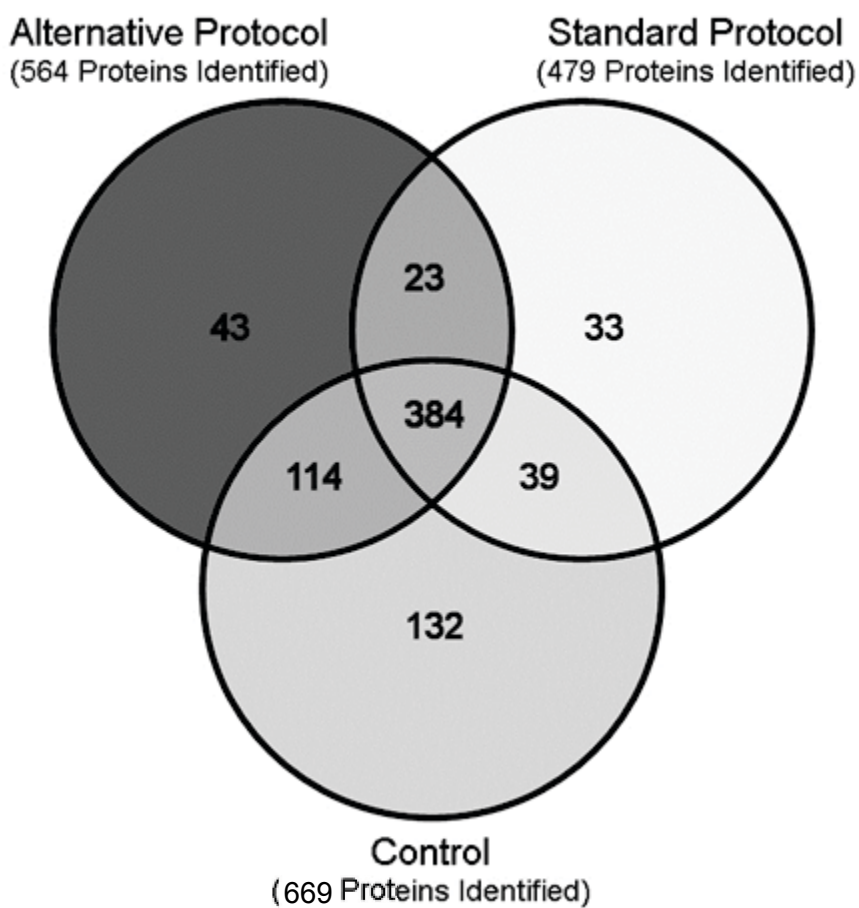


Figure 3.8: Venn diagram of proteins identified from MS analysis of 1 μ g trypsin-digested yeast injected onto a nanoLC-ESI-MS. Prior to mass spectrometry, Control had no SDS present, while Alternate and Standard were treated for SDS removal by the alternate and standard protocols, respectively.

components; of particular note are GELFrEE fractions, wherein separated proteins are collected in Laemmli running buffer (tris, glycine and 0.1% SDS). To ensure that the presence of buffers did not affect protein recovery, detergent removal from such samples was investigated. Analysis of 5 μ g trypsin-digested yeast in Laemmli running buffer was performed. Recovery was determined to be $74.7 \pm 4.0\%$, similar to that previously obtained for trypsin-digested yeast proteins in aqueous 0.1% SDS. It was therefore clear that the alternate protocol is also effective for cleanup of samples containing salts or buffers, such as those obtained from GELFrEE.

3.4 Conclusions

From determination of the minimum volume of DR-beads required for sufficient SDS reduction, to optimization of protein recovery for three different sample types, this chapter presented the development of a new method for SDS removal. The alternate protocol developed employs the Pierce Detergent Removal Spin Column technology, and is an attractive option for depletion of detergent while maintaining protein recoveries. Such a consideration is of particular importance when analyte is limited since it works to ensure that sufficient material is available for subsequent analyses. Use of an automatic agitator also allowed for high-throughput SDS removal. Additionally, the cost-per-sample using the standard protocol was \$4.00. However, reducing the volume of DR-beads used by 12.5-fold resulted in a much lower cost-per-sample at \$0.32, allowing for the treatment of a large number of samples at a significantly lower cost.

3.5 Future Work

The alternate protocol developed for SDS removal succeeds in providing higher protein recoveries, particularly when the sample is diluted to 1000 μL ; however, this causes SDS removal to be greatly reduced. Therefore, it would be worthwhile to investigate the effect of larger DR-bead volume in diluted solutions as an attempt to optimize detergent depletion in dilute samples while retaining very high protein recoveries.

SDS removal was significantly less effective in diluted solutions, and it was observed that such an effect may be due to an equilibrium process. The effect should be further explored by analysis of samples diluted into a wide range of volumes (*i.e.*, 50, 100, 200, 500, 1000 μL) so as to extrapolate equilibrium constants. Additionally, under the listed conditions, both detergent depletion and protein recovery should be determined.

The saturation point for any given volume of DR-beads is currently unknown. However, determination of the saturation point is important because it would provide information about the minimum volume of DR-beads required for any given concentration of SDS. Using an automated agitator, overnight agitation could be performed for a sample containing high SDS and low DR-bead concentration. The extensive agitation time should be sufficient to saturate the DR-beads, and subsequent analysis of remaining SDS will allow for calculation of bead saturation point.

To obtain sufficient SDS removal, agitation times of greater than ten minutes were deemed unnecessary. However, given the low correlation between protein recovery and increased agitation, greater agitation times should be investigated in an effort to further optimized SDS removal.

Although standard proteins exhibited variable recoveries, the exact effect of such variation in complex proteomes is unclear. To determine such, sample loss of intact yeast proteins, pre- and post-treatment, should be analyzed using SDS-PAGE with silver staining.

The standard protocol recommends washing the DR-beads with a buffer having pH 4 – 10, indicating that pH may influence the protocol's effectiveness. However, the alternate protocol does not use a buffer, but merely uses water. Thus, it is advisable to examine protein recovery and SDS removal in the alternate protocol using a buffer (*i.e.*, 50 mM ammonium bicarbonate), instead of water, to wash the storage solution from the DR-beads.

Chapter 4

Automated Strong Cation Exchange-Reversed-Phase Platform for the Removal of Sodium Dodecyl Sulfate

4.1 Introduction

Proteomics experiments commonly involve the analysis of multiple complex samples. Methods for offline analysis often result in sample handling which is time-consuming, and which provides increased probability for contamination and sample loss. Automation is a means by which these shortcomings may be circumvented, where instruments are employed for the online coupling of separation techniques to mass spectrometry, such as liquid chromatography-mass spectrometry (LC-MS) [30]. It also allows for the application of such methods to high-throughput analysis, wherein the labour required for each step (*i.e.*, protein separation, removal of salts and detergents, protein identification and characterization) is minimized.

Sodium dodecyl sulfate (SDS) is a detergent commonly employed in proteomics experiments at concentrations at or above 0.1%. However, to avoid signal suppression during mass spectral analysis, it must be depleted to levels below 0.01% [51]. As discussed in Chapter 1, strong cation exchange (SCX) is a method which may be employed for SDS removal, wherein protonated (positively-charged) proteins are retained on the stationary phase and the negatively-charged SDS elutes in the void volume. In addition to reduction of SDS, SCX has been successfully applied for the separation of intact proteins, and is particularly advantageous for the separation of trypsin-digested samples [16, 17]. Through judicious choice of elution buffer, proteins or peptides which differ by only one charge may be separated and recovered with high yield [21].

Online (automated) coupling of orthogonal separation techniques is commonly performed; as compared to one-dimensional LC-MS analysis, such separations have been shown to greatly enhance identification and characterization of proteins originating from a complex sample [15]. Two-dimensional LC-MS (2D-LC-MS) is popular, particularly through the coupling of SCX ahead of reversed-phase (RP) [15, 18]. The two chromatographic methods may be combined into a biphasic column, wherein only a single column is required for both modes of separation [155]. However, if one desires to avoid injecting contaminants (*i.e.*, SDS) into the second dimension, a switching valve is required to couple the two techniques in sequence. The applicability of current automated SCX-RP-MS separation techniques to SDS removal is limited; eluent from SCX may only be diverted to RP, and because SDS binds to RP, such methods are unsuitable. Although offline methods have successfully employed strong cation exchange for the removal of SDS, they are not amenable to high-throughput analysis. Additionally, SDS removal by strong cation exchange has been quantified only by the change in observed signal during subsequent analysis [15, 59, 118, 119, 157]. As such, its effectiveness at detergent depletion has not been empirically determined.

Herein, a fully automated and high-throughput system, which employs two switching valves, was utilized for the online coupling of SCX ahead of reversed-phase chromatography. A condition for diverting SCX eluent directly to waste was implemented, thus allowing for the online removal of SDS by cation exchange. SDS removal was quantified and loading conditions optimized for maximum SDS reduction. Additionally, all steps were optimized to maintain the high yields associated with SCX.

As with 2D-LC-MS, the full automation presents the additional advantage of online coupling to mass spectrometry for top-down or bottom-up proteomics.

4.2 Materials and Methods

4.2.1 Reagents and Solutions

Sodium phosphate tribasic, protease inhibitor cocktail (cat. P8849) and *Saccharomyces cerevisiae*, Type II (cat. YSC2) were purchased from Sigma-Aldrich Canada Inc. (Oakville, Canada). Sodium dodecyl sulfate, tris, and urea were obtained from Bio-Rad (Mississauga, Canada). Sodium sulfate was from Caledon Laboratory Chemicals (Georgetown, Canada) and sulfuric acid from EMD Chemicals (Gibbstown, USA). Milli-Q grade water was purified to $18.2 \text{ M}\Omega\text{cm}^{-1}$. Chloroform, methylene blue, and HPLC grade solvents were purchased from Fisher Scientific (Ottawa, Canada). Cyanogen bromide and formic acid were obtained from Fluka (Oakville, Canada), and anhydrous sodium carbonate from ACP Chemicals (Toronto, Canada). The BCA protein assay was obtained from Pierce (Rockford, Illinois, USA). The Waters Spherisorb $5 \mu\text{m}$ C18 beads for HPLC were acquired from Waters Corporation (Milford, Massachusetts, USA). The $5 \mu\text{m}$ SCX beads employed were from The Nest Group, Inc. (Southborough, USA).

4.2.2 Yeast Proteome Extraction

Yeast proteins were prepared as described in Chapter 3, Section 3.2.2.

4.2.3 Cyanogen Bromide Protein Digestion

Cyanogen bromide-digest was performed as described in Chapter 3, Section 3.2.3

4.2.4 SDS Removal by HPLC

SDS was removed on an Agilent 1200 HPLC system wherein strong cation exchange and reversed-phase were coupled online using a Waters 11 Port, 10 Position switching valve and a Waters 10 Port, 2 Position switching valve. The setup is illustrated in Figure 4.1 (Full Setup). Strong cation exchange (SCX) was performed on a self-packed polysulfoethyl column (1 mm × 10 cm, 5 μm), and reversed-phase on a self-packed C18 column (1 mm × 10 cm, 5 μm). A flow rate of 100 μL/min and UV detection at 214 nm was employed.

4.2.4.1 Workflow for SDS Removal System

Figure 4.1 provides a schematic of the valve system employed to couple SCX to reversed phase. Prior to detergent depletion, the HPLC system was purged, and flow diverted directly to waste (Figure 4.1, Bypass to Waste). SDS was then removed in a three step process.

- (1) Step 1: Sample loading onto strong cation exchange (Figure 4.1, Step 1: SCX Column), and washing to remove SDS.
- (2) Step 2: Peptide elution from strong cation exchange to reversed-phase (Figure 4.1, Step 2: SCX to RP Column), with additional washing to remove elution buffer.
- (3) Step 3: Recovery of peptides from reversed-phase (Figure 4.1, Step 3: RP Column) through use of a standard acetonitrile gradient.

A detailed description of each step is provided below.

Step 1: Switching valves were set to Position 1. Following sample injection onto SCX, the gradient consisted of a 15 minute hold at 40% acetonitrile (ACN) and 0.1%

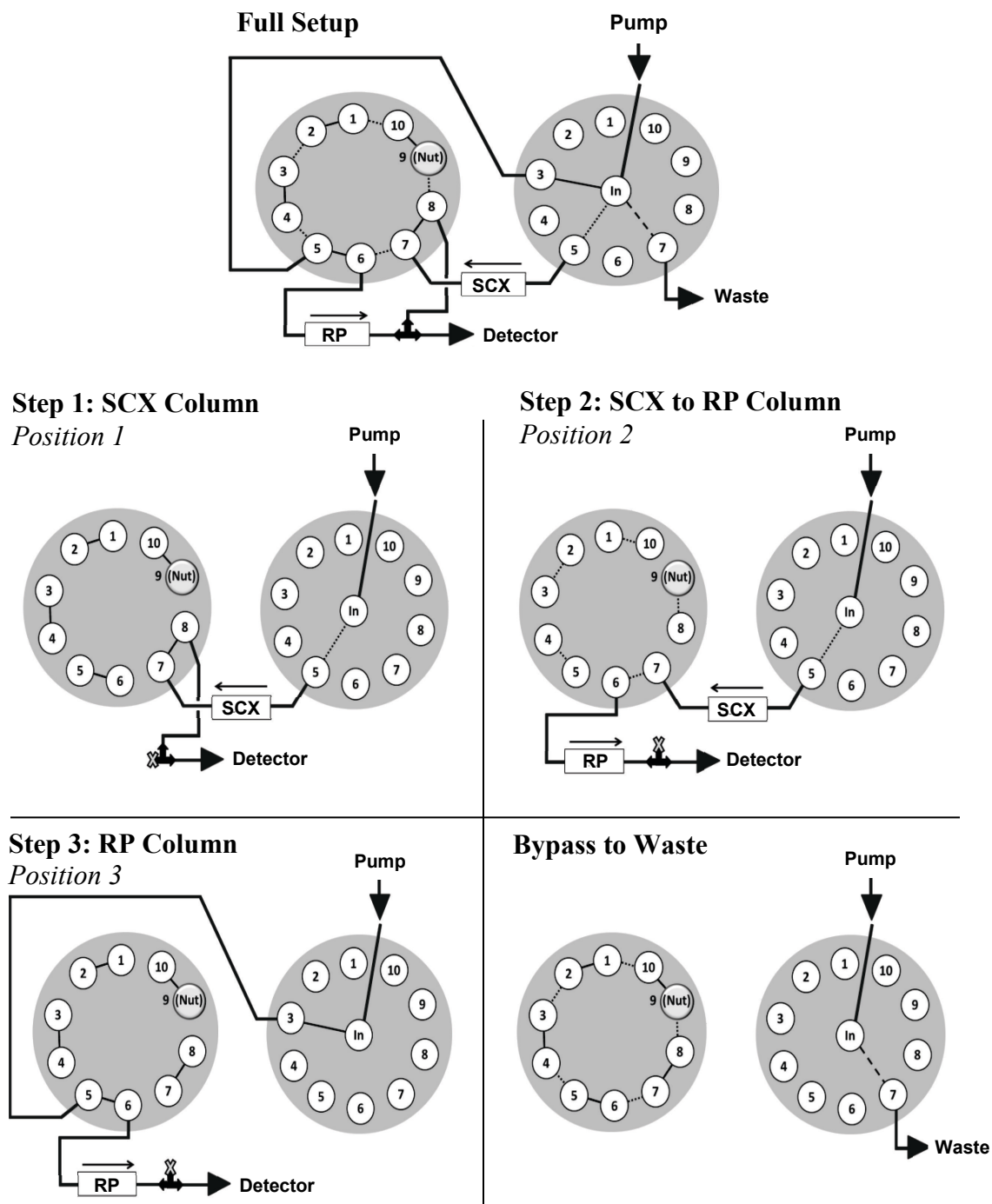


Figure 4.1: Schematic representation of the switching valve setup and workflow for SDS removal by online strong cation-exchange-reversed-phase liquid chromatography. The left valve is 2-Position, 10-Port and the right valve is 10-Position, 11-Port. **Full Setup** demonstrates all connections. **Step 1** Strong cation-exchange trapping of peptides and elution of SDS; **Step 2** elution of peptides onto reversed-phase; **Step 3** reversed-phase recovery of peptides. **Bypass to Waste** allows for purging of solvents in the HPLC system.

trifluoroacetic acid (TFA) in water, then an instantaneous decrease to 0% ACN for 20 minutes.

Step 2: Switching valves were set to Position 2. Initial solvent composition of 100% water, 0.1% TFA was held for 25 minutes to allow for equilibration of the SCX column, before instantly increasing to 100% of aqueous 20 mM sodium carbonate, pH 11. The system was held at such conditions for 20 minutes, followed by an instant switch to 100% water, 0.1% TFA for 35 minutes.

Step 3: Switching valves were set to Position 3. The gradient consisted of a 20 minute hold at 5% ACN (0.1% TFA/water) to equilibrate the reversed phase column, then an increase from 5% to 95% ACN over 22.8 minutes. The system was held at 95% ACN for 5 minutes before instantly dropping to initial conditions for 27.15 minutes to regenerate the column. At 60 minutes, solvent composition was instantaneously increased to 40% ACN, and held for 20 minutes. At 65 minutes, flow was diverted (Position 1) to allow for equilibration of the SCX column prior to injection of another sample. Eluting peptides were collected as two fractions over 15 minute intervals, beginning 18 minutes into the run (18 – 33 minutes, 33 – 48 minutes). The samples were completely dried in a SpeedVac.

4.2.5 Spectrophotometric SDS Assay

The SDS assay was performed as described in Chapter 3, Section 3.2.9.

4.2.6 Pierce BCA Assay

Pierce BCA assay performed as described in Chapter 3, Section 3.2.12.

4.3 Results and Discussion

Strong cation exchange shows promise as an excellent strategy for SDS removal. To determine optimal conditions, consideration was given not only to the efficiency of SDS depletion, but also to the recovery of protein from the column-based clean-up strategy. Thus, a complete optimization of the various stages of sample loading, detergent washing, and protein elution from the cation exchange column was performed by selecting solvent conditions which maximized protein yield. The online clean-up strategy was automated using a series of switching valves connected in sequence (Figure 4.1), in a manner similar to that seen in other multidimensional separation techniques [157, 158]. The goal of employing such a system was to fully bind proteins to SCX while washing SDS from the sample, followed by elution of peptides to reversed phase for recovery or for separation.

To fully optimize the protocol, several variables were of concern, an overview of which are supplied below.

- (1) Determination of optimal conditions for SDS removal.
- (2) Determination of optimal conditions for binding of proteins to SCX.
- (3) Determination of optimal conditions for protein elution.

4.3.1 Step 1: SDS Removal and Peptide Retention

The aims of Step 1, wherein sample was loaded onto the SCX column (Figure 4.1, Step 1), were to minimize SDS binding while simultaneously maximizing protein binding.

4.3.1.1 SDS Removal

Because SDS-containing samples were loaded onto an SCX column, the negative charge on dodecyl sulfate should have precluded its binding to the similarly charged

column support. However, it is possible that hydrophobic interactions may occur between the alkyl chain of the detergent and the solid support of the SCX resin. To test this, a protein-free SDS sample (100 μ L of 0.1% SDS) was injected, and bound SDS eluted by application of 100% acetonitrile. The result of this experiment revealed that approximately 1% of the injected SDS was retained, corresponding to 1 μ g SDS. The implications of binding such an amount are severe, wherein detergent depletion would not be sufficient for subsequent mass spectrometric (MS) analysis. Thus, a strategy must be developed to prevent the binding of SDS to the SCX column.

Given that the application of 100% acetonitrile removed SDS from the SCX column, it was considered that loading conditions containing acetonitrile may increase the efficiency of detergent depletion. Therefore, 100 μ L of protein-free sample containing 0.1% (100 μ g) SDS was injected at loading conditions of increasing acetonitrile concentrations (0% – 50%); the results are illustrated in Figure 4.2. As expected, at higher acetonitrile concentrations, less SDS was retained on the column. Above 30% ACN, detergent depletion was below the limit of detection for the SDS assay used (0.2 μ g in 200 μ L, equivalent to 1×10^{-4} % SDS). Thus, based on this experiment, one would recommend using at minimum 30% acetonitrile as loading conditions for the SCX column. However, the effect of >30% ACN on protein binding was unclear, therefore necessitating investigation prior to determination of optimal loading conditions.

4.3.1.2 Protein Binding

For determination of protein recoveries, samples of cyanogen bromide-digested yeast proteins were chosen. Because they are of intermediate molecular weight

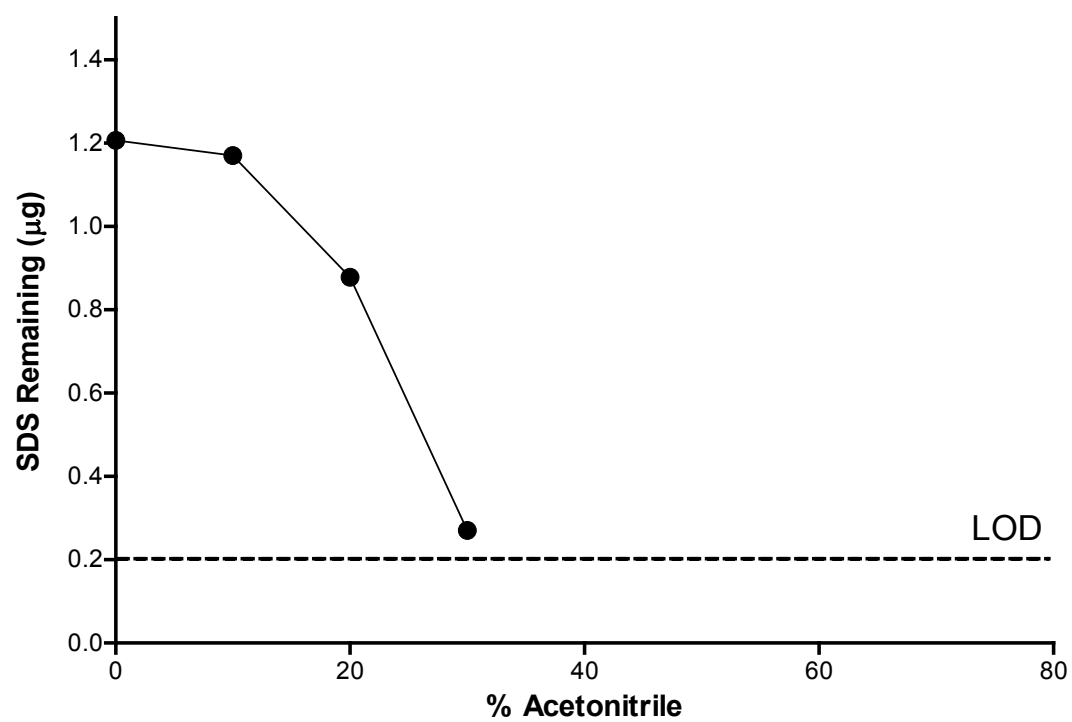


Figure 4.2: Mass of SDS remaining after injection of 100 µg SDS onto strong cation exchange at increasing concentrations of acetonitrile. At greater than 30% acetonitrile, the mass of SDS bound to the column was below the limit of detection for the colorimetric SDS assay used.

(approximately 5 kDa – 20 kDa), it was considered that optimal conditions for such samples may also be ideal for trypsin-digested and intact (undigested) protein samples (<5 kDa and <1 kDa – >50 kDa, respectively). Additionally, the strength of binding to a cation exchange column is correlated to the number of positive charges contained on a protein, which is directly related to solution pH. As such, all protein samples were adjusted to approximately pH 2 prior to injection.

The first consideration to the first step (sample loading), as previously discussed, was to minimize the binding of SDS. The second consideration during the first step was to determine optimal conditions for maximum protein binding. It was considered that the acetonitrile concentration required for optimal SDS elution could also result in the elution of SDS-protein complexes and, therefore, negatively impact protein recovery; such an effect has been previously reported for several different systems [115, 117, 144].

The effect of acetonitrile on protein binding was determined; 100 μ L of 30 μ g CNBr-digested yeast proteins in 0.1% SDS were injected under loading conditions of 0, 30, 40 and 50% ACN. The protein recovered after elution from the SCX column is shown in Figure 4.3. It is clear that conditions for maximum removal of SDS (loading at >30% ACN) resulted in extremely poor yields. Measurement of protein concentration in the injection peak indicated that the majority of unrecovered protein eluted immediately, and did not bind to the column, likely due to the elution of SDS-protein complexes. However, approximately 17% was not recovered, indicating that it did not elute from the column. Under current conditions, the use of acetonitrile is not recommended. However, dissociation of SDS-protein complexes may allow for high protein recoveries

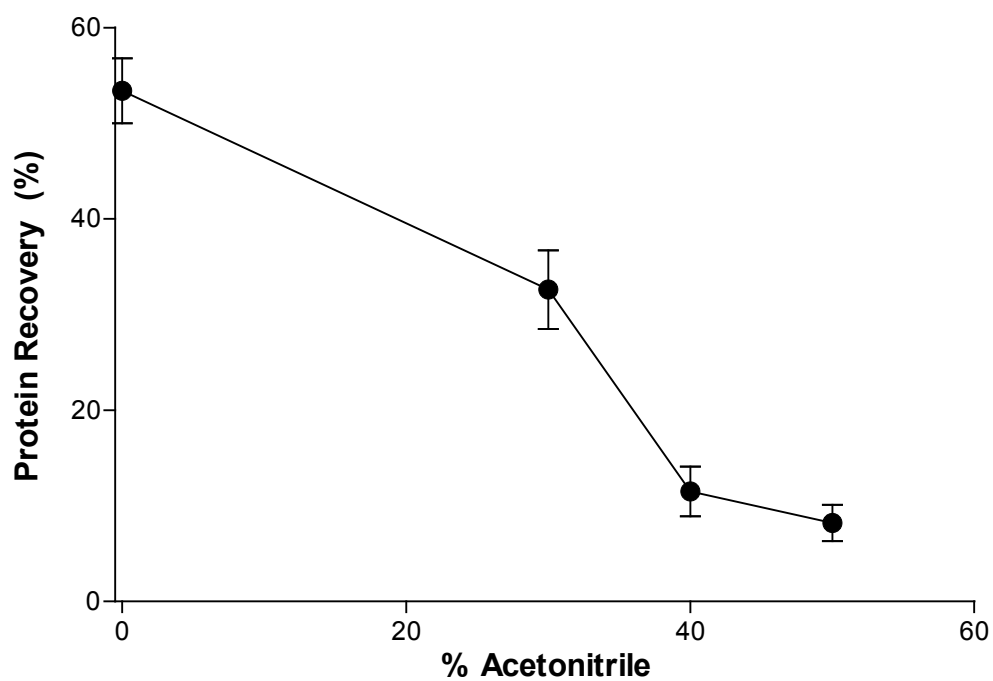


Figure 4.3: Protein recovery obtained after 100 μL of 30 μg CNBr-digested yeast proteins, 0.1% (100 μg) SDS were injected onto strong cation exchange at loading conditions of 0, 30, 40 and 50% acetonitrile.

even under loading conditions of $\geq 30\%$ ACN. Urea is a chaotrope known to interfere with non-covalent interactions (such as those between SDS and protein). Because incubation of sample with urea has been shown to increase protein recovery in several alternate systems (including strong anion exchange) [115, 117, 144], the addition of urea to sample was therefore investigated as a means for improving yields.

4.3.1.3 Urea for Enhanced Protein Binding

Samples of 30 μg CNBr-digested yeast protein, 0.1% SDS, were partially dried in a SpeedVac and diluted with 8 M urea to a final concentration of 6 M urea. They were not dried completely so as to avoid protein loss due to inefficient solubilisation. Urea-containing samples were injected under loading conditions of 0, 30, 40 and 50% ACN; the recoveries observed after elution from the SCX column are illustrated in Figure 4.4.

Within the errors of the experiment, protein recoveries were consistently high at all acetonitrile concentrations. This is a significant improvement over the low recoveries observed for non-urea samples which were injected at 30-50% ACN (Figure 4.3). Additionally, incubation of the sample with urea resulted in recoveries comparable to the benchmark recovery of 50% observed in the absence of urea (0% ACN loading). It is therefore likely that addition of urea for disruption of SDS-protein interactions minimizes the loss of protein due to the elution of SDS-protein complexes. The majority of unrecovered protein was found to elute in the injection peak and did not bind to the column. However, approximately 17% was not observed, indicating it did not elute from the SCX resin. For optimal SDS removal and maximum protein binding, a loading condition of 40% acetonitrile is recommended.

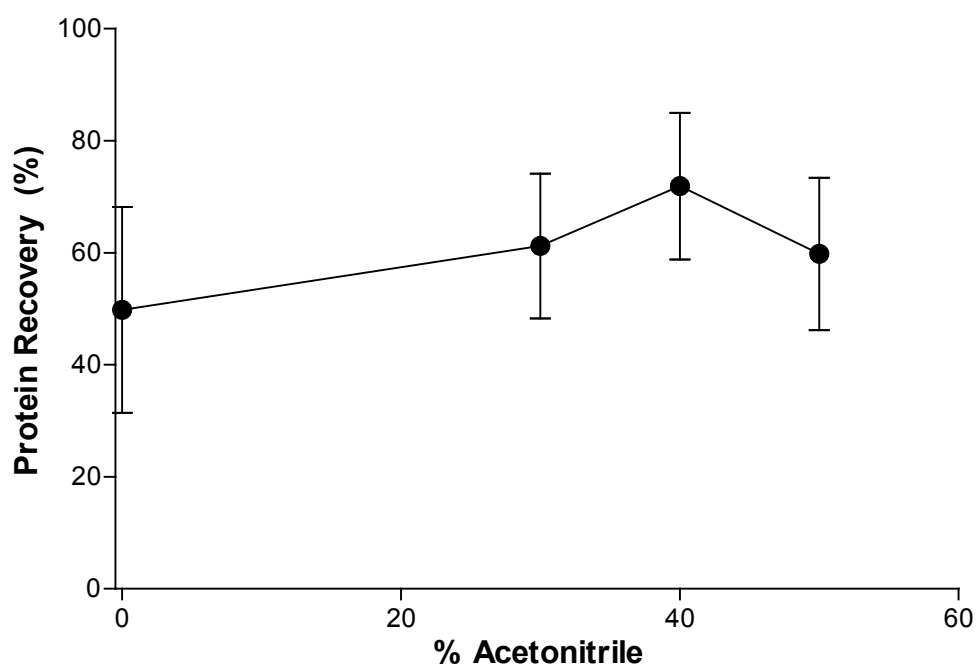


Figure 4.4: Protein recovery obtained after 100 μ L of 30 μ g CNBr-digested yeast protein, 0.1% (100 μ g) SDS were incubated in 6 M urea and subsequently injected onto strong cation exchange at loading conditions of 0, 30, 40 and 50% acetonitrile.

To further optimize protein binding, the effect of urea concentration was investigated. Samples of 30 μg CNBr-digested yeast proteins, 0.1% SDS, were partially dried and diluted with urea to final concentrations of 2, 4, and 6 M urea. The recoveries obtained after injection and elution of the urea-containing samples are shown in Figure 4.5. The influence of urea is clearly evident, wherein higher concentrations result in greater recovery. The result is as expected, given that higher concentrations of urea allow for greater disruption of non-covalent bonds such as those which occur between protein and SDS. Prior to injection, dilution of sample into a final concentration of 6 M urea is therefore recommended.

4.3.2 Step 2: Protein Elution from SCX to RP

The second step of the protocol for strong cation exchange as an SDS removal method elutes proteins from the cation exchange column directly onto the reversed-phase column (Figure 4.1, Step 2). For this step, it was considered that the recovery of protein may be maximized through optimization of the elution buffer. Characteristics of commonly used cation exchange buffers [15, 119, 158], such as pH, salt concentration, and acetonitrile concentration were therefore investigated. All fractions were collected directly after elution from SCX to avoid potential interference from reversed-phase.

4.3.2.1 Effect of Salt and pH

Protein recovery of samples containing 30 μg CNBr-digested yeast protein in 0.1% SDS were determined after elution with a buffer (100 mM tris) of varying solvent pH and NaCl concentrations. Results are illustrated in Figure 4.6. It is evident that yields increase as pH increases, and that addition of NaCl is advantageous only if the pH is < 10 .

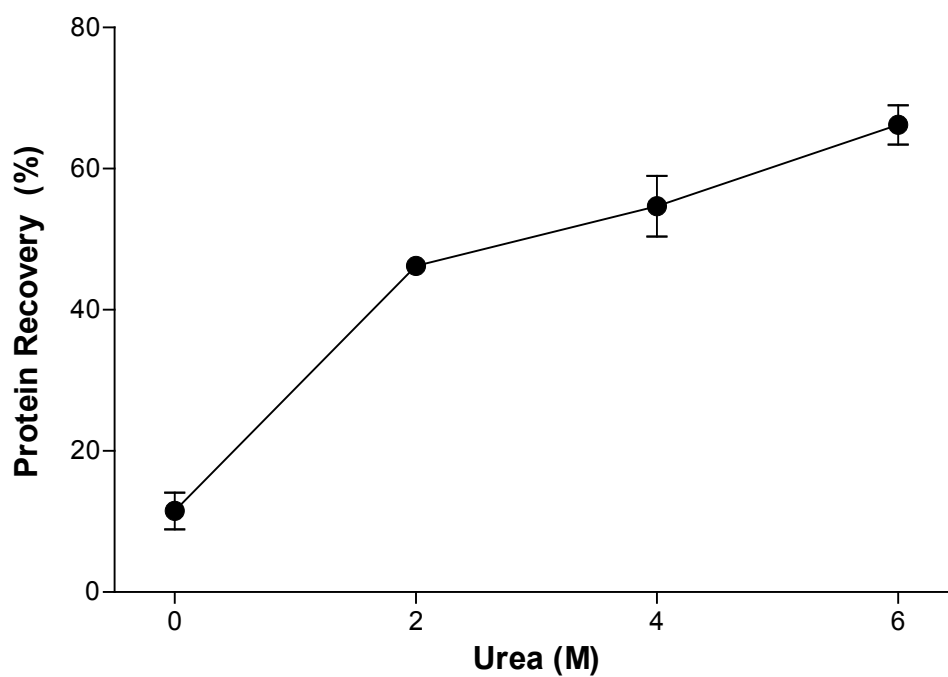


Figure 4.5: Protein recovery obtained after 100 μL of 30 μg CNBr-digested yeast, 0.1% (100 μg) SDS was incubated in increasing concentrations of urea and injected onto strong cation exchange at loading conditions of 0% acetonitrile.

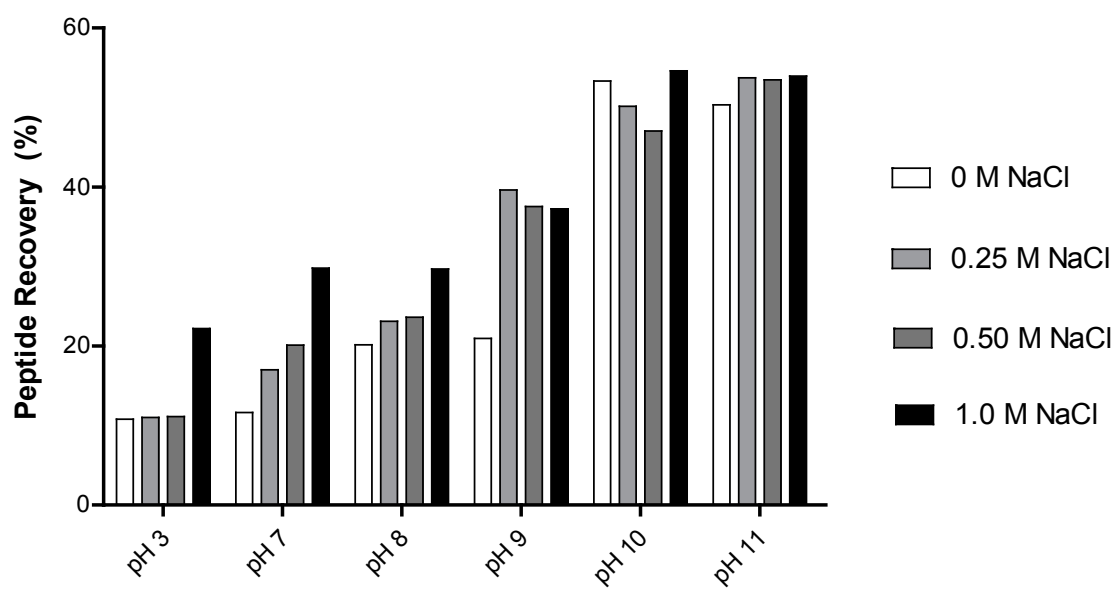


Figure 4.6: Protein recovery obtained after elution of 30 μg CNBr-digested yeast protein from a strong cation exchange column, using 100 mM tris buffer at various pH and salt concentrations.

This is likely due to protein charge at basic pH. The majority of amino acid side chains have isoelectric points at acidic or neutral pH; at basic pH, they carry a net negative charge and thus are no longer able to interact with the negatively-charged strong cation exchange column. It is therefore clear that, at pH 10, elution is predominantly due to deprotonation of protein molecules, thus eliminating the need for elution based on displacement with a compound of high ionic strength (*i.e.*, NaCl). A pH of 11 exhibited the highest recoveries, likely because such a highly basic pH is above the isoelectric point of all amino acids, and therefore each protein has a net negative charge.

4.3.2.2 Effect of Buffer

Although elution with 100 mM tris, pH 11, exhibited the highest recoveries, maximum yield was only 50%. Given that tris has a pK_a of 8.30, and pH 11 is outside its buffering range, it was considered that the pH of solvent which reached the column may have been lower than in the originating solution. Therefore, further increased recoveries could be obtained by employing an elution buffer with buffering range at highly basic pH (wherein solvent would remain at pH 11).

In addition to tris (pK_a 8.30), sodium phosphate tribasic (pK_a 2.2, 6.8, 12.3) and sodium carbonate (pK_a 6.3, 10.3) were investigated. Tris was prepared at 100 mM, while the latter two were prepared at 20 mM; all were adjusted to pH 11. Buffer concentrations were kept low so as prevent interferences with the BCA Assay (Section 4.2.6) used for determination of protein recoveries in the succeeding sections. To determine buffering efficiency at pH 11, the SCX column eluent was collected over the course of buffer elution, in fractions of 2.5 minutes each, and the pH measured. The results are illustrated in Figure 4.7.

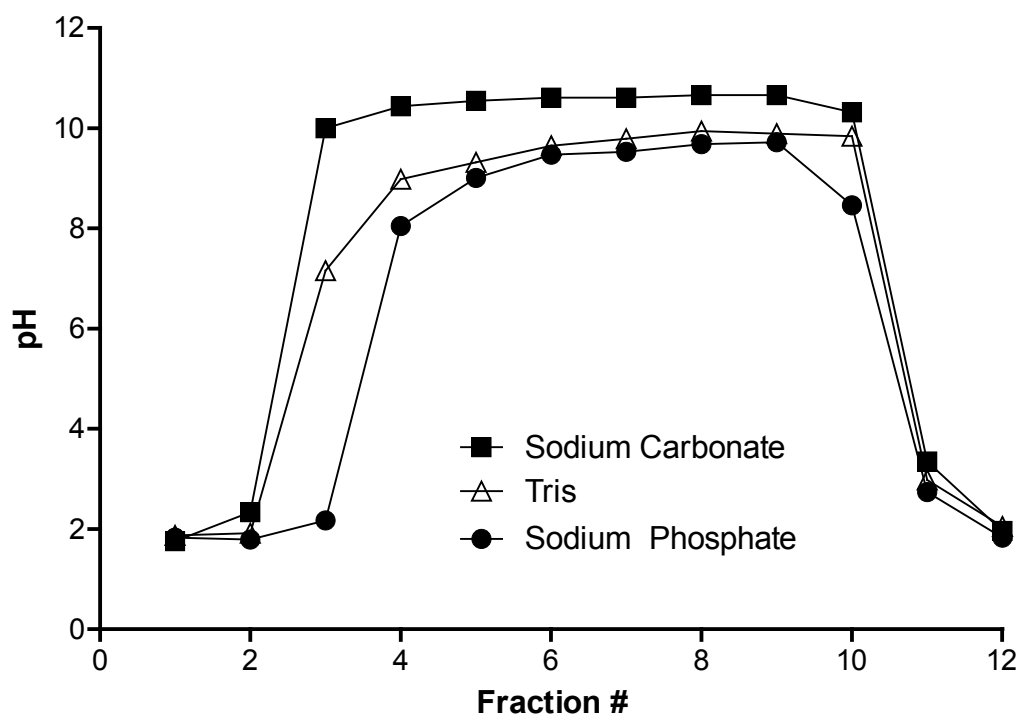


Figure 4.7: CNBr-digested yeast protein (30 μ g) was eluted from the strong cation exchange column using 20 mM sodium carbonate, 100 mM tris and 20 mM sodium phosphate tribasic, all adjusted to pH 11. Fractions were collected in 2.5 minute increments over the course of elution, and the pH measured.

The pH exhibited by sodium phosphate and tris eluents had maximum values of 9.6 and 9.8, respectively, while sodium carbonate had a pH of 10.7. Tris is ineffective at buffering at pH 11 because such a highly basic pH is outside its buffering range. The decrease in observed pH, as compared to that of the original solution, was likely due to residual TFA remaining in the system from Step 1: Sample Loading. However, pH 11 was within the buffering range for the other two buffers tested. Because sodium carbonate has a pK_a closer to the desired pH than does sodium phosphate, it exhibited a greater resistance to pH changes. Additionally, the pK_a for sodium carbonate is lower than the desired pH. Because peak buffering capacity occurs when $pH = pK_a$, a decrease in pH due to the presence of acid therefore produced an increase in buffering capacity for sodium carbonate; the opposite was true for sodium phosphate. Based solely on effective pH, a buffer solution of 20 mM sodium carbonate, pH 11, is therefore recommended.

To determine if higher effective pH would result in increased protein yields, 30 μ g CNBr-digested yeast was eluted from the SCX column using each of the studied buffers. Results are summarized in Table 4.1, where it is clear that sample recovery increased at higher effective pH. Because sodium carbonate demonstrated the highest effective pH, a buffer solution of 20 mM sodium carbonate, pH 11, is therefore recommended for protein elution.

4.3.2.3 Effect of Acetonitrile

A common component of elution buffers for strong cation exchange is acetonitrile [118, 119], which may be employed at concentrations up to 25% ACN [15] for enhanced

Table 4.1: Protein recovery and effective pH obtained after CNBr-digested yeast protein (30 μ g) was eluted from strong cation exchange using 20 mM sodium phosphate tribasic, 100 mM tris and 20 mM sodium carbonate, all buffered to pH 11.

Buffer (Concentration)	Protein Recovery (%)	Effective pH
Sodium Phosphate (20 mM)	54.7 \pm 3.4	9.6
Tris (100 mM)	61.3 \pm 2.8	9.8
Sodium Carbonate (20 mM)	72.5 \pm 3.6	10.7

protein separation [159]. However, separation *via* SCX is not of concern in the presented protocol for detergent depletion; overall protein recovery is of much greater importance. Furthermore, ACN is commonly employed to elute proteins during reversed-phase chromatography. Because SCX is coupled directly to a reversed-phase column (Figure 4.2, Step 2), the ACN concentration must be below 5% to ensure protein retention on the reversed phase matrix. However, higher concentrations of ACN may be employed if solvent is diluted after the SCX column, but prior to reversed-phase chromatography. Such could be accomplished through use of a T-valve, wherein water is added to the system for ACN dilution. The effect of ACN on protein yield was therefore investigated at concentrations up to 25%. Samples were collected directly after elution from SCX to avoid potential losses due to incomplete recovery from reversed-phase.

Figure 4.8 illustrates the observed recoveries from samples of 30 μ g CNBr-digested yeast protein, 0.1% SDS, 6M urea, after elution from the SCX column with 20 mM sodium carbonate, pH 11, with varying ACN concentrations. Although there was little variation in protein recovery at differing acetonitrile concentrations, there was a slight increase at 5% ACN. With a two-tailed p-value of 0.0110, the observed increase is statistically significant. Therefore, subsequent experiments were performed using an elution buffer of 20 mM sodium carbonate, 5% ACN, pH 11. Such an ACN concentration is also advantageous as it does not require dilution prior to reversed-phase chromatography.

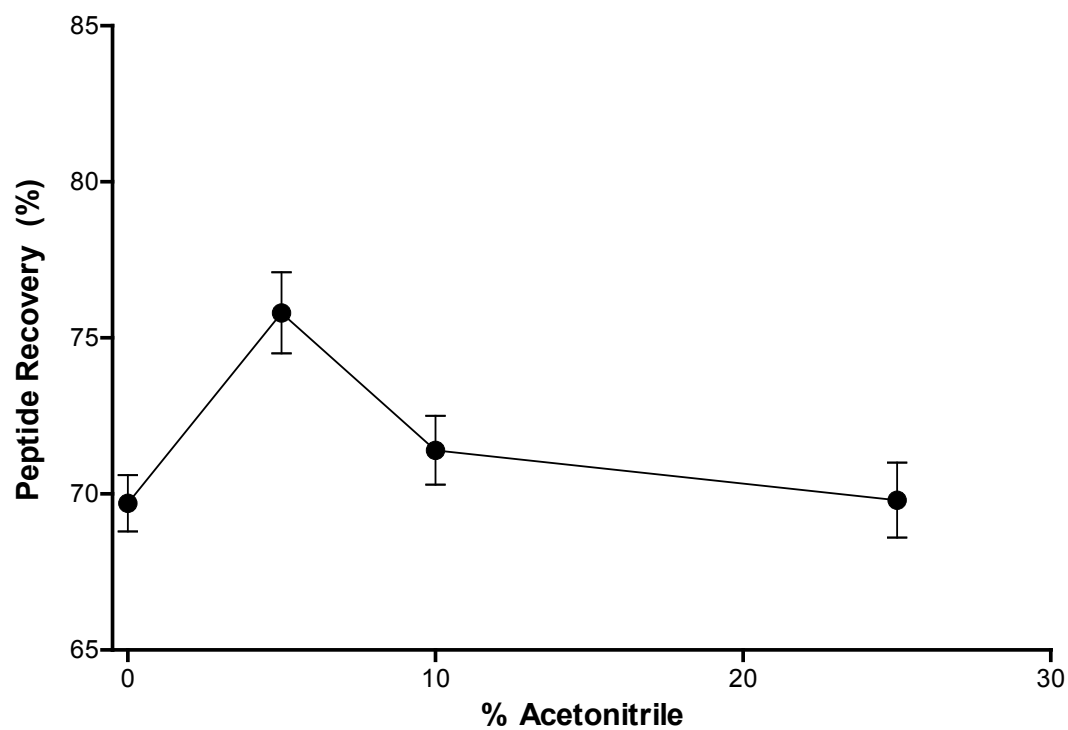


Figure 4.8: Protein recovery obtained after 100 μ L of 30 μ g CNBr-digested yeast protein, 0.1% (100 μ g) SDS, 6 M urea was injected onto strong cation exchange column and subsequently eluted with 20 mM sodium carbonate, pH 11, under loading conditions of 0, 5, 10 and 25% acetonitrile.

4.3.3 Step 3: Recovery of Protein from Reversed Phase

Peptides were eluted from the reversed-phase column (Figure 4.1, Step 3) using a standard acetonitrile gradient (Section 4.2.4.3), known to have high (>90%) recovery for trypsin-digested proteins. However, it was unclear if CNBr-digested proteins would exhibit similarly high recoveries. Injection of 30 μg CNBr-digested proteins yielded recovery of $91 \pm 3\%$, signifying that the gradient was suitable for use in the SCX-RP SDS removal method.

4.3.4 SDS Removal by SCX: From Loading to Protein Recovery

Having optimized each step for the removal of SDS by strong cation exchange, the efficiency of the entire system was examined using the optimized conditions, outlined in Table 4.2. The efficiency of SDS removal and protein recovery (100 μL injection) are summarized in Table 4.3. Depletion of SDS was effective, with remaining levels below the limit of detection for the assay used ($<1 \times 10^{-4}\%$ SDS), which is significantly lower than the 0.01% required for subsequent MS analysis. Additionally, protein recoveries were high (70 – 75%) for samples containing both abundant (50 μg) and limited (5 μg) amounts of CNBr-digested yeast protein; the latter consideration is of particular importance, since high recovery at low analyte concentration works to ensure that sufficient material is available for subsequent analysis. Determination of protein concentration in the injection peak showed that the majority of unrecovered protein eluted immediately and did not bind to the SCX column. It is therefore clear that the fully automated method for removal of SDS from strong cation exchange is effective for samples at both high and low concentrations of protein.

Table 4.2: Summary of the optimal chromatographic conditions determined for each step of the automated SCX-RP method for SDS removal.

	Sample	Solvent
Loading Conditions	6 M Urea 0.1% TFA	40% ACN 0.1% TFA
Elution from SCX	--	20 mM Na ₂ CO ₃ 5% ACN pH 11
Elution from Reversed Phase	--	Standard ACN Gradient 0.1% TFA

Table 4.3: Recovery of CNBr-digested yeast protein and mass SDS remaining after detergent depletion using the optimal conditions determined for the fully automated SCX-RP method.

Sample Amount (μg)	Protein Recovery (%)	SDS Injected (%)	SDS Remaining (%)
5	76 \pm 4	0.1	< 1×10^{-4} *
50	69 \pm 2	0.1	< 1×10^{-4} *

*Below LOD for the colorimetric SDS assay used

4.4 Conclusions

This chapter presented the development of a fully automated method for SDS removal employing strong cation exchange chromatography coupled to reversed-phase chromatography. Full automation allows for less sample handling, therefore decreasing the probability of sample loss or sample contamination, while coupling to reversed-phase permits online coupling to mass spectrometry. The method developed herein is therefore an attractive option for depletion of detergent while maintaining protein recoveries.

4.5 Future Work

Reversed-phase chromatography often employs packing material which is sensitive to pH extremes, such as those which were used for elution of proteins from strong cation exchange phase to the reversed-phase. However, there do exist materials which are stable at high pH; investigation of such may lead to increased protein recovery from the reversed-phase matrix, and therefore higher protein yield overall.

Chapter 5

Conclusions

5.1 Thesis Summary

This thesis described the development of techniques for the quantification and removal of sodium dodecyl sulfate (SDS) from protein-containing samples. In Chapter 1, the advantages and limitations of working with SDS were discussed. Both the mechanism by which SDS works to denature and solubilize proteins and the mechanism by which SDS causes signal suppression in liquid chromatography-electrospray ionization-mass spectrometry were discussed (LC-ESI-MS). Although generally employed at concentrations of 0.1% to 2%, SDS negatively affects LC/MS analysis at concentrations greater than 0.01%. Methods for SDS removal are therefore required, wherein detergent depletion is efficient and protein recoveries are high. The latter is of concern so as to avoid loss of potentially important sample components (*e.g.*, a biomarker), and to ensure that sufficient protein is available for subsequent analysis. The development of such techniques is an ongoing area of interest in proteomics.

Chapter 2 presented the optimization of an isocratic negative-mode LC-ESI-MS technique for sensitive and selective quantification of SDS. The technique is not sensitive to the presence of protein and allows for removal of salt and buffer contaminants by reversed-phase HPLC prior to analysis of SDS. It was determined to have a limit of quantification of 0.5 ng, and a limit of detection of 0.15 ng.

In Chapter 3, the Pierce Detergent Removal Spin Columns were evaluated and found to provide efficient detergent depletion but variable and often quite poor protein

recoveries. However, it was determined that reducing the volume of detergent removal beads (DR-beads) led to increased protein yield. An alternative protocol was therefore developed, employing manual agitation of the sample with significantly reduced volumes of DR-beads. The protocol was found to increase protein and peptide recoveries by upwards of 30% while maintaining efficient removal of SDS.

Lastly, Chapter 4 described the development of a fully automated strong cation exchange-reversed-phase (SCX-RP) method for the removal of SDS. It possesses the ability for online coupling to mass spectrometry, which is also advantageous due to the minimal amount of labour required. This method affords efficient detergent depletion while maintaining protein recoveries of >70%.

The work presented herein provides an attractive option for removal of SDS from protein samples prior to mass spectrometric analysis. The ability for high-throughput analysis increases the applicability of the techniques to proteomics experiments, wherein the analysis of many samples is often required. Additionally, the high protein recovery exhibited by both the Pierce Detergent Removal Spin Columns alternate protocol and the SCX-RP protocol allows for detergent depletion from samples containing limited analyte. Although the methods may be improved through further optimization (see Chapter 3, Section 3.5 and Chapter 4, Section 4.5), the techniques may be successfully employed 'as is' for SDS removal prior to protein identification and characterization by mass spectrometry.

5.2 Future Work

The main purpose of this thesis was to develop techniques for efficient removal of SDS while maintaining high protein recoveries. While application of the developed methods to real samples (*e.g.*, human plasma) was beyond the scope of this thesis, high recovery was observed for the complex yeast proteome. Future work using the developed techniques will therefore provide high-throughput detergent depletion with concurrent high protein yields. Although much of the future work was presented in Chapter 3, Section 3.5 and Chapter 4, Section 4.5, additional experiments for further optimization are suggested below.

In the field of proteomics, there are many sample types which require detergent depletion prior to MS analysis. Of particular interest are samples collected from Gel-Eluted Liquid Fractionation Entrapment Electrophoresis (GELFrEE), wherein proteins and peptides are collected in solution containing approximately 0.1% SDS. As such, the developed techniques were investigated only for samples containing the aforementioned concentration of detergent. Although the methods are theoretically applicable to samples where SDS is employed at concentrations up to 2% (*i.e.*, for protein denaturation and solubilisation), further work is required to ensure that in such samples SDS is depleted to below the threshold value of 0.01% required for subsequent mass spectral analysis.

GELFrEE is advantageous because proteins are separated and collected in solution at the intact level, thus allowing for top-down analysis. Due to the large number of samples generated from even a single GELFrEE run, a high-throughput method for SDS removal is required. The alternative protocol developed for the Pierce Detergent Removal Spin Columns is ideal for detergent depletion of such samples prior to MS

analysis. However, further work is required to extend application of the SCX-RP system to intact proteins, since the C18 beads employed in the reversed-phase column often provide high recovery only for peptides. It is likely that investigation of different reversed-phase column packings (*e.g.*, C4 beads) will allow for efficient removal of SDS while maintaining high protein recovery.

5.3 Conclusions

Sodium dodecyl sulfate is an indispensable tool for protein pre-fractionation, denaturation, and solubilisation. However, its negative effect on LC-ESI-MS analysis necessitates its removal prior to LC-MS analysis. Although many techniques for its removal currently exist, none are applicable to all sample types. As such, the development of techniques for detergent depletion is still an area of great interest. Presented in this thesis is work towards the development of methods for high-throughput removal of SDS while maintaining high protein recoveries. Although work remains to tackle issues of SDS concentration and recovery of intact proteins after reversed-phase chromatography, it is anticipated that the methods developed will be applicable to all SDS-containing samples in the near future. This will help to allow for the improved analysis of complex proteomes, thus aiding in the search for disease biomarkers which may be used to design treatments for improving human health.

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