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Mass Spectrometric Analyses of Surface Proteins from Candida albicans After Treatment with Histatin 5

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Graduate Program in Chemistry
A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science

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by

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Abstract

Current drug therapies have lost their effectiveness in controlling the fungal species known as *C. albicans*; therefore, the need for newer drugs becomes crucial. Histatin 5 (Hst 5), a naturally occurring peptide found in the oral cavity, has demonstrated strong effects on controlling candidiasis. Here, we have proposed that the adhesion of cells through surface proteins is an important process. To allow the analysis of surface proteins, the concept of on-whole cell tryptic digestions were developed. This on-target approach allows for isolation of the peptides of interest, while greatly reducing digestion times.

To study the effects of Histatin 5 on surface proteins, live cells were adhered to slides pre-coated with human serum albumin (HSA); and subsequently, treatments with Hst 5 were done. Mass spectrometry was used to correlate changes in ion counts to protein expressions. Preliminary work done by MALDI MS verified that changes in ion counts occurred, as well as tentative protein identifications. Then, HPLC-ES-MS/MS was used to verify proteins and their biological processes. A few proteins were seen from the cell surface, where their presences indicated the success of our on-target approach. Three other processes were found; virulence, mitochondrial associated and nucleic acid binding. Hst 5 was shown to decrease the expression of proteins involved in the virulence, supporting Hst 5 as an antifungal agent. The identified proteins involved in the last two pathways have provided additional support for the speculation that cellular respiration in the mitochondria may be the true target of Hst 5.

Keywords
mass spectrometry, *Candida albicans*, Histatin 5, surface proteins, adhesion, trypsin digestions, protein expression

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List of Abbreviations

AIDS	acquired immune deficiency syndrome
ATP	adenosine triphosphate
BCA	bicinchoninic acid
BSA	bovine serum albumin
CHCA	α-cyano-4-hydroxycinnamic acid
CID	collision induced dissociation
DC	direct current
DHB	2,5-dihydroxybenzoic acid
DNA	deoxyribonucleic acid
EI	electron impact
ESI	electrospray ionization
HPLC	high performance liquid chromatography
HSA	human serum albumin
Hst	histatin
LC	liquid chromatography
m/z	mass-to-charge
MS/MS	tandem mass spectrometry
MALDI	matrix-assisted laser desorption ionization
MS	mass spectrometry
Nd:YAG	neodymium-doped yttrium aluminum garnet
NPLC	normal phase liquid chromatography
OD	optical density

PBS	phosphate buffered saline
PMF	peptide mass fingerprinting
PSD	post source decay
RF	radio frequency
RPLC	reverse phase liquid chromatography
RNA	ribonucleic acid
ROS	reactive oxygen species
s.d.	standard deviation
SA	sinapinic acid
SDA	Sabouraud dextrose agar
TPCK	tosyl phenylalanyl chloromethyl ketone
TOF	time-of-flight
UV	ultraviolet
YNB	yeast nitrogen base

Section 1 Introduction

1.1 Mass Spectrometry (MS) of Microorganisms

1.1.1 Protein Profiling for Taxonomy

The ability to differentiate microbial species can be beneficial, as it allows for rapid clinical identification of pathogenic and non-pathogenic species. Traditional identification methods are based on morphology and biochemistry. Unfortunately, this led to sample preparations spanning several days, which can hinder clinical applications. As such, identification using the genome sequence is now the standard. Indeed the genome does provide the most accurate identification; however, analysis of the genome is time-consuming and challenging. The analysis of proteins has been recognized as a post-genomic science, allowing one to indirectly study the genome. Therefore, protein analysis performed by mass spectrometry (MS) is an excellent alternative for microbial speciation. Two "soft" ionization techniques, electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI), allow for protein analyses. The detailed mechanisms of both ionization techniques coupled with MS are discussed in Appendix 1.

In microbiology, MS offers significantly simplified sample preparations, a high degree of certainty, and more importantly, rapid analyses of proteins. MS has been shown to effectively determine taxonomy down to the genus, species and strain levels [1]. In this approach, the process of profiling either the complete proteome or a sub-fraction of proteins, are used for identification. This can be achieved by developing key mass-to-charge (*m/z)* signals, known as biomarkers, which are characteristic to specific microorganisms. These biomarkers are like the microorganism's fingerprint, and so by incorporating them into a database, rapid identification is possible [2].

In 1998, Liu *et al.* successfully used this biomarker method for the identification *Escherichia coli* from crude lysates [3]. In their work, cells were lysed open and the

resulting lysate was analyzed via MS. Due to the complexity of using cell lysates, separation techniques like liquid chromatography (LC) are often used, therefore making ESI MS more favourable. In Liu's work, however, they developed their own cellulose-based molecular weight cut off microdialyzer for fractionation. Their work demonstrated the need for a sample cleanup prior to mass spectral analyses. Despite this success, more rapid methods are sought after as LC-ESI-MS has a lower sample throughput and is still time-consuming.

MALDI MS offers several advantages with regards to biomarker fingerprinting. The main improvement is the rate of data acquisition. In MALDI MS, larger amounts of data can be analyzed over a set period of time. This is because many samples can be spotted onto a single plate. Protein profiling for microorganisms using MALDI MS was first introduced by Cain *et al.* in 1994 [4]. In their work, cells from the *Pseudomonas* species were lysed using sonication, and the released proteins were analyzed. By comparing spectra obtained from pure bacterial cells to those obtained from mixtures, they developed biomarkers characteristic to each species. As a result, these biomarkers were able to accurately identify the *Pseudomonas* species within mixtures.

Despite these successes, there was still one drawback in MS of microorganism speciation. This would be the use of crude lysates. Lysates are biologically complex and almost always require a sample cleanup to help improve detection. Because of this, methods using whole intact cells, rather than lysates, became of interest. Anhalt and Fenselau were able to use whole microbial cells for biomarker identification [5]. In their work, cells from several bacterial species were directly inserted into the ion source of a pyrolysis mass spectrometer. The gentle heating of the cells lead to partial vaporization, and the resulting spectra were able to differentiate the species. Their success demonstrated that using whole cells directly, instead of cellular fractions, was possible with the same results.

With whole cells, both the use of ESI and MALDI, have been explored. Vaidyanathan *et al.* reported using ESI MS by injecting suspensions of whole cells directly into the mass spectrometer [6]. In addition, they were able to identify strains of bacteria without the use

of LC. Unfortunately, for ESI MS clogging by the intact cells may occur. And yet again, sample throughput is still limited due to rinsing and sample loading steps. Because of this, MALDI MS seemed more as a suitable candidate for whole cell analyses.

With MALDI MS, intact cell sample handing and analyses become simpler. In general the procedure for these analyses can be completed in three steps [7]. To start, fresh cells of a colony are transferred from the media plate to the MALDI target. Samples are then overlaid with a matrix, where α-cyano-4-hydroxycinnamic acid (CHCA) and sinapinic acid (SA) were proven to produce better signals [8, 9]. And lastly, once dried, they can be introduced into the mass spectrometer. Holland *et al.* successfully carried out this described procedure towards identifying five bacterial strains in a mixture [8]. With cells only needing to be transferred directly to the MALDI target, the application of MALDI for microorganisms is limitless. It does not encounter needle clogging and MALDI MS is more tolerant to higher salt concentrations. Together, MALDI MS seems to be more appropriate in whole cell analysis.

1.1.2 Surface Proteins and Enzymatic Cleavages

Out of all proteins encoded by the genome, interest has shifted towards investigating the surface proteome. The surface proteome encompasses all proteins present at the cell surface under a specific condition and time [10]. These proteins localize to the cytoplasmic membrane, membrane-cell wall interface or the cell wall [11]. There they may be partially or completely exposed to the external environment. This meant that they can be freely soluble, held within vesicles or surface-attached [12]. Surface proteins have multiple functions that are vital to cell survival which include; transportation of nutrients, acting as receptors for signalling pathways, acting as binding factors to other cells and surfaces, and lastly, they have enzymatic activities [12].

Within microbiology, surface proteins are of particular importance as they play crucial roles in establishing pathogenesis. The first step in causing an infection is to adhere to host epithelial tissues [13]. Once attached, they can grow and ultimately invade the host system [14]. Upon invasion, they can release toxins and cause serious health concerns.

Additionally, surface proteins allow for microorganisms to send appropriate signals to the cytoplasm for defense against toxins and host immune responses [15]. For the host, the first encounter is the surface proteome, and because of this, surface proteins may be important for future drug therapies.

While the need for understanding surface proteins is crucial, not much is known about them. This is majorly due to difficulties in defining their protein compositions and surface topologies [14]. There are three methods that have been used for identifying surface proteins. The first method involves the use of algorithms with genome analysis to predict where surface proteins may be located [16]. This technique albeit rapid, was neither fully reliable nor quantitative. A second approach would be to isolate membrane and cell wall fractions from cytoplasmic ones [17]. Unfortunately, this suffered contamination from cytoplasmic proteins. And lastly, antibodies that bind surface proteins may be used [18]. In this method, surface proteins are predicted and tested against antibodies to see whether binding occurs. When binding occurs, then there is conformation of a surface protein. This has been shown to be successful, however, it was very expensive and labour intensive. Overall, convenient methods are available for identifying surface proteins, but, to minimize complications, others should be investigated.

It was not until 2006 where the concept of limited proteolysis was coupled with MALDI MS. In 2006, Rodriguez-Ortega *et al.* established a protocol for what was termed “cell shaving", where proteins partially exposed on the outside of bacterial cells were isolated [14]. In their protocol, live cells of group A *Streptococcus* were analyzed. Group A referred to the sub-classification for specific carbohydrates present on the cell wall of the β-hemolytic groups of microorganisms. β-hemolytic groups are those that completely rupture red blood cells in their pathogenesis. Cells of group A *Streptococcus* were subjected to the proteolytic enzymes, trypsin or proteinase K, to cleave peptides at the surface. The use of enzymes to "shave" the cell surface allows for peptides to be released into the surrounding buffer, allowing them for their separations from remaining cells. The peptide-buffer mixture was collected and then analyzed with MADLI-MS. In theory,

these digestions should only target regions protruding outside of cells, hence, the peptides can be said to belong to surface proteins.

Although studies involving surface digestions center on the use of MALDI MS for analyses, it should be clarified that the use of ESI MS iwa possible. Phinney *et al.* used limited proteolysis to understand how proteins of the Sindbis virus are arranged on the cell surface [19]. It was determined that under different environmental conditions, different protein regions are exposed and can be readily digested using trypsin. The resulting peptides were released, separated by HPLC and then analyzed by ESI MS. In addition, the recent development by Randall *et al.* demonstrated analyses of surface digestions using ESI MS without a need for any separation [20]. They directly coupled on-target surface protein sampling with nano-ESI inlet needle for mass spectral analyses.

1.2 *Candida albicans* (*C. albicans)*

Through collaboration with Dr. Walter Siqueira in Dentistry at UWO, our research has become interested in studying microorganisms occupying the oral cavities and their associated salivary proteins. Further knowledge can allow for a long term goal of promoting oral health and defense against oral pathogens. From all of the microorganisms in the oral cavity, our focus is towards the fungal species *Candida albicans (C. albicans),* with its high increasing infection rates and ineffective oral therapeutic treatments.

C. albicans can be found within the gastrointestinal and genitourinary tracts, as well as on the surface of skins [21]. It exists within humans in a commensal relationship, where it benefits without causing any damage. However, in individuals with compromised immune systems, *C. albicans* can cause dangerous infections known as candidosis [22]. This includes individuals with acquired immune deficiency syndrome (AIDS), transplant recipients, and those receiving chemotherapy [23]. There are two types of infections that may occur, superficial and systemic. Superficial infections affect the skin, hair, nails and

mucosal membranes [24]. Whereas systemic infections occur within major body organs such as the kidney, heart and brain [25].

C. albicans exists in three distinct morphological forms; the unicellular yeast, pseudohyphae with the starting growth of filaments, and as hyphae with fully grown filaments. All three forms can be observed under varying growth; below 30°C or at pH 4.0, 30°C at pH 6.0., and 37°C at pH 7.0 or in N-acetylglucosamine, respectively. It is because of this ability to adopt three forms, that *C. albicans* can be fatal. It enters the host as the budding yeast, growing into the hyphal state to penetrate tissues [21]. And it is only the hyphae that can initiate pathogenesis. As a result, the hyphal form carries the most resilience to current therapeutic treatments [26]. In addition to these three states, *C. albicans* can also form surface-attached communities called biofilms [21]. Biofilms are cell communities encased in a matrix of extracellular polymeric substances. They can be found on medical devices such as dentures, shunts, implants, peacemakers and catheters [27]. Once growth starts on them, *C. albicans* becomes extremely difficult to remove, where the only solution is to remove the device completely.

The most common way to treat candidiasis is to administer oral antifungal drugs. Four categories of antifungal agents are available, classified according to their working mechanism. The first class aims to disrupt the cell membrane, which the drug amphotericin belongs [22]. The second group, including drugs like fluconazole, works to inhibit the synthesis of ergosterol for the cell membrane [28]. Ergosterol is a key component for maintaining cell membrane integrity. Third, is a group of drugs aimed towards inhibiting the synthesis of the cell wall polysaccharide known as β-1,3-glucan [22]. Lastly, is a group targeted towards the induction of incorrect ribonucleic acid (RNA) synthesis and deoxyribonucleic acid (DNA) replication [22]. Despite these different types, antifungal agents are losing their effectiveness in treatments. One significant problem with using antifungal agents is that *C. albicans* has developed resistance towards them [28]. In addition, the drug dose at the target site is not high enough to illicit a response [22]. One simple solution to these complications may be to develop additional drugs, however, this is challenging in itself. Fungal species are eukaryotic which means they share common biological process as humans [29]. The

development of new drugs must be done in such a way that no serious side effects occur in humans. Consequently, these drugs can cause serious side effects in humans. As such, newer approaches targeted towards controlling *C. albicans* are needed.

1.3 Histatin 5 (Hst 5)

For infections to start, adhesion to oral surfaces must occur where *C. albicans* can begin to colonize [30]. The process of adhesion is controlled by the development of an acquired pellicle. This acquired pellicle develops on surfaces composed of about 130 proteins, carbohydrates and lipids [31]. Of all the proteins, salivary proteins like histatins, statherin and acidic proline-rich ones are of interest as they exhibit antifungal properties [32]. Vukosavljevic *et al.* have suggested that a newer approach to control *C. albicans* can be done at the pellicle level by increasing the amount of these proteins [32]. One family of proteins, the histatins, are of particular interest.

The histatin family differs from other proteins due to three factors; their unusually high histidine content, lack of disulfide bonds, and their weak amphipathic nature [33]. They are cationic peptides ranging in length from 7-38 amino acids [34]. These peptides are naturally found within the human body, being secreted from only the partoid and submandibular glands [35].

Histatin peptides come in three forms; Histatin 1 (Hst 1), Histatin 3 (Hst 3) and Histatin 5 (Hst 5), all containing 7 histidine residues, as shown above [35]. Hst 1 is composed of 50 amino acids with a molecular weight of 4929 Da. Hst 3 contains 51 amino acids with a mass of 4063 Da. And Hst 5, has 25 amino acids with the smallest mass at 3037 Da. Hst 1 and 3 are derived from different structural genes, and Hst 5 is the proteolytic product of Hst 3. The amino acid sequences of all three histatins are shown in Figure 1.1. The effects of histatin peptides have been explored by Oppenheim *et al.*, where they were shown to inhibit the conversion of *C. albicans* to its pathogenic form. [36]. As a consequence of this, Vukosavljevic *et al.* noticed the inability of cells to colonize denture materials [30].

Hst 1 MKFFVFALVL ALMISMISAD SHEKRHHGYR RKFHEKHHSH REFPFYGDYG

Hst 3 MKFFVFALIL ALMLSMTGAD SHAKRHHGYK RKFHEKHHSH RGYRSNYLYD N

Hst 5 DSHAKRHHGYKRKFHEKHHSHRGY

Figure 1.1. The histatin family is comprised of three histidine-rich peptides; Hst 1, Hst 3 and Hst 5. Hst 1 and Hst4 are derived from different structural genes, and Hst 5 is a proteolytic product of Hst 3. Histatin peptides are naturally-occurring, with synthesis and secretion from the parotid and submandibular glands.

From this family, Hst 5 has gained attention due to its higher potency. Oppenheim discovered that using Hst 5, in comparison to the others, showed a 90-100% killing effect at physiological conditions [37]. Two general theories have been proposed; the first affects the cell membrane and the second, affects cellular respiration at the mitochondria. The first theory stems from the general mechanism of how antibiotics work. In 1996, Raj *et al.* discovered that antimicrobial agents work by adopting an amphipathic structure to allow their insertion into the cell membrane [38] However, structural analysis of Hst 5 indicated it being weakly amphipathic [33]. Therefore, it cannot insert itself into the cell membrane. Others have suggested that if Hst 5 is not being inserted, then perhaps the cell membrane is being lysed instead [39]. Unfortunately, this concept was proven incorrect by Baer *et al*. [40].

The theory regarding Hst 5 and its effects on the mitochondria was first defined by Helmerhorst *et al.* [41]. They discovered that when Hst 5 was applied towards respiring and non-respiring cells, the non-respiring ones were unaffected. In addition, they showed that when Hst 5 is taken up by *C. albicans*, the peptides are localized towards the mitochondria. This may be due to the similarities between mitochondria pre-sequences and Hst 5. Recent work by Komatsu *et al.* further supplemented the effects of Hst 5 on the mitochondria [42]. By investigating the expression before and after Hst 5 treatments, changes in proteins involved in cellular respiration were observed. The inhibition of the

Krebs cycle and loss of adenosine triphosphate (ATP) from cells lead to cell death. Overall, this does provide further insight towards how Hst 5 works. However, the exact mechanistic nature of Hst 5 on *C. albicans* remains unclear. The whole process is thought to possess multiple steps with many missing links and uncertainties.

1.4 Thesis Overview

MS for studying microorganisms has been gaining attention. The identification of proteins expressed by these microbes is a newer approach into developing new therapeutics against them. The fungal species, *C. albicans*, has become increasingly effective in combating current oral antifungal agents. As a result, infection rates have reached high numbers. One factor potentially contributing to its virulence is in its ability to adhere to host surfaces. Once adhered, cells can easily colonize and penetrate tissues for pathogenesis. With adhesion being the initial encounter between host and fungus, one theory of how Hst 5 works can be hypothesized. Hst 5 may be altering the expression of surface proteins, causing cells to no longer have the ability to adhere. Previous proteomic work has been done by the Siqueira Lab. They have grown cells of *C. albicans* with Hst 5, and performed in-solution tryptic digestions resulting in the lysis of cells. Analyses of the lysates were performed using HPLC-ESI-MS and database searches; however, the quantity of resulting peptides was extensive. This made it challenging to identify key proteins that may have any relevance.

Recent work accomplished by Moffa *et al.* in the Siqueira Lab has shown that upon introducing Hst 5 within ninety minutes of cell adhesion, the number of cells remaining has decreased [43]. Concurrently, they observed the inhibition of *C. albicans* into the hyphal form in the presence of Hst 5. The precise cause of the decrease in adhesion has yet to be explained; however, here we hypothesize the influence of Hst 5 on surface proteins. By working concurrently with the Siqueira Lab, we can develop sample preparations to mimic the adhesion process in the oral cavity. Then by performing digestions on whole cells, we can reduce the sample pool and specifically isolate surface proteins. Overall, this research will specifically address the following:

a. The development of on-cell digestions for direct performance on *C. albicans*.
b. The adhesion of *C. albicans* to a solid support followed by treatment with the Hst 5 peptide
c. Analyses by MS to detect changes in the expression of the identified proteins following treatment.

Our results may help to identify key surface proteins and their roles in particular biological pathways. Our combined results may supplement the understanding of Hst 5 and its influence on *C. albicans*. Furthermore, our developed surface digestion approach may have potential applications towards understanding the pathogeneses of other microorganisms.

Section 2 Experimental

2.1 Reagents

Deionized water (18.2 Ω grade) was used for all experiments. Purification was done by the Milli-Q Gradient A10 System (Millipore, USA).

A 0.1 mg/mL solution of human serum albumin (HSA) was used for cell adhesion (Sigma Aldrich, USA). Frosted 76 x 26 mm glass microscope slides (VWR, Canada) were used as the solid support for adhesion. Stock cultures of *C. albicans* in glycerol were provided by the Siquiera Lab from Dentistry at University of Western Ontario. The strain provided was ATCC90028. BD Difco Sabouraud dextrose agar (SDA) medium (Becton Dickson and Company, USA) was used as the agar base for cultivating fungal microorganisms. The growth of cells in solution was done using the media called yeast nitrogen base (YNB) broth (Sigma Aldrich, USA) supplemented with sucrose (BioShop Canada Inc, Canada). The media was sterilized and filtered using a BD 10 mL syringe (Becton Dickson and Company, USA) through 25 mm IC Acrodisc syringe filters with a 0.45 µm Supor (PES) membrane (PALL Corporation, USA). 1X phosphate buffer solution (PBS) was used in between for washings (Sigma Aldrich, USA). Histatin 5 (China Peptides Co., China) peptides were also provided by the Siquiera Lab.

Tosyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin from bovine pancreas (Sigma Aldrich, USA) was used for all digestions. To activate digestions, trypsin was resuspended in 50 mM ammonium bicarbonate at pH 8 (Sigma Aldrich, USA). Two standard proteins, horse heart cytochrome c (Sigma Aldrich, USA) and horse heart myoglobin (Sigma Aldrich, USA), were used for the development of on-target digestions. During incubations and digestions, the Dako delimiting pen composed of 60-100% 1-bromopropane and 5-10% dipentene (Cedarlane, Canada) was used to draw hydrophobic circles.

For MALDI MS analyses, peptides were mixed in a 1:1 (v/v) ratio with 5.5 mg/mL α-cyano-4-hydroxycinnamic acid (CHCA) matrix solution containing 6 mM ammonium phosphate monobasic, 50% acetonitrile and 0.1% trifluoroacetic acid (Sigma Aldrich, USA).

Bovine serum albumin (BSA) was used as the standard for the bicinchoninic acid (BCA) assays (Sigma Aldrich, USA). The Pierce BCA protein assay kit (Life Technologies, USA) was used for peptide quantification. Both standards and the peptides were mixed with the BCA reagent in a 96-well polystyrene microplate (Life Technologies, USA).

Before injection into the HPLC-ESI mass spectrometer, peptides underwent sample cleanup via ZipTip (Millipore, USA). There were four solutions used during this procedure. 50% acetonitrile (Sigma Aldrich, USA) was used to decontaminate the C_{18} column. 0.1% trifluoroacetic acid (Sigma Aldrich, USA) was then used to remove excess acetonitrile. 0.5% trifluoroacetic acid was added to acidify peptides to allow binding to the negatively-charged column. And lastly, a solution of 80% acetonitrile (Sigma Aldrich, USA) was used to elude peptides off the column. After drying, peptides were resuspended in 0.1% formic acid (Sigma Aldrich, USA). Solvent B (Sigma Aldrich, USA), composed of 97.5% acetonitrile and 0.1% formic acid, was used in HPLC.

2.2 Instrumentation

2.2.1 MALDI MS

Samples were mixed in a 1:1 (v/v) ratio with 5.5 mg/mL CHCA matrix solutions prior to analyses. All sample-matrix mixtures were spotted on a 384 well Opti-Tof 123x83 mm SS MALDI plate (AB Sciex, MA, USA). The instrument is equipped with a 349 nm OptiBeam On-Axis laser with a pulse rate at 400 Hz. Data acquisitions and processings were done using TOF TOF Series Explorer and Data Explorer. The spectra were acquired

in Reflectron positive mode, in the *m/z* range of 500-3500. Peak lists were created using the following parameters; a peak density of 10 per 25 Da, minimal signal-to-noise (S/N) of 10, minimum area of 50 and a maximum peak per spot of 200. Second fragmentation, MS/MS, was also performed via post-source decay (PSD) using the 1 kV Positive acquisition mode.

2.2.2 HPLC-ESI-MS/MS

The drying of peptides and analysis by HPLC-ESI-MS/MS were completed by the Siqueira Lab. This setup allows for online liquid chromatography to be coupled with direct introduction to the mass spectrometer for analyses.

Prior to mass spectral analyses, peptides underwent sample cleanup using C_{18} ZipTip pipette tip columns. For the detailed protocol, please refer to Appendix 4. The eluted peptides then drying to remove solvents and concentrate the peptides. This was accomplished with the Vacufuge vacuum concentrator (Eppendorf, Germany). The drying of peptides was done at ambient room temperature for 10 minutes at 14000 rpm. Once dried, the peptides were resuspended in 0.1% formic acid.

HPLC was carried out on the Thermo Scientific Easy nLC II instrument (Thermo Scientific, CA, USA). The 85 minute gradient composition ranged from 5 to 55% of solvent B. The flow rate used was 200 nL/min at a pressure of 280 bar. The volume of sample injected was 8 µL. Electrospray ionization was completed using a voltage of 2.0 kV with an ion transfer capillary temperature of 250.00°C.

Mass spectral analyses were performed on the Thermo Scientific linear trap quadrupole (LTQ) Velos ion mass spectrometer (Thermo Scientific, USA). Positive-mode data acquisitions and processing were done using Thermo Xcalibur 2.1.0 SP1.1162 software (Thermo Scientific, USA). The MS scan range used was 0-2000 m/z. MS/MS was done via collision induced dissociation (CID) using helium (He) as the inert gas.

2.3 *C. albicans* Growth and Cell Culturing

Stock cultures of *Candida albicans* were provided by the Siqueira Lab from Dentistry at The University of Western Ontario. The strain of cells used throughout all experiments was ATCC90028. Prior to cell growth, SDA prepared plates were placed into a 37°C oven overnight to ensure the absence of other microorganisms. The exact protocol for preparing SDA plates is described in Appendix 2.

To start growth, cell colonies were grown on the plate containing the SDA medium. Without thawing, a pipette tip of stock *C. albicans* was inoculated on the SDA plate. The inoculated plate was incubated in a 37°C oven for 48 hours. Figure 2.1 is the resulting colonies grown on the SDA plate.

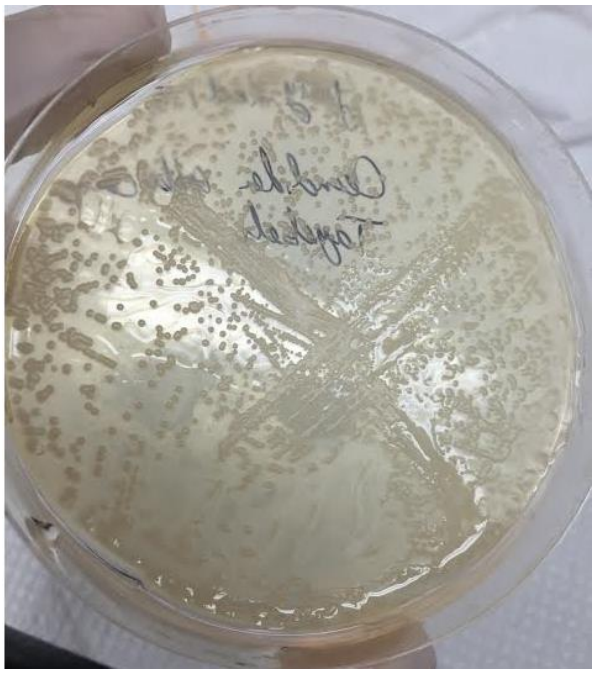

Figure 2.1 *C. albicans* budding yeast colonies grown on SDA plates. Each dot corresponds to a single colony.

One-third of a loop of the colonies on the SDA plate was transferred into about 45 mL of YNB100. The protocol for preparing YNB100 is described in Appendix 2. The cells

were then incubated at 37°C for 21 hours with shaking at 200 rpm. After this incubation, optical density (OD) measurements were taken at 600 nm, giving what is known as OD_{600}. OD_{600} correlates the amount of cells present in the solution to the level of light absorption. For *C. albicans*, the OD reading should be around 0.300. The measurements were performed on the NanoDrop 2000C spectrophotometer (Thermo Fisher Scientific Inc, USA). Following 21 hours, the solution was centrifuged (Avanti J-251 Centrifuge, Beckman Coutler, USA) for 5 minutes at 6000 rpm. The pellet was then resuspended in 1X PBS, and the final *C. albicans* solution was stored at 4°C for no longer than one week. For the cell lysate, the solution was centrifuged at 13 000 rpm for 5 minutes instead. The supernatant was collected and analyzed by MALDI MS.

2.4 Adhesion of *C. albicans* to Glass Slides

The protocol for cell adhesion onto a solid support was adapted from Vukosavljevic *et al.*[30]. In all adhesion experiments, glass slides were used as the platform in which cells were adhered. The setup for the incubations is shown in Figure 2.2. Here inverted glass vials containing wet cotton was used to cover the spots and retain humidity. vials containing wet cotton was used to cover the spots and retain humidity. A maximum of three spots per slide was used to prevent overcrowding of the vials. The Dako delimiting pen was used to draw hydrophobic circles (shown in blue), therefore minimizing spreading. The digestions were contained within hydrophobic circles of an inner diameter of 0.60 mm. The volume of HSA, *C. albicans* and Hst 5 used was 25 µL. All incubations were carried out on the bench top at ambient room temperature (RT).

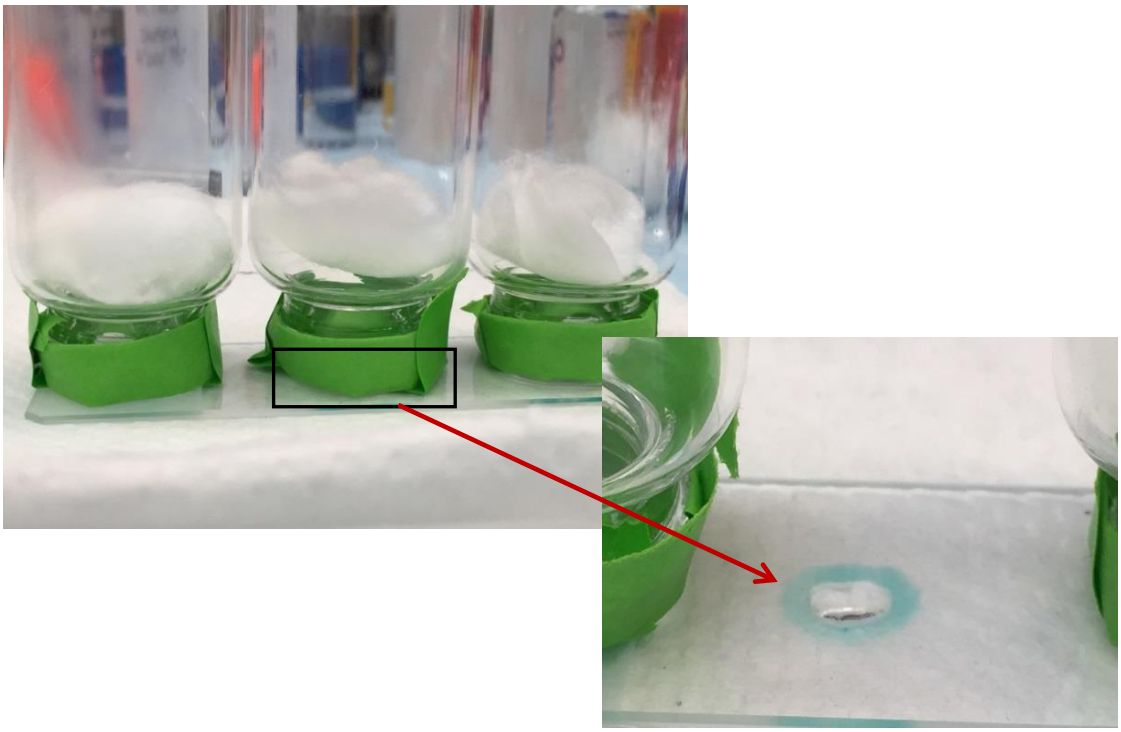

Figure 2.2. The setup for the preparation of slides. The attachment of cells, treatment with Hst 5 and on-target digestions were carried out within the hydrophobic circle. The spots were covered with an inverted vial, where soaked cotton was used to retain moisture. A maximum of three spots per slide was used.

In order for the cells of *C. albicans* to adhere, a pre-existing layer of HSA was needed. Before introducing solutions, the hydrophobic circles were drawn on the bare glass slide first. To each circle, 25 µL of 0.1 mg/mL HSA was added and the spots were covered with the vials. HSA incubation was done for 2 hours at RT. Once done, the spots were washed three times with water. To verify that cells of *C. albicans* did attach, microscope images of the slides were taken. Images were taken using the Leica DME basic microscope with the imaging component DFC295 (Leica Microsystems, Canada).Upon confirmation, Hst 5 was introduced next for the treatment.

2.5 Treatment of Hst 5 on Adhered Cells

Two application methods of Hst 5 were investigated, pre-application and post-application. The concentration of Hst 5 was 0.030 mg/mL, as suggested by the Siqueira Lab. In the pre-application method, as shown in Figure 2.3C, Hst 5 was introduced prior to adhesion of *C. albicans.* The HSA-coated slide was prepared as discussed above. Hst 5 was added for incubation next. This was carried out at RT for 2 hours. Afterwards, the spots were washed thrice with 1X PBS. Last, 25 µL of the prepared *C. albicans* solution

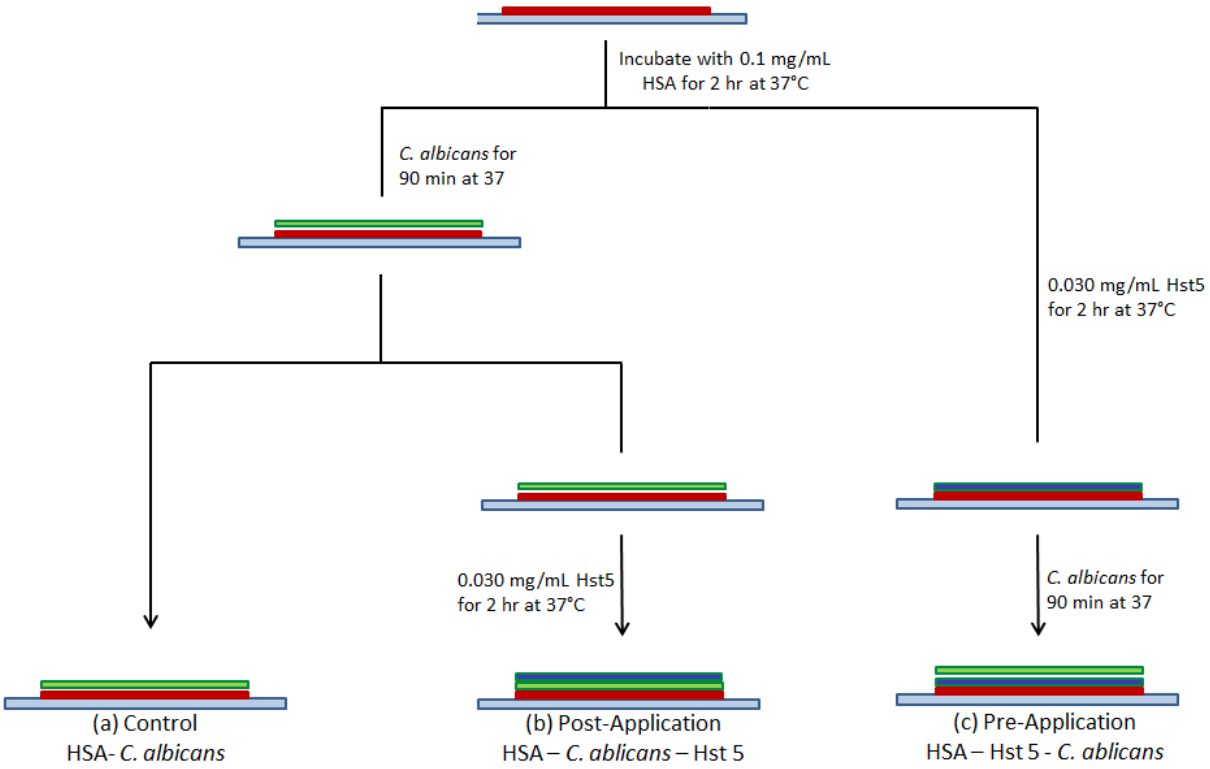

Figure 2.3. Schematic for the preparation of *C. albicans* adhered slides. Red represents HSA, green for *C. albicans*, and blue for Hst 5. (A), the control used was a HSA-*C. albicans* slide. In (B), the post-application slide of HSA-*C. albicans*-Hst 5 is prepared. And lastly in (C), is the pre-application slide of HSA-Hst 5-*C. albicans.*

was introduced. The incubation was performed for 90 minutes at RT. Again, once completed the spots were washed thrice with 1X PBS. This completed the attachment and treatment, where on-target digestions were performed afterwards. This will be discussed later.

For the post-application method, HSA was first added as already discussed. Following this, 25 µL of the *C. albicans* solution was introduced next. The incubation was done for 90 minutes at RT. Washing of the spots were done three times with 1X PBS. And lastly, 25 µL of 0.030 mg/mL Hst 5 was applied for a 2 hour incubation at RT. The spots were washed thrice before on-target digestions were performed. The preparation of the spots in post-treatments are shown in Figure 2.3B.

Lower concentrations of Hst 5 used were also tested to determine the lowest threshold that could be used. Two lower concentrations, 0.025 and 0.020 mg/mL, were considered using both application methods. Glass slides were prepared according to Figure 2.3, and the varying concentrations of Hst 5 were introduced in place of the 0.030 mg/mL dose. Once prepared, the slides underwent on-target tryptic digests and peptides were collected for MALDI MS analyses.

2.6 Tryptic Digestions

2.6.1 Standard Overnight Digestions for Test Proteins

Standard in-solution tryptic digestions were carried out prior to on-target digests. These served as the basis for comparison of the developed on-target digestions. Two proteins, cytochrome c and myoglobin, were chosen as the standard proteins. A ratio of 1:50 enzyme-to-protein (v/v) was used. The concentration of trypsin was 1.0 mg/mL in 50 mM ammonium bicarbonate with a pH of 8. Ammonium bicarbonate was incorporated for the activation of trypsin. The concentration of proteins used for digestions was 1.0

mg/mL in water. Once the standard proteins and trypsin were mixed together, the resulting solution was placed in a 37°C oven for an overnight digestion. The peptides were then lyophilized overnight and resuspended in water to a final concentration of 0.1 mg/mL. This peptide solution was mixed with CHCA for MALDI MS analyses.

2.6.2 Development of On-Target Tryptic Digestions

It should be noted that when the term "on-target" was used, it referred to performing the digestions directly on the analyte. On-target digestions were developed as a substitution to the standard overnight protocols. To start, they were first performed using the standard proteins. These digestions were done on the MALDI plate on the bench top at room temperature. They were initially designed using the standard proteins, cytochrome c and myoglobin. 0.36 µL of 0.1 mg/mL standard protein was spotted onto the MALDI plate, whereby, 0.36 µL of 1.0 mg/mL trypsin was layered on top for digestions. The digestions occurred at ambient room temperature for ten minutes. Once completed, CHCA matrix was laid over top and mass spectral analyses were performed. The digestion time was also investigated to determine the optimal duration. To start, digestion times ranging from; 15, 30 and 45 seconds; and 1, 2, 3, 4, 5, 10, 15, 20, 25, 30 and 60 minutes, were explored on three replicates.

With success from the standard proteins, the on-target surface digestions were applied towards the prepared *C. albicans* slides from Figure 2.3. Tryptic digestions with the following conditions were used; a concentration for trypsin of 0.0015 mg/mL and a digestion time of 10 minutes at RT. Again, during the digestions the spots were covered with the glass vials. Once digestion was completed, the peptide-containing buffer was recollected for mass spectral analyses.

The conditions described above represented the optimal experimental factors. Prior to this, four experimental conditions were optimized. The first was to ensure the on-target digest was not lysing the cell to produce unwanted background noise. To do so, a cell lysate was prepared for comparison. Here, instead of centrifuging cells at a speed of 6000

rpm, they were spun at 13000 rpm. This faster speed would cause cell lysis. The supernatant was then collected for analyses. Next, five replicates of four final concentrations of trypsin were tested; 0.0005, 0.0075, 0.001, 0.0015 and 0.002 mg/mL. Lastly, the digestion time was re-evaluated for *C. albicans*. Here, digestion times of 5, 10, 15 and 20 minutes were investigated. Three replicates were performed for each.

In the case of MALDI MS, Fenselau reported that organic solvents can cause lysis which would incorporate signals from internal cellular components [44]. Here, the cells were in contact with three organic solvents from CHCA matrix. They were ammonium phosphate monobasic, trifluoroacetic acid and acetonitrile. Figure 2.4 is a schematic of how the digestions were carried out. The digested peptides as shown (step B) were separated from the remaining cells. CHCA matrix was only incorporated afterwards (step C) As a result, the influence of organic solvents on the cells was not an issue.

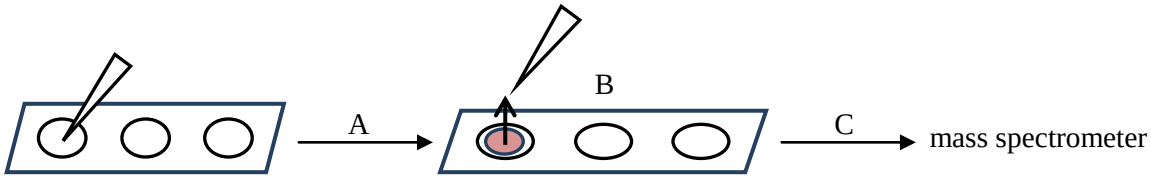

Figure 2.4 Schematic for on-target digestion followed by the introduction of CHCA matrix. (A) trypsin was added to spots for digestion (B) Digested peptides were removed from remaining cells (C) mixing of peptides with CHCA matrix for analyses

Depending on the mass spectral analyses, the next sample handlings varied. In the case of MALDI MS, each spot was mixed in a 1:1 ratio with CHCA before spotting on the plate for analyses. As for ESI MS/MS, it will be discussed in the following section.

2.7 HPLC-ESI-MS/MS

Before analyses with HPLC-ESI-MS/MS, quantification for the cleaved peptides was carried out. This was to ensure that the amount of our peptides were within the level of detection of the mass spectrometer. The minimal amount of peptides needed was 2 µg. For peptides, quantification can be accomplished using the BCA assay. The standard protein used was bovine serum albumin (BSA). The exact protocol for the BCA assay is presented in Appendix 3.

For the analyses with ESI MS/MS, the slides were prepared in the droplet method. On-target digestions using the optimal conditions of; 0.0015 mg/mL of trypsin and the duration of ten minutes at ambient room temperature. Of the two application methods, only slides prepared in the post-application were analyzed by MS.

2.8 Database Searches

All MALDI MS spectra were searched against the SwissProt protein database on MASCOT provided by Matrix Science (Peptide Mass Fingerprint, http://www.matrixscience.com). The search parameters used for *C. albicans* were: (1) trypsin with one missed cleavage, (2) fungi as the taxonomy, (3) a variable modification for the oxidation of methionine and (4) a mass tolerance of ± 70 ppm.

All ESI MS/MS spectra were run through the Thermo Proteome Discoverer version 1.3.0.339 software (Thermo Fisher Scientific Inc., USA), using the algorithm known as SEQUEST. The *C. albicans* database chosen to search results against was the UniProt Knowledgebase, more commonly known as UniProtKB. (UniProt Consortium, http://www.uniprot.org/, UK, Switzerland, USA). The data analysis program known as SEQUEST was used for protein identification. The search parameters were as follows:

(1) enzymatic cleavage by trypsin with 2 missed cleavages, (2) S/N of 1.5, (3) precursor mass tolerance of ± 2 Da, (4) fragment mass tolerance of ± 0.8 Da, (5) modifications for phosphorylation on serine and threonine, and oxidation on cytosine and methionine, and (6) a minimum of 2 peptides per protein with high peptide confidences.

Section 3 Results and Discussion

In Section 1, the use of MS in rapid whole cell analyses for microorganisms was discussed. Of all microorganisms, infections caused by *C. albicans* have rapidly progressed due to the development of resistant strains against current oral dugs. The peptide Hst 5 has shown to have antifungal properties in controlling *C. albicans in vivo*, but the precise mechanism of how Hst 5 is able to kill cells is not known. Current research has proposed that Hst 5 affects cellular respiration at the mitochondria. This may be so, but the influence of Hst 5 can be hypothesized to affect additional pathways. Here, we hypothesize that Hst 5 may exert influence on surface proteins as well. Surface proteins play crucial roles in allowing *C. albicans* to adhere to the oral cavity to initiate pathogenesis. The treatment of *C. albicans* with Hst 5 may alter the expression of these proteins. By performing surface digestions, we can quickly isolate surface exposed peptides and protein identification via MS can allow further insight towards their role in Hst 5.

3.1 Development of On-Target Digestions of Test Protein Standards

In order to analyze surface proteins, a common approach is to study the peptides removed from the surfaces of the intact cell. To accomplish this, site-specific enzymes are used to cleave them off. Trypsin is commonly used for microorganism digestion, where it cleaves on the C-terminal end of lysine and arginine residues. A typical workflow is to subject cells to overnight proteolysis under conditions where cell integrity remains intact [45]. This allows for peptides to be released, which can be collected for mass spectral analyses. Although this protocol was successful, it does have the drawback of being tedious and time-consuming. Additionally, the longer incubation of cells with the enzyme may potentially cause cell lysis. Because of these reasons, the use of on-target surface digestions may be an improved substitution.

With no established protocol for an on-target approach, digestions were initially developed using standard proteins. First, myoglobin and then cytochrome c were tryptically digested overnight at 37°C, serving as a standard to which the completeness of the on-target digestions was compared. For the on-target digestions, the standards were digested for ten minutes at ambient room temperature. A total of three replicates were prepared for both proteins and the two digestion approaches. To begin, reference to myoglobin will be discussed. The comparison of digested myoglobin from both digestion methods are indicated in Figure 3.1. The asterisks indicated peptides belonging to myoglobin.

Based solely on the number of asterisks, one can see that indeed the in-solution digestions yielded a few more peptides than on-target. In addition, the relative peak intensities were lower from on-target digestions. Although the appearances of the spectra seemed identical, this was not the only indication of whether they were equivalent. To supplement the spectra, MASCOT database searches were also completed. The results were able to identify the protein, as well as determine the amount of sequence coverage. The sequence coverage refers to the number of observed amino acid residues relative to the total number in the protein. The database results for both digestions were successful in identifying myoglobin as the protein. The average sequence coverage with standard deviation (s.d.) of myoglobin was determined to be 77 s.d. 3% for in-solution, and 62 s.d. 5% for on-target. Likewise, the average coverage for cytochrome c was 83 s.d. 8% and 67 s.d. 3%, respectively. Despite the loss in the number of peptides and sequence coverages, it can be concluded that the on-target method developed was sufficient. Lastly, the on-target approach offers advantages such as; greatly reducing digestion times from overnight to ten minutes, and minimizing the volumes of materials.

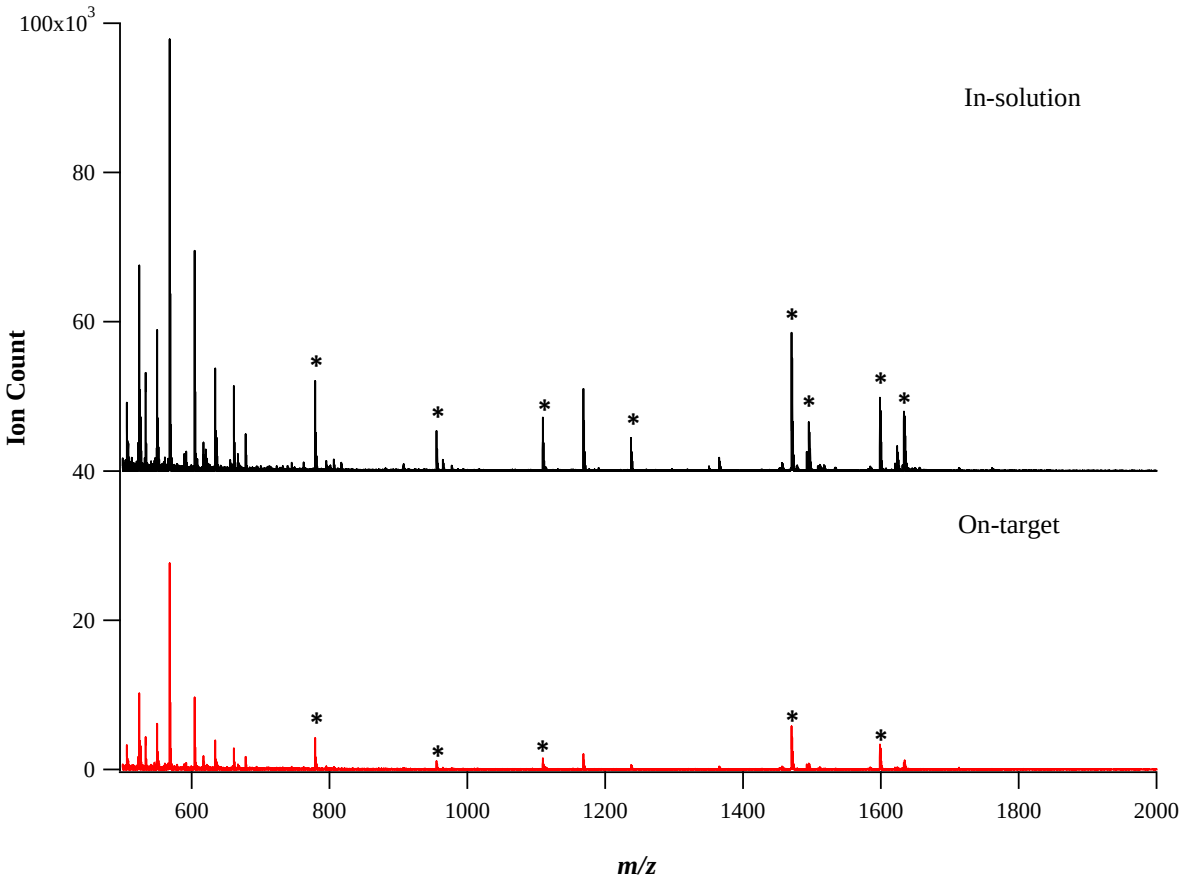

Figure 3.1 MALDI MS spectra of the in-solution and on-target tryptic digests of myoglobin. Three replicates were performed in total. The in-solution digestion was the standard protocol used for digesting proteins. In the on-target approach, the digestion was carried out for 10 minutes at room temperature. Asterisks (*) represent peptides of myoglobin. The average sequence coverage with standard deviation for in-solution was 77 s.d. 3% and on-target was 62 s.d. 5%.

To help improve the completeness of the digestions, the investigation of digestion times were explored. Both standard proteins were analyzed; however, here we will discuss cytochrome c. The times ranging from seconds to one hour were tested. These included times at 15, 30 and 45 seconds; and 1, 2, 3, 4, 5, 10, 15, 20, 25, 30 and 60 minutes. Three replicates of cytochrome c digestions were prepared for each time mentioned. It was determined that the shorter digestion times, from 15 seconds to 4 minutes, were not long enough to allow trypsin to digest the standards. Likewise, increasing the digestions from 20 minutes until 1 hour provided no additional peaks relevant to cytochrome c. As such,

it was determined that the critical times ranged from 5 to 15 minutes. To start the comparisons, focus will be placed on three of the easily observed peptides with no missed cleavages, *m/z* of 1168.62, 1598.56 and 1633.41, from cytochrome c. At five minutes, the ion counts with standard deviations (s.d.) were; 30000 s.d. 75000, 2600 s.d. 150 and 17000 s.d. 2900 respectively. Between five and ten minutes, it was determined that at ten minutes, the presence and intensity of peaks had increased. At ten minutes, the counts were; 440000 s.d. 25000, 4800 s.d. 580, and 6500 s.d. 4700 respectively. This large of an increase meant that trypsin was given enough time to effectively cleave off peptide molecules. Therefore, this indicated that ten minutes allowed for a more completed digestion. Five minutes was still not long enough for trypsin to reach and cleave the peptides. At fifteen, a large drop in the ion counts was observed. The counts were; 380000 s.d. 26000, 430 s.d. 120, and 3800 s.d. 770 respectively. A possible explanation for this would be the adsorption of peptides to the solid surface. The longer time would allow for more adsorption, and consequently, less would be recovered for MS detection. As a result, a loss in the ion counts would be expected. Due to this reason, it was concluded that ten minutes was optimal for the digestion of cytochrome c. However, the optimal digestion time is specific to each substrate, and thus it needs to be re-examined as will be the case for proteins of *C. albicans*.

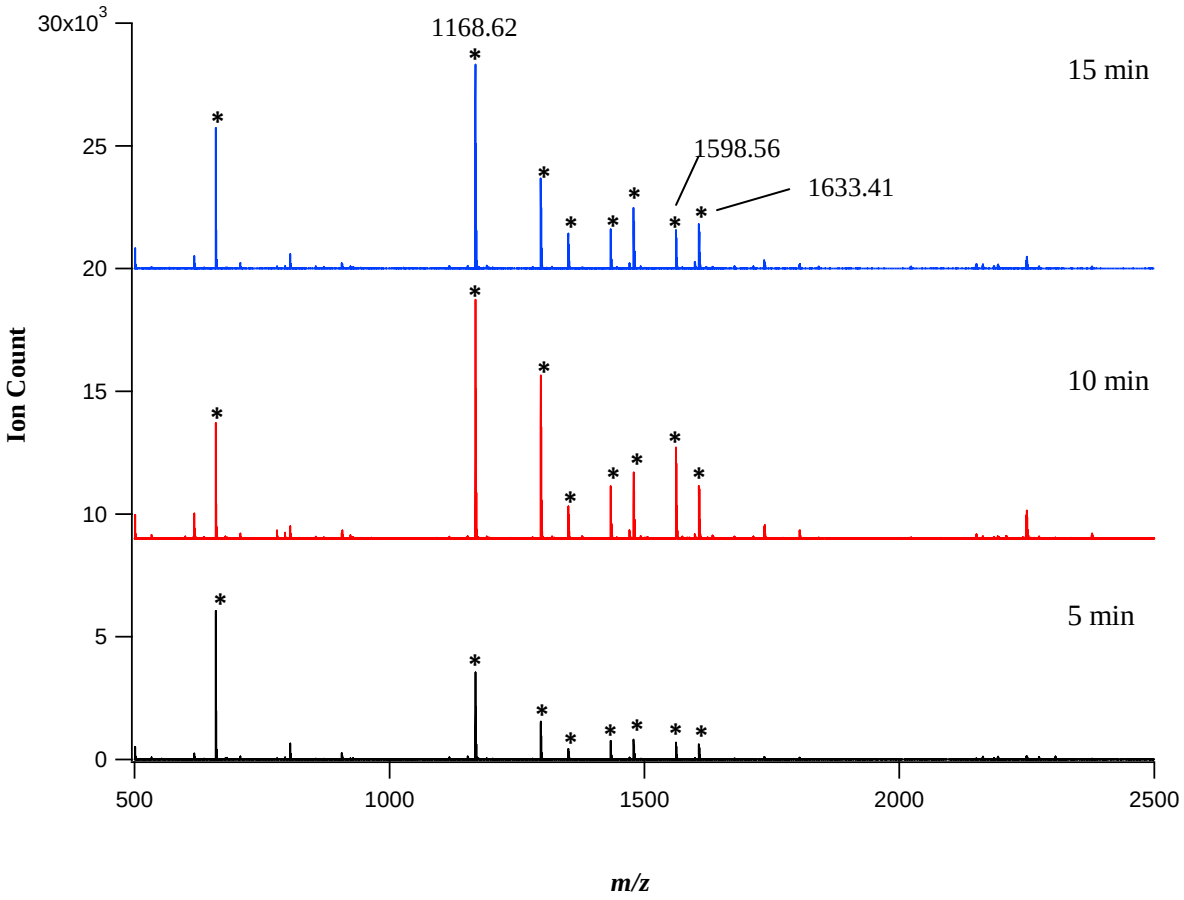

Figure 3.2 MALDI MS spectra for the comparison of digestion time for the on-target digestions of cytochrome c. Three replications of each time were analyzed. The digestion times were varied from 5-15 minutes. Peaks belong to cytochrome c are marked with asterisks. The three *m/z* peaks belong to peptides of cytochrome c discussed are labelled with; 1168.62, 1598.56 and 1633.41.

3.2 *C. albicans* Adhesion on Glass Slides

The analyses of surface proteins for *C. albicans* were commonly done while in-solution. In this workflow, cells were grown on SDA media, resuspended in a buffer and enzymatically digested overnight. This may work as it will allow for the identification of surface proteins; however, it does not truly represent the adhesion process. The adhesion process becomes crucial as a requirement for *C. albicans* to penetrate the oral cavity. In order for the expression of these specific proteins, the process of adhesion must have

occurred or is occurring. Unfortunately, the factors driving the adhesion process remains unknown. Factors such as the type of microorganism, material surface characteristics and the presence of other proteins, can influence attachments.

To start, the ability of *C. albicans* to attach to a solid support was tested. For this, a bare glass slide was immersed into a solution of cells, with the resulting image shown in Figure 3.3A. As can be seen, no cells have attached to the slide, which indicated another component was needed for adhesion. Chaffin demonstrated when beads were coated with any of the extracellular matrix (ECM) proteins, the yeast and pseudohyphae forms bound [46]. ECM proteins include albumin, fibronectin, fibrinogen, laminin and denatured collagen. The way in which these proteins aid in adhesion remained unclear. They have been hypothesized to help in three ways; first, to bind the substrata surfaces to allow for colonization, second, to bind the surface of the microorganism and then colonize, and lastly, to be present in the solution during the adhesion process [47].

Previous work completed by the Siqueira Lab has shown that *C. albicans* can bind to one of the ECM proteins known as human serum albumin (HSA) [30]. HSA has been identified as a relevant component in the oral cavity. It was reported to serve as a receptor for initial adhesions [46]. By applying HSA to the glass slide first, thereby creating a pre-coated surface, *C. albicans* has the potential to attach. An image of a slide containing the pre-existing layer of HSA was shown in Figure 3. It can be clearly seen that HSA was necessary for cells to bind. The larger spots observed were cells that have begun to adhere to one another, forming groups called colloids by a process known as flocculation [2]. Despite washings of the slides, colloids remained, as highlighted by the red circle. This was negligible, as the amount of cells was minimal.

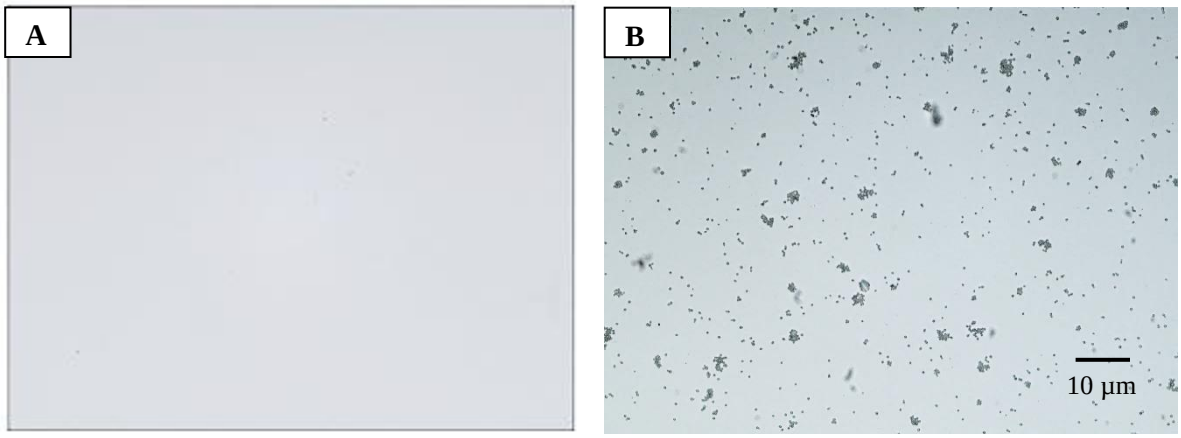

Figure 3.3 Microscope images of *C. albicans* adhered glass slides. (A) No *C. albicans* adhesion on glass without HSA coating. (B) *C. albicans* adhered to HSA.

3.3. Influence of Hst 5 on Adhered Cells

With successful adhesion of *C. albicans*, Hst 5 was introduced into the system next. As discussed in Section 2, Hst 5 was applied in two different methods; pre-application which meant Hst 5 was applied before cell introduction and, post-application where Hst 5 was introduced after cells. The 0.030 mg/mL concentration of Hst 5 will be used to discuss the differences in both methods. By applying Hst 5 prior to *C. albicans* adhesion, cell removal was observed. An image from the pre-application method is shown in Figure 3.4B. A control, where no Hst 5 was used, served as the basis to which the concentrations were compared. The average values with standard deviations (s.d.) were listed in Table 3.1. The control had an average cell count of 110 s.d. 41, whereas pre-application was 13 s.d. 2. The reduction in cells may be attributed to the pre-existing layer of Hst 5. This layer of Hst 5 seemed to act as a preventive layer, allowing fewer cells to attach. Equally in its ability to cause cell removal was the post-application approach. Figure 3.4E was an image of the resulting slide. The average cell counts for post-application was 16 s.d. 8, which was roughly the same as pre-application. These results indicated that the influence of Hst 5 on *C. albicans* was independent on when Hst 5 was introduced to the cells.

Next, the effects of varying concentrations Hst 5 were also investigated. The physiological concentration of Hst 5 found in the human body was reported to be 10-30 µM [48]. Thus far, only a concentration of 10 µM (0.030 mg/mL) has been applied towards treating *C. albicans.* Lower Hst 5 concentrations became of interest in hopes of

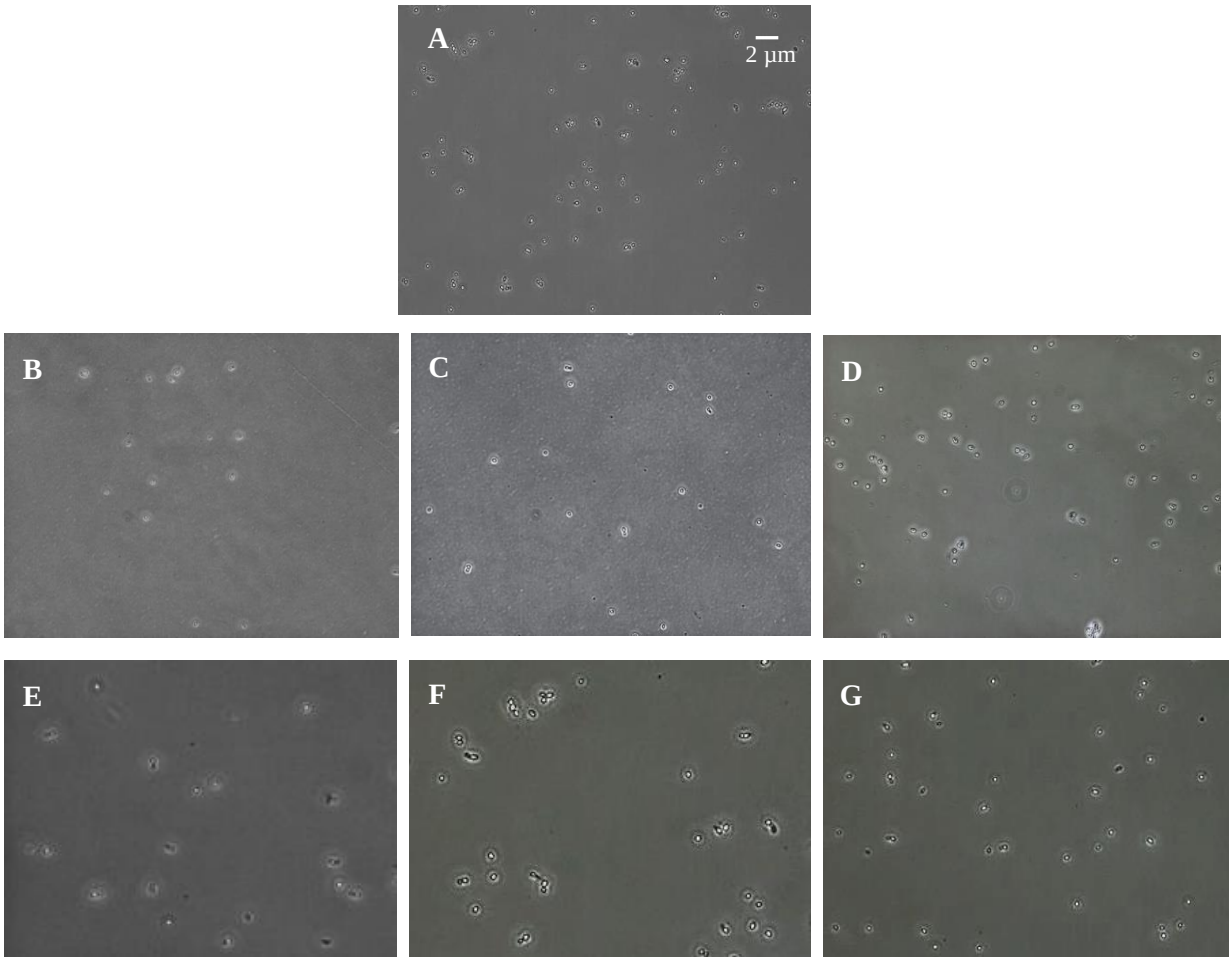

Figure 3.4 Microscope images of *C. albicans* adhered to slides. Four replicates were imaged and cells were counted. Both pre-application and post-application methods were investigated (A) Control, (B-D) Pre-application; 0.030, 0.025 and 0.020 mg/mL respectively (E-G) Post-application; 0.030, 0.025 and 0.020 mg/mL respectively. The corresponding average counts for each condition is shown in Table 3.1

Table 3.1 The average number with standard deviations (s.d.) of cells remaining when exposed to Hst 5 from four replicates. Pre-application, Hst 5 before cells attachments, and post-application, Hst 5 after cell attachments, were analyzed. The control had an average of 110 s.d. 41 cells.

	0.030 mg/mL Hst 5	0.025 mg/mL Hst 5	0.020 mg/mL Hst 5
Pre-Application	(A) 13 s.d. 2	(B) 18 s.d. 4	(C) 47 s.d. 7
Post-Application	(D) 16 s.d. 8	(E) 25 s.d. 5	(F) 42 s.d. 4

determining the lowest threshold that may be reached before no changes in adhesion were observed. Two lower concentrations, 6.6 µM (0.020 mg/mL) and 8.2 µM (0.025 mg/mL) were tested. Four repeats of each concentration was analyzed. Microscope images taken for both concentrations in both application methods are shown in Figure 3.4C-D and F-G.

Again, our results showed that no difference between pre- and post-application of Hst 5. For the determination of the lowest threshold of Hst 5, Table 3.1 indicated that 0.025 mg/mL was the lowest we could go. When compared to the control, it was determined that all three of the Hst 5 concentrations demonstrated the ability to reduce the number of adhered cells. Between 0.030 and 0.025 mg/mL, the two concentrations appeared to have the same cell removing ability, as the average number of cells was similar. However, upon using 0.020 mg/mL, the number of cells remaining increased greatly. The cell counts results revealed that 0.020 mg/mL was significantly weaker.

3.4 On-Target Digestions of *C. albicans*

Ideally, on-target digestion will allow for the removal and sampling of peptides on the surface of *C. albicans* without lysing the cells. The internal components from lysed cells complicate mass spectral data, essentially by acting as non-informative background ions. In the work done by Rodriguez-Ortega *et al.*, cells of *C. albicans* were simply incubated

with trypsin, thereby separating them from remaining cells [14]. Their work yielded only proteins with origins at the cell surface. Because our developed digestion protocol varied from theirs, the absence of cell lysis needed to be verified. By centrifuging cells at a speed of 13000 rpm, the cell lysate was prepared. As a preliminary experiment, the mass spectrum of the cell lysate was obtained. The results indicated that the spectrum for on-target digestions produced substantially fewer peaks (spectrum not shown). Therefore, it appeared that the on-target digestion was able to at least reduce the extent of cell lysis, if not entirely preventing it.

The next task was to optimize the trypsin quantity for the on-target digestion of proteins on adhered *C. albicans.* A total of five concentrations of trypsin were tested; 0.0005, 0.00075, 0.0010, 0.0015 and 0.0020 mg/mL. For each concentration explored, five replicates were done. Of the five concentrations, the optimal concentration of trypsin was determined to be 0.0015 mg/mL. Based on the intensity of a resulting peptide characteristic to *C. albicans*, at *m/z* of 1758, insignificant intensities were obtained at the two lowest concentrations studied. At 0.0010 mg/mL, the ion count and standard deviation for this peak was 4000 s.d. 300. Then at 0.0015 mg/mL, it was 7000 s.d. 2000. Finally, at 0.0020 mg/mL, the ion count decreased to 4000 s.d. 300. This indicated that 0.0015 mg/mL provided a more complete digestion than 0.0010 mg/mL.

3.4.1 Determining the Optimal On-Target Digestion Time for *C. albicans*

As already discussed in Section 3.1, the optimal digestion time determined from the test standard proteins was ten minutes. Because the sample used here was different, the time required for digestion was re-evaluated. Previous work done by Hernaez *et al.* performed the isolation of surface peptides from *C. albicans* with variations in digestion times [49]. They investigated different times ranging from five to twenty minutes in five minute intervals, whereby they concluded that five minutes was sufficient for the release of easily accessible surface peptides. They also determined that as the time was increased, the number of resulting peptides increased as well. Paralleling their work, times of five, ten, fifteen and twenty minutes were tested and the resulting spectra were shown in

Figure 3.5. Because the peaks pertaining to *C. albicans* were not known at that time, specific comparisons of five replicates were made based on the peak with a *m/z* ratio around 1500. This peak was neither an autolytic trypsin peak nor one from HSA; therefore it was hypothesized to be one from *C. albicans.*

At five minutes, few peaks were observed in the mid to upper mass range. This was likely due to the fact that trypsin was not given enough time to diffuse to the cells for the removal of surface proteins. At ten minutes, a more completed digestion was observed. This statement was made based on the appearance of the spectrum, and was supported by the ion counts of the *m/z* peak around 1500. At five minutes, this peak was not present. But at ten minutes, the count and standard deviation was 5100 s.d. 1300. Not only did the count increase, but the appearance of more peaks signified a more completed digestion; therefore, concluding five minutes was too short. Likewise, at fifteen minutes, the same peaks seen at ten minutes were observed, however the intensities have decreased. This was indicated by the reference peak which had an ion count of 3400 s.d. 1100. At twenty minutes, it was expected the intensities of the peaks should have increased. However, what happened was in fact the opposite. Here, not only did the peak at 1500 appear lower in Figure 3.5, but the ion counts decreased to 3100 s.d. 1100, which was similar to fifteen minutes. Together, the ion count and appearance of the spectra indicated that twenty minutes was not better than fifteen. Interestingly, this produced parallel results to the standard proteins in Section 3.1, where no additional information was gained as the digestion times increased. Again, the same reasoning from before can be applied here. The nonspecific adsorption of peptides to the slide decreases the number of ions available for MS analyses.

Hernaez *et al.* have also reported that the longer the digestion, the greater the number of peaks observed [49]. Our results here have shown otherwise. The numbers of peaks have not increased by a significant amount when comparing ten, fifteen and twenty minutes. Instead, only the ion counts have changed. It should be stressed that their digestions were performed in-solution with 37ºC incubations. Digestions carried out while in-solution offers a significant advantage as the number of cells is much larger. In contrast, our digestions here become strongly influenced by the total number of cells attached to each

slide, which easily differ between replicates. Based on these results, it was determined that ten minutes provided the better digestion time.

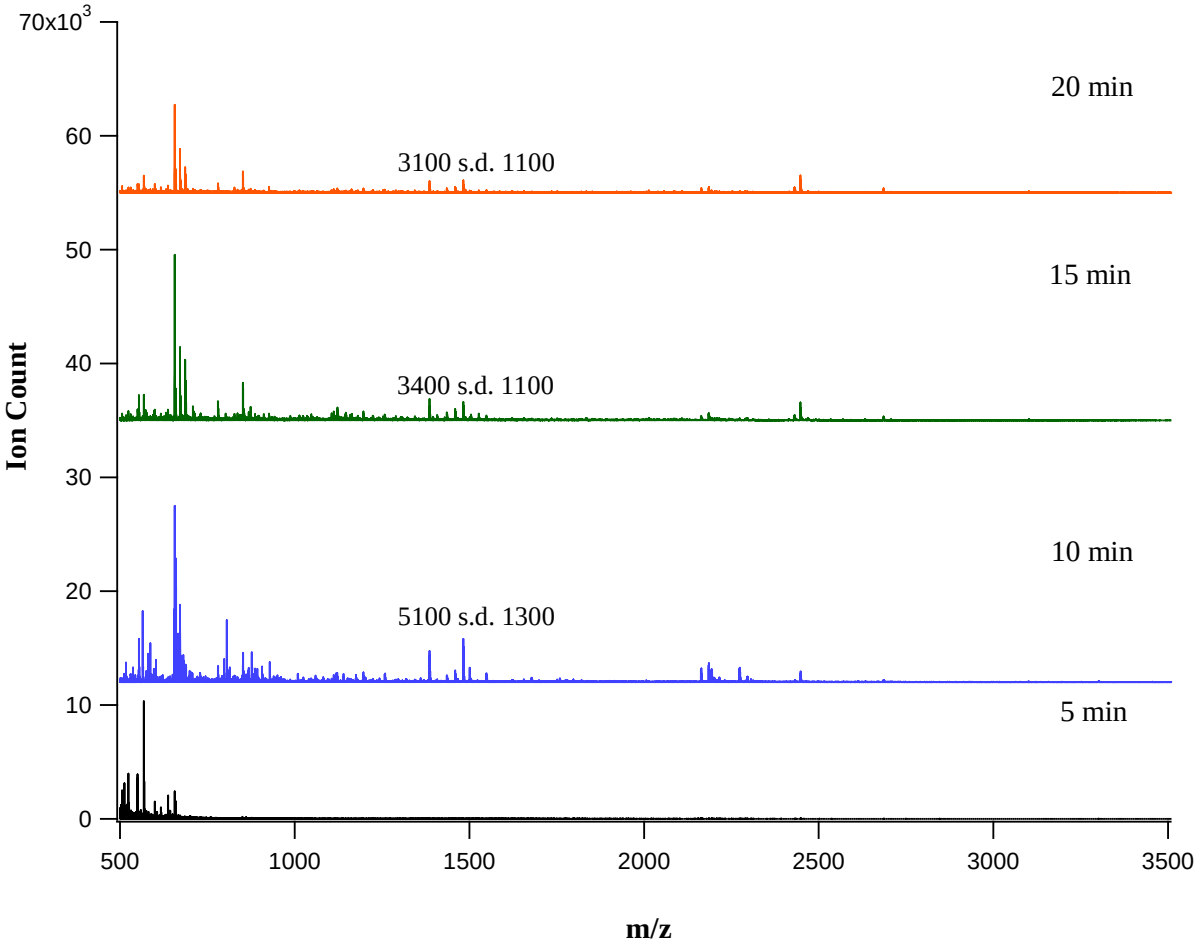

Figure 3.5 MALDI MS spectra for the comparison in surface digestion times of adhered *C. albicans* treated with Hst 5. Five replicates were analyzed. Average ion counts and standard deviations (s.d.) for the peak around 1500 were listed.

3.5 Analysis of Peptides from *C. albicans* by MALDI MS Following Treatments with Hst 5

With the capability of performing on-target surface digestions on *C. albicans*, we were able to determine changes in the resulting peptides due to treatments with Hst 5, and hopefully the results may provide insight towards which proteins and pathways are involved in the mechanism of Hst 5. Both application methods of Hst 5 were analyzed where four replicates were performed for each. From each replicate, the removal of background signals such as keratin, matrix peaks, trypsin autolytic peaks, and peptides of HSA was done. The data was searched against the *C. albicans* genome database, with tentative protein identification. This led to a list of twenty *m/z* peaks assumed to be characteristic only to *C. albicans.* All peaks have been assigned except for the *m/z* peak at 2294.92 in Table 3.2. This was because the *C. albicans* database remains incomplete.

The number of *m/z* peaks obtained was significantly lower than those when done in-solution. In 2010, Hernaez *et al.* carried out a similar workflow to identify surface proteins, and they were capable of identifying fifty proteins [49]. In their work, *C. albicans* was treated with Hst 5, digested by trypsin and separated by HPLC. Mass spectral analyses were performed using a MALDI TOF-TOF mass spectrometer. Because their digestions were performed while in-solution, they were not limited the number of cells. In our studies, the number of cells exposed to trypsin was limited. Only those that have successfully adhered to HSA would have undergone digestion. As a result, it was expected that fewer proteins would be identified. Nonetheless, with our rapid and more targeted approach, a large number of peaks were observed.

The presence of antibiotics decreases microorganism adhesion depending on the susceptibility of the species and the concentration of antibiotic used [47]. This can regulate adhesion at both the cellular and molecular level. As a result, these changes were elicited via which proteins were expressed. Hst 5, although not an antibiotic, may be classified as an antimicrobial agent capable of causing changes in protein expressions. In

MS, changes in protein expression can be linked semi-quantitatively via the ion counts of the detected peptides. In this experiment, we are interested in peaks that either significantly decreased or increased after treatment with Hst 5.Hence, *m/z* peaks with no changes were disregarded.

To begin, peaks with large decreases in their ion counts are discussed. Table 3.2 contains the identified *m/z* peaks and their corresponding counts. The tentative protein identification, amino acid sequences and their biological functions are listed as well. With regards to the Hst application method, the results here demonstrated that both pre- and post-treatments had a decreasing trend. It can be suggested that these proteins are the cell's defense mechanism against antifungal agents. For these proteins, it became difficult to conclude that a decrease in ion count was truly representative of down regulation. This was because the decrease may have also occurred as a result of the loss in cells. A loss in cells would mean less peptides are available for analyses, therefore already constituting a decrease in ion counts. Therefore, the use of ESI-MS/MS may provide validation of these decreasing changes.

Table 3.2. Database identified proteins from four replicates of on-target digestions of *C. albicans* treated with Hst 5. Both pre- and post-application of Hst 5 have been included. Decreasing average ion counts and standard deviations (s.d.) have been listed. The period (.) represents the site of enzyme cleavage and the dash (-) represents the end of the terminus.

m/z	Before Treatment (Avg. Counts and s.d.)	Pre-Application (Avg. Counts and s.d.)	Post-Application (Avg. Counts and s.d.)	Amino Acid Sequence	Protein	Functions
802.00 1433.70 1677.80 1758.60	150 s.d. 30 1400 s.d. 90 350 s.d. 70 770 s.d. 20	30 s.d. 10 0 220 s.d. 40 180 s.d. 180	0 700 s.d. 100 0 150 s.d. 60	IAQQDNGK.G R.YGAGAGHPHRALAR.R -.MSWFGFFDPDFDDFFGRPR.K K.GQQVSRYGAGAGHPHR.A	Small heat shock protein 21	Virulence and stress response
797.33	440 s.d. 120	0	230 s.d. 160	K.YDDEKK.Q R.ALFSGFR.F	Cell wall protein RHD3 Presequence translocated-associated motor subunit PAM17	Pathogenesis and cell wall organization Transport of peptides from inner membrane to mitochondrial matrix
2184.74	6400 s.d. 30	1700 s.d. 2300	1940 s.d. 260	K.TGHSGTLDPKVTGCLIVCIDR.A	Centromere/microtubule binding protein CBF5	Chromosome migration
2214.50	3600 s.d. 400	600 s.d. 80	630 s.d. 50	K.CVVVGDGAVGKTCLLISY TTSK.F	Cell division control Protein 42 homolog	hyphal formation, virulence, morphogenesis
2214.50	3600 s.d. 400	600 s.d. 80	630 s.d. 50	K.LALVGISFVPLVLLVTVLYGK.I	Alpha-factor-transporting ATPase	ATPase activity and binding
2294.92	7700 s.d. 3200	610 s.d. 910	580 s.d. 260			
2304.80	1600 s.d. 900	340 s.d. 4	280 s.d. 50	K.ENLDWFKPFDLARFPVDPK.D R.VTDEILQLVPKPTVFKHALR.C	Acetyl-coenzyme A synthetase 1 Poly(A) polymerase PAPa	ATP binding mRNA polyadenylation and biofilm formation
2310.00	1300 s.d. 200	0	420 s.d. 60	K.ETDESYHELINKWENEMK.- + Oxidation (M)	Uncharacterized protein YAE1	

2322.82	1200 s.d. 120	200 s.d 30	640 s.d. 140		Adherence factor	surface antigen mediating adhesion and aggregation
2326.96	1200 s.d. 110	0	430 s.d. 40	K.FVSISVPILYGNHAIKLTPEK.R R.VDVLLIIVHAITFSVSDKATGK.Y R.AYMMDPWWSPSIEDQAIDR.I + Oxidation (M)	Protein AF-9 homolog Probable ferric reductase transmembrane component DNA repair protein RAD5	DNA repair, transcription and histone acetylation Ion transport DNA, ATP and Zn binding
2334.50	1400 s.d. 600	300 s.d. 500	630 s.d. 50	K.LTSQEFWEHIFNIFEINK.F K.DTFAAAALACGEKYYQVASSIR.T K.ENLDWFKPFDLARFPVDPK.D K.VQALILVPTRELALQTSQVVR.T	Alpha-1,2-mannosyltransferase Acetyl-coenzyme A synthetase 1 ATP-dependent RNA helicase DHH1	protein mannosylation acetyl-coA biosynthesis ATP and RNA binding (body assembly)
2571.95	820 s.d. 90	0	310 s.d. 160	K.DEEDDESSDDDDSDDDDTALLLK.E	Pre-mRNA-splicing factor WC15	Pre-mRNA splicing
3175.19	1300 s.d. 130	0	100 s.d. 10	MSEVNETCKPQACAIQNCLEKNGYNESR.C + Oxidation (M)	Cx9C motif-containing protein 4	Involved in the transfer of disulfide bonds to proteins entering mitochondria

In the second part, we sought peaks with large increases in ion counts following Hst 5 treatment. Higher emphasis may be placed on these peaks because despite the loss of cells due to Hst 5, an increase in ion counts was observed. From the peaks identified, the only increases in counts were for proteins who made new appearances after treatment. There was no increase for peaks already present prior to treatment. Table 3.3 contains the four peaks and their changes in ion counts. In addition, their tentative protein assignments, amino acid sequences and their biological functions were listed. Both application methods were used and the ion counts from both are listed. The five peaks verified the results seen in Table 3.2, in that both the pre- and post-application method revealed the same increasing pattern. Based on this trend, it can be hypothesized that the tentative proteins are undergoing an up-regulation in expression. It can be proposed that these proteins aid Hst 5 in accessing *C. albicans* whereby it can elicit its antifungal effects. Higher emphasis may be placed on these peaks because despite the loss of cells due to Hst 5, a noticeable increase in counts was observed. Therefore, confirmation of their identities will provide interesting insight on how Hst 5 works.

Table 3.3. Database identified proteins from four replicates of on-target digestions of *C. albicans* treated with Hst 5. Both pre- and post-application of Hst 5 have been included. Increasing average ion counts and standard deviations (s.d.) have been listed. The period (.) represents the site of enzyme cleavage and the dash (-) represents the end of the terminus.

m/z	Before Treatment (Avg. Counts and s.d.)	Pre-Application (Avg. Counts and s.d.)	Post-Application (Avg. Counts and s.d.)	Amino Acid Sequence	Protein	Functions
641.24 953.50	0 0	280 s.d. 80 560 s.d. 70	300 s.d. 10 420 s.d. 80	K.MESMK.Q + Oxidation (M)	Increased recombination centers protein 6	Chromosomal rearrangement and telomere healing
669.24	0	710 s.d. 750	610 s.d. 190	K.YDDEK.K	Cell wall protein RHD3	Pathogenesis and cell wall organization
695.35 841.44	0 0	1000 s.d. 100 170 s.d. 70	980 s.d. 110 43000 s.d 110	-.MAKTTK.V + Oxidation (M)	ATP-dependent RNA helicase HAS1	40S ribosomal subunit biogenesis

The proteins identified in both tables have cellular origins relating back to the cell surface. These proteins were cell wall protein RHD3, small heat shock protein 21, cell division control protein homolog 42, adherence factor and alpha-1,2-mannosyltransferase. This supported the assumption that the protocol developed for digestion proteins on cell surfaces was successful. The experimental conditions chosen were adequate to cleave surface proteins, yet mild enough to disfavour cell lysis. For some of the identified proteins, origins pertaining to the nucleus were observed. Initially, this contradicted the theory of digestion without cell lysis; that is, the only way nucleus-associated proteins can be identified was if the cells were lysed. It has been recently discovered that certain proteins belong to a group known as moonlighting proteins. Moonlighting proteins are multifunctional proteins that originate from one cellular compartment, and then are transported to another whereby they perform different functions. The protein, alpha-1,2-mannosyltransferase, has a potential of being a moonlighting protein. Two locations, the Golgi apparatus and cell membrane, were determined for this protein. However, its listed functions have roles associated at the cell surface. Because of this, there may be a possibility for the presence of other moonlighting proteins.

Another protein of particular interest was the small heat shock protein 21 (Hsp21). Not only did it have an origin and functions at the cell surface, but its identification seemed more reliable. In Table 3.2, four different masses and peptide sequences have been shown to identify this protein. This not only meant that Hsp21 could be identified more accurately, but there was a high possibility for its presence. Hsp21 was determined to have functions as a coping mechanism for *C. albicans* in coping with thermal and oxidative stresses [50]. Hst 5 may have functioned as a stressor, therefore, *C. albicans* would have responded by changing the expression of Hsp21 in hopes of allowing cell survival. With the mention of oxidative stresses, the expression of Hsp21 strongly suggested the theory that Hst 5 affects cellular respiration may be correct.

3.5.1 Limitations of MALDI MS Analyses

Thus far, we have demonstrated the ability of MALDI-MS to analyze large changes in the protein expression after Hst 5 treatment. This was observed through large changes in ion counts. The identities of these proteins and their roles in biological pathways, allows focus on the key biological processes Hst 5 may influence. Unfortunately the proteins identified in Tables 3.2 and Tables 3.3, were only tentative. To confirm their identities, further identification would be required. Tandem MS (MS/MS) would allow for the determination of their amino acid sequences. These sequences would be more specific, allowing for a more specific identification.

MALDI MS/MS has been performed on the *m/z* peaks observed; however, the results were inconclusive. One problem was the intensities of the observed peaks. To obtain a sufficient MS/MS spectrum, the precursor peak must have an ion count of at least 2000. When it is less, fragmentation becomes difficult and the spectrum can become noisy. This was observed for the majority of the peaks. Their counts were not high enough to undergo a second fragmentation. Figure A.1 was the spectrum obtained for the *m/z* peak of 1677.86. Here, the second fragmentation was possible but as one can see, the spectrum greatly suffered from background signals. The sequencing of the amino acids was attempted, but due to the noise, it was difficult to distinguish between a signal from the background and one from the amino acid. Automatic sequencing such as de novo sequencing, can be used instead, however, this did not work for the acquired spectra.

To overcome this, additional sample cleanup was necessary. This will help remove any signals from salts and buffers, allowing improved fragmentation. With that in mind, ESI became a more suitable option as it can be easily coupled with LC. LC will allow for the cleanup via further separation of the components. And with its ability to operate in a continuous online mode, MS/MS can easily be acquired for all precursor ions as the fractions leave the column. One focal point with ESI was its ability to automatically run MS/MS spectra through databases such as SEQUEST, allowing for all probable amino acid sequences within a selected species.

Between the different Hst 5 application methods, both pre- and post-application offered similar results. Both identified approximately the same number of *m/z* peaks, and the changes in ion counts demonstrated the same trends. For further research however, with interest towards clinical applications, it was decided that focus would be placed on post-application. For example, take the situation where an individual has already contracted the pathogen. This can represent the situation of *C. albicans* adhered to HSA in the oral cavity. For them, administering Hst 5 would occur next, which is parallel to our post-application method. In this aspect, we would be addressing the role of Hst 5 as a treatment, rather than used as a prevention. In the next section, ESI MS/MS will be used on post-application prepared slides. The appearance of the same list of *m/z* peaks from MALDI MS will be searched in hopes of further confirming their identifications via tandem MS.

3.6 Analysis of Peptides from *C. albicans* by HPLC-ESI-MS/MS Following Treatment with Hst 5

3.6.1 Quantification of Total Peptide Concentration Prior to Sample Injection

Prior to injecting the sample into the instrument, quantification of the peptides was carried out. The linear trap quadrupole (LTQ) linear ion mass spectrometer used had a lower level of detection at 0.002 mg. Since, peptides have molecular weights less than 4000 Da, a BCA assay was used. In the BCA assay, quantitation of proteins with low molecular weight and peptides were done via reactions with Cu^{2+} ions. The specific reactions involved in the BCA assay are discussed in Appendix 3. In addition, the protocol for standard preparation and measurements are explained. Absorbance values of the standard protein, bovine serum albumin (BSA), was used to extrapolate the quantity of peptide. This assay was performed on the digested peptides from a single droplet spot with a volume of 1.50 µL. The standard curve had an equation of $y = 0.000x-0.021$, $R^2 = 0.9662$. It was therefore determined that from a digestion volume of 1.50 µL, the mass of

peptides was 0.5050 mg/mL. Therefore, for a single spot with a volume of 1.50 µL, the mass of the peptides was 0.7575 mg. To meet the lower level of detection, three spots were combined as one replicate. From MALDI MS results, one significant limitation was quantitative comparisons between data sets due to different cell counts. Therefore the BCA assay, may somewhat act as a normalization in minimization large variations.

3.6.2 Database Search Results for Protein Identification

Prior to injection into the instrument, peptide solutions were dried and resuspended in 0.1% formic acid. Together, both HPLC and ESI MS/MS data were produced. However, what was of more importance was the MS/MS data acquired. MS/MS spectra allowed for the direct input into SEQUEST, an algorithm that searches against selected databases. All spectra were run against the *C. albicans* database acquired from UniProt. In this way, all mass spectral peaks were used, tentatively identifying all possible proteins correlating to the observed masses. To be considered a protein of interest, it must have been identified in both the untreated and treated samples with the same ascension number. In this way, we can relate a change in protein expression as significant.

A total of six replicates were prepared and analyzed by HPLC-ESI-MS/MS. All resulting spectra were searched against the *C. albicans* database, with the tentative protein identifications shown in Table 3.4. Again, without completion of the database, *m/z* assignments would not be possible. As an alternative, the concept of identifying peaks via protein families was used. Those identified in this manner were represented with an asterisk. Protein families include proteins who share similarities in sequences, structures and functions. Unfortunately, even when assignments were done via protein families, several proteins were still not identified. New protein entries are entered daily into these databases, therefore when needed; the spectra can be searched again more frequently in hopes of identification.

Upon comparison between MALDI and ESI MS results, the list of masses identified was different. This can be due to different sample bias during both ionizations. Another

probable explanation may be due to the sample cleanup during the ZipTip protocol. When the ZipTip was used, the loss of peptides during numerous binding and washing steps was expected. In the case of the digested peptides, the washings may have potentially removed the peaks observed previously from MALDI MS results. This would then produce a set of new masses following analyses. Another reason may be because sample cleanup may have removed abundant contaminants. The presence of these contaminants would mask the peptides of interest, which were already of lower concentrations. As a result, this would allow for new peaks to be observed in ESI MS/MS only. And lastly, there may be a possibility that the peaks identified in MALDI MS may have been by chance. The performance of MS/MS would have allowed for us to determine their amino acid sequences, and subsequently whether or not they were truly peptides. However as already discussed, MS/MS was not possible with MALDI MS.

Similar to the MALDI MS results, Table 3.4 was examined for changes in ion counts. For a change in ion count to be concluded, the standard deviations were taken into consideration. When changes fell outside the standard deviation, a change was concluded. Again, the two trends of decreasing and increasing counts were observed. Ion counts that have decreased following Hst 5 exposure were bolded in blue, and those with increases were bolded in red. Similar to the results from MALDI MS analyses, those with increasing counts were of more interest. This was because even with the reduction of cells adhered, an increase in expression for these proteins was seen. For those that did not show changes outside the standard deviation, a change could not be concluded. As a result, Table 3.4 categorizes the results according to which biological pathway they were associated with.

Due to the difficulties in basing our results solely on ion changes, more emphasis will be placed towards the biological pathways affected. Being able to identify specific biological pathways may allow for better focus on how Hst 5 works. In addition, it will allow research to become more targeted towards proteins in these key processes. Our results in Table 3.4 have been classified into eight categories; (A) virulence, (B) surface-associated, (C) mitochondrial-associated, (D) DNA binding, (E) protein synthesis, (F) signalling, (G) miscellaneous and (H) uncharacterized. For those in the miscellaneous

group, no common pathway was found amongst the proteins. Those within the uncharacterized group have no identification due to the incomplete *C. albicans* database. However, frequent searches may be done as the new entries are added in hopes of identification. The first four pathways will be our focus for the remaining of the discussion.

Before discussing each specific pathway, the conflicting changes in each group will be addressed. Conflicting changes are situations where one or more *m/z* peak exhibited an opposite trend compared to the majority within same group. Table 3.4 contains number in parenthesis beside each amino acid sequence. These values, known as XCorr, correspond to the scoring system of the database searches. XCorr will score the number of fragment ions common to two different peptides of the same precursor mass [51]. The cross-correlation score is then presented from all peptide candidates provided from the database. To be considered a "good" match, the XCorr value must be two or greater. In the surface-associated proteins (Group B), only the mass of 1297.59 indicated an opposing trend. As listed, the XCorr value was 1.82. This lower value suggested that the identification of the protein, and consequently, its ion change, was not reliable. The same can also be said for the proteins with masses 575.44 and 1297.59 of the DNA binding proteins (Group D).

With previous work indicating the antifungal ability of Hst 5 in controlling *C. albicans* [35] , proteins involved in virulence were assessed. The ion counts of these proteins were expected to decrease following Hst 5 exposure, which was observed for all listed in (A). The decrease for these proteins indicated that the ability of Hst 5 to affect the pathogenesis of *C. albicans.* For example, the protein of the β-lactamase family (*m/z* of 4115.51) had a function with regards to resistance of *C. albicans.* This protein may potentially aid *C. albicans* in withstanding the host's immune system. Therefore, a decrease in its expression would lower the defense of *C. albicans*, making it susceptible to the immune system in the host. Similarly, the protein kexin was identified to be involved in the production of toxins. A decrease in expression could correlate to a decrease in the amount of toxin produced, therefore weakening the pathogenesis of *C.*

albicans. Overall, these results demonstrated the ability of Hst 5 to decrease the virulence of *C. albicans.*

Table 3.4. Database identified proteins from on-surface digestions by HPLC-ESI-MS/MS. Six replicates were analyzed from *C. albicans* treated with Hst 5 in the post-application method. Average ion counts are listed with standard deviations (s.d.). Proteins indicated with an asterisk (*) have between determined using their protein families. Blue represented those with decreasing ion counts, and red for increasing ones. XCorr score values are listed in parenthesis.

Biological Pathways	Mass	Ion Counts		Amino Acid Sequence	Protein Name	Functions
		Before	After			
A) Virulence	1284.83	**27000 s.d. 9500**	**14000 s.d. 2800**	K.sLYTISPNKGK.R (2.52) K.SKYNLPFAMK.E (3.75)	Transcriptional regulator STP3 Candidapepsin-7	Filamentous growth Supplies nutrients and degrades proteins involved in host's immune system
	1295.87	**66000 s.d. 1100**	**19000 s.d. 7000**	R.tKKWGLGWIK.N (1.61)	Integrase family*	DNA recognition between phage and bacterial attachment sites
	3579.79	40000 s.d. 5000	31000 s.d. 36000	HPQYsEACsAVmVVTYSSGSGEHIH TTDIK (3.33)	Kexin	Enzyme that produces killer toxins
	3895.36 4659.96	36000 s.d. 4100 **15000 s.d. 1100**	26000 s.d. 31000 **3200 s.d. 2700**	ELKTTVIVTSCFNNVCSETsITT PKtAVtATtSK (3.1) K.HLTLKSSTPASTLEYSTSIPPALATTSSS LStESTtLttISR.S (3.25)	Flocculin*	Needed for invasion and pseudohyphae formation in nitrogen starved environment
	4115.51	**51000 s.d. 12000**	**24000 s.d. 17000**	VTFVEKAtSTSTTNtTttTTTTTTTTTTTTTI PVKR (3.23)	β-lactamase family*	Resistance

	4121.03	**31000 s.d. 23000**	**17000 s.d. 7200**	K.NVKVITTTTTtSPSSFSSSSSLMsPITPQT PNIPKTPK.T (3.94)	PX domain*	Phosphatidylinositol binding
B) Surface-associated	1175.88	**64000 s.d. 3900**	**22000 s.d. 1100**	R.tINLDsQVK.Y (3.16)	3'(2'),5'-bisphosphate nucleotidase 2	Phosphatidylinositol phosphorylation
	1297.59	**18000 s.d. 8800**	**51000 s.d. 5400**	K.KPsTEDtFSK.Y (1.82)	Peroxisomal protein family*	Membrane component, lipid metabolism and decomposition of H_2O_2
	1295.87	**66000 s.d. 1100**	**20000 s.d. 7000**	mQTSIStTIEDHLHHYsPEESQKLLSRESSI NTDLFK.E (3.13)	Bud site selection protein BUD4	Establishes the axial budding pattern, induces hyphal growth and has role in epithelial adherence
C) Mitochondrial-associated	1175.88 1295.87	**64000 s.d. 3900** **66000 s.d. 1100**	**22000 s.d. 1100** **19000 s.d. 7000**	K.YMLLTLLtK.L (2.96) K.LTtLISSIENK.I (2.96)	YAP-bd/ALF4/Glomulin family *	Stress response and redox homeostasis, induced by H_2O_2
	1297.59	**18000 s.d. 8800**	**51000 s.d. 5400**	R.NsIttEsVK.A (2.03)	Aldehyde hydrogenase family*	Ooxidoreductase activity, acting on the aldehyde or oxo groups of donors, NAD or NADP as acceptor
	1374.01	16000 s.d. 1300	10000 s.d. 3000	R.TASGNIIPSSTGAAK.A (2.86)	Glyceraldehyde-3-phosphate dehydrogenase	NAD^+ phosphorylation and cell biogenesis/degradation
	4659.96	**15000 s.d. 1100**	**3200 s.d. 2700**	K.StIVEEIYsNARSHLVQGNKEmGmALFN ELLAINESIYGK.V (3.37)	Clustered mitochondria protein Homolog	Intracellular distribution of mitochondria

D) DNA binding	575.44	**9700 s.d. 1000**	**28000 s.d. 6200**	K.SLLSR.I (1.54)	Telomerase reverse transcriptase family*	DNA binding
	804.77	42000 s.d. 22000	19000 s.d. 3100	K.QPAVFSR.I (2.56)	DNA polymerase	DNA-directed DNA polymerase activity
	1284.83	**27000 s.d. 9500**	**14000 s.d. 2800**	R.VASHsLsTsR.R (2.67)	Minichromosome maintenance protein 10	DNA replication
	1297.59	**18000 s.d. 8800**	**51000 s.d. 5400**	K.NVIPAtItK.V (1.54) K.EFTIGPFKcIK.W (1.56)	Spt6 Transcriptional regulatory protein family *	Nucleic acid binding Zn-ion binding & DNA binding RNA polymerase II transcription factor activity
	2806.76	47000 s.d. 36000	30000 s.d. 23000	R.TTPPTVsITGPNPsSSPAsASTNtSK.S (3.60)	Phosphatase family*	Chromosome segregation
	3376.60	43000 s.d. 12000	32000 s.d. 28000	K.SSPTSsATTTATtSVsISSLSLtMGKPKNSK.L (3.15)	Mediator complex*	Cofactor to modulate transcription
	3331.28	**63000 s.d. 1200**	**14000 s.d. 1400**	K.GFTNTMISHIGFDPTGtsLNQNsTSLGsK.S (3.21)	Minichromosome maintenance protein 10	DNA replication
	3498.15	**34000 s.d. 8900**	**18000 s.d. 16000**	K.LGSVLTtRSQLIEYELtTRtIFINCSAALK.I (3.11)	Spt6	Nucleic acid binding
	3503.40	43000 s.d. 8300	31000 s.d. 25000	R.EVIIKLSITRtHtPPPDSTtTTTPTTSIEK.T (3.57)	Zn finger domain *	Zn ion binding
	3939.14	36000 s.d. 24000	17000 s.d. 18000	VSSYILDGNNSTKLPsPVLtHtTFDSRsDEGQR (3.99)	Basic leucine zipper domain*	DNA binding transcription factor activity

	4115.51	**51000 s.d. 12000**	**24000 s.d. 17000**	R.APQsIQLPPIQsFtKsQAVFPQSVRDSAPAANFNR.Y (3.12) K.sStQMSSCtNsVTQTLDRLPKIVSTQQNNLTPTSK.I (3.03)	Transcription factor and DNA binding protein families* Zn(2)-DNA binding domain *	DNA binding RNA polymerase II transcription factor activity and Zn ion binding
	4173.60	**24000 s.d. 1200**	**13000 s.d. 1200**	K.tVTsINGSPPPLEtAPsSHHNVPIDFIHFKKESDR.T (3.12)	Uncharacterized protein	DNA binding RNA polymerase II transcription factor activity and Zn ion binding
	4368.59	**33000 s.d. 21000**	**7100 s.d. 1300**	K.NMQFPPYQVSsHNsSEtSQsIPNTPSITRQVESNTR.S (3.54) K.tPTTTTTTTTtANGNTSNGNTSNGNsTGKTATAATATKSNtK (3.19)	Transcriptional regulatory protein LEU3 Transcription protein family*	Sequence-specific DNA binding RNA polymerase II transcription factor activity and Zn ion binding
	4659.96	**15000 s.d. 1100**	**3200 s.d. 2700**	MKImmIPTHHQtYNINTHQPPQQHQYLPPPGtSYTSPR.A (3.12) R.RSVSYSPGPsSIKSQLPHLTSSSTTtssVQSPPPPPPSQPPR.G (3.61)	Transcription factors and DNA binding protein* Zn (2) – DNA binding domain*	DNA binding RNA polymerase II transcription factor activity and Zn ion binding
E) Protein synthesis	804.77	**42000 s.d. 22000**	**19000 s.d. 3100**	K.QTSLNDK.C (2.54) R.VKIDSDK.S (1.51)	Mannosyltrans-ferase family* Protein transporter SEC24	Protein glycosylation ER to Golgi vesicle-mediated transport
	1175.88	**64000 s.d. 3900**	**22000 s.d. 1100**	K.TKQFNDsKK.K (2.01)	Vesicle tethering protein family*	Intracellular protein transport
	1284.83	**27000 s.d. 9500**	**14000 s.d. 2800**	K.tAGsNHNsEK.K (2.51)	Ribosomal RNA-processing protein 12	Protein binding

	4121.03	**31000 s.d. 23000**	**17000 s.d. 7200**	K.FFsDNIANDLAtTTTTTTTTNTGAtSVHP ILQVDAIK.Y (3.24)	CAS/CSE protein family*	Intracellular protein transport
	4368.59	**33000 s.d. 21000**	**7100 ± 1300**	K.ESSSTADQPSVVPPQESHKDTVETPKPE VtEtsVEAtK.E (3.24) K.NPTPTPTPTPTPTPNNLAQGVDsSSTLD VEtTLtGLtRR.I (3.21)	Translation initiation factor 4G Imidazoleglycerol phosphate synthase cyclase subunit	Protein biosynthesis Histidine biosynthesis
F) Signalling	3503.40	43000 s.d. 8300	31000 s.d. 25000	K.RSSITtPtPPLTTTHSSNGNGNGNVNVN VNsK.R (3.30)	Peroxisome transmembrane receptor *	Protein import
	4115.51	**31000 s.d. 20000**	**17000 s.d. 7200**	K.QSTTNTsTLssTtAASTLATSNNTQPDTY TSTSTSIR.G (3.33)	Pleckstrin homology domain *	Small GTPase mediated signal transduction
	4416.51	29000 s.d. 27000	16000 s.d. 1100	R.QHPDPLSNQsNFNsNTINNYSNYRSsTR SGLDPsQR.H (4.12)	Rho GTPase activating protein domain*	Signal transduction
	4659.96	**15000 s.d. 1100**	**3200 s.d. 2700**	K.DLPIGYILHmINLcPNIVsLNLGNLSLsT DYEISRSTIHK.Y (3.68) K.WNKEKIELDsPLIVSYVSSLCNGGGGGI ITNsTNSTttNSK (1.11)	F-box protein COS111 Pentatricopeptide repeat (PPR) protein family*	Signal transduction Termination of G-protein coupled receptor
G) Miscellaneous	575.44	**9700 s.d. 1100**	**28000 s.d. 6200**	R.SLNSR.I (3.15)	ATPase family*	ATP binding
	1284.83	27000 s.d. 9500	14000 s.d. 2800	R.NDsDtsLsK.E (1.66) R.YtmNEVFK.V (2.02)	Insulin induced protein family* GYF domain*	Cholesterol synthesis
	1901.71	**7800 s.d. 3900**	**13000 s.d. 2300**	K.NKATssSSStsRDTR.W (3.19)	Leucine rich repeats*	Protein-protein interactions

	3331.28	**63000 s.d. 1200**	**14000 s.d. 1400**	ETsQFMGNAESEDLtGNGLLTSTL AVLSSIS (3.15)	Orf in C. albicans Major Repeat Sequence, RB2 Region	
	3331.28 3376.60	**63000 s.d. 1200** 43000 s.d. 12000	**14000 s.d. 1400** 33000 s.d. 28000	K.LSSLGNHGTtTTSSLSSSsSsSIsNNT SIAK.I (3.46) R.KQQDQNEVAGAAAATTTTAtAtATAAT NWKPK.N (3.21)	CBS domain*	Sensing of intracellular metabolites
	3498.15	**34000 s.d. 8900**	**18000 s.d. 16000**	K.SSsLIKNsTsSNQSsPATSTNTSIVDVPIE K.S (3.21) KAFSSsKLTSDSANStNStNsTSMSILGNDK .D (3.16)	WD repeat protein* CDK inhibitor PHO81	Coordination of multi-protein complexes Lipid metabolism
	3895.36	36000 s.d. 4100	26000 s.d. 31000	R.ISRPNGVGGISTSGSSSPTTEFVTPQAsK sSVDQNKK.R (3.29)	Ubiquitin interacting motif*	Ubiquitin metabolism
	4173.60	**24000 s.d. 1200**	**13000 s.d. 1200**	R.INNNNDKssILsNITTTNTTTGTGTTNTtT VPSIKTK.R (4.24)	WD repeat protein*	Coordination of multi-protein complexes
	4421.03	32000 s.d. 19000	17000 s.d. 7200	R.HLILGYKItVVtDHQSLTsVMTSSSRPEN NRMIR.W (3.83)	Aspartic peptidase family*	Proteolysis
	3939.14 4372.57	36000 s.d. 24000 9600 s,d. 4600	17000 s.d. 18000 5400 s.d. 1800	R.NVNGSGStNtNTMtRLDsTTIASSLFCRQ LYFNLLSK.D (3.11) R.NNsTVSSTNSLmSNNsDTNtAATAATAA TSGSTTNNVKR.M (3.38)	Globin family*	Heme and oxygen binding
	4416.15	29000 s.d. 27000	16000 s.d. 1100	R.NGtYSSsStSSSsTSSVSSSSATTANGESL NSTTHNIQLER.T (2.89)	Sec2p*	Vesicular transport

	4659.96	**15000 s.d. 1100**	**3200 s.d. 2700**	K.QNYVDPGQIAIKGLKGFVNLGAtCFMss ILQtLIHNPLI.K.Y (3.69) K.YDKPmEDTEEIDDVtSISKsINEQID DPFsQFNSVTLR.Y (3.51)	Ubiquitin carboxyl-terminal hydrolase Protein-serine/threonine kinase	Anti-ubiquitination ATP binding
H) Uncharacterized	1175.88	**64000 s.d. 4000**	**22000 s.d. 1100**	K.tImtItIK.I (2.07)		
	1297.59	**18000 s.d. 8800**	**51000 s.d. 5400**	R.NNTtVSKGRIK.V (1.51) K.KHQtHILNVK.S (1.60)		
	3331.28	**63000 s.d. 1200**	**14000 s.d. 1400**	R.IsNIITLNSSLSSSsSsSSSsSSLLLL TLK.S (3.18)		
	3895.36	36000 s.d. 4100	26000 s.d. 31000	K.TTPVMGNSStPSTVtANtNtGAYSTSSDT AAKPTKK.A (3.14)		
	3939.14	36000 s.d. 24000	17000 s.d. 18000	K.TTPVMGNSStPSTVtANtNtGAYSTSSDT AAKPTKK.A (3.46)		
	4115.51	31000 s.d. 20000	17000 s.d. 7200	K.TVCNWQtLGHTDEFEEstQFARGVSDtA LVGGITK.L (2.18) T.CGKSCFINSttANKLIsYNLFQSStEVDSI GPTGSK.T (3.86) K.TTPVMGNSsTPSTVTANTNTGAYSTSs DtAAKPTKKATK.R (3.91) R.ILLLLPLLGVtILTSKSSLESISLNsPSDSI ALssSK.S (2.34)		
	4416.51	29000 s.d. 27000	16000 s.d. 1100	K.STSSANIKtKPKPNTATtATTPTAtTATTt ATTSSIDPTEK.D (2.59)		

	4421.03	32000 s.d. 19000	17000 s.d. 7200	R.LSGtNNPGsGsGsGGGGGGGANNNSLP GYSVGSSIGRGRGLGR.G (1.89)		
	4368.59	**33000 s.d. 21000**	**7100 s.d. 1300**	K.NYNQFKLIELDDSMNTTTTTTTTTTtTtt TtTSTIGK.F (3.76) K.SKsTTIHNQSNIHSEQISINDENNNKStst STSTDTK.K (1.32)		
	4372.57	9600 s.d. 4600	5400 s.d. 1800	K.SSGGSSDTKSVWIAtTGSDFASQSNSDS sStASRNSSSSASR.Q (2.46) K.EsEGVWGWsLQCLLLLFTKVNLRscDQ LLICALVIR.E (2.99)		
	4659.96	**15000 s.d. 1100**	**3200 s.d. 2700**	K.VILIGNSMTsRTTsSVRFFSIMLTVSLPIt NILVsSELSK.V (3.11)		

Our original hypothesis was that surface proteins were affected following Hst 5 treatments. Hence, our results were examined for their presence and functions. Group (B) in Table 3.4 listed the proteins found to have either cellular origins or functions pertaining to the cell surface. The bud site selection protein BUD4 showed direct surface association with functions directly associated with adhesion. With surface proteins, Hst 5 would affect the ability of *C. albicans* to adhere; therefore a decrease in ion counts was expected. This did indeed occur as the ion counts went from 66000 ± 1100 before treatment, to 20000 ± 7000 following treatment. This supported the microscope images taken in Section 3.3.

At first glance, the number of proteins correlated to the cell surface may be discouraging, but the chances of moonlighting proteins was probable. The protein glyceraldehyde-3-phosphate dehydrogenase (GADPH) was discovered to be an anchorless surface protein [52]. Anchorless surface proteins are similar to moonlighting proteins, in that they carry functions at one cellular location and then are transported to the cell surface of unknown function, but differ in that they originate at the cytoplasm. GADPH has been shown to be involved in the Krebs cycle within the cytoplasm. But has also participated in the colonization, persistence, and invasion of host tissues [53]. This dual functionality demonstrates that perhaps the proteins identified may carry multiple roles, where the effects at the cell surface still occur.

Current research has shown that Hst 5 influences *C. albicans* at the mitochondria through energy metabolism, either through reactive oxygen species (ROS) formation, or via ATP release [54, 55]. Similar to the work carried out here, Komatsu *et al.* used MS to measure changes in protein expression at the mitochondrial level [56]. By comparing the level of expression before and after Hst 5 treatment, they determined that Hst 5 caused the down regulation of proteins participating in cellular respiration and adenosine triphosphate (ATP) metabolism. Table 3.4 listed any mitochondrial-associated proteins in Group (C). For all entries, it was also determined that the general trend of ion counts was decreasing following Hst 5. This showed parallel results to Komatsu *et al*. Surprisingly, relatively few proteins were listed. Only two the protein belonging to the aldehyde hydrogenase

family and GADPH showed direct correlation to cellular respiration. The YAP-bd/ALF4/Glomulin, did not show direct involvement to cellular respiration. However, the protein family it belonged to proved otherwise. It is involved in a stress response induced by hydrogen peroxide. Hydrogen peroxide has been shown to participate in the formation of ROS at the mitochondrial level, thereby killing the cells [54]. The YAP-bd/ALF4/Glomulin family protein may be involved as a defense mechanism against hydrogen peroxide and ROS. This could imply that Hst 5 affects the expression of this protein, therefore weakening the defense of *C. albicans* against ROS. Together, this would indicate the possibility of Hst 5 affecting cellular respiration.

Unexpectedly, what was observed for the majority of the proteins was their connection to the nucleic acid. These proteins were listed in Group (D) of Table 3.4. They have functions relating to acid (DNA) or ribonucleic acid (RNA) binding, transcription and regulation. Previous work has discovered that the structure of Hst 5 was determined to have two main metal binding motifs, making it a metallopeptide [57]. The motif at the N-terminal, binds Cu^{2+} and Ni^{2+}, and is known as the ATCUN-motif with the general sequence of NH_2-X-X-His. The second motif at the C-terminal, binds Zn^{2+}, and has the general sequence of His-Glu-X-X-His (HEXXH). It has been discovered that peptides with these motifs behave as a DNA intercalator, delivering the peptide and metal cofactor close enough to the DNA for binding [58]. Long *et al.* discovered that when the Ni/Cu-ATCUN peptide complex formed, it had the ability to cleave nucleic acids in the presence of co-reactants [59]. The ATCUN motif would bind the minor groove of DNA, and the binding of Zn^{2+} ions at the HEXXH motif will increase the exposure of the side chains of basic residues to increase DNA binding. Hst 5 has been shown to bind DNA, however the exact roles of the two metal motifs remains unclear. The presence of both motifs and its cationic nature favours the likelihood of its interaction with DNA for a nuclease effect. With DNA damage and without DNA replication, the survival of *C. albicans* is low. Many of the Group (D) proteins had DNA and Zn binding, which are key participants in the ATCUN complex model. Therefore, based on these results, it may be suggested that the nuclease ability of Hst 5 has occurred.

At first, it seemed very plausible that Hst 5 as a metallopeptide, had another mechanism whereby it only exerted influence at the nucleus. However, what was discovered was that the affects at the nucleus were working in conjunction with effects on cellular respiration at the mitochondria. Research done by Melino *et al.* showed specific function of the ATCUN motif in oxidative and hydrolytic cleavage, through its nuclease activity [60]. The presence of the ATCUN motif bound to DNA led to a triple increase in ROS production [61]. ROS can lead to organelle damage via DNA damage. Furthermore, it was shown that the Cu-ATCUN complex was a necessary prerequisite for the oxidative cleavages [62]. The mitochondria has been shown to house a large pool of Cu^{2+} ions [40]. This demonstrated that affects at the nucleus, were only side influences, where the true processes affected were those pertaining to mitochondria and cellular respiration.

Section 4 Concluding Remarks and Future Work

4.1 Conclusions

With the rapid increase in infections caused by *C.* albicans, new methods in minimizing its effects are needed. The peptide known as Hst 5 has been shown to kills cells *in vivo,* however, its mechanism remains unclear due to its complex multistep nature. Research has become focused towards Hst 5 affecting cellular respiration at the mitochondria. In this work, it was proposed that the adhesion of *C. albicans* to the oral cavity is crucial for virulence. That Hst 5 may be affecting the surface proteins allowing for attachment. By using MS and changes in ion counts, changes in protein expression may highlight which proteins are affected.

The adhesion of live cells of *C. albicans* and treatment with Hst 5 was successful. Following this, on-target tryptic digestions were developed to potentially isolate surface proteins. In this way, digestions were shorter and can be quickly executed. Both MALDI MS and ESI MS/MS showed the ability to detect changes in ion counts, and protein expressions. MALDI MS and ESI MS/MS resulted in different *m/z* peaks, what was prominent was that both techniques showed the same biological pathways were affected. Both MS results contained proteins associated at the cell surface. This supported the original assumption that the developed on-target digestions were able to cleave surface proteins. This also suggested that surface proteins and adhesion may one of the many ways in which Hst 5 controls *C. albicans.* Additionally, what was seen for the majority of the remaining tentatively identified protein was their association with the nucleus. Dr. Siqueira's previous work showed that the mitochondria was where Hst 5 exhibits its therapeutic effects. The presence of these nucleus-associated proteins suggests that Hst 5 behaves like a metallopeptide. The binding of Hst 5 to DNA has been linked to produce ROS. The presence of this species supports that cellular respiration was likely the biological process that was affected.

4.2 Future Work

In hopes of still exploring the influence of Hst 5 on surface proteins, the determination of where the resulting peptides come from may be more sufficient. Indirectly, this can be done through the use of immobilized trypsin. Trypsin can be immobilized on beads of various materials such as glass or metal. With trypsin immobilized, the chances of diffusion into the cell would be minimized. This would have the potential to then limit protein cleavage within the cell and subsequently, their incorporation into our MS analyses. Therefore, the observed peaks would only correlate to surface proteins.

A large complication seen with the ion counts, specifically, with the determination of whether or not a change was observed. This was mainly because the degree of adhesion could not be controlled and so, the number of cells present for each replicate remains unknown. To address this, what can be done next is to incubate the slides with the same number of cells. This way, variability in ion counts are more controlled and changes would be more representative of the effects of Hst 5.

The tentative identification of proteins has been achieved, however, as the results indicated, there were too many possibilities for a single observed *m/z* ratio. To address this, additional replicates could be performed. The proteins identified from these additional analyses may highlight which of the already observed proteins are more significant.

Thus far, ESI MS/MS has been applied towards the post-application method of introducing Hst 5. The results have identified proteins with changing expressions after treatment. MALDI MS results have shown that the same proteins were identified independent of when Hst 5 was introduced. The use of ESI MS/MS on pre-application slides will be of interest next. The identification from this may result in the same proteins as the post-application, provide more proteins, or they may identify a whole different set.

And lastly, the use of the developed on-target surface digestion can be applied towards other microorganisms. By using this approach, it offers a fractionation technique for

samples in which peptides may be analyzed. In this way, the sample pool becomes more limited, therefore allowing focus on a specific set of proteins.

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Appendices

Appendix 1: Mass Spectrometry

Mass spectrometry (MS) is an analytical technique capable of correlating measured mass-to-charge (*m/z*) ratios of ions generated from samples to their intensities. From acquired measurements, a mass spectrum is generated whereby the *m/z* ratios are plotted along the x-axis, and the relative ion intensities on the y-axis. From the spectra, information such as masses, isotopic patterns and even structures can be determined. In MS, the sample is first introduced into the instrument, whereby it undergoes ionization under high vacuum to produce either singly or multiply charged ions. These ions are then accelerated, and it is in the mass analyzer, where the ions are separated based on their *m/z* ratios. As a result, the separations allow for ions to reach the detector at different times leading to the mass spectrum.

Small molecules (<500 Da) with a single functional group predominantly give rise to singly charged molecules. They can either be $[M+H]^+$ in positive mode, or $[M-H]^-$ in negative mode. Larger molecules containing several functional sites give rise to multiply charged ions with a general form of $[M+nH]^{n+}$, such as $[M+2H]^{2+}$. Additionally, adducts can also occur with salts such as; $[M+Na]^+$, $[M+K]^+$, $[M+formate]^-$, and $[M+acetate]^-$.

Once ions are produced, they must be separated according to their *m/z* ratios. This is accomplished by mass analyzers. The choice of which mass analyzer is suitable for which mass spectrometer depends on factors such as mass range, resolution and sensitivity [63]. There are four commonly seen mass analyzers in research today; quadrupole and ion traps, time-of-flight (TOF), orbitrap and cyclotron. Focus will be placed on quadrupole and ion traps commonly seen with ESI systems, and TOF seen with MALDI systems.

The quadrupole mass analyzer consists of four oppositely charged, two are positive and two are negative, parallel rods in which radiofrequency (RF) and direct current (DC) can

be applied [63]. At one end is the ion source, with the detector at the other. When only RF is applied, all masses are able to pass through. And when only DC is applied, all masses are neutralized by coming into contact with the charged rods. When using a combination of RF and DC, ions of certain masses are able to pass through and reach the detector, therefore acting as a mass filter. Additionally, ions can become trapped within the quadrupole to function as an ion trap. There are two types, linear and nonlinear, where emphasis will be placed on linear ion traps. In a linear ion trap, both ends of the quadrupole are set to carry the same charge as the ion that one wishes to trap. The entrance will be set at a lower voltage than the exit. Damping gas will then be introduced, causing ions to lose kinetic energy. As a consequence, ions cannot pass through and become trapped. The quadrupole carries its own advantages; it has a low cost, allows for easy automation and is physically small in size.

TOF analyzers, commonly seen with MALDI mass spectrometers, are well suited for pulsed ion sources [64]. In TOF analyzers, ions are accelerated into a drift region of known length. Lower masses travel down the tube in shorter times due to faster speeds. In comparison, heavier masses take longer to travel due to slower speeds. These analyzers can operate in two modes, linear and reflectron. In linear mode, ions simply travel down the flight tube until they reach the detector. Ions with the same mass will have different potential energies, reaching the detector at different times. Consequently, this causes a dispersion effect leading to low resolution. To correct for this occurrence, a reflector, which will causes ions to reverse directions, can be used in the reflectron mode. In this mode, ions with higher kinetic energies penetrate the reflector more deeply, reaching the detector later. Ions with lower kinetic energies do not penetrate as much and so will reach the detector early. By operating in the reflectron mode, dispersion is minimized leading to improved resolution.

The initial ions produced, known as the precursor ions, can undergo a second fragmentation in what is known as tandem MS (MS/MS). In MS/MS, precursor ions from the first ionization are chosen and undergo fragmentation. The second fragmentation can be done in two ways, collision-induced dissociation (CID) and post source decay (PSD). In CID, fragmentation is accomplished by colliding the excited precursor ions with an

inert gas such as nitrogen, helium or argon [65]. As a result, the precursor ions become unstable and will decompose into the fragment ions. MS/MS spectra will give *m/z* ratios and intensities for all fragment ions of single precursor. By operating CID at lower energies, fragmentation occurs at the amide bond between amino acids, instead of at the side chain groups [66]. In PSD, fragmentation is accomplished by the laser. The precursor ion is selected using an ion gate, where it then travels to a field free region for fragmentation [67]. In both cases, the resulting spectra allows for the amino acid sequences of peptides to be determined.

Many types of ionization techniques exist today; where they can are categorized as "hard" or "soft", depending on their ability to keep the analyte molecules intact during ionization. "Hard" ionization fragments analytes into their daughter ions which can provide structural information. One "hard" ionization technique commonly used is electron ionization (EI). In this, an electron beam is based through the gas-phased sample, whereby molecules collide with the neutral analyte to give fragmented ions. An application of this would be to study the structures of organic and inorganic molecules. However, with a developing interest in studying whole intact molecules, "hard" ionization was incapable of doing so. As such, with the development of "soft" ionization where little fragmentation occurs, molecules remain intact. This then allows for ions to be generated from large, non-volatile compounds. Overall this makes the analyses of biological compounds such as polysaccharides, drug metabolites and proteins possible. Today, there are two "soft" ionization methods, electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI), widely used in scientific fields. Both will be discussed in greater details in section below.

A.1.1 Electrospray Ionization (ESI)

Since 1968, ESI has been incorporated largely into analyzing biomolecules. Figure A.1 is a schematic describing the process of ionization. To start, a dilute polar solution containing the analyte first flows through a hypodermic needle at a low flow rate [68].

This needle will charge the surface of liquid passing through, where Coulombic forces disperse it into charged droplets. As the droplets drift towards the end of the chamber, evaporation of the surrounding solvent occurs, thereby decreasing its diameter. As a consequence, the charge density on the surface will increase until it reaches its Rayleigh limit. Once this limit occurs, a Coulombic explosion occurs were daughter droplets are produced. This process of solvent evaporation and explosion repeats until the magnetic field is strong enough to desorb ions. The resulting charged droplets are then suitable for mass spectral analyses. The ions produced in ESI carry multiple charges with the general formula of $[M+nH]^n$ in positive mode, and $[M-nH]^n$ in negative mode. This multiple charging allows for analysis or proteins and peptides with limited *m/z* range analyzers. For small molecules (< 2000 Da) such as peptides; singly, doubly or triply charged ions are common seen. For large molecules (> 2000 Da) such as proteins, series of multiply charged ions are observed.

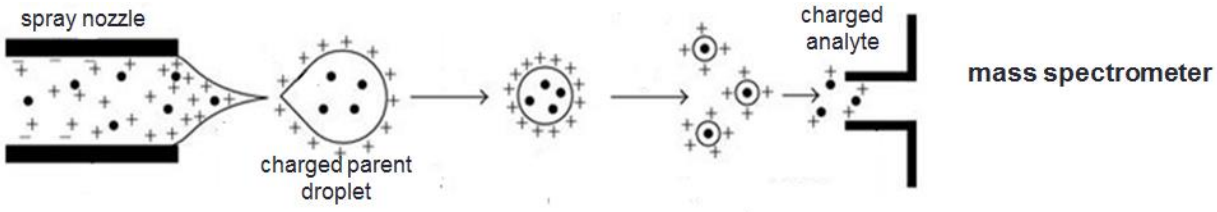

Figure A.1. Schematic for electrospray ionization (ESI). The sample-containing solution exits the charged needle as an aerosol. Upon desolvation, the aerosol diminishes in size until a Coulombic explosion occurs, producing daughter ions which move towards the inlet of the mass spectrometer.

With the development of ESI, studying large biomolecules became an area of interest. However, along with these analytes, came their complex cellular mixtures. Because of this complexity, spectra became too difficult to interpret. As a result, this led to a need for chromatographic separations. With the coupling of liquid chromatography (LC) and ESI, giving LC-ESI, a continuous stream of liquid sample can be directly introduced into the mass spectrometer. Individually, LC is described below followed by a detailed explanation of LC-ESI-MS as a hyphenated technique.

To start, the sample mixture first undergoes analytical separation by LC. In LC, the partitioning of analyte molecules is based on physical factors such as their solubility in water and organic solvents, net charge and size [69]. In particular, separations are accomplished by using the varying affinities of compounds towards two phases, either the mobile or stationary phase. The mobile phase is a solution mixture containing the analyte. This is injected into the column and continuously flows throughout. In contrast, the stationary phase consists of porous beads, packed and held within the separating column. As the components elute off the column, their signals are detected using UV, fluorescence or electrical conductivity. The resulting measurements are presented as a chromatogram, which plots the signal response of the components against their retention times. With the combination of retention times, peak intensities and peak areas, components can be successfully identified. In 1970, a variation of LC known as high performance liquid chromatography (HPLC) was developed. In HPLC, high pressure is used to generate the flow needed for the solution to pass through the column. Today, HPLC is one of the most commonly used chromatographic techniques used. In LC, the mobile phase moves along the column with very little pressure. However, HPLC differs as it uses much high pressures to generate the flow rate of the mobile phase.

Within LC, there are two types known as normal phase liquid chromatography (NPLC) and reverse phase liquid chromatography (RPLC). They are differentiated by the type of mobile and stationary phases used. In NPLC, the stationary phase commonly used is silica or organic materials with functionalities like cyano or amino groups. Because of these, the stationary phase is a polar phase. The mobile phase will then be nonpolar, where water and organic solvents are common. As the molecules partition between the two phases, the order of elution will be least polar molecules first, and the more polar ones later on. In order to elute the polar compounds, solvents of increasing polarity are used. In RPLC, the stationary phase is composed of hydrocarbons such as C_{18}, making it nonpolar . As for the mobile phase, it is typically a mixture of water and organic solvents. Therefore, from this alone, one can see that this type of chromatography has been termed "reverse" as it is the opposite of NPLC. The order of elution is thus, from highly polar compounds to nonpolar ones. For nonpolar compounds, solvents of higher hydrophobicity will be needed to elute compounds from the column.

By using LC alone, separation of mixtures can be done via the competition of the mobile or stationary phases for analytes. However, a significant problem that may arise is when components elute at very similar retention times. This leads to overlap of peaks and identification is not as accurate. By introducing MS as the detector, mass spectra will supplement the chromatographic separations. In a LC-ESI-MS setup, as separations elute off the column, they undergo the "soft" ionization next. This can be achieved in either the online or offline modes. In online, the elution of compounds and ionization are continuous. In offline, eluted fractions are collected first and then introduced into the mass spectrometer. Of these two modes, online continuous ionization is preferred. In this mode, a metal capillary is used instead of the hypodermic needle described previously. The sample solution is pumped through the HPLC inlet and nebulized at the end to form the spray. The resulting ionic species will then enter a capillary that carries them into the high vacuum for mass spectral analyses. Overall, this will provide sufficient information where masses and structures from MS, will complement the observed retention times of LC.

A.1.2 Matrix-assisted Laser Desorption Ionization (MALDI)

The term "matrix-assisted" laser desorption was first introduced by Karas and colleagues in 1985 [70]. In their work, a 1:1 ratio mixture of the amino acids alanine and tryptophan underwent ionization and mass spectrometry at a wavelength at 266 nm. At this wavelength, alanine was incapable of ionizing. However, when they analyzed the mixture, a strong signal was observed for both alanine and tryptophan. Because of this observation, they proposed that tryptophan acted as an absorbing matrix which aided ion formation of alanine. It was not until 1989 where laser desorption ionization of large intact molecules was possible. Tanaka *et al.* developed the concept of MALDI that is used in research today [71]. In their research, the new sample preparation called "ultrafine metal plus liquid matrix method" was developed. By combining the use of a conductive metal and the "matrix-assisted" laser desorption discovered by Karas, they were able to ionize an organic compound up to 34 kDa. Tanaka and his colleagues were

able to demonstrate the capability of MALDI for ionizing large molecules, in particular, biomolecules. In research today, the concepts of MALDI discovered by Karas and Tanaka remain the same. Both a conductive metal plate and a matrix are used to aid in ionization.

One large difference between MALDI in the past and present is the matrices being used. As a matrix, the compound must absorb light in the UV region. As such, matrices must absorb at the same wavelength as the laser being used. Consequently, in the past, only molecules carrying aromatic residues were analyzed using MALDI [72]. Because not many aromatic residues may be present, it was clear that the need for a compound to aid ionization was necessary. Therefore, due to this lack of one, matrices were developed. The theory behind developing matrices was to turn non-ultraviolet (UV) absorbing molecules to ones that could. To qualify as a matrix, the compound must be relatively water-soluble, not too volatile to prevent rapid evaporation, and it must not be too

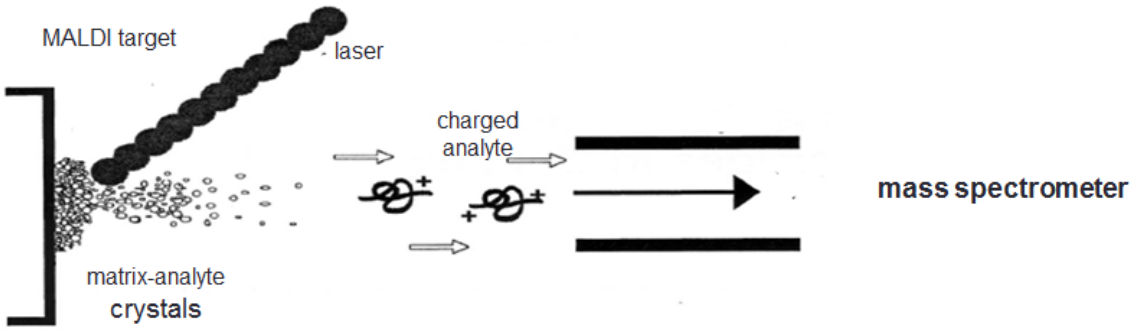

Figure A.2. The schematic for matrix-assisted laser desorption ionization (MALDI). Here, the analyte is mixed with a matrix in a 1:1 ratio and spotted onto the target. Upon air-drying, crystallization occurs between matrix and analyte. A laser is used to desorb these molecules, producing singly-charged ions that can be introduced into the mass spectrometer.

chemically reactive [72]. Following these requirements, there are three matrices commonly used today; α-cyano-4-hydroxycinnamic acid (CHCA), 2.5-dihyroxybenzenoic acid (DHB) and sinapinic acid (SA). They can be divided into two

categories; derivatives of cinnamic acid which include CHCA and SA, and derivatives of aromatic compounds where DHB lies. Matrices contain functional groups such as –OH, –SH or –NH, which can supply the proton needed for protonation [73].

In MALDI, analytes are dissolved in a solution containing excess matrix, where typically a 1:1 volume-to-volume ratio is used. The amount of matrix must be excessive enough to completely solvate the analyte. This sample-matrix mixture is then spotted onto a MALDI target, typically a conductive metal plate, where the spots are allowed to air-dry. Upon drying, crystallization between the analyte and matrix occurs. Once dried, the target can be introduced into the mass spectrometer. During ionization, as shown in Figure A.2, a laser beam will hit the spots where absorption of light by the matrix occurs. Two common lasers are seen today; the first is nitrogen laser with a wavelength of 337 nm, and second the neodymeium-doped yttrium nitrogen aluminum garnet (Nd:YAG). Nd:YAG comes in two types, frequency tripled which absorbs at 355 nm and quadrupled at 266 nm [74]. The energy absorbed is transferred to the analyte, causing it to desorb from the target plate. This creates a plume of ions which can be accelerated towards the detector. In comparison to ESI, MALDI predominantly produces singly charged ions of the general formula $[M+H]^+$ and $[M-H]^-$, independent of the mass. The ions will carry different *m/z* ratios, therefore reaching the detector at different times to produce a mass spectrum.

A.1.3 Comparison between ESI and MALDI

Both ESI and MALDI are "soft" ionization techniques allowing ionization of whole biomolecules. As discussed, the methods of ionization vary drastically. Each carries its own advantages and disadvantages. One of the major differences between ESI and MALDI is the ability to couple ESI with LC. MALDI, unfortunately, does not possess the capacity and is called an offline method. A second difference is that MALDI has a higher tolerance towards salts, whereas ESI cannot have samples that contain too many. When a sample has a higher concentration of salts, then MALDI may be the better choice. Lastly,

both differ in the types of ions formed. ESI has the capacity to form multiply charged ions, and because of this, is said to have an unlimited m/z range [74]. Analytes with larger mass can simply appear as lower *m/z* peaks carrying multiple charges. The production of multiply charged ions allows for the production of a larger number of daughter ions in MS/MS. For instance, if the multiply charged ion is $[M+4H]^{4+}$, then it can be expected that a maximum of four daughter ions can be produced. With the increase in the number of daughter ions, the greater the chances of confirming the amino acid sequence. MALDI, in comparison, predominantly favours the formation of single charged ions. Consequently, the MS/MS can only produce a maximum of one daughter ion. Therefore, in comparison with ESI, the determination of the amino acid sequences may not be as reliable. What is commonly seen in research today is to use LC-ESI-MS for complex mixtures and MALDI MS for simpler ones. However, this need not be the ultimate deciding factor. Which one should be used depends on what the technique is applied towards, and what outcomes are being sought.

A.2 Proteomics

With the successful development of ESI and MALDI, the applications of MS became focused on studying biological components. Emphasis was originally placed on studying genes, collectively known as the genome; however, a shift towards studying proteins has occurred within the past decades. The genome provides information corresponding to the expression of proteins. The study of the proteome, that is all proteins expressed by the cell at a given time and state, is what is called “proteomics” today. This allows for large-scale investigations of the genome and cellular functions indirectly at the protein level [75]. As such, proteomics is known to be as a post-genomic science [76].

In proteomics, the first crucial step is to isolate proteins from complex biological mixtures. This alone is extensive and more challenging when compared to the genome. Complications arise due to the fact that one gene can easily encode for several proteins

differing anywhere from function to location [76]. In addition, the isolation of proteins is difficult as a result of complex backgrounds and low abundances. And so, it is because of these difficulties that an analytical technique such as mass spectrometry is needed. By incorporating MS into the proteomic work flow, numerous types of biological information can be explored.

One widely used application of MS is peptide mass fingerprinting (PMF). In PMF, protein mixtures are subjected to site-specific enzymes, which will generate peptides. These experimentally-determined peptides are then analyzed using MS. The resulting spectra are searched against databases containing theoretical peptides produced from in silico digests. Protein sequence databases, such as UniProtKB, are widely available. These are libraries that house the genomes, and likewise the proteins they encode, for a significant amount of species. Unfortunately, when the genome for a species has not been completely identified, the protein database is also incomplete. As a consequence, when running experimental data through them, matches may not occur. Of course, this does not mean the obtained peptides are irrelevant.

When PMF does not provide sufficient information for identification, MS/MS can be used to determine the amino acid sequences. Hunt *et al.* were able to sequence peptides from apolipoprotein B [77]. Apolipoprotein B (apoB) is one of the proteins involved in lipid metabolism. In their work, apoB was digested with the enzyme trypsin. Trypsin cleaves on the C-terminal side of lysine and arginine, except when followed by proline. The resulting peptides underwent MS/MS in a triple quadrupole mass spectrometer, producing spectra where sequences were determined. The determination of these sequences allowed for confirmation of the apoB protein.

Another common application of MS in proteomics is for the detection of post-translational modifications of proteins. Once proteins are folded, they may undergo further modification before being transported to their cellular location. For instance, proteins undergo common modifications such as phosphorylation, the addition of phosphate groups, or glycosylation, which is the addition of sugars. The addition of these

groups will change the masses of said proteins, which can then be successfully determined by MS [78].

Appendix 2: Preparation of Media Used

Stock cultures of *C. albicans* were grown on Sabouraud dextorse agar (SDA) plates. They were prepared as following:

1. Prepare a 6.50% (m/v) solution in water. Heat with stirring to aid in dissolution
2. Autoclave at 121°C for 25 minutes with a 10 minute steam down
3. Once cooled to room temperature, sterile polystyrene 100 mm x 15 mm Petri dishes with stackable lids
4. The cooled plates are then placed into a 37°C oven overnight to ensure no microbial growth has occurred

Yeast nitrogen base (YNB) broth was use for the cell growth of *C. albicans.* It can be prepared as following:

1. Dissolve 6.70 g in 100 mL H_2O to make the solution known as 10X YNB
2. Dissolve 18.00 g of sucrose in 900 mL H_2O.
3. Supplement the sucrose solution with 100 mL of 10X YNB to make a final volume of 1.00 L
4. Sterilize by autoclaving
5. Then filter the final solution

1X phosphate buffered saline (PBS) solution was for washings of cells and preparation of adhered slides. It can be made according to the protocol below: :

1. Dissolve the four components in 800 mL H_2O:

    8.00 g of sodium chloride (NaCl)

    0.20 g of potassium chloride (KCl)

    1.44 g of sodium basic diphosphate (Na_2HPO_4)

    0.24 g of monopotassium phosphate (KH_2HPO_4)

2. Bring the pH to 7.4 using hydrochloric acid (HCl)

3. Then bring the final volume to 1.00 L using water

4. If necessary, sterilize the media via autoclaving

Appendix 3: MALDI MS/MS Raw Spectrum

Once a list of peaks characteristic to *C. albicans* was determined, tandem mass spectrometry (MS/MS) was performed. This second fragmentation would be able to provide the specific amino acid sequence of the selected precursor ion. For a majority of the peaks, second fragmentation was not possible due to the low ion counts. For an adequate spectrum, the precursor ion should have an ion count of 2000. In the cases of peaks that did meet this requirement, their spectra suffered significantly from contamination and background noise. Figure A.3 is the MS/MS spectrum for the *m/z* peak of around 1677, demonstrating this scenario. The presence of the *m/z* peak of around 175 did indicate that this was a peptide, as this corresponds to C-terminal arginine from a tryptic digest. However, other than this, sequencing could not be done. The presence of this many peaks did not allow for the differentiation between relevant amino acids and non-informative background ions.

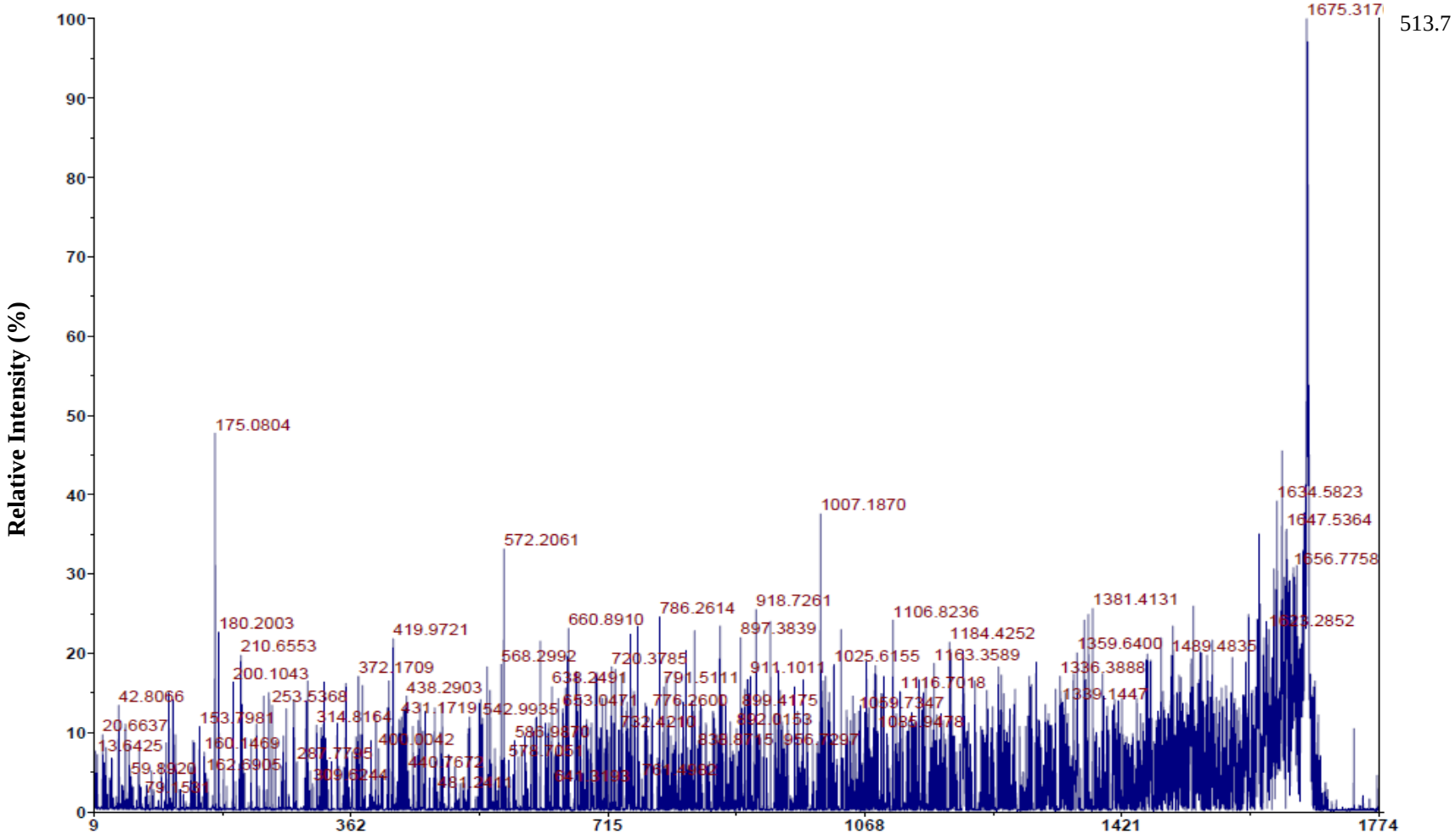

Figure A.3. MALDI MS/MS spectrum for the precursor ion with *m/z* = 1677.80.

Appendix 4: Bicinchoninic Acid (BCA) Assay for Peptide Quantification

To meet the detection limit of the linear trap quadrupole (LTQ) mass spectrometer, 2 µg of peptides were required. When working with peptides, who have molecular masses of 4000 Da or lower, the bicinchoninic acid (BCA) assay is ideal. The BCA assay relies on the generation of a coloured complex between peptide bonds and the Cu^{2+} ion in alkaline environments [79]. The concept of the BCA assay stems from the initially used Lowry reagent, and today it is substituted as it has a higher salt tolerance. The BCA assay is composed of two major reactions; the first is known as the biuert reaction, and the second is chelation [79]. The biuret reaction is where an organic compound know as biuret reacts with Cu^{2+} to form a light blue coloured complex [80]. This reaction is shown in Figure A.4. The major product of this reaction is the Cu^{+} ion, which will be needed in the second step of the BCA assay.

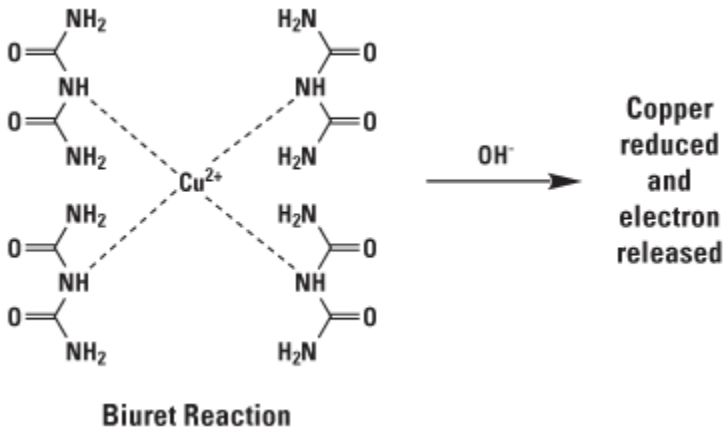

Figure A.4 The first reaction, known as the biuret reaction, of the BCA assay. This reaction reduces Cu^{2+} to Cu^{+} through the binding of the amide bond, which will be needed for the second half of the BCA assay [79].

As seen in Figure A.4, a single Cu^{2+} ion has the ability to bind to four to six amide bonds [79]. In the second reaction shown in Figure A.5, the BCA reagent is chelated to the Cu^{+},

producing the BCA-Cu complex. And it is this complex that allows for indirect measurement of protein. Essentially, the amount of complex produced reflects how much Cu^{+} is produced, which then ultimately indicates how many amide bonds were present to cause the copper reduction. This complex absorbs strongly at a wavelength of 562 nm, however measurements can be taken anywhere between 550 to 570 nm.

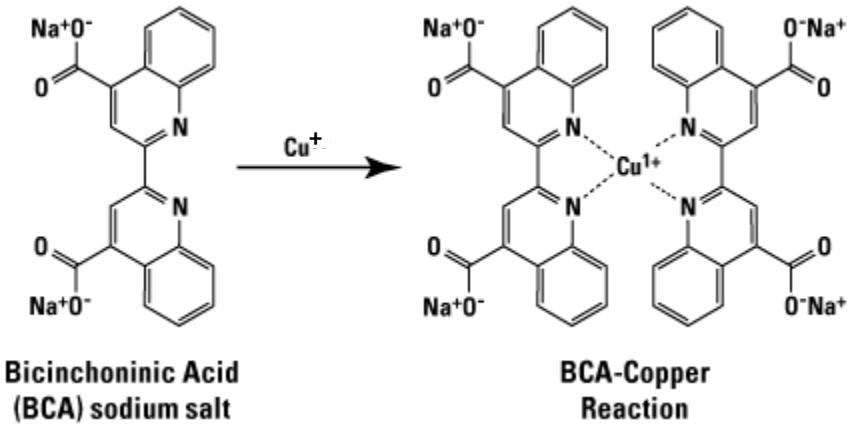

Figure A.5 The second reaction of the BCA assay. Here, Cu^{+} reacts with the BCA salt. The production of the complex corresponds to the amount of protein originally bound to the biuret reagent [79].

To create the standard curve, known concentrations of bovine serum albumin (BSA) were used. A total of eight standards were used. The preparation volumes and final concentrations are displayed in Table A.1.

Table A.1 Standard BSA concentrations and volumes used to determine the standard curve in the BCA assay.

Vial	Volume of H_2O (µL)	Volume OF BSA (µL)	Measured BSA Concentration (µg/mL)
A	400	0	0
B	325	325 from C	125
C	325	325 from D	200
D	325	325 from F	500
E	175	325 from G	750
F	325	325	1000
G	125	325	1500
H	0	300	2000

The protocol for performing the BCA assay was provided by the manufacturer (Fisher Scientific, USA). The buffer in which the peptides are suspended in, in this case ammonium bicarbonate, was diluted by a factor of ten. The diluted ammonium bicarbonate was then mixed in a 1:1 (v/v) ratio with the BSA standards. 20 µL of this mixture was pipetted into sixteen wells, two per concentration, in a 96 microwell plate. For the digested peptides, they were diluted by a factor of ten using the diluted ammonium bicarbonate. 10 µL of the diluted peptides were then pipetted into two wells each, and were further diluted by the addition of 10 µL of water. Overall, this ensures the total volume of standards and analytes were consistent. To each well, 180 µL of the BCA reagent was added. The microwell plate was incubated at 37°C for 30 minutes. After 30 minutes, the plate was cooled to room temperature before measurements were taken. Readings were taken on 1420 Multilabel Counter Victor^{3}V (Perkin Elmer, Canada) at a wavelength of 560 nm. The corresponding software used was the Perkin Elmer 2030 Manager.

Appendix 5: ZipTip Protocol

Prior to analyses by HPLC-ESI-MS/MS, our digested peptides underwent sample cleanup and concentration. This was accomplished by using 10 µL ZipTip C_{18}. The protocol used between different samples should be optimized. For our purposes, the protocol used in our research will be discussed.

The list of solutions is as follows:

- wetting solution: 50% acetonitrile
- equilibration and wash solution: 0.1% trifluoroacetic acid
- elution solution: 80% acetonitrile

The protocol consists of five steps, which are described below:

1. Decontamination of the ZipTip using the wetting solution. This will remove non-specific peptides.
    a. Using 40 µL, wet the resin by pipetting and expelling the waste. Perform this twice
2. Equilibration with the equilibration and was solution. This will remove acetonitrile
    a. Using 30 µL, introduce the solvent by pipetting and expelling the waste. Repeat this two times
3. Acidification making the peptides negatively charged to bind the resin.
    a. To acidify peptides, 0.5 µL increments of 0.5% trifluoroacetic acid are added. The pH is checked after each addition until a pH of around 4 is reached
    b. The acidified peptides are then pipetted up and down ten times
4. Removal of ions from the ZipTip using the equilibration and wash solution. Peptides will remain bound to the resin
    a. Using 20 µL, the solution is pipetted up and down five times
5. Elution of peptides using the elution solution

a. Using 5 µL of the solution, the peptides on the resin are pipetted up and down ten times
b. The solution is kept as it contains the peptides

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April 19, 2012. DNA-modified screen-printed electrodes for the electrochemical detection of mercury. Poster presented at the 11th Annual Chemical Biophysical Symposium, Toronto, CA.

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9504 Protein Folding Grade: 88
9658 Seminar Grade: 82