

Development of Mass Spectrometry Methods for Direct Analysis of Proteins in Microbes

By

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A thesis submitted to the University of Birmingham
for the degree of DOCTOR OF PHILOSOPHY

School of Biosciences
College of Life and Environmental Sciences University of Birmingham
November 2024

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ABSTRACT

Mass spectrometry (MS) is a powerful technique used to analyse proteins. A specific version, native liquid extraction surface analysis (LESA) mass spectrometry (MS) allows us to study proteins in their natural form, providing information on their tertiary and quaternary structure directly from different types of samples. In this thesis, this technology was developed for the analysis of proteins from living colonies of microorganisms, including bacteria and yeast. This design enables a deeper understanding of microbial proteomes in their native state, which is essential for studying biological processes, discovering new drug targets, and improving biotechnological applications.

To improve the approach of detecting different native proteins, methods were designed combining native LESA MS with various technologies, including electroporation (which helps extract proteins from cells), a filtering method named high-field asymmetric waveform ion mobility spectrometry (FAIMS), colony washing and a specialised particle system known as styrene maleic acid lipid particles (SMALPs).

This work demonstrates that the combination of native LESA MS and electroporation is effective for detecting native intact proteins in *Escherichia coli* K12 and *Saccharomyces cerevisiae* living colonies. Proteins were identified by

use of a variety of fragmentation techniques. In some cases, use of FAIMS filtering improved detection, allowing for a more detailed analysis of proteins in yeast *Saccharomyces cerevisiae*.

The integration of native LESA MS and prior colony washing enabled detection of outer membrane proteins in gram-negative bacteria. Native LESA MS was also applied to the analysis of two membrane proteins (ZipA and Sav1855) encased in SMALPs. The results show detection of truncated versions of the proteins.

This research demonstrates the potential of LESA MS for studying proteins directly from living microorganisms, which could be valuable for relative disease research and drug development.

ACKNOWLEDGEMENTS

I would like to thank my incredible supervisor, Professor Helen J. Cooper, for her patience, expert guidance, and encouragement during my PhD, and my supervisor, Professor Robin C. May, for his support. I am also deeply grateful to the Darwin Trust for funding my PhD and this work.

I would also like to thank the people from the Cooper and Leney groups for their support and help with my work and life in the lab: Dr Aneika Leney, Dr Oliver Hale, Dr Emma Sisley, Dr James Hughes, Dr Sarah Vickers, Jaspreet Sound, and Danielle Kay. I truly appreciate the time I spent with everyone. I would like to thank Jana Havlikova for teaching me the skill of culturing bacteria and thank Dr. Todd Mize and Dr. Jinglei Yu for their help with the instrument. I also thank Professor Timothy Dafforn, Pooja Sirdhar, Dr Joan Geoghegan, and Dr Saverio Brogna for kindly providing samples for this work.

Finally, I would like to thank my friends and family for their love, support and encouragement throughout my PhD.

OUTPUTS

Peer Reviewed Journal Articles

- Du, Yuying, Robin C. May, and Helen J. Cooper. "Native ambient mass spectrometry of intact protein assemblies directly from *Escherichia coli* colonies." *Chemical Communications* 58.48 (2022): 6857-6860. DOI: 10.1039/D2CC02085H
- Du, Yuying, and Helen J. Cooper. "Native ambient mass spectrometry of membrane proteins directly from bacterial colonies." *Chemical Communications* 61 (2025): 4168-4171. DOI: 10.1039/D4CC03881A

Oral presentations

- Du, Yuying and Helen J. Cooper. "Native mass spectrometry of intact protein assemblies from bacterial colonies" flash oral. 41st British Mass Spectrometry Society Annual Meeting.
- Du, Yuying and Helen J. Cooper. "Native mass spectrometry of intact protein assemblies from microbe colonies". BMSS Ambient Ionisation & EFA SIG Meeting 2022.
- Du, Yuying and Helen J. Cooper. "Native mass spectrometry of intact protein assemblies from microbe colonies". 42nd British Mass Spectrometry Society Annual Meeting.

Poster presentations

- Du, Yuying and Helen J. Cooper. "Native LESA mass spectrometry of Intact Proteins and Protein Complexes Directly from Living Bacterial Colonies" Emerging Analytical Professionals 2022.
- Du, Yuying and Helen J. Cooper. "Native LESA mass spectrometry of Intact Proteins and Protein Complexes Directly from Living Bacterial Colonies" 24th International Mass Spectrometry Conference.
- Du, Yuying and Helen J. Cooper. "Analysis of Intact Protein Assemblies Directly from Living Yeast Colonies by Native LESA Mass Spectrometry" 71st ASMS Conference on Mass Spectrometry and Allied Topics.
- Du, Yuying and Helen J. Cooper. "Native mass spectrometry of intact protein assemblies from microbe colonies" 43rd British Mass Spectrometry Society Annual Meeting.
- "Analysis of intact membrane proteins directly from living bacterial colonies by native LESA mass spectrometry" Native Mass Spectrometry SIG 2023.
- Du, Yuying and Helen J. Cooper. "Analysis of intact membrane proteins directly from living bacterial colonies by native LESA mass spectrometry" 72nd ASMS Conference on Mass Spectrometry and Allied Topics.

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ABBREVIATIONS AND GLOSSARY

AC - Alternating Current

ACBP - Acyl-CoA-Binding Protein

AGC - Automatic Gain Control

AHP1 - Peroxiredoxin

AP-MS - Affinity-Purification Mass Spectrometry

APCI - Atmospheric Pressure Chemical Ionisation

Aqp-0 - Aquaporin-0

C8E4 - Tetraethylene Glycol Monooctyl Ether

CAT - Cationic Amino Acid ABC Transporter

CE - Capillary Electrophoresis

CEM - Chain Ejection Model

CF - Compensation Field

CHCA - α -Cyano-4-Hydroxycinnamic Acid

CI - Chemical Ionisation

CID - Collision-Induced Dissociation

CMC - Critical Micelle Concentration

CRM - Charged Residue Model

CSF - Compensation Scaling Factor

CSP - Cold Shock-like Protein

CV - Compensation Voltage

CYPH - Peptidyl-Prolyl Cis-Trans Isomerase

DC - Direct Current

DD - Dried Droplet

DDM - n-Dodecyl- β -D-Maltoside

DESI - Desorption Electrospray Ionisation

DHB - 2,5-Dihydroxybenzoic Acid

DIBMA - Diisobutylene Maleic Acid

DTIMS - Drift-Time Ion Mobility Spectrometry

DV - Dispersion Voltage

ECD - Electron Capture Dissociation

EI - Electron Ionisation

ENO - Enolase

EPS - Extracellular Polymeric Substances

ESI - Electrospray Ionisation

ESI-MS - Electrospray Ionization Mass Spectrometry

ETD - Electron Transfer Dissociation

EThcD - Electron Transfer Higher-Energy Collision Dissociation

ETnoD - Nondissociative Electron Transfer Dissociation

FAB - Fast Atom Bombardment

FADL - Long-Chain Fatty Acid Transport Protein

FAIMS - Field-Asymmetric Waveform Ion Mobility Spectrometry

FD - Field desorption

GPMA - 2,3-Bisphosphoglycerate-Dependent Phosphoglycerate Mutase

HCD - Higher-Energy Collision Dissociation

HPr - Phosphocarrier

HSP 12 - 12 kDa Heat Shock Protein

HSP31 - Glutathione-Independent Glyoxalase

ICP-MS - Inductively Coupled Plasma-High Resolution Mass Spectrometry

IEM - Ion Evaporation Model

IM-MS - Ion Mobility Mass Spectrometry

IMS - Ion Mobility Spectrometry

IRM - Ion Routing Multipole

IRMPD - Infrared Multiphoton Dissociation

LB - Lysogeny Broth

LC - Liquid Chromatography

LESA - Liquid Extraction Surface Analysis

LILBID - Laser-Induced Liquid Bead Ion Desorption

LPS - Lipopolysaccharides

MALDI - Matrix-Assisted Laser Desorption Ionisation

MALDI-TOF - Matrix-Assisted Laser Desorption Ionization Time-Of-Flight

MaIE - Maltodextrin-Binding Protein

MD - Molecular Dynamics

MS - Mass Spectrometry

MSPs - Membrane Scaffold Proteins

NAMS - Native Ambient Mass Spectrometry

NCE - Normalised Collision Energy

Nano-DESI - Nanospray Desorption Electrospray Ionisation

Nano-ESI - Nanoelectrospray Ionisation

OMPA - Outer Membrane Protein A

PCKA - Phosphoenolpyruvate Carboxykinase ATP

PD - Plasma Desorption

PGK - Phosphoglycerate Kinase

Psi - Phosphate Starvation-Inducible

PTCR - Proton Transfer Charge Reduction

PTM - Post-Translational Modification

RF - Radio Frequency

RP - Reverse-Phase

SA - Sinapic Acid

SDS-PAGE - Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis

SEC - Size Exclusion Chromatography

SHMT - Serine Hydroxymethyl Transferase

SMA - Styrene Maleic Acid

SMALPs - Styrene Maleic Acid Lipid Particles

SOD - Superoxide Dismutase

SPB - Solute-Binding Protein

TEV - Tobacco Etch Virus

TOF - Time-Of-Flight

TPIS - Triosephosphate Isomerase

TRX2 - Thioredoxin-2

TSB - Tryptic Soy Broth

TSI - Thermospray Ionisation

TWIMS - Traveling-Wave Ion Mobility Spectrometry

UBI4P - Polyubiquitin),

YPD - Yeast Extract Peptone Dextrose

Chapter 1

Introduction

This thesis focuses on the development of mass spectrometry methods for in situ analysis of intact proteins in their native, i.e., folded state in microorganisms, including bacteria and fungi.

Understanding proteins in bacteria and yeast is important, as microbes play a crucial role in public health, disease progression and biotechnologies. Many microbes contribute to processes such as antibiotic resistance, pathogenicity, and industrial enzyme production. Analysing their proteins at the ambient level could provide more in-depth information on their functions, interactions, and potential applications in medicine and biotechnology. By analysing native proteins directly from microbe colonies, this research aims to bridge the gap between laboratory-based biochemical studies and microbial behaviour in a natural environment.

The first chapter details the background by giving an overview of techniques and ideas employed in the research. The second chapter briefs the materials, and the specific technical procedures applied to achieve research goals. Chapters 3-5 present the results. In Chapter 3, a workflow combining native liquid extraction surface analysis (LESA) mass spectrometry and electroporation was designed to

detect native intact proteins in a commonly used non-toxic bacterial strain model *Escherichia coli* (*E. coli*) K12. Chapter 4 describes extension and development of the method for analysis of native proteins in yeast, specifically *Saccharomyces cerevisiae* (*S. cerevisiae*). Chapter 5 describes the development of mass spectrometry methods for the detection and identification of intact native membrane proteins in microorganisms. Chapter 6 presents a summary of the research, along with a discussion of future endeavours and application of mass spectrometry to the identification of proteins in microorganisms.

1.1 Introduction to mass spectrometry

Mass spectrometry is a technique that can be used to identify the components of chemical or biological samples. First, gas-phase ions are generated before being separated on the basis of their mass-to-charge (m/z) ratio by magnetic or electric fields. The key components of a mass spectrometer are the ion source, mass analyser and detector.

1.1.1 Ionisation and sampling

1.1.1.1 Overview

Ionisation is the first step in mass spectrometry analysis [1]. This process involves charging of molecules and transferring them into the gas phase using different physical techniques. It is a critical step, as it fundamentally affects the types of analytes detected and the efficiency of ion detection.

There are various ionisation techniques, including soft ionisation techniques, which mainly generate intact ions of the molecular species, and hard ionisation techniques, which are highly energetic and result in extensive fragmentation. Electron ionisation (EI), which uses an electron beam to ionise molecules, is the most common hard ionisation technique [2]. EI can ionise compounds in the gas phase that are sufficiently volatile and thermally stable. Apart from EI, other hard ionisation methods include Plasma Desorption (PD), where the sample is ionised by fission fragments or high-energy particles [3, 4], and thermospray ionisation (TSI), which causes ionisation by passing a heated liquid sample through a heated capillary [5-7].

As mentioned above, hard ionisation techniques result in extensive molecular fragmentation. Soft ionisation methods involve lower energy, resulting in less fragmentation during ionisation, making it suitable for biological samples that require the structure of intact biological molecules to be preserved. For example,

chemical ionisation (CI) can be used. In CI, charge is transferred from reagent gases such as methane or ammonia, to molecules to form protonated ions with minimal fragmentation [8]. Whereas CI is performed in vacuum, a further development Atmospheric Pressure Chemical Ionisation (APCI) was designed to be conducted in atmospheric pressure and makes use of a corona discharge to ionise molecules [9, 10]. Field desorption (FD), another soft ionisation technique, is used for ionising non-volatile, thermally labile compounds by use of a surface coated-emitter and strong electrical field [11, 12]. Fast Atom Bombardment (FAB) makes use of a beam of neutral atoms to ionise sample mixed with matrix to avoid thermal degradation of compounds, and is suitable for detecting non-volatile, thermally labile, or polar compounds [13]. In addition to the soft ionisation methods discussed above, the two most commonly used soft ionisation methods are matrix-assisted laser desorption ionisation (MALDI) and electrospray ionisation (ESI).

1.1.1.2 Matrix-assisted laser desorption ionisation (MALDI)

MALDI is one of the most widely used soft ionisation technologies [14]. It is suitable for analysis of large biomolecules including proteins, peptides, nucleic acids, and polymers [14-17]. The sample is placed on a metal plate to dry and crystallise after being mixed with a small compound that acts as a matrix which can absorb laser radiation of 266 nm, 337 nm or 355 nm [18-22]. Traditional

MALDI is a vacuum technique in which the dried matrix on the plate is exposed to a pulsed laser, absorbs the energy and transfers protons to analyte [18]. After energy absorption, the matrix is desorbed simultaneously transferring charged analytes into the gas phase, see Figure 1.1. With the protection that the matrix provides for analyte, the ionisation usually happens without extensive fragmentation and singly charged positive ions are produced.

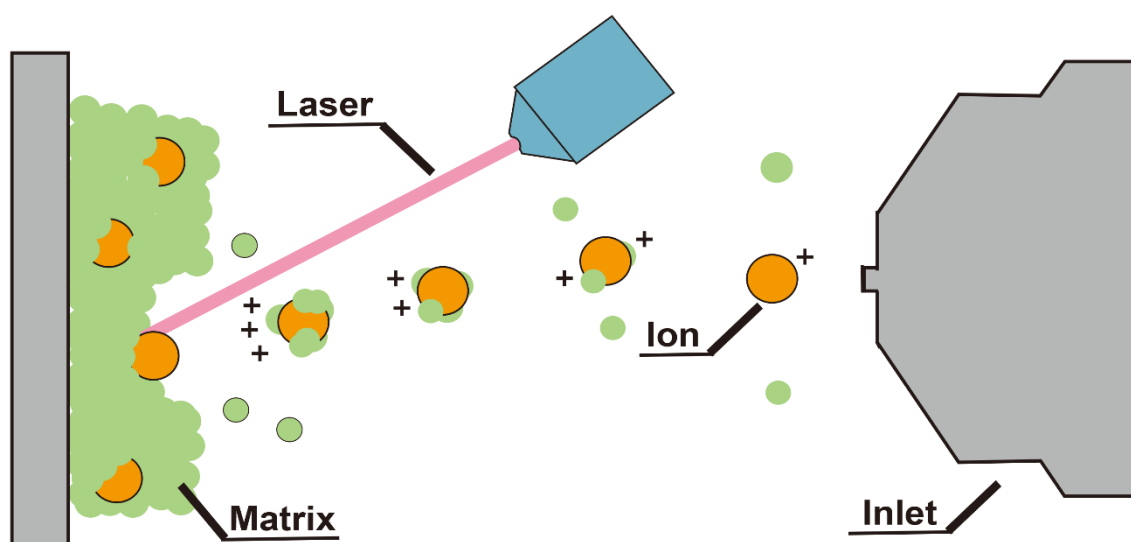


Figure 1.1 Schematic representation of a MALDI source. A laser is directed at a sample coated with an energy-absorbing matrix. Upon laser irradiation, the matrix and analyte are desorbed from the surface for further analysis by mass spectrometry.

The matrix plays a critical role in MALDI. An ideal matrix should evaporate easily, absorb the laser efficiently, form small and uniform crystals, and be compatible with both the analyte and the mass spectrometer [23]. The matrix performs key

functions, including promoting soft ionisation and facilitating effective proton transfer for successful ionisation. The commonly used matrixes for protein and peptide detection include 2,5-dihydroxybenzoic acid (DHB), α -cyano-4-hydroxycinnamic acid (CHCA), and sinapic acid (SA) [23, 24].

Sample preparation for MALDI involves incorporating a matrix into the sample system prior to laser desorption. The commonly used preparation methods include dried droplet (DD) method, layering methods and spray coating method. Among these methods, the dried-droplet technique is the most traditional, recognised for its simplicity and reliable results. In this approach, a mixture of matrix and analyte is applied to the MALDI plate, where it is left to evaporate for analysis. However, for certain samples, this method presents limitations in surface uniformity, signal reproducibility, and detection sensitivity [23]. To address these issues, various layering techniques have been developed, including the thin layer method [25], two-layers method [26, 27], sandwich method [28, 29], fast evaporation method [30, 31] and ultra-thin layer method [32]. Moreover, the spray coating method, which involves spraying the matrix onto the target prior to the application of the sample, is commonly used in MALDI imaging [33-38].

The theory for MALDI ionisation process is still inconclusive, however, the current model includes two steps [39]. In brief, the formation of ions is initially carried out by irradiation of the matrix sample mixture. These ions are further irradiated in the gas plume generating secondary ions for MS detection. This process tends

to generate singly charged ions which are suitable for detection by time-of-flight (TOF) analysers, due to their advantages of a wide m/z range a compatibility with the non-continuous ion beams generated by MALDI.

1.1.1.1 Electrospray ionisation (ESI)

ESI is a soft ionisation method that produces ions by applying a high electric field between the sample solution and the MS inlet [40]. The analyte, dissolved in an appropriate solvent, is delivered through a syringe and directed toward the inlet via an emitter. The applied electric potential, combined with the pump's backing pressure, causes the charged solution droplets to overcome surface tension, forming a Taylor cone [40, 41], shown in figure 1.2. As the solution exits the emitter, it disperses into fine droplets, which move toward the inlet. During this movement, the droplets undergo evaporation and fission, leading to an increase in charge density as they approach the MS inlet [40].

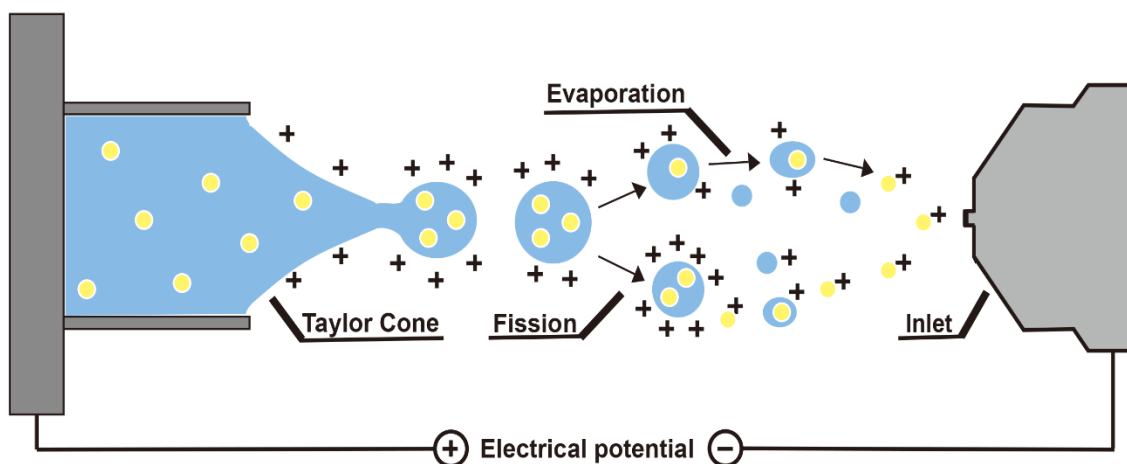


Figure 1.2 Schematic representation of Electrospray ionisation (ESI) source. The left of diagram illustrates the Taylor cone at the tip of an electrospray device. As the solvent evaporates from the droplets, the charge density increases, eventually causing fission. This cycle of evaporation and fission produces progressively smaller droplets until the solvent is completely evaporated.

To explain the mechanism of ion release and ejection from a charged droplet, three models have been proposed, shown in figure 1.3. Among them, the charged residue model (CRM) is the most relevant for this thesis, as it is commonly associated with the ionisation of large proteins [42-45]. In this model, as the solvent evaporates and fission occurs, the droplet became smaller and the charge carried by the droplet is directly transferred to the analytes [42]. During this process, the proteins are cooled and do not face an energy barrier when they are later accelerated in the electric field, thus preserving their structural integrity [44].

Low molecular weight ions and small organic ions are thought to be transferred by the ion evaporation model (IEM) [42, 45-48]. The ion experiences two

opposing forces: one that pulls it back toward the droplet due to the image charge created by solvent polarization, and another that pushes it away from the droplet due to the repulsion from the excess charge present in the droplet [42, 49]. The emission of ions happens via the creation of an extended solvent connection at the droplet's surface, which ultimately breaks down, leaving a charged droplet and a cluster composed of the analyte along with a few solvent molecules [42, 49].

The chain ejection model (CEM) describes the process by which denatured proteins are released from a charged solvent droplet [42, 45, 48]. When proteins denature and unfold (due to exposure to acidic conditions), the disordered chain will expose hydrophobic residues which were previously hidden in the protein's core. This hydrophobic nature makes it unfavourable for the unfolded protein to stay inside a charged nanodroplet, causing it to migrate to the surface. From there, the protein chain is sequentially ejected into the vapour phase, beginning with one terminus [42, 48].

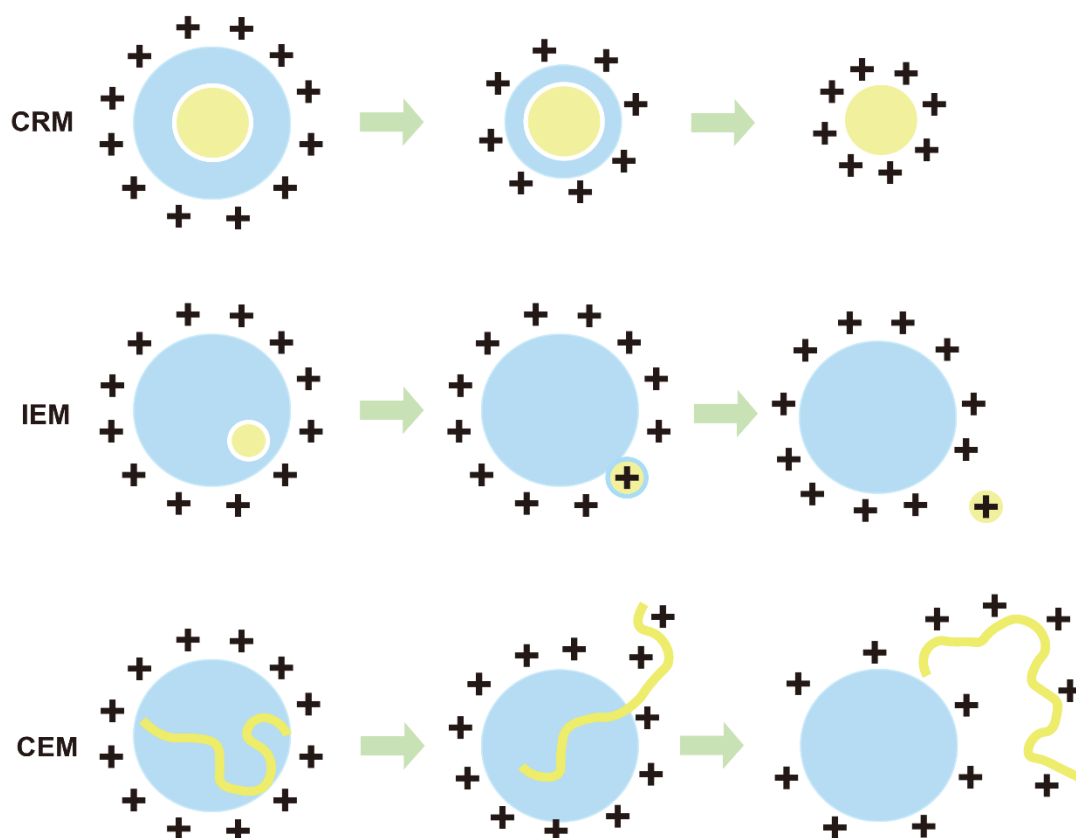


Figure 1.3 Illustration of ion desorption from a charged droplet during electrospray ionisation (ESI), demonstrating the CRM, CEM, and IEM models.

ESI is suitable for coupling with separation techniques such as liquid chromatography (LC) and capillary electrophoresis (CE) [45, 50]. Electrospray ionisation is particularly well-suited for determining the molecular weight of large biomolecules, as it allows for multiple charge states to be detected. This results in high mass measurement accuracy when analysing proteins within the 10,000-100,000 Da range [45].

As ESI technology became more miniaturised, low-flow-rate electrospray was developed to improve sensitivity [45, 51, 52]. Nanospray ESI was then designed,

where the sample is electrosprayed from a metal-coated capillary (or needle) or a platinum wire coiled capillary with a tiny opening (1–10 μm) by applying a voltage [45, 53, 54]. This method is particularly suitable for samples that are available in limited quantities.

1.1.1.4 Ambient ionisation

Ambient ionisation techniques offer the benefits of rapid analysis and the flexibility to examine various sample types, sizes, and shapes in an open environment, while allowing for independent control of desorption and ionisation, providing additional insights beyond traditional methods [55]. Compared to other ionisation techniques, it requires simpler (or no) sample preparation. Solid-liquid extraction-based ionisation techniques are the most prevalent in ambient sampling, as they are easy to set up and compatible with commercial ESI mass spectrometers, with all ambient surface sampling methods using some form of electrospray ionisation [55-58]. These techniques include LESA [59], desorption electrospray ionisation (DESI) [60] and nanospray desorption electrospray ionisation (nano-DESI) [61-64]. LESA is the main technique used in this work.

1.1.1.5 Desorption electrospray ionisation (DESI)

DESI is a commonly used ambient ionisation technique related to ESI [60]. In the DESI process, shown in Figure 1.4, an electrohydrodynamically nebulised

solution (typically a water-alcohol mixture, sometimes with added reagents) is accelerated by high nebulizing gas velocities and applied directly onto a solid sample surface [65]. These droplets then hit the sample surface, causing analyte ions to splash, which are then collected by the atmospheric pressure ion transfer capillary and sent into the MS [65].

Figure 1.4 Schematic of a typical DESI experiment. The sample is deposited and dried on a solid surface; then, a solvent is sprayed at a controlled flow rate under high voltage to create charged droplets. These droplets impact the surface, desorbing and ionizing the analytes, which are then transferred into the mass spectrometer for analysis.

LESA is a liquid microjunction surface sampling technique usually paired with nano-electrospray ionisation [56, 66]. It was first detailed by Kertesz and Van

Berkel in 2010 [59], and was commercialised as the TriVersa NanoMate robotic pipette system (Advion, Ithaca, NY) [56, 66]. This sampling technique requires less complex sample preparation [55, 67] and avoids the dilution associated with other continuous-flow liquid sampling approaches [59], making low-abundance analyte detection easier.

In the sampling process, a small volume (μL) of solvent is aspirated by a robotic pipette from the solvent well and dispensed onto the sample surface. After holding the solvent for a short time (on the order of seconds) during which the analytes dissolve in the solvent, the droplet is re-aspirated and introduced into the MS through a nano-electrospray chip comprising 400 individual nozzles [56].

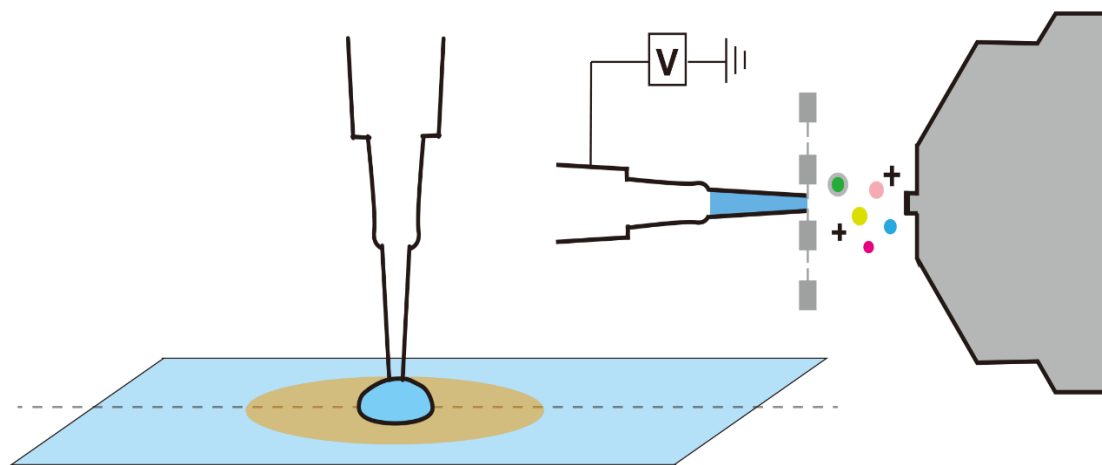


Figure 1.5 Schematic of a LESA experiment. The sample (represented by ellipse in orange) is extracted by solvent (represented by ellipse in blue) using liquid microjunction and then different analytes (represented by circles in different colours) are directly introduced into MS through a nano-electrospray nozzle on the chip.

Compared to DESI which is suitable for detecting small molecules, LESA MS has proven to be efficient in extracting and ionising intact proteins [66, 68-79].

Previous research shows that LESA MS can be applied for top-down intact protein detection in living bacterial colonies by use of an aqueous organic mixture as the solvent [76, 78]. The work demonstrates the potential this technique has to rapidly analyse intact proteins *in situ* from microbial colonies, although higher order structural information is absent in this earlier work due to the denaturation of proteins.

1.1.1.7 Nanospray desorption electrospray ionisation (nano-DESI)

Nano-DESI is a liquid junction-based technique suitable for applying to protein imaging [61, 80-84]. The device consists of two capillaries positioned at an angle to each other, with a small gap between them at the sample surface [56]. The careful positioning of the two capillaries to minimise the liquid junction provides the highest spatial resolution among liquid sampling-based techniques. A controlled liquid junction forms as the solvent flows continuously through the first capillary, spilling onto the sample. It is then drawn into the second capillary and emitted as a nanoelectrospray mist directed at the mass spectrometer inlet [56].

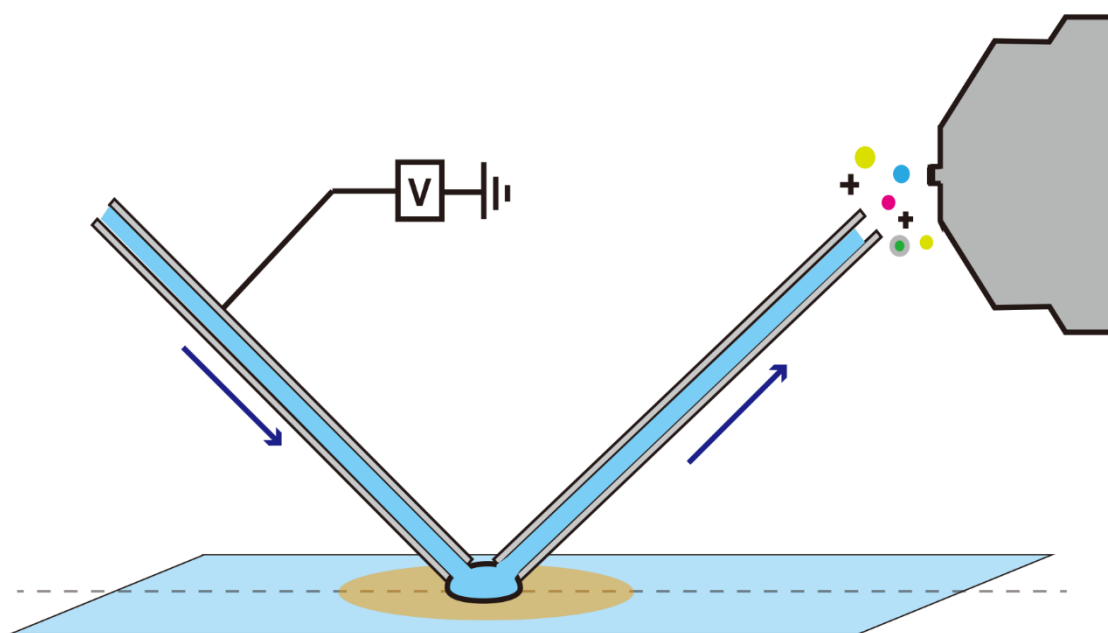


Figure 1.6 Schematic of a nano-DESI experiment. The solvent flows (blue) from the left capillary onto the sample (represented by ellipse in orange) surface and is then pulled into the right capillary, forming a liquid junction that enables analytes (represented by circles in different colours) extraction from the sample.

Nano-DESI is a valuable technique for high-resolution imaging of native proteins in tissue [61]. It has also proven effective for imaging membrane proteins in mouse tissue when combined with a tissue washing protocol [85].

1.1.2 Mass analysis

The mass analyser is the part of the MS which differentiates ions depending on their mass-to-charge. In this thesis, three types of mass analyser/filter were applied: quadrupole mass filter, ion trap analyser and orbitrap analyser. The mass

spectrometers used in this work were the Thermo Fisher Orbitrap Eclipse and the Thermo Fisher Q-Exactive HF.

1.1.2.1 Quadrupole mass filter

The quadrupole is widely used as an ion guide, mass filter, or mass analyser in various mass spectrometry instruments such as quadrupole mass spectrometer, triple quadrupole mass spectrometer, quadrupole time-of-flight mass spectrometer, quadrupole ion trap mass spectrometer and tribrid mass spectrometer. A quadrupole comprises four metal rods to which different voltages applied, combining direct current (DC) and radio frequency (RF) to isolate ions within the space between them.

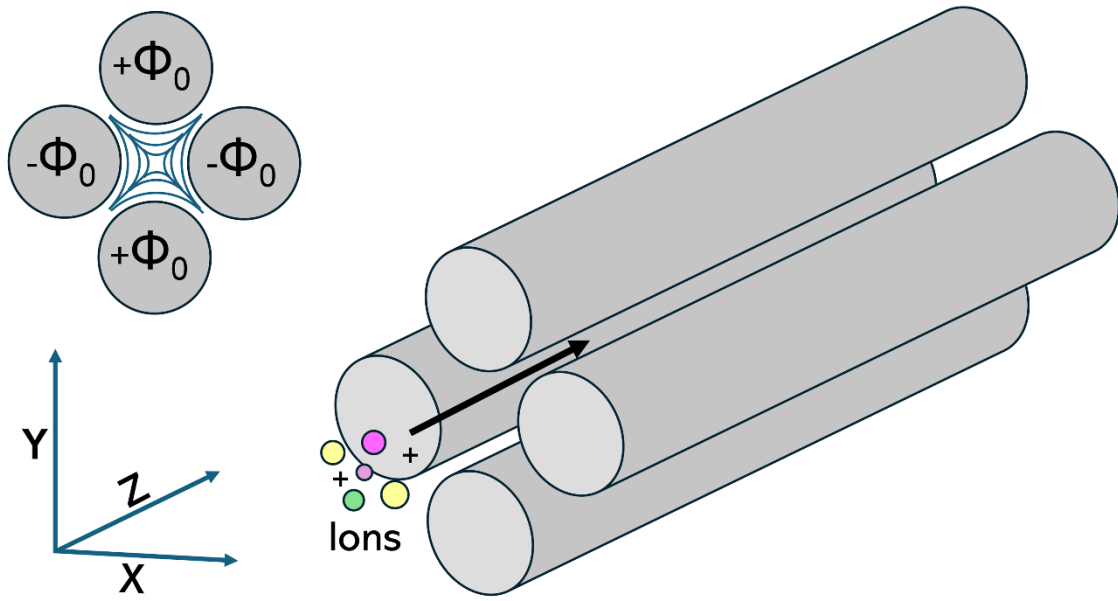


Figure 1.7 Schematic of a quadrupole. Four metal rods (gray) are positioned in opposing pairs, each pair alternating between positive and negative voltage polarity, maintaining a 180-degree phase difference relative to the other pair. Ions (represented by circles in different colours) travel through the central gap between the rods along the z-axis.

The four rods are lined in parallel, positioned as four long edges of a cube. Opposing pairs of rods are subjected to the same voltage. As the rods are equally spaced and form a square cross-section, a parallel hyperbolic electric field is generated within the structure.

$$\Phi_0 = U + V \cos \Omega t \quad (1.1)$$

Equation 1.1 defines the potential on the surface of the quadrupole rods. In this equation, U represents DC voltage and V represents RF voltage. Ω is the driving

frequency and t is time. While the positive ions are in the alternating electric field caused by pole voltages oscillating between positive and negative, they will flow towards the negative pole. If the ions reverse direction due to the oscillating voltage before floating out of the electric field, they are pulled back toward the device centre and continue travelling through the field until they reach the detector. During this process, the ions oscillate as the electric field changes, and only ions with specific m/z values remain stable and reach the detector. The m/z of the detected ions depends on the values of U and V in the equation.

To determine the range of m/z values for stable ions in the electric field, the Mathieu equations (Equations 1.2 and 1.3), based on Equation 1.1, are presented. The a in equation 1.2 is the DC voltage and the q represents the RF voltage. r_0 represents the radius of the quadrupole trap, z is the charge of the ion, and m is the mass of the ion.

$$a_u = \frac{8eU}{mr_0^2\Omega^2} \quad (1.2)$$

$$q_u = -\frac{4zeV}{mr_0^2\Omega^2} \quad (1.3)$$

When only RF is applied without DC voltage, the quadrupole acts as an ion guide; when a higher DC voltage is applied, it functions as an ion analyser or ion filter,

enabling selected ions to remain stable and reach the MS detector.

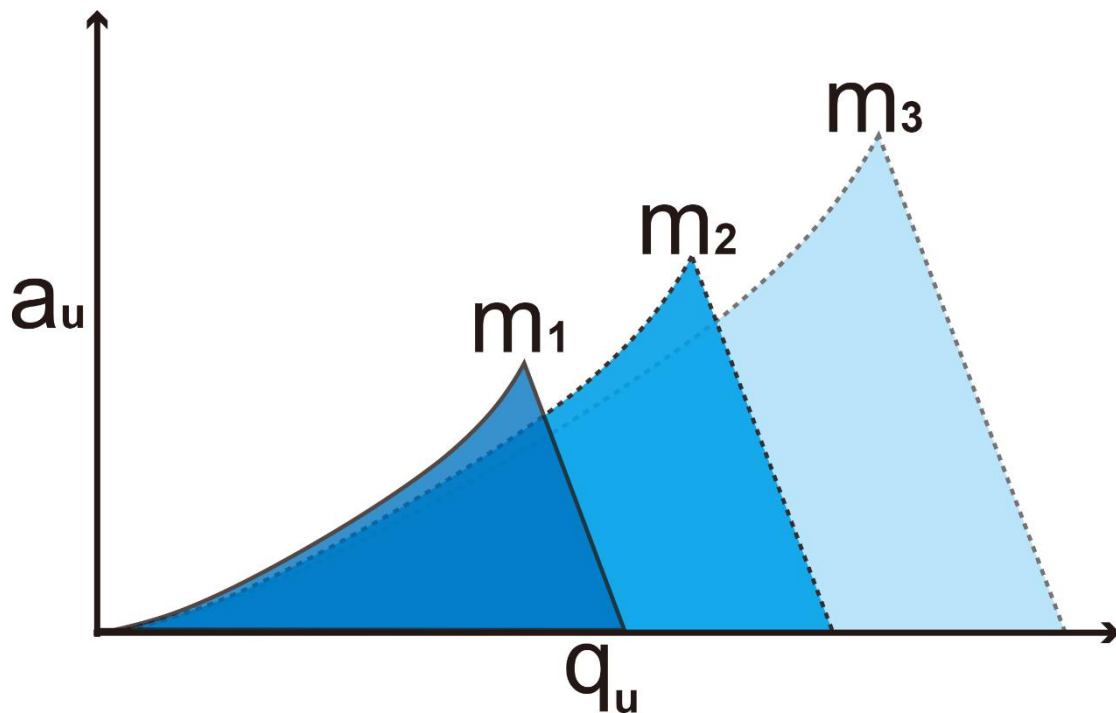


Figure 1.8 Plot showing ion stability based on the solutions to the Mathieu equations. For ion transmission, the values of U and V must be selected so that a_u and q_u lie within the stable region (represented by different blue colours) for ions.

1.1.2.2 Ion trap

A modern linear ion trap is designed according to ion isolation using a quadrupolar field. In the ion trap, the four rods are applied with a quadrupole-like voltage (described in Chapter 1.1.2.1) to create a hyperbolic electrical field, trapping the ions in x and y dimensions. The linear ion trap differs from the quadrupole in that its four-rod structure is divided into three sections: front, centre,

and end. The reason for this design is that the front and end sections can act as trapping plates by applying independently adjustable voltages. For example, as ions pass through the trap with oscillating voltages on the four rods, the voltages on the front and end sections are increased while the centre section remains at a lower voltage, effectively isolating ions at the device centre. This configuration enables efficient three-dimensional (x, y, and z) ion trapping.

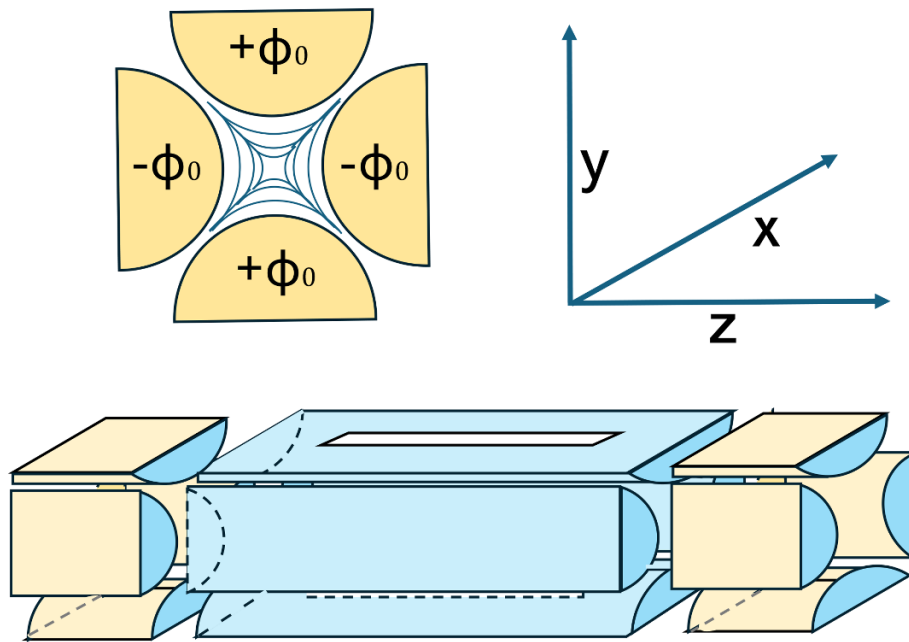


Figure 1.9 Schematic of a linear ion trap. It contains three parts including front, centre and end. Same voltages with quadrupole are applied on the metal rods. Ions pass through the central gap between the rods and are trapped within the centre of the device.

After trapping the ions, the voltages are adjusted to allow ions with specific m/z values to exit the device through the central gap and be analysed by the detector. The linear ion trap is a commonly used mass analyser in instruments such as the Orbitrap Eclipse and QE-HF, applied in this thesis, and is particularly suitable for MS^n detection.

1.1.2.3 Orbitrap

The orbitrap mass analyser is a Fourier transform mass analyser. It was first demonstrated by Alexander Makarov and coworkers in 2000 [86]. The device includes a central spindle-shaped electrode and an outer barrel electrode with a slit at the centre for collection and amplification of the image current generated by ion oscillation [86, 87]. It operates using static electrostatic fields. The axially symmetric electrodes of the Orbitrap generate a distinctive 'quadro-logarithmic' electrostatic potential, represented by the following equation [87]:

$$U(r, z) = \frac{k}{2} \left(z^2 - \frac{r^2}{2} \right) + \frac{k}{2} (R_m)^2 \ln \left(\frac{r}{R_m} \right) + C \quad (1.4)$$

In this equation 1.4, r and z are cylindrical coordinates, k is a constant representing field curvature, and R_m denotes the characteristic radius.

When ions are introduced into the analyser from a position offset from the central spindle's long axis ($z=0$) and enter tangentially to the outer electrode, they begin to spin around the central electrode while oscillating along its length [87]. The central spindle electrode is negatively charged in positive ion mode to attract incoming ions and bend their trajectory into an orbit around the spindle, effectively trapping them near the central electrode, shown in Figure 1.10.

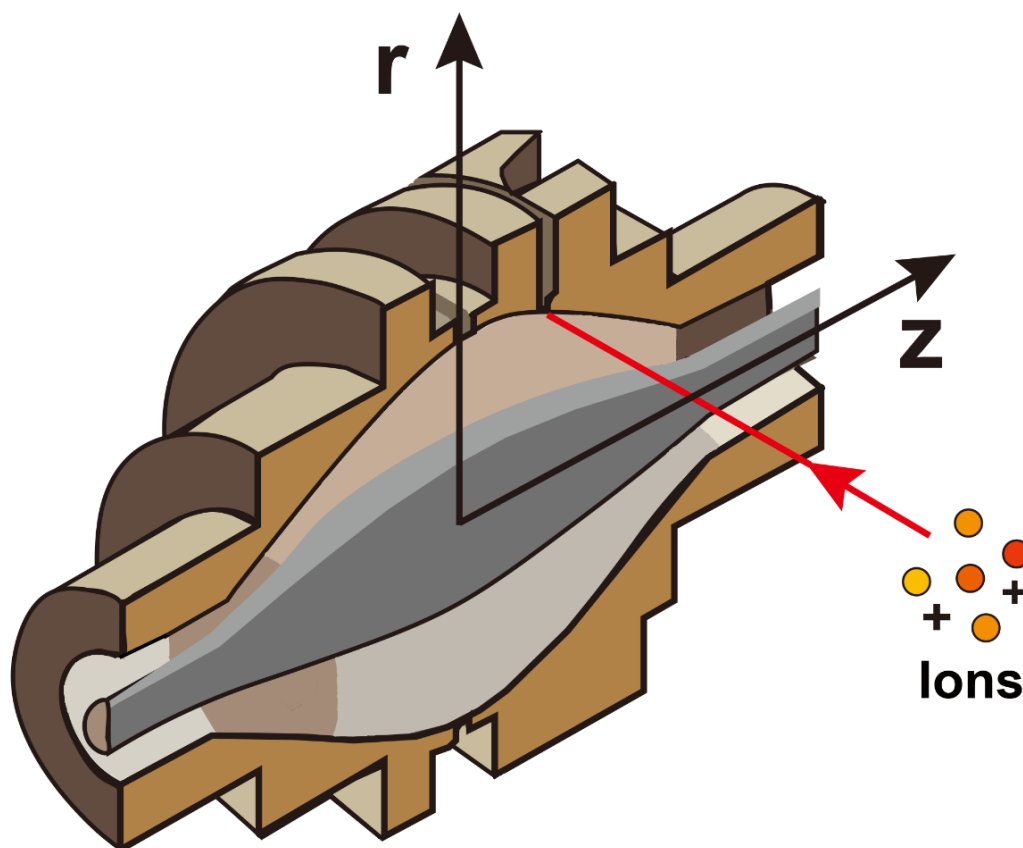


Figure 1.10 Schematic of one half of an Orbitrap. The ions (represented by circles with different colours) travel through a position offset from the central spindle's long axis ($z=0$) and oscillate along its length, allowing for separation based on different m/z values.

In this electrostatic field, stable ions undergo both orbital motion around the central electrode in the r -direction and oscillation along the z -axis. According to equation 1.4, the electrodes with special shape generate an electrostatic potential with no cross-terms in r and z , making the z -direction potential purely quadratic. Thus, the ion motion along the z -axis behaves as an independent harmonic oscillator [87]. The frequency of this axial oscillation is closely related to the ion's m/z , as shown in Equation 1.5, where the oscillation frequency ω is proportional to the m/z ratio.

$$\omega = \sqrt{\frac{z}{m}} \times k \quad (1.5)$$

k represents the curvature of the field as an independent value in this equation. The Orbitrap offers a simpler and more compact design, with mass resolution reach up to 1,000,000 at m/z 200 [88], just behind that of FT-ICR [86].

1.2 Ion mobility spectrometry

1.2.1 Overview

Ion mobility spectrometry (IMS) is a gas phase-based technology designed to separate ions according to their shape and size [89, 90]. Commonly-used IMS techniques that can be coupled with MS include drift-time ion mobility spectrometry (DTIMS), field-asymmetric waveform ion mobility spectrometry (FAIMS) and traveling-wave ion mobility spectrometry (TWIMS) [89]. These IMS techniques allow ion separation before MS analysis (IM-MS) [90-92]. IM-MS allows differentiation of isomeric ions [89], and can improve the signal-to-noise ratio of low abundance analytes [93-95]. DTIMS and TWIMS both enable calculation of the ion's rotationally averaged collision cross section. In this work, FAIMS was coupled with MS for detection of intact proteins from yeast (Chapter 4).

1.2.2 High Field Asymmetric Waveform Ion Mobility (FAIMS)

FAIMS is an IMS device that achieves ion separation on the basis of their

differential mobility in low and high electric fields. Alternating low and high fields are generated through application of voltages between two parallel plates [96, 97].

The voltage cycle consists of a short, high-amplitude positive pulse followed by a longer, low-amplitude negative pulse, creating an oscillating electric field perpendicular to the ion flow [97]. Ions that remain stable within this oscillating field experience no net movement, as their motions in response to the positive and negative voltages balance each other. This indicates that these ions have equal mobility in both high and low electric fields [97]. Otherwise, the ion moves toward one of the electrodes, with this movement increasing with each cycle until the ion eventually reaches an electrode and is neutralised [97]. This displacement of the ion from its original trajectory can be corrected through application of a voltage, the compensation voltage (CV). This feature enables FAIMS to function as a filter, selectively allowing specific ions to pass through based on electrical field, in contrast to other IMS techniques, which separate ions by gas flow and permit all positively/negatively charged ions to pass and be detected.

The DC compensation voltage is a crucial parameter in FAIMS, creating a compensation field (CF) that is superimposed on the asymmetric electric field [96-99]. This adjustment selectively corrects the trajectories of ions with specific differential mobilities, allowing only these ions to pass through the device. However, there is not a method for using FAIMS to determine the CCS of ions

unless it is combined with other IMS techniques. The ion moving in FAMIS is shown in Figure 1.11.

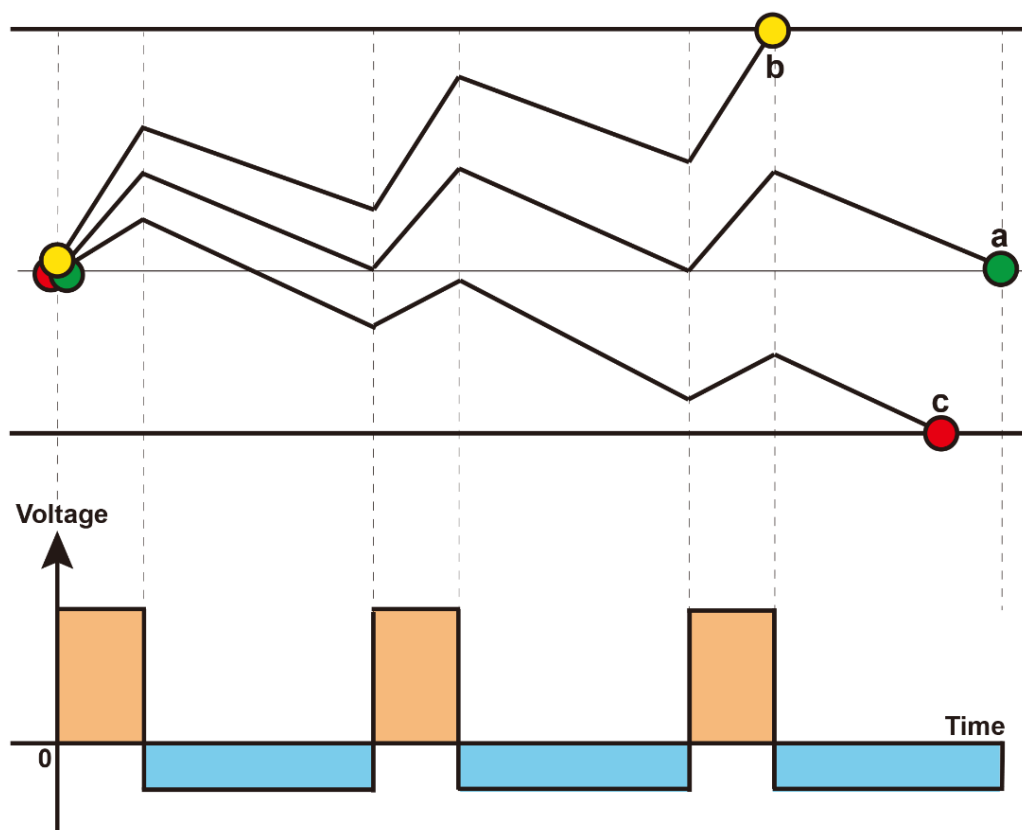


Figure 1.11 (Top) Schematic of ions moving in a FAIMS device. An ion represents the stable ion selected by FAIMS with a certain alternating electrical field; b and c ions represent the unstable ions neutralised on the electrodes. (Bottom) Alternating electric field applied between the parallel electrodes through which ions move.

The ion's mobility within an electric field can be represented mathematically by:

$$K_h(E) = K_0[1 + f(E)] \quad (1.7)$$

K_h represents the ion mobility at high field, K_0 describes the ion mobility at zero field, and $f(E)$ represents ion mobility as a function of the electric field strength [96]. This equation includes three types of ion behaviour in FAIMS. The first one, type A ions, show increased mobility as field strength rises and are usually small ions. The second, type B ions, initially exhibit an increase in mobility as field strength rises; however, as the field strength continues to increase, their mobility begins to decrease. Lastly, type C ions experience a consistent decrease in mobility as the field strength becomes stronger [96]. These differences are primarily related to variations in CCS, ion structure, and instrument settings [96].

There are three general models used for explaining the different behaviours of ions, including rigid scattering, ion–dipole and clustering models [99, 100]. Ion clustering with drift gas molecules or dopants, where ion-neutral clusters form in low fields and dissociate in high fields to enhance mobility differences, is the most relevant hypothesis explaining mobility dependence on electric field associated with small ions [101], while rigid sphere scattering is suitable for discussing higher interaction energy associated with heavy ions, strong electric fields, and high temperatures [102]. It is regarded as a suitable model for explaining proteins and protein complexes in ion mobility spectrometry [103]. Moreover, while the long-range ion-dipole interactions contribute minimally to the dependence of mobility on the electric field [100], this model may still relate to the behaviour of proteins in ion mobility spectrometry [104-106].

1.3 Protein detection by mass spectrometry

1.3.1 Top-down protein analysis

Traditional mass spectrometry methods in proteomics, known as bottom-up approaches, often begin with one-dimensional or two-dimensional (2D) electrophoretic separation of a sample mixture. Spots or gel slices containing individual proteins are then excised for further enzymatic digestion [107, 108]. Subsequently, peptides are extracted and collected for analysis by MS. The result of this process, called a "mass fingerprint," includes all detected peptide masses and can be used for protein identification by searching a peptide mass fingerprint database [109-111]. This method is particularly suitable for relatively pure proteins [112]. Another common application of the bottom-up approach is called "Shotgun mass spectrometry", which is used to identify and characterise complex mixtures of proteins or metabolites. In this approach, proteins are purified and digested into peptides before LC separation, ionisation, MS detection and MS/MS analysis. The analytes can be analysed and then identified by comparing against proteomics and metabolomics database [113-116].

The contrary concept to the bottom-up method is the top-down approach [112], which focuses on analysing intact protein ions within the mass spectrometer [117].

This method does not require enzymatic digestion in advance, enabling the direct detection of intact analytes [118, 119].

Direct analysis of intact proteins using the top-down approach can provide extra information, such as connectivity between post-translation-modifications [117, 120-126], compared to bottom-up method [119]. In the bottom-up approach, low peptide recovery from digestion could cause limitations in identifying protein modifications [119, 127]. This leaves certain modifications on unrecovered regions unobserved and disrupts the ability to identify connections between modifications across various regions of the protein [119]. The top-down method can address this technical issue effectively by keeping the protein structure intact [118]. Furthermore, digesting proteins into small peptides increases sample complexity [119], whereas direct detection by top-down retains the advantage of getting information with a simpler and more straightforward sample.

Top-down analysis is typically associated with some level of sample preparation, with desalting as a crucial step, especially when working with samples directly from cell or tissue lysates [119]. Common desalting methods include the use of reverse-phase (RP) columns or buffer exchange techniques such as dialysis or ultrafiltration [119]. To separate and purify proteins, key LC techniques that may be employed include affinity chromatography, ion-exchange chromatography, size-exclusion chromatography, and RP chromatography [119, 128]. Among these, affinity chromatography often considered as one of the most precise and

efficient approach for protein purification by isolating a particular protein from complex mixture with high selectivity [119, 129]. Moreover, the rapid progress made in instrument development has undoubtedly provided powerful support for the development of top-down proteomics.

1.3.2 Native mass spectrometry

Native MS is an electrospray ionisation based method in which analytes are sprayed from a non-denaturing solution into the gas phase [130]. Thus, the protein ions can retain much of their native structure, as well as inter- and intramolecular interactions [130-135]. Furthermore, due to the direct measurement of molecular weight, the stoichiometry of any protein complexes or assemblies can be readily determined from the mass spectrum [132]. This technique has primarily been employed to analyse a diverse array of soluble proteins, including protein assemblies and their interactions with various ligands, such as small-molecule drugs, cofactors, lipids, nucleotides, DNA, and RNA [135]. To date, membrane proteins are gradually becoming accessible to native MS by electrospraying these assemblies from detergent micelles, nanodiscs, or native lipid membranes [136, 137].

Though in the past few years, significant progress has been made in native MS, there are still some challenges. With the increasing need to analyse larger

proteins with greater heterogeneity and more complex structures, the demand for more advanced instruments for native MS has become an urgent priority [137]. Compared to denatured conditions, native proteins generated from electrospray of non-denaturing solvents exhibit fewer charge states, requiring a higher m/z detection range for the instrument [137]. In the early years, TOF based MS instruments were widely used due to their capability to detect over an extended m/z range. As time went on, ion trap-based instruments made significant progress in applications for native MS [137]. So far, instruments based on FT-ICR and Orbitrap are all developed for native MS detection [138-140].

Another challenge focuses on achieving higher mass resolution, which would enhance the accuracy of mass analysis. In native MS, this depends not only on the instrument but also on the ionisation process. Complete desolvation of proteins and protein complexes during ionisation can improve resolution by removing all solvent molecules and salt adducts as they transition from the electrospray solution into the (ultra) high vacuum of the mass analyser [137, 141]. This process can be challenging due to the strong binding between small molecule adducts and target analytes. To enhance desolvation, protein activation through heating or neutral gas collisions without causing dissociation in the instrument can be applied [137].

Native MS is suitable for combining with ambient MS, creating a faster, more straightforward method for detecting analytes directly from biological samples.

This combination is especially suitable for research that requires fast and in-situ analysis.

1.3.3 Native ambient mass spectrometry

Native ambient mass spectrometry (NAMS) is a combination of ambient mass spectrometry and native mass spectrometry. It preserves noncovalent interactions into the gas phase and allows for the direct analysis of many biological substrates without the need for complex sample preparation [82, 85, 130, 142, 143]. This approach involves sampling of a biological substrate using ambient techniques, followed by electrospray ionisation from a solvent that mimics native conditions, thereby preserving non-covalent interactions [130]. It allows for the direct analysis of tertiary and quaternary protein structures, providing valuable structural information during analysis [82, 85]. Ambient MS techniques were introduced in Chapter 1.1.1.4., and native MS is described in Chapter 1.3.2.

Previous research has demonstrated that intact proteins from living microbial colonies can be analysed in situ by using LESA MS [72, 76-78, 144-146]. Early studies indicated that by using a modified sampling strategy in which the pipet tip is brought into contact with the colony to improve protein signal detection, LESA mass spectrometry combined with a high-resolution orbitrap mass spectrometry

allows for the direct study of denatured intact proteins from a variety of colonies [77]. This approach makes it feasible to identify proteins not detected by other techniques and enabled the identification of several clinical pathogens [76, 78]. It also allowed identification of bacterial and host proteins in an infected wound skin model [146]. Other work demonstrated that enhanced protein signal-to-noise ratios could be gained by combining LESA mass spectrometry and a FAIMS device, resulting in quick single-scan protein detection in bacterial colonies [72] and extension of the protein detection range [144]. Further work developed an electroporation technique which successfully used in denatured protein detection on yeast colony [145]. In this thesis, NAMS was applied and combined with LESA for in situ analysis of native intact proteins from living microorganisms, including both soluble proteins and membrane proteins.

1.3.4 Tandem mass spectrometry

Tandem mass spectrometry is an approach comprising three steps: isolation of target ions, fragmentation of the selected ions, and subsequent analysis of their fragments [147]. The MS/MS or MSⁿ spectra produced from one or multiple rounds of this approach is mainly used for characterizing target ions, including

determining molecular weight, analysing structure, and identification. MS^n is only possible with instruments equipped with certain types of mass analysers, such as linear ion traps, enabling in-depth analysis through further fragmentation of the ions. Theoretically, the number of MS^n rounds is unlimited; however, in practice, it is restricted by ion loss during transitions and the complex setup of the instrument. In tandem MS, when proteins fragment, it typically occurs at specific sites along the protein backbone, depending on the fragmentation method used [148-151], shown in Figure 1.12.

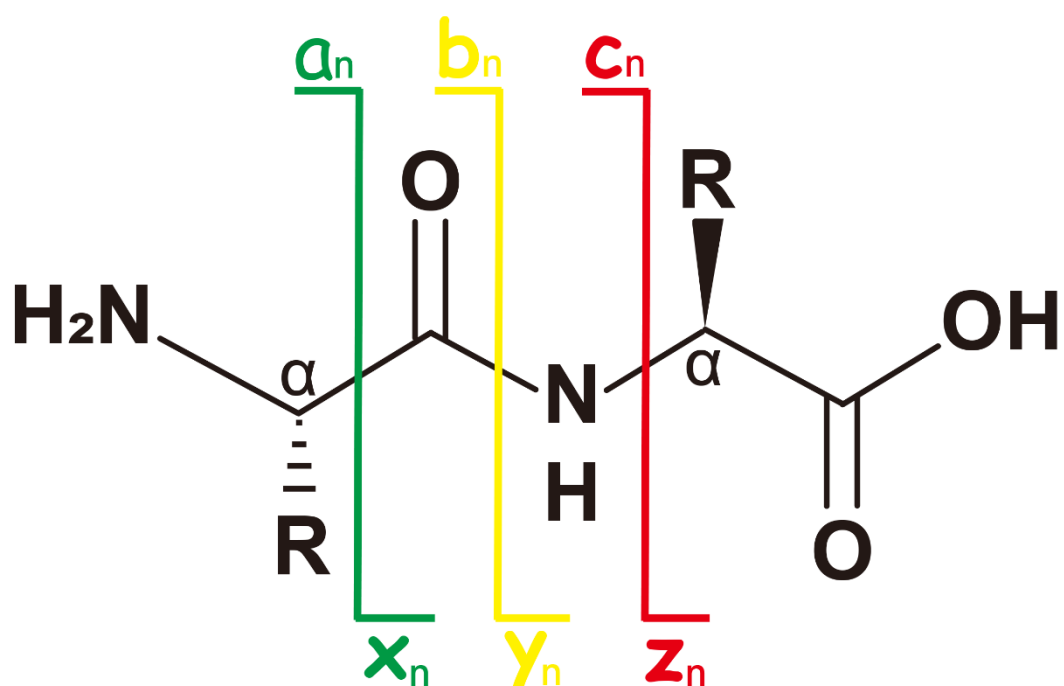


Figure 1.12 Fragmentation nomenclature: Cleavage sites along a peptide backbone [148, 150]. a, b and c ions contain the N-terminus; x, y, and z ions contain the C-terminus. n denotes the number of amino acid residues contained within the fragment.

Several tandem MS methods were used in this work. Higher-energy collision dissociation (HCD) was mainly used for native intact protein fragmentation and identification. Electron transfer/higher-energy collision dissociation (ET_hcD) was used to investigate the presence of disulphide bonds in some proteins. Proton transfer charge reduction (PTCR) was used to determine the intact molecular weight of some proteins.

1.3.4.1 Higher-energy collision dissociation (HCD)

HCD is a fragmentation technique similar to collision-induced dissociation (CID) but uses higher energy collisions. In ion trap CID, ions experience resonance excitation through the addition of a supplementary alternating current (AC) voltage, based on the RF voltage used for ion trapping, to excite ions with a certain m/z , resulting in energetic collisions between the ions and the neutral gas and fragmentation [152].

Unlike ion trap CID, HCD is a non-resonant fragmentation technique that operates at higher energy levels. Originally, HCD was designed to occur in the C-trap, where ions are collimated, discretised, and then ejected in pulses into the orbitrap mass analyser [152]. The ions dissociated in the C-trap by collisions with neutral gas (approximately 1 mTorr) molecules after acceleration. However, due to the curved design of the C-trap, high-mass ions with high kinetic energy may

easily escaped trapping, and low mass ion analysis are unsatisfactory due to high radiofrequency voltage [152]. A subsequent design to address this problem was to relocate the collision site to an octupole collision cell positioned after the C-trap. The collision octupole, with nitrogen at approximately 5 mbar pressure, allows collisions with accelerated ions, after which the ions are directed back to the C-trap for ejection into the mass analyser [152, 153]. This design modification reduced ion loss, removed the low-mass cut off and allowed for an increased gas pressure, which was not feasible in the C-trap due to its impact on the connected mass analyser [152, 153].

Though CID and HCD both break the peptide backbone and generate b and y ions, HCD often provides more complicated fragmentation because of its greater fragmentation energy, providing more comprehensive information in protein identification [154, 155]. It is particularly suitable for use with high-resolution analysis.

1.3.4.2 Electron Transfer/Higher-energy Collision Dissociation

ETHcD is a combination of electron transfer dissociation (ETD) and HCD. ETD is a fragmentation method based on ion-ion interactions and energy redistribution within the analytes [156-158]. During the ETD process, multiply charged cations of peptides or proteins interact with a singly charged reagent anion, forming odd-

electron radical cations that undergo cleavage driven by free radicals [156-158]. The principle is similar to electron capture dissociation (ECD), breaking the protein or peptide backbone in N-C α bond and generating c and z ions [157-159]. This fragmentation method is more effective for fragmenting more highly charged ions compared to ions in lower charge states. In ions of low charge state, ETD may cleave the N-C α bond but the fragments remain bound through non-covalent interactions due to insufficient Coulombic repulsion—a process known as nondissociative electron transfer dissociation (ETnoD) [156, 158]. ETnoD and ECCR share a similar principle for reducing the precursor's charge state via electron transfer, but in ETnoD, the electron is transferred from an electron donor, whereas in ECCR, the electrons captured are free, separate single electrons [160, 161]. In ETnoD, product ions remain as a single species with lower charge states in the mass analyser, but no information regarding the protein sequence is obtained [156]. To address the issue and achieve better results in peptide and protein identification, EThcD was developed. EThcD introduces a supplementary beam-type HCD broadband activation step following ETD to activate all ETD reaction products, generating both b/y ions and c/z ions [156-158]. The supplementary activation is applied to both ETnoD ions and non-activated ions, making a higher sequence coverage of protein and peptide identification [158, 162].

In this thesis, EThcD was used because ETD preferentially fragments disulfide

bonds, allowing for the determination of disulfide bonds in proteins.[163, 164].

The following HCD activation could help with dissociation of peptide or proteins after disulfide bond cleavage, providing further information by a more comprehensive fragmentation [156].

1.3.4.3 Proton transfer charge reduction (PTCR)

PTCR is one of the charge reduction methods. Another commonly use charge reduction method is electron transfer charge reduction (ECCR) in which the protein positive ion's charge states reduced by capturing free electrons without dissociation electrons [160, 161]. Compare to ECCR, PTCR transfers protons from multiply-charged protein cations to reagent anions ions that have high proton affinity, thereby reducing the charge state of the protein ions [165]. This technique is commonly used to analyse large biomolecules without dissociation, such as proteins or protein complexes, to provide more detailed information [166-170].

By PTCR, the mass analysis of large intact or native proteins becomes more straightforward and achievable [61, 82, 169-172] compared to traditional methods, which suffer from complicated overlapping peaks and are processing-intensive, often requiring manual work [173]. A series of peaks with lower charge states of the same protein appear in the spectrum following PTCR. The PTCR mass spectrum can then be deconvoluted using the UniDec software, which considers

the charge-state distribution and applies a Bayesian approach [173]. The charge reduction of biomolecules enables their detection at higher m/z values, which is beneficial for analyses with limited m/z ranges and helps reduce peak overlap [165, 169, 174].

1.3.5 Membrane protein analysis by native mass spectrometry

Accounting for about 30% of the proteome and approximately 60% of drug targets, membrane proteins are embedded within amphipathic lipid bilayers and play essential roles in biological processes [175-178]. Due to the low abundance [179-181] and hydrophobic nature [178, 182], membrane proteins are particularly difficult to analyse by MS.

In Chapter 5 of this thesis, the work focuses on bacterial membrane proteins. The bacterial cell membrane contains a diverse group of membrane proteins, varying in size, structure, and function [183]. Using native MS to analyse membrane proteins can provide structural information and insights into protein-protein interactions [184]. The difficult task of extracting these membrane proteins during sample preparation presents challenges in maintaining their native structure and stability. Moreover, bacterial samples typically contain a complex mixture of

cellular components like lipids, nucleic acids, and soluble proteins, which can affect the detection of membrane proteins via native MS.

Before the analysis, membrane proteins need to be solubilised by membrane mimetic. Approaches to solubilisation of membrane proteins include use of detergents, nanodiscs, styrene maleic acid lipid particles (SMALPs) and lipid vesicles [184]. In this thesis, detergent and SMALPs were explored.

1.3.5.1 Detergent micelles

In native MS, detergent micelles are frequently employed as membrane mimetics. A solvent with one to two times the critical micelle concentration (CMC) of detergent is used to solubilise membrane proteins, resulting in protein–micelle complexes ready for ionisation [184, 185]. The detergent adducts are then removed in the MS by activation [178, 184]. Non-ionic detergents are typically preferred for solubilising membrane proteins in native MS, as they better preserve native protein states, minimise ion suppression, and are easier to remove in the gas phase [184, 186, 187].

In this thesis, the application of charge-reducing detergent tetraethylene glycol monooctyl ether (C8E4), which is one of the most commonly used detergents and has been successfully used for nanoDESI imaging of membrane proteins [85], for native MS is explored. This detergent is chosen for its enhanced ability to

preserve membrane protein structure during ionisation by lowering proteins' average charge states [188, 189].

1.3.5.2 Nanodiscs and styrene maleic acid lipid particles

The nanodisc technique uses two copies of amphipathic membrane scaffold proteins (MSPs) to encompass a nanoscale section of lipid bilayer, forming solubilised nanoscale discs for MS detection [184, 190, 191]. It is particularly suitable for native MS detection with the features of good homogeneity and low size variability [184, 192, 193]. Owing to the presence of lipid bilayers, membrane proteins solubilised in nanodiscs demonstrate high stability and effectively maintain their stoichiometry [194, 195]. Although the nanodisc technique is useful for solubilisation of membrane proteins, it is worth noting that detergents are still commonly used (though not necessarily) with this technology to initially solubilise membrane proteins before MS analysis [184, 196].

SMALPs provide a more membrane-mimicking approach by using styrene maleic acid (SMA) copolymer to encapsulate the natural lipid bilayer around the protein, creating a soluble SMALP complex ready for MS detection [184]. However, due to its heterogeneous nature, SMALPs are difficult to resolve by native MS [197]. Laser-induced liquid bead ion desorption (LILBID) has been shown to effectively ionise SMALPs, enabling the determination of the oligomeric states of membrane

protein complexes and addressing existing challenges [198]. The principle of formation of SMALPs is shown in Figure 1.13.

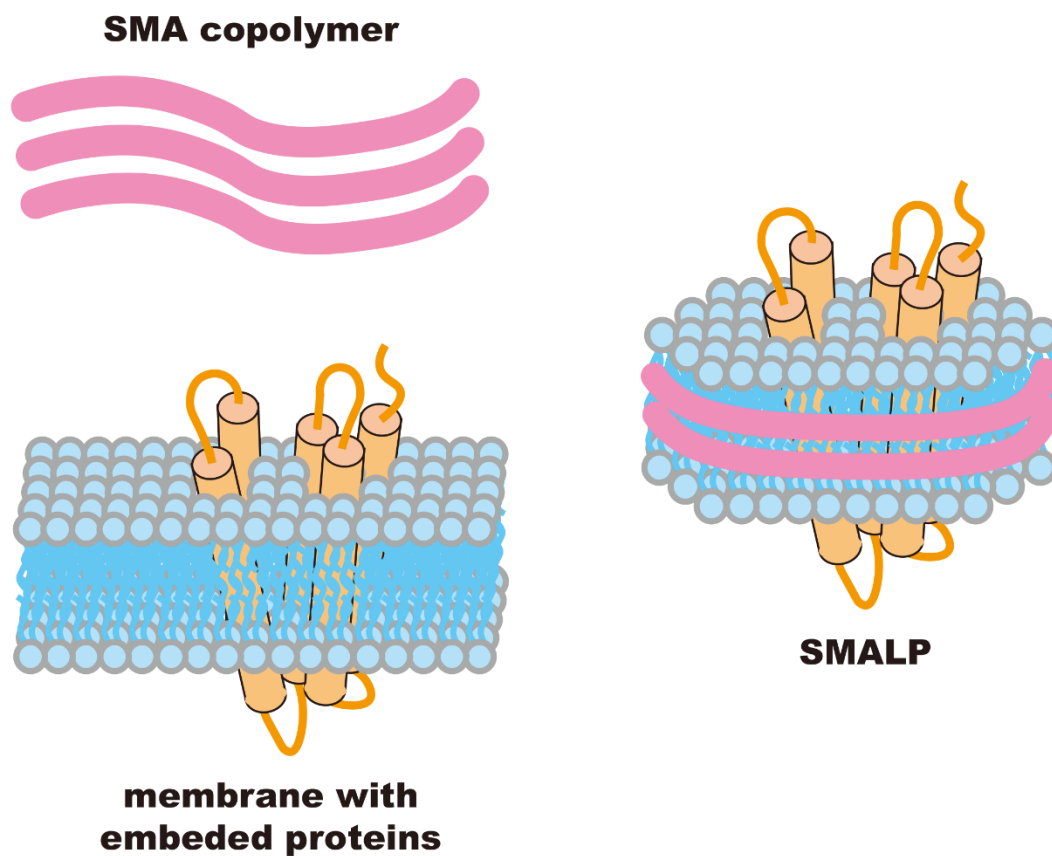


Figure 1.13 Formation of SMALPs to solubilise membrane proteins.

In this thesis, SMALPs was applied and discussed for bacterial membrane protein detection.

1.3.5.3 Lipid vesicles

An alternative approach used lipid vesicles directly from natural membranes for

MS detection [199, 200]. Large membrane fragments are first generated by ultracentrifugation, then sonicated into smaller liposomes, maintaining the native composition of lipids and membrane proteins. Although this method bypasses the need for purification or overexpression, the heterogeneity of protein assemblies presents significant challenges for MS analysis [201].

1.4 Microorganisms

Microorganisms include various species, such as bacteria, archaea, fungi, protists, viruses, algae, and prions. Among them, bacteria are the most well-known. In this thesis, the focus is primarily on native LESA MS protein detection in bacteria (both Gram-positive and Gram-negative) and yeast (Chapters 3 and 4), as well as native MS detection of membrane proteins in Gram-negative bacteria (*Escherichia coli*). The structure of the different microorganisms had varying influences on sampling and detection approaches developed.

1.4.1 Structure of bacteria

The gram-negative bacterial cell is surrounded and protected by a multi-layered structure composed of three main parts: the inner membrane, the peptidoglycan

layer, and the outer membrane [202], shown in Figure 1.14. Periplasm is the space between the inner and outer membranes and is abundant in proteins [203-207], corresponding with the detection of many periplasmic proteins in this thesis. These two layers of membrane structure of gram-negative bacteria provide extra difficulty in extracting proteins in its natural state.

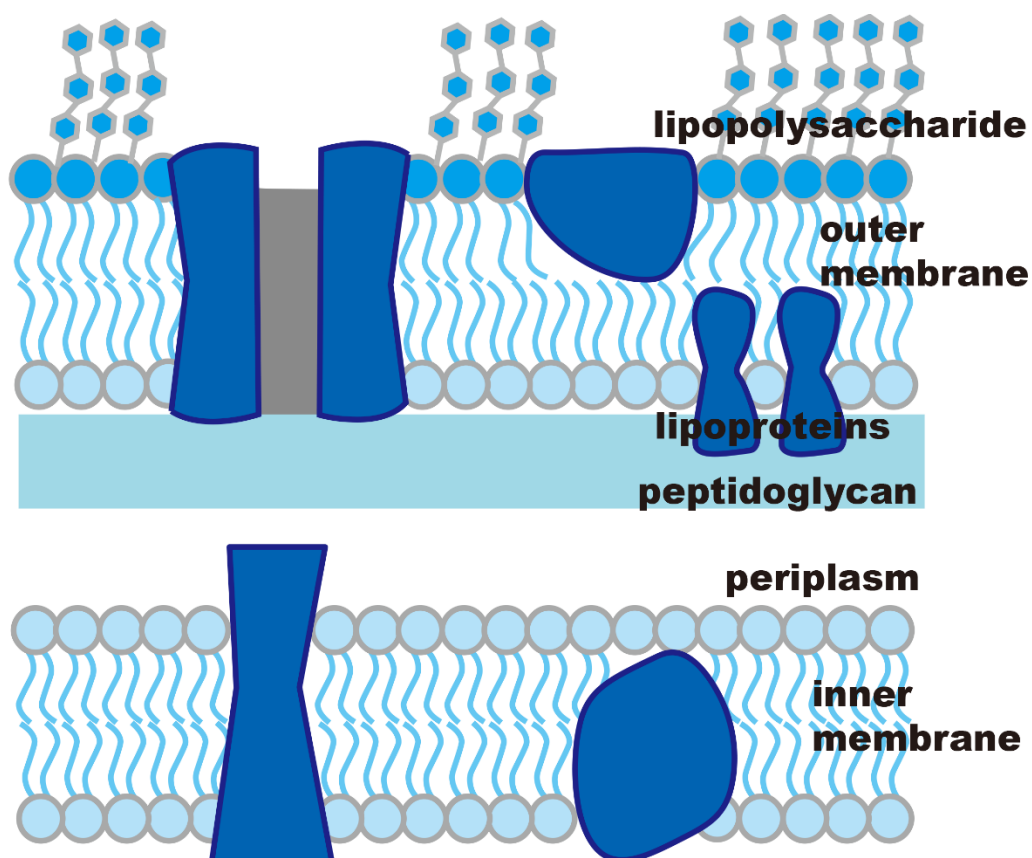


Figure 1.14 The Gram-negative structure of membrane and cell wall based on the structure of *Escherichia coli*.

A defining feature of Gram-negative bacteria is their outer membrane, an asymmetric lipid bilayer with lipopolysaccharides (LPS) on the outer leaflet and phospholipids on the inner leaflet [206, 208]. It plays a key role in nutrient absorption and help with bacterial resistance in an environment with detergents and antibiotics [205, 206, 208-210]. This outer membrane is anchored to the peptidoglycan layer by lipoproteins and contains proteins that serve as transport channels [211, 212]. The peptidoglycan layer beneath maintains the cell's rigidity and shape from internal osmotic pressure within the cytoplasm [213-216]. The primary defence of bacteria against acid stress is the inner membrane, which has a tightly packed, highly ionised lipid layer that is more robust than the outer membrane and periplasm [215]. So, the detection of native inner membrane proteins is more challenging than native outer membrane proteins.

The thick peptidoglycan cell wall serves as the outermost layer of the Gram-positive cell envelope, which lacks the outer membrane found in Gram-negative bacteria [217, 218]. Although much thicker that could reach up 30-100 nm, this peptidoglycan layer shares structural similarities with that of Gram-negative bacteria with only a few nanometres thickness [218]. While the purpose of this charge distribution is still unknown, teichoic acids within the peptidoglycan contribute to a net negative surface charge [219].

1.4.2 Structure of yeast

Selective permeability is made possible by the strong, intricate cell wall of *S. cerevisiae*, which is composed of glycoproteins and polysaccharides. Mannoproteins, chitin, glucans $\beta(1-3)$ -glucan and $\beta(1-6)$ -glucan are present in this wall, providing it with robustness and resistance to chemical lysis [220-222], shown in Figure 1.15. The distinct composition of these structural elements preserves the cell's selective barrier capabilities while enhancing its strength, making it extremely difficult to be sampled and analysed by native LESA MS.

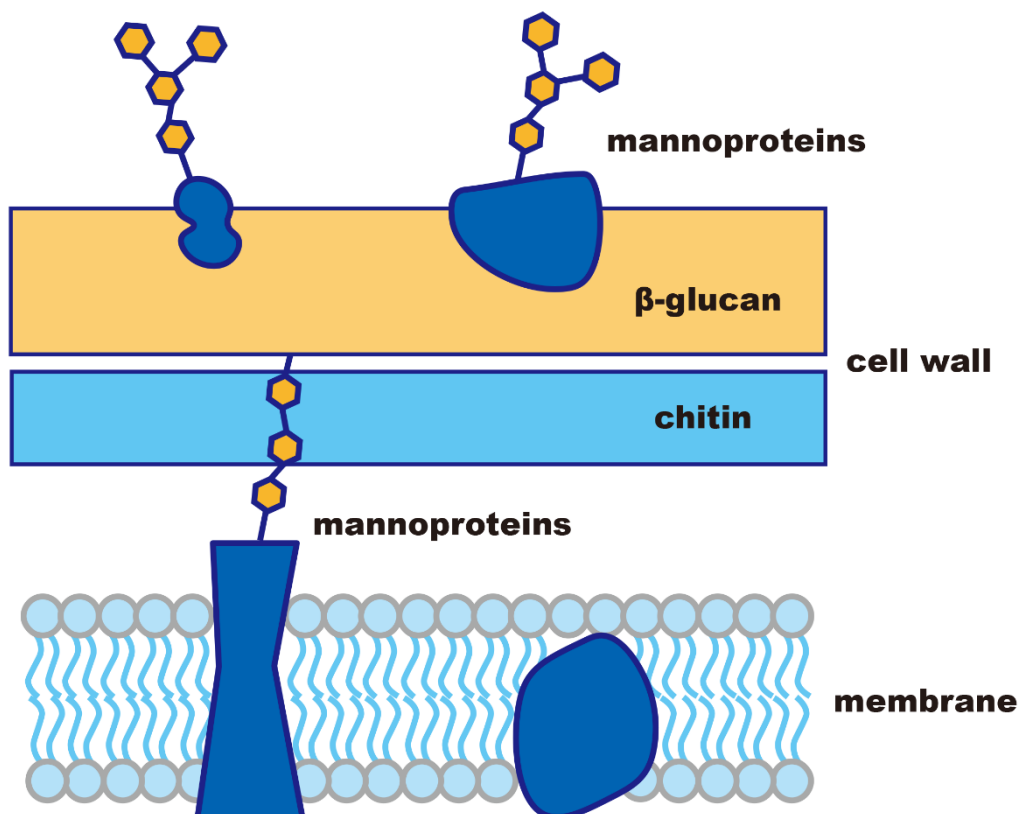


Figure 1.15 The yeast structure of membrane and cell wall based on the structure of *Saccharomyces cerevisiae*.

1.4.3 Structure of colony

The extracellular matrix, or glycocalyx, consisting of exopolysaccharides, holds together adherent cells in microbial colonies (including bacteria and fungi) and biofilms, which are organised formations [223-225]. This matrix encases the colony, preserving its structural stability with strong environmental resistance [226, 227] and facilitating cell communication, and collaboration [225, 228-231]. Unlike planktonic cells, microorganisms within a biofilm exhibit distinct behaviour alterations and attachment to surfaces and adjacent cells [231, 232].

A key characteristic relevant to this study is the microbial biofilms increased natural resistance to antimicrobial treatments [226, 227, 233-236]. Possible reasons for this resistance include restricted solute entry into the biofilm matrix, dormant cells, and a stress response that reduces cell permeability [233-236]. Furthermore, its extracellular polymeric substances (EPS), consisting of nucleic acids, proteins, lipids, and polysaccharides, shield biofilm bacteria, increasing their resistance to disinfectants and antimicrobial agents [237]. To access cytosolic proteins during LESA sampling on agar, disruption is needed [77, 78, 144], as these factors likely influence the microbial colony's vulnerability and permeability to chemicals.

1.4.4 Mass spectrometry analysis of microorganisms

After being developed as an ionisation technique for mass spectrometry, MALDI was soon used to analyse bacterial biopolymers [238]. The early studies of MALDI MS detecting protein in bacteria is usually on recombinant proteins [238-241]. Gradually, based on recombinant proteins, unknown protein identification by MALDI MS in bacteria was developed [242-246]. Subsequently, MALDI MS was used in proteomic detection on bacteria [238, 247, 248], and now, rapid bacterial intact organism identification has been widely used in clinical work by MALDI MS to analyse the unique bacterial fingerprints [236, 238, 249].

Ambient ionization methods have made it possible to conduct direct mass spectrometry on entire microbial colonies in their native substrate. With minimal sample preparation, DESI-MS has been proved can produce reproducible, bacteria-specific mass spectra suitable for pattern recognition [250]. DESI-MS also shows potential for applications like food contamination screening by effectively distinguishing major bacterial species and strains through lipid profiling and principal component analysis in both positive and negative ion modes, with minimal interference from growth media [251]. DESI-MS usually used for small molecular detection on bacteria and difficult to provide spatial information of colony.

Nano-DESI has been successfully used in metabolic imaging on living bacterial colonies [252]. Though achievable, the uneven surface of agar presents difficulties for imaging. The dried agar was used for addressing this issue [253], however, not keeping the bacteria alive.

As described above in section 1.1.3, LESA is an ambient ionisation method capable of directly extracting proteins from microbial colonies. Building on previous studies, this thesis explores native LESA MS detection for bacterial colonies, enhancing the characterization of microbial proteomes.

1.4.5 Electroporation of microorganisms

In native detection by MS, whole bacteria and yeast cells present difficulties due to their structure with cell walls and cell membranes. A LESA MS method incorporating electroporation, which involves applying high-voltage electrical field directly to microbial colonies, was shown to successfully detect and identify denatured proteins in living yeast colonies [145]. For yeast cells only 5 kV/cm is required to lose viability [145, 254-256]. This process can disrupt cell membranes, generating small holes while the cells are still alive, making them permeable.

Aqueous pore formation represents the most acceptable explanation of what is happening in the electroporation process [257]. When an electric field induces

pore formation in a cell membrane, molecules can pass through, which is a process called electroporation [258, 259]. Aqueous pore formation suggests that applying electrical field on lipid bilayer could reduce the energy need to form the pores, consequently, increase the number and stability of pores [258, 259]. The molecular dynamics (MD) simulations indicate that pore formation begins with a "water wire," (or a water column) a chain of water molecules spanning the membrane's thickness [260-263], shown in Figure 1.16. This alignment causes hydrophilic lipid headgroups to reorient around the water, stabilizing the pore. In an electric field, water molecules align according to their dipole moment, further penetrating the bilayer and aiding pore formation, shown in the Figure 1.16.

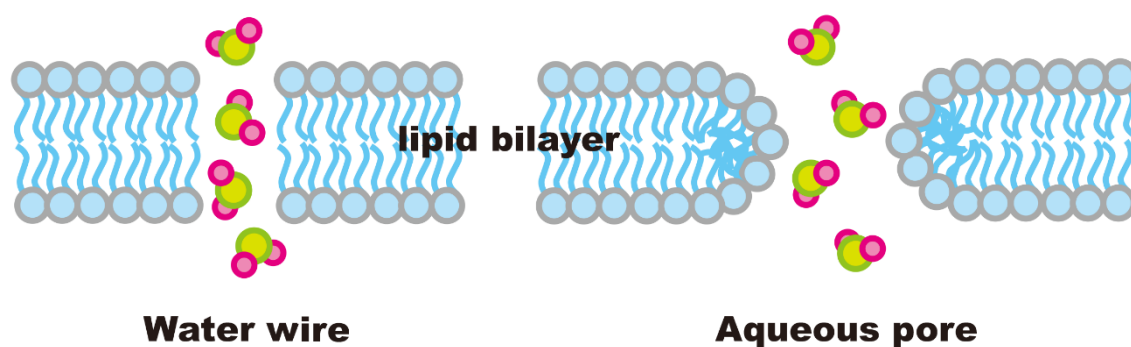


Figure 1.16 The diagram of pore formation principle in a lipid bilayer. (left) A water wire penetrates and spans the bilayer; (right) Polar head groups rearrange around the water wire, creating a stable aqueous pore.

Unlike solid insulators, lipids will gradually return to their original state once the

electric field is removed. Consequently, if the lipid bilayer is not exposed to an excessively high electric field or for too long, the electroporation state is reversible. [257, 261, 264, 265].

It has been demonstrated that applying an electric field can facilitate the delivery of exogenous molecules into cells and extract endogenous proteins from within the cell [266-269]. Previous work has shown that electroporation can detect denatured intact proteins in living yeast colonies [145]. Due to the feature that electroporation would be applied with minimal heating and can preserve cell viability [145, 269, 270], the combination of electroporation and native MS would potentially be able to detect native intact proteins from living bacterial colonies.

1.5 Research aims

The aim of this research was to develop mass spectrometry techniques for the direct analysis of bacterial and fungal proteins in their native state. It specifically focuses on integrating mass spectrometry with electroporation, ion mobility spectrometry, SMALPs and LESA. The purpose is to establish methods that enable the direct extraction of protein structural information, including tertiary and quaternary structures, from the physiological setting as living microbial colonies.

The research objectives include:

- To develop a method, combining LESA and electroporation for analysis of intact native proteins in gram-negative bacteria. (Chapter 3)
- To investigate if the method combining LESA and electroporation could be applied to analysis of intact native proteins in gram-positive bacteria. (Chapter 3)
- To optimise the combined electroporation/LESA MS method to enable the detection of intact native fungal proteins. (Chapter 4)
- To develop a method for detection of intact membrane proteins directly from bacteria. (Chapter 5)

Chapter 2

Material and methods

2.1 Materials

2.1.1 Strains

Bacterial strains *E. coli* K12 were sourced from Willem Van Schaik at the Institute of Microbiology and Infection (IMI), University of Birmingham, UK. The stock of *E. coli* BL21 (DE3) with ZipA protein overexpression and SMALP with ZipA and Sav1866 protein was provided by Professor Timothy Dafforn, University of Birmingham, UK. *Pseudomonas aeruginosa* PS1054 was provided by Mark A. Webber from the library of clinical isolates of the Queen Elizabeth Hospital Birmingham. *Staphylococcus epidermidis* RP62a and *Staphylococcus epidermidis* HB strains were obtained from Joan Geoghegan, also at IMI, University of Birmingham, UK. The donor plates containing *S. cerevisiae* were generously provided by Dr. Saverio Brogna from the University of Birmingham,

UK.

2.1.2 Sample preparation

Lysogeny broth (LB) broth (yeast extract from VWR, Lutterworth, UK; peptone from Sigma-Aldrich, Gillingham, UK; sodium chloride from Fisher Scientific, Loughborough, UK), LB agar (LB broth with added bacteriological agar), and tryptic soy broth (TSB) agar were prepared to culture bacterial species. In detail, 20g/L lysogeny broth base or 30g/L tryptic soy broth base was dissolved in 18.2 MΩ water prior to sterilisation through autoclaving (121 °C, 0.5 bar). Yeast extract peptone dextrose (YPD) broth (Sigma-Aldrich, Gillingham, UK) and YPD agar prepared by dissolving 50 g/L yeast extract peptone dextrose broth base in 18.2 MΩ water followed by sterilisation through autoclaving (121 °C, 0.5 bar) were formulated to support the growth of yeast species. Biospin column 6kDa (Bio-Rad, Watford, UK) was used for buffer exchange of SMALPs.

2.1.3 Chemicals

Acetonitrile was purchased from J.T. Baker in Deventer, Netherlands. Ammonium acetate and formic acid were acquired from Sigma-Aldrich in Gillingham, UK. MS-

grade water was purchased from Fisher Scientific in Loughborough, UK. The detergent C8E4 was purchased from Sigma-Aldrich (Gillingham, UK).

2.1.4 Nanoelectrospray emitters

Gold-coated borosilicate nanoelectrospray emitter was fabricated using a P-1000 pipette puller (Sutter Instruments) and coated with gold using a sputter coater (Agar Scientific Ltd.).

2.2 Methods

2.2.1 Microorganism colony culture

250mL of the prepared agar in a glass bottle was melted in a microwave oven and transferred into 6 cm diameter petri dishes in the environment without microorganisms (within 10cm from the flame) with agar height of 4-6mm. After cooling down and solidifying at room temperature, the petri dishes were stored at 4°C until use.

1 μ L of frozen bacterial glycerol stock (liquid bacterial culture in an appropriate

growth medium (optical density w600 ~0.6-1.0) mixed with 50% glycerol solution in a 1:1 ratio) or 5-10 single colonies (diameter 1-2mm) of yeast was mixed with 5–10 mL of liquid broth. For gram-negative *bacteria E. coli K12*, *E. coli BL21 (DE3)* and *Pseudomonas aeruginosa PS1054* and gram-positive strains *Staphylococcus epidermidis RP62a* and *Staphylococcus epidermidis HB*, LB broth was used, and for yeast *S. cerevisiae*, YPD broth was used. The mixture was then placed in a dark environment and incubated at 37°C for bacteria or 30°C for yeast with shaking at 200 rpm for approximately 18 hours. Subsequently, 10 µL of the liquid culture for each colony (diameter of 5mm) was then transferred onto solid LB (for gram-negative bacteria), TSB (for gram-positive bacteria) or YPD (for yeast) medium in 6 cm diameter Petri dishes by 10 µL culture loop in a non-microorganism environment and incubated overnight at 37°C for bacteria or 30°C for yeast until the colonies exceeded 0.3mm thickness and were ready for the following processes.

2.2.2 Colony washing

In the Chapter 5, before the following process, colonies were subjected to different washing protocols to remove the soluble protein allowing for the detection of membrane proteins.

A single round of washing, as defined in this thesis, refers to application of 20 μ L of the washing solvent (defined elsewhere in the relevant results chapter) such that the entire colony was covered. The wash solvent remained on the colony for 30 seconds before being gently aspirated.

The initial experiment in Chapter 5 tested the impact of different washing solvents, including 200mM ammonium acetate, 200mM ammonium acetate with 0.5 x CMC (0.125% v/v) C8E4 and 200mM ammonium acetate with 2 x CMC (0.5% v/v) C8E4, by washing colony for one round before protein extraction.

The following two washing processes were assessed to detect membrane proteins. One process was carried out by the washing solvent consisting of 200mM ammonium acetate with 0.5 x CMC C8E4 for three rounds, followed by another round by 200mM ammonium acetate, resulting in a (3+1) washing process with totally four rounds of washing. The other washing process was carried out by repeating above (3+1), resulting in a process with 2 x (3+1).

In the assessment of combining colony washing and electroporation, a twelve rounds washing step was established. It consists of washing by 200mM ammonium acetate with 0.5 x CMC C8E4 for five rounds, followed by another round of washing by 200mM ammonium acetate, forming a (5+1) process and repeating into a 2 x (5+1) process.

2.2.3 Electroporation

The electroporation procedure described in this thesis was carried out using a custom-built device detailed in the work by Kocurek et al [145], shown in Figure 2.1.

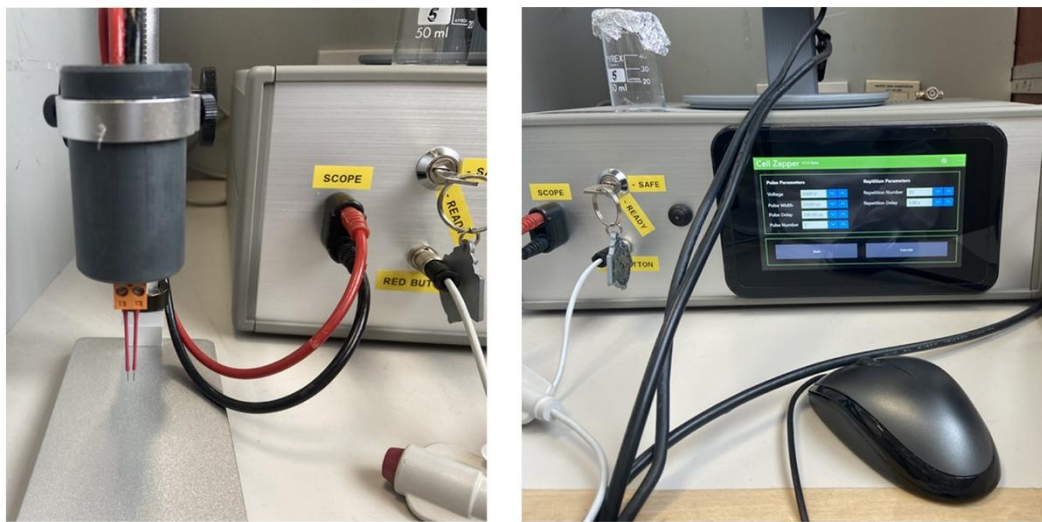


Figure 2.1 Custom-built electroporation device with (left) electrode and (right) operating panel.

In Chapter 3, for the denaturing control, colonies of *E. coli* K12 were electroporated directly by bringing them gently into touch with the colony. The height of electrodes needed to be controlled carefully to avoid the penetration of agar. The electroporation settings were voltage of 3 kV, a field strength of 15 kV/cm, 30 pulses, and a pulse length of 20 microseconds each duty cycle.

For the native MS experiments (Chapter 3, 4 and 5), 2 μ L of 200mM ammonium acetate was carefully dispensed onto the colony surface prior to electroporation. The electrodes were then adjusted using the lifting lever until they made gentle contact with the colony. The electroporation settings for bacteria were: 3 kV, a field strength of 15 kV/cm, 30 pulses, a pulse length of 20 microseconds as a duty cycle, and 1s between two duty cycles; while for yeast, they were voltage 2 kV, corresponding to a field strength of 10 kV/cm, number of pulses 35, pulse length 20 μ s and 1s between two duty cycles.

2.2.4 Liquid extraction surface analysis (LESA) sampling

LESA was conducted using a TriVersa NanoMate robotic pipet system (Advion, Ithaca, NY) controlled by the advanced user interface (AUI) feature (NanoMate ChipSoft Manager software 8.3.3) with tip sampling depth optimised. It is worth mentioning that when extracting proteins from the colony, the pipette tip had to make a contact with the colony surface but should not penetrate the agar.

In Chapter 3, the LESA solvent composition for the denaturing control was a mixture of acetonitrile, water, and formic acid, with a ratio of 39.5% acetonitrile to 59.5% water to 1% formic acid. The LESA sampling process involved aspirating 3 μ L solvent from well, dispensing 2 μ L onto the colony between the imprints of

the electroporator electrodes, maintaining the liquid microjunction for 15 seconds, reaspirating 2.5 μ L, and finally introducing the sample to the mass spectrometer via chip-based nanoelectrospray ionisation (nano-ESI) at 1.65 kV and 0.3 psi. For native protein detection on bacterial colonies, the LESA sampling solvent was 200mM ammonium acetate. The extraction process was the same as above.

In Chapter 4, to extract proteins from the yeast colony, a total of 3 μ L of LESA sampling solvent containing 200 mM ammonium acetate with 2 x CMC C8E4 and 3% methanol was aspirated from a reservoir, and 2 μ L was dispensed onto the colony and aspirated for 10 cycles, with each cycle maintaining microjunction for 15s. The sample was placed into a well and subsequently transferred to a gold-coated borosilicate nanoelectrospray emitter for introduction into the mass spectrometer for detection. The voltage of nano-ESI was set up as 1.1 kV.

In Chapter 5, to extract membrane proteins from bacterial colonies, the LESA sampling solvent was 200 mM ammonium acetate (pH 6.8-7) with 2 x CMC C8E4. The whole process is the same with the process used in Chapter 4.

2.2.5 Sample preparation of styrene maleic acid lipid particles (SMALPs)

In Chapter 5, SMALPs samples needed to be prepared. The sample preparation prior to buffer exchange was carried out by Pooja Sirdhar, School of Biosciences, University of Birmingham. In detail, the extracted membranes from *E. coli* BL21 (DE3) overexpressing the ZipA or SAV1866 protein were solubilised by 50 mM Tris (pH 8.0), 500 mM NaCl, 5% w/v SMA2000P, and 10% glycerol (or 10% diisobutylene maleic acid (DIBMA) or 2% n-Dodecyl- β -D-Maltoside (DDM)) for ZipA (30 mg/mL) or 50 mM Tris (pH 8.0), 150 mM NaCl, and 5% w/v SMA2000P (or 10% DIBMA or 2% DDM) for SAV1866 (40 mg/mL). The samples were incubated at 4°C for 2 hours, centrifuged at 100,000 $\times$ g for 30 minutes to remove insoluble components, went through a 0.45u syringe filter, and bound to Ni-NTA agarose beads overnight at 4°C. After washing, the protein was eluted with 5 column volume of elution buffer (50mM Tris pH 8.0, 500 NaCl, 0.5M Imidazole, 10% Glycerol (for DDM purification add 0.05% DDM) for ZipA, 50mM Tris pH 8.0, 150 NaCl, 0.5M Imidazole (for DDM purification add 0.05% DDM) for SAV1866).

Target protein fractions were identified by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), pooled, concentrated with an Amicon concentrator, and purified via size exclusion chromatography (SEC) (S200 column). After verification by another SDS-PAGE, the pooled fractions

were concentrated, measured with a NanoDrop (1-5 mg/ml), aliquoted, flash-frozen, and stored at -80°C.

Buffer exchange of SMALPs (in elution buffer) with protein ZipA and Sav1866 was carried out by Biospin columns 6kDa. The column was spun at 1000g for 2 minutes to remove excess Tris buffer. Then 500 μ L 200mM ammonium acetate was added into the column and spun at 1000g for 1 minute. This step was repeated for another 4 times to replace 99.99% of the previous buffer. After that, 15 μ L of SMALP sample was added into the column, spun at 1000g for 4 minutes and then collected in a clean Eppendorf tube.

2.2.6 High Field Asymmetric Waveform Ion Mobility (FAIMS) Mass Spectrometry

For the identification of some proteins in Chapter 4, a Thermo Fisher FAIMS-Pro device connected to a Thermo Fisher Eclipse Tribrid mass spectrometer (Thermo Fisher, San Jose, CA) was configured. The FAIMS dispersion voltage (DV) was maintained at -5 kV. To achieve optimal identification of target proteins, the CV was adjusted to -35 V.

2.2.7 Mass spectrometry detection

All experiments were performed on a Thermo Fisher Orbitrap Eclipse tribrid mass spectrometer (Thermo Fisher Scientific, San Jose). The instrument, equipped with the HMR_n option, was operated in positive intact protein mode.

In Chapter 3, full scan spectrum was acquired within the 900-4000 m/z range (initial full scans were acquired within 900-3000 m/z range), in ion routing multipole (IRM) standard pressure mode (8 Torr), achieving a resolution of 120,000 (m/delta m) at m/z = 200, with automatic gain control (AGC) set at 100%. For further investigation of identifying larger proteins, 80V of in-source collision energy was applied.

In Chapter 4, initial full scan spectrum was collected in IRM standard pressure mode with a range of 1000-5000 m/z at a resolution of 120,000 (m/delta m) at m/z 200, with a normalised AGC target of 100%, and an in-source collision energy of 80V. Subsequent full scan spectrum was obtained in IRM high-pressure mode (20 Torr), with the 2000-6000 m/z range, at a resolution of 75,000 at m/z 200, with a source collision energy optimised to 120V.

In Chapter 5, for colony detection, full scan mass spectrum of was first conducted in in IRM standard pressure mode with a range of 1000-6000 m/z at a resolution of 120,000 (m/delta m) at m/z 200, with a normalised AGC target of 100%, and a

source fragmentation energy of 100V. The following spectrum was conducted in IRM high-pressure mode within the range of 2000-8000 m/z at a resolution of 75,000 at m/z 200, with a normalised AGC target of 100%, with an in-source collision energy of 120V or with 120V in-source collision energy along with 30% HCD normalised collision energy (NCE) to cleavage the detergent micelles. The compensation scaling factors were set as 5%, 7% and 14%. For SMALPs, the full scan was applied in IRM high-pressure mode within the range of 2000-8000 m/z at a resolution of 75,000 at m/z 200, with a normalised AGC target of 100%, and a source collision energy of 100V.

2.2.8 Protein fragmentation

2.2.8.1 Higher-energy collision dissociation (HCD) mass spectrometry

In Chapter 3, precursor ions were isolated by quadrupole ($m/z < 2000$) and ion trap ($m/z > 2000$) for fragmentation with 4-6 m/z isolation window, and HCD was employed with 5-40% normalised collision energy, optimised for each protein to maximise fragmentation efficiency and obtain the highest number and best signal-to-noise ratio of fragments, prior to detection in the orbitrap at a resolution of 120,000 at m/z 200. Based on the peak detected at m/z 1813.45 in this study, the actual resolution at this point, calculated using a full width at half maximum

(FWHM) of 0.0420, was approximately 43177.38.

In Chapter 4, ions targeted for fragmentation were isolated with a 10 m/z window in the linear ion trap and subjected to 20-60% normalised collision energy in the HCD cell prior to detection in the orbitrap at a resolution of 120,000 at m/z 200, with an in-source collision energy 80V-120V.

In Chapter 5, MS/MS or MS3 was performed to fragment the ions. The linear ion trap was used to isolate the precursor ions which were chosen to fragment to be identified with an isolation window of 15-20 m/z. The HCD NCE was 20-60% and fragments were detected in the orbitrap at a resolution of 240,000 at m/z 200.

2.2.8.2 Proton transfer charge reduction (PTCR) mass spectrometry

PTCR experiments were conducted in positive-ion mode at a resolution of 7500 at m/z 200, employing 80V-120V in-source collision energy. Precursor ions were isolated via ion trap with a 6-20 m/z isolation window, and fragments were analysed in the orbitrap analyser. The reaction time was set at 2-10 ms, covering a mass range of 3000-8000 m/z.

2.2.8.3 Electron transfer higher-energy collision dissociation EThcD mass spectrometry

EThcD was only applied in Chapter 3 for HdeB protein. The analysis was configured with a range of m/z 500–4000 and a resolution of 120,000 at m/z 200. Precursor ions were isolated using the ion trap with a 5 m/z isolation window, and fragment analysis was carried out in the orbitrap analyser. The ETD irradiation time was 25 ms and HCD NCE was 38%. Other parameters remained consistent with full scan mass spectrum.

2.2.9 Protein Deconvolution using UniDec

PTCR mass spectra were deconvoluted using UniDec software [173]. The parameters were set up as: Charge Range: 1 – 30 (z), Mass Range 1000 – 8000 (Da), Sample Mass Every: 0.1 (Da), Smooth Charge State Distributions: Yes, Use Automatic m/z width: Yes, Smooth Nearby Points: Some, Peak Detection Range: 10 (Da) and Peak Detection Threshold: 0.01 (Da). The other parameters were set as default.

2.2.10 Protein identification

For protein identification, ProSightPC software version 4.1 alpha (Thermo Fisher Scientific, Bremen, Germany) was used search fragmentation data against the whole organism proteome databases *E. coli* K12 (UniProt ID UP000000625, 4448 protein entries), *S. cerevisiae* (strain ATCC 204508 / S288c) (UniProt ID UP000002311, 6060 protein entries) and for *E. coli* BL21 (DE3) (UniProt ID UP000002032, 4156 proteins entries) from the UniProt website (uniprot.org). Identification was conducted in absolute mass mode, with MS/MS spectrum deconvoluted using the THRASH algorithm at a signal-to-noise ratio of 3. A standard top-down database was applied considering initial methionine cleavage and N-terminal acetylation, along with single nucleotide polymorphisms and all available posttranslational modifications. Fragment ion tolerance was set at 20 ppm, and protein identities were further validated through manual analysis using Protein Prospector (<http://prospector.ucsf.edu/prospector/mshome.htm>).

Chapter 3

Native LESA MS detection of native intact protein directly from bacterial colonies

The work presented in this chapter has been published in Y. Du, R.C. May and H.J. Cooper; “*Native ambient mass spectrometry of intact protein assemblies directly from Escherichia coli colonies*”, Chemical Communications, 2022, 58, 6857-6860. A copy of the paper is provided in **Appendix 1**.

3.1 Introduction

The work presented in this Chapter aimed to integrate native MS [130] into the LESA workflow to enable the structural characterisation of proteins directly from bacteria. Previous work on LESA analysis of proteins from living bacterial colonies made use of aqueous organic solvents [76, 78]. These solvents were

essential to make the bacterial cell wall and cell membrane permeable, enabling the detection of cytoplasmic proteins, but also resulting in the unfolding of the proteins. In this work, an alternative approach focused on preserving higher-order protein structure during LESA extraction by avoiding the use of organic solvents. Instead, electroporation was used to render the cell wall and membrane permeable with preserved cell viability, facilitating the release of endogenous proteins [269, 270]. Electroporation is associated with minimal heating [254] and has previously been demonstrated for LESA analysis of denatured proteins in yeast [145].

Three workflows incorporating electroporation were trialled and compared with a denaturing control. The most suitable workflow was applied to analysis of proteins in living bacterial colonies of *E. coli K-12*. The same method was subsequently applied for *Staphylococcus epidermidis RP62a* and *Staphylococcus epidermidis HB* to assess the possibility of this method being applied to gram-positive bacteria.

3.2 Experimental Section

3.2.1 Bacteria Culture

Colonies of *E. coli* K-12, *Staphylococcus epidermidis* HB and *Staphylococcus epidermidis* RP62a were cultured as described in Chapter 2, section 2.2.1.

3.2.2 Native LESA sampling of *E. coli*

Three distinct workflows were considered for electroporation on *E. coli* K-12, shown in Figure 3.1. To ensure accuracy in the assessment, each workflow was performed at least twenty times. In the first approach, a small volume (2 μ l) of 200 mM ammonium acetate was pipetted onto the bacterial colony, followed by electroporation and subsequent LESA sampling. The initial addition of ammonium acetate was designed to prevent air breakdown during electroporation by increasing colony surface humidity. The second workflow involved deposition of 2 μ l of 200 mM ammonium acetate onto the colony prior to LESA sampling without electroporation. In the third workflow, the bacterial colony was sampled by LESA without prior electroporation. Control experiments were also performed, in which denaturing solvents including 39.5% acetonitrile to 59.5% water to 1% formic acid

were used. In these, the colonies were either sampled directly by LESA or were sampled by LESA following electroporation. Experimental details are given in Chapter 2, sections 2.2.3 and 2.2.4.

The workflow that was successfully used to detect proteins in *E. coli* K-12 was also applied to the Gram-positive strains *Staphylococcus epidermidis* HB and *Staphylococcus epidermidis* RP62a.

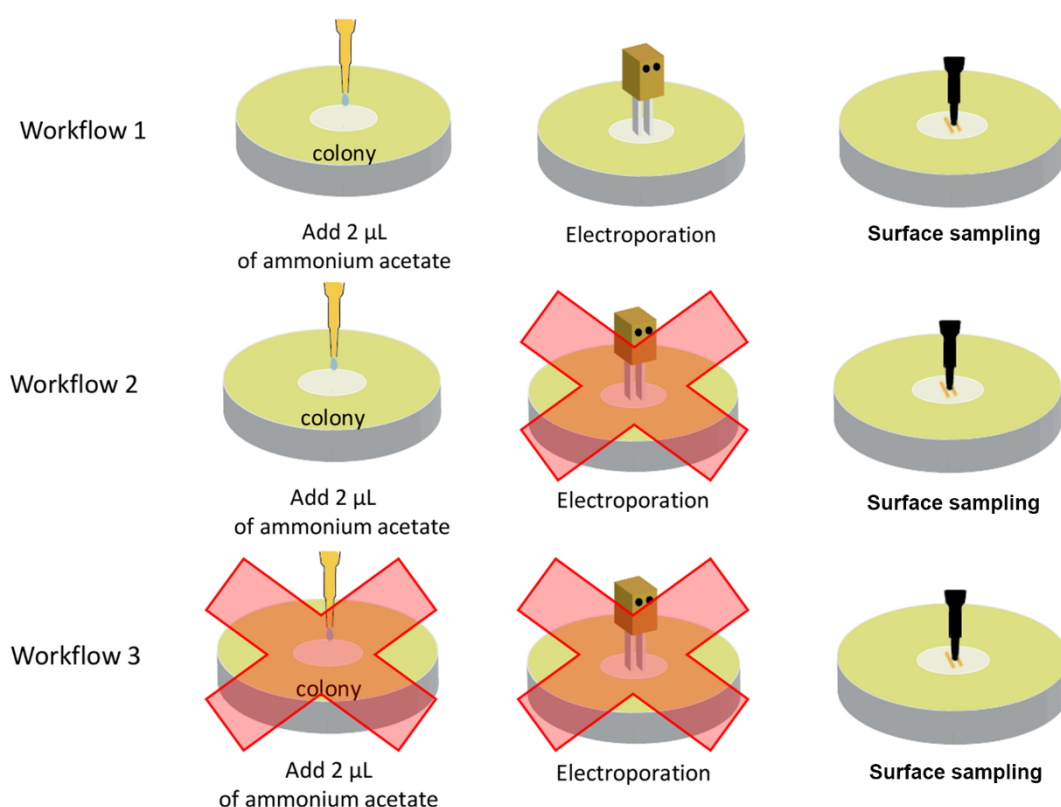


Figure 3.1. Three workflows for native ambient mass spectrometry of bacterial colonies.

3.2.3 Protein Identification

Protein ions detected in the mass spectra were isolated subjected to MS/MS by HCD, EThcD and PTOR (as described in Chapter 2, section 2.2.8). Details of exact fragmentation technique used for each protein are given in the Results (section 3.3). The resulting fragments were searched against protein databases as described in Chapter 2, section 2.2.10.

3.3 Results and discussion

3.3.1 LESA MS of *E. coli* by use of denaturing solvent

To investigate the effect of native-like solvents in LESA of bacterial colonies, initial baseline experiments were performed in which *E. coli* K-12 colonies were subjected to LESA with denaturing solvent. The experimental setup involved two separate control groups, with each group being repeated three times. In the initial control group, colonies were extracted using the denaturing solvent immediately after being cultured at 37°C overnight. The extraction solvent comprised 39.5:59.5:1 acetonitrile-water-formic acid, as detailed in Chapter 2, section 2.2.4.

The second control group was subjected to electroporation followed by LESA sampling with denaturing solvents. Figure 3.2 A) shows the LESA mass spectrum obtained in the absence of electroporation and Figure 3.2 B) shows the LESA mass spectrum obtained after prior electroporation.

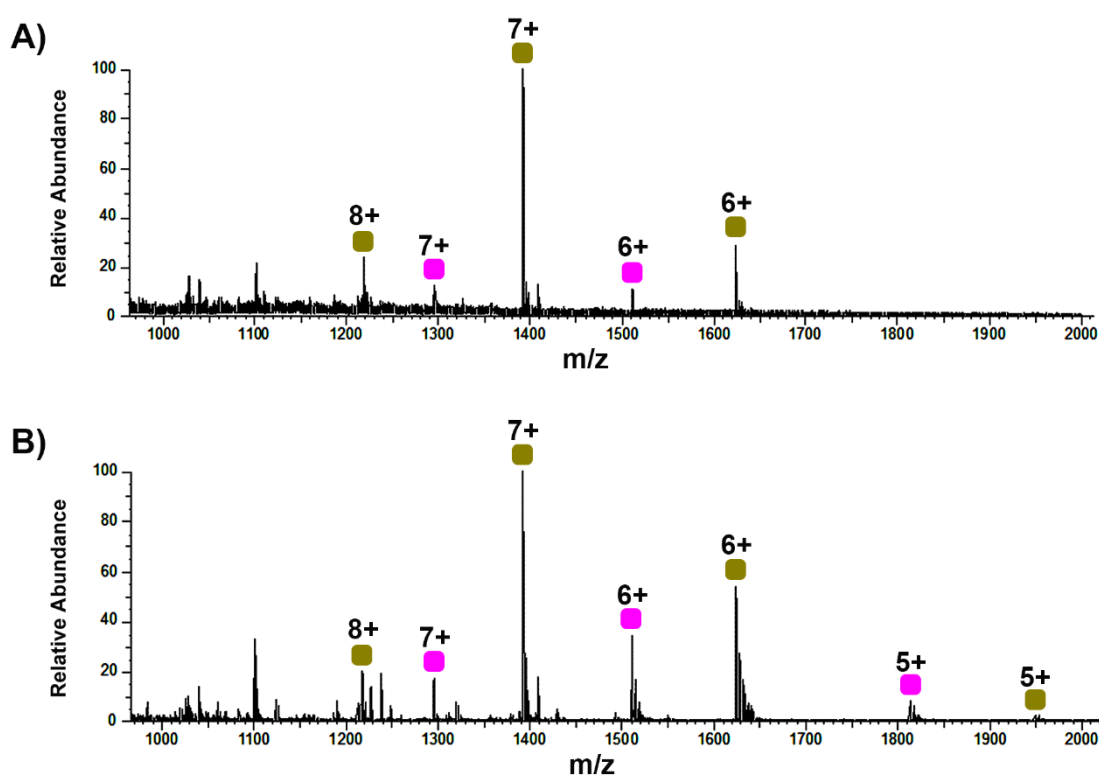


Figure 3.2 LESA mass spectrum obtained following sampling of *E. coli* colonies by use of denaturing solvent. A) Full scan mass spectrum obtained in the absence of electroporation. B) Full scan mass spectrum obtained following prior electroporation of the sample. The tan-green squares indicate the peaks corresponding to the protein HdeA, while the pink squares indicate peaks corresponding to the protein HdeB.

Proteins were observed in both denaturing control experiments. The mass spectrum shown in Figure 3.2 A) illustrates the efficacy of using an acetonitrile-based solvent for independent extraction and detection of bacterial proteins. Without electroporation, nearly all protein observed in the control group within the m/z range of 900-1300 matched the masses of proteins identified in previous research [77, 78]. The identification of proteins was confirmed by HCD MS/MS (as described in Appendix 2. Chapter 3). For peaks above m/z 1300, two new proteins, HdeA and HdeB, with high signal intensities were observed, as shown in Figure 3.2. Furthermore, when coupled with electroporation, the same proteins were detected. A higher signal-to-noise ratio and wider charge state distributions were observed, especially above m/z 1300, which mainly refer to HdeA and HdeB, shown in Figure 3.2 B). The results suggest that electroporation, which acts on bacterial cells by disrupting the cell wall and cell membrane, prompts the release of cytoplasmic proteins and leading to higher signal intensity for these peaks in the mass spectrum. The combination of using an acetonitrile-based solvent and electroporation proves beneficial as they assist in breaking down the cell wall and cell membrane. Due to the quick process without complicated sample preparation, this method facilitates the immediate and efficient in-situ detection of proteins from bacterial colony right after sample collection. The robust methodology with high repeatability of the procedure makes it a valuable control for further experiments on native protein detection.

3.3.2 Native LESA MS of *E. coli*

The next step was to design a new process to analyse native proteins from living bacterial colonies by integrating electroporation with native LESA MS. In the initial experiment, the bacterial colony was subjected to an electric field strength of 15 kV/cm (equivalent to 3 kV across a 2 mm gap); however, a significant challenge appeared due to air breakdown caused by the high electric field strength. This not only decreased the effectiveness of permeabilising the cell wall and cell membrane but also posed a risk of damaging the equipment. To address this issue, 2 µl of 200 mM ammonium acetate was deposited onto the top of the colony to increase colony surface humidity level before electroporation, as illustrated in Figure 3.1 workflow 1. To investigate the impact of this additional step, two control protocols were assessed. The first involved LESA sampling of the colony immediately after the addition of 2 µl of 200 mM ammonium acetate, while the second involved LESA sampling without any prior treatment, as depicted in Figure 3.1 workflow 2 and 3. The representative mass spectrum of three replicates obtained following the three workflows are shown in Figure 3.3.

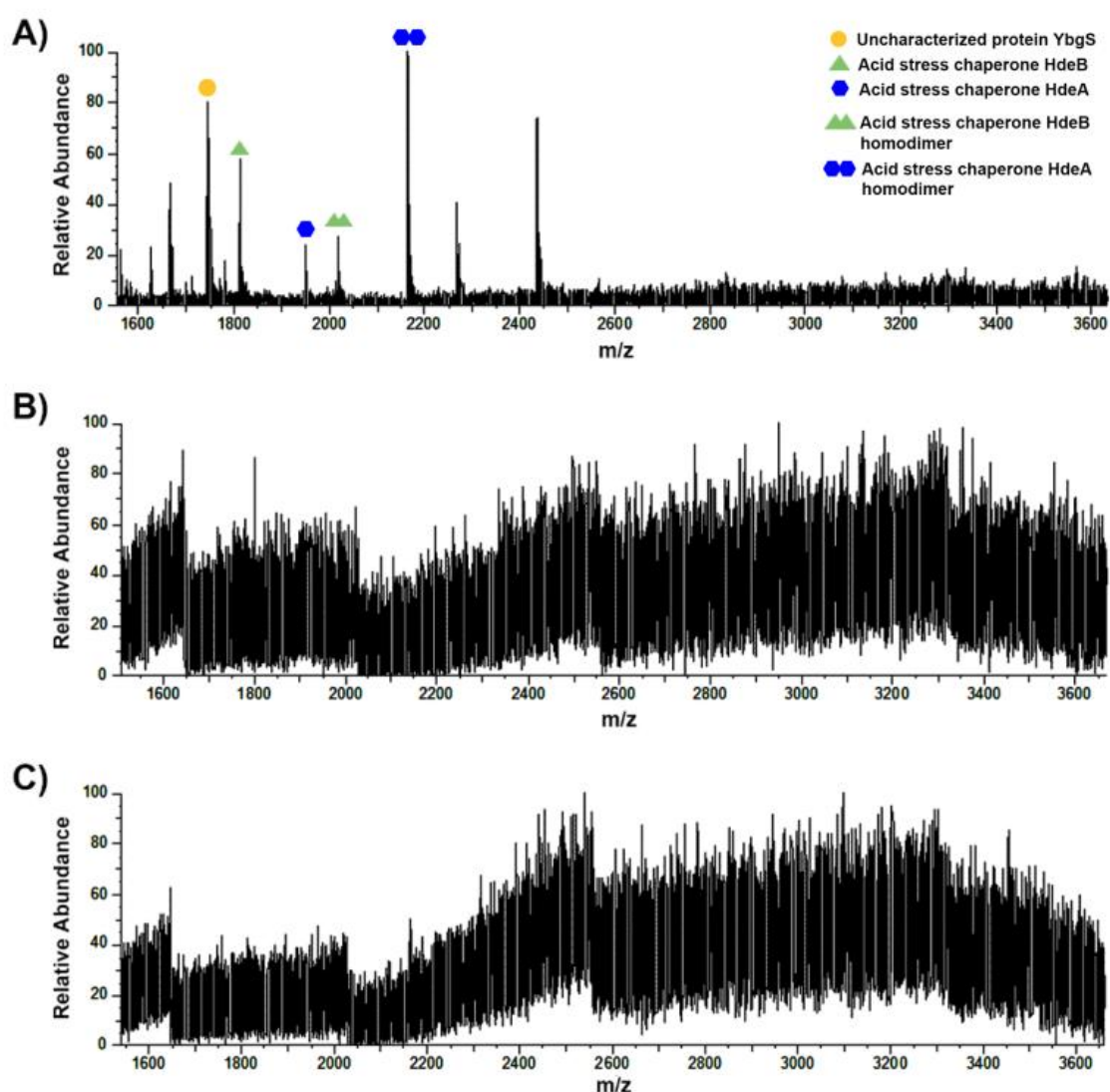


Figure 3.3 Representative LESA mass spectrum (from three replicates) obtained following the three workflows outlined in Figure 3.1. A) LESA mass spectrum obtained following electroporation after deposition of 2 μ l of 200 mM ammonium acetate onto the bacterial colony. B) LESA mass spectrum obtained following deposition of 2 μ l of 200 mM ammonium acetate onto the bacterial colony and no electroporation. C) LESA mass spectrum obtained without pre-treatment.

As the mass spectrum in Figure 3.3 demonstrate, the workflow in which a small volume (2 μ l) of 200 mM ammonium acetate was deposited onto the bacterial

colony prior to electroporation, proved to be the optimal method for detecting proteins from bacterial colonies by LESA using native-like solvents. Nevertheless, it should be noted that occasionally peaks corresponding to proteins were detected in the absence of electroporation. Therefore, a comparative analysis of the success rates of the three methods was conducted. Success rate, defined as the ratio of sampling events resulting in mass spectrum containing protein peaks to the total sampling events, was found to be 77% for the addition of solvent and electroporation (workflow 1), 14% for the addition of solvent without electroporation (workflow 2), and 31% for no solvent addition and no electroporation (workflow 3). The result is demonstrated in Table 3.1. With electroporation following the addition of the ionic solution ammonium acetate, as shown in workflow 1, bacterial cells came into direct contact with the ions and were more easily disrupted, resulting in a higher rate of successful protein detection. For workflow 2, when sampling directly after adding ammonium acetate, some cells from the colony became suspended, diluting the sample without disrupting bacterial cell wall and leading to a lower protein detection success rate. When using direct sampling without any pretreatment, illustrated in workflow 3, the colony was not diluted. Therefore, the success rate is higher than simply adding ammonium acetate but lower than when using electroporation to disrupt the colony.

Table 3.1 Comparison of the success rates of the three workflows

Workflow	Number of sampling events	Number of sampling events resulting in mass spectrum containing protein peaks	Success rate
Workflow 1 (w/ electroporation)	35	27	77.14%
Workflow 2 (w/o electroporation)	22	3	13.64%
Workflow 3 (LESA control)	35	11	31.42%

3.3.3 Identification of proteins

The workflow in which 2 μ l of 200 mM ammonium acetate was dispensed on the colony prior to electroporation, resulted in detection of a number of proteins in *E. coli* K12. Figure 3.4 shows a representative mass spectrum (from 27 replicates) obtained following native LESa MS with electroporation. The majority of the protein peaks fall within the m/z range 1300-2500. Protein identification relied on intact mass and MS/MS data, with specific details outlined in Chapter 2, section 2.2.7, section 2.2.8 and section 2.2.9.

Overall, eleven multiply charged protein peaks were successfully observed and identified during the process, with tandem mass spectrometry (MS/MS) (top 11

shown in Table 3.2). Full identification data are shown in appendix 2. Notably, eight proteins (Acid stress chaperone HdeA, Acid stress chaperone HdeB, Cold shock-like protein CspE, Acyl carrier protein, Uncharacterized protein YibT, Phosphocarrier protein HPr, Phosphate starvation-inducible protein PsiF, and protein YgiW) were significant as they had never been detected using LESA MS in previous research. Observing both the monomeric and dimeric forms of the same protein, including HdeA and HdeB shown in the Figure 3.4, within the same full scan mass spectrum indicates that it is possible to obtain structural information about proteins by this method.

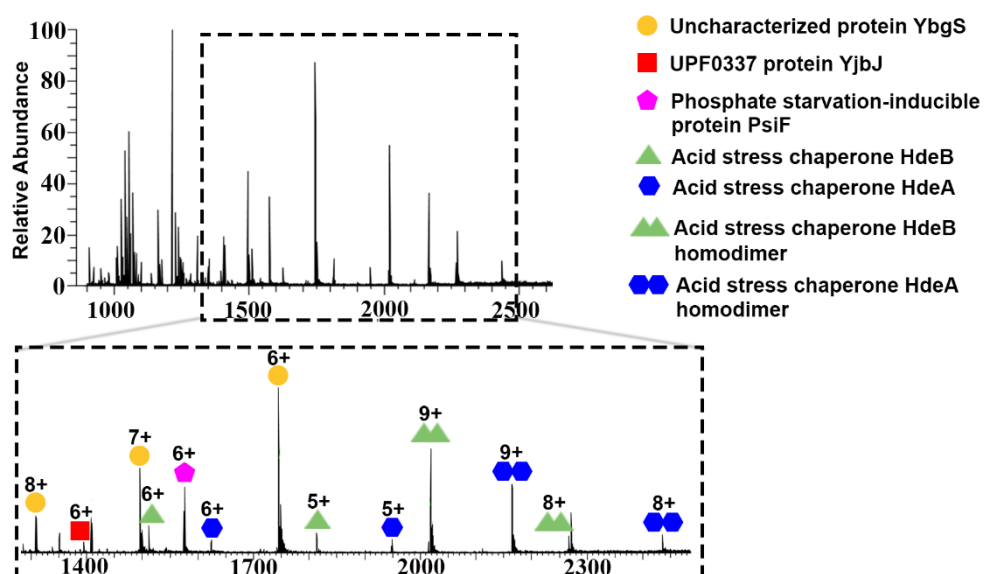


Figure 3.4 Representative full scan native LESA mass spectrum (of 27 replicates) obtained following electroporation of a living *E. coli* K12 colony.

Half of proteins identified using the current method, i.e., CspE, YibT, HdeA, HdeB, YbgS, and YgiW, are periplasmic proteins. These proteins are located and function within the region between the inner and outer membranes of the bacterial cell [271]. Among these proteins, HdeA and HdeB are protein dimers, while others are all monomeric proteins. The identification of monomeric periplasm proteins is shown in Figure 3.5.

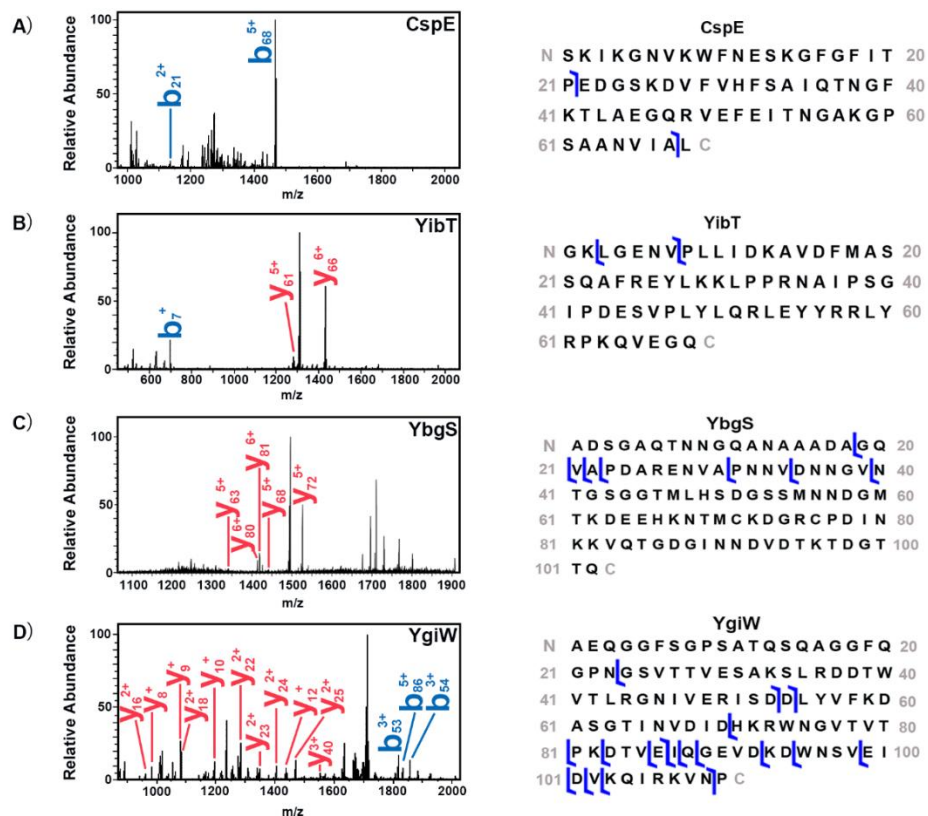


Figure 3.5 The identification of monomeric periplasm proteins; A) (left) HCD MS/MS spectrum of protein CspE 5+ monomer ions ($m/z\ 1466.7 \pm 4$) and (right) summary of sequence fragments observed; B) (left) HCD MS/MS spectrum of protein YibT 6+ monomer ions ($m/z\ 1311.0 \pm 4$) and (right) summary of sequence fragments observed; C) (left) HCD MS/MS spectrum of protein YbgS 7+ monomer ions ($m/z\ 1495.5 \pm 4$) and (right) summary of sequence fragments observed; D) (left) HCD MS/MS spectrum of protein YgiW 7+ monomer ions ($m/z\ 1710.9 \pm 4$) and (right) summary of sequence fragments observed.

The remaining proteins identified are cytoplasmic, including CspA, YjbJ, Acyl carrier protein, HPr and PsiF. Aside from the acyl carrier protein, which will be discussed later, the identification details of other cytoplasmic proteins are presented in Figure 3.6. These proteins reside within the cell cytoplasm and

engage with various cellular components to fulfil their roles, thereby contributing significantly to the cell overall functions and viability.

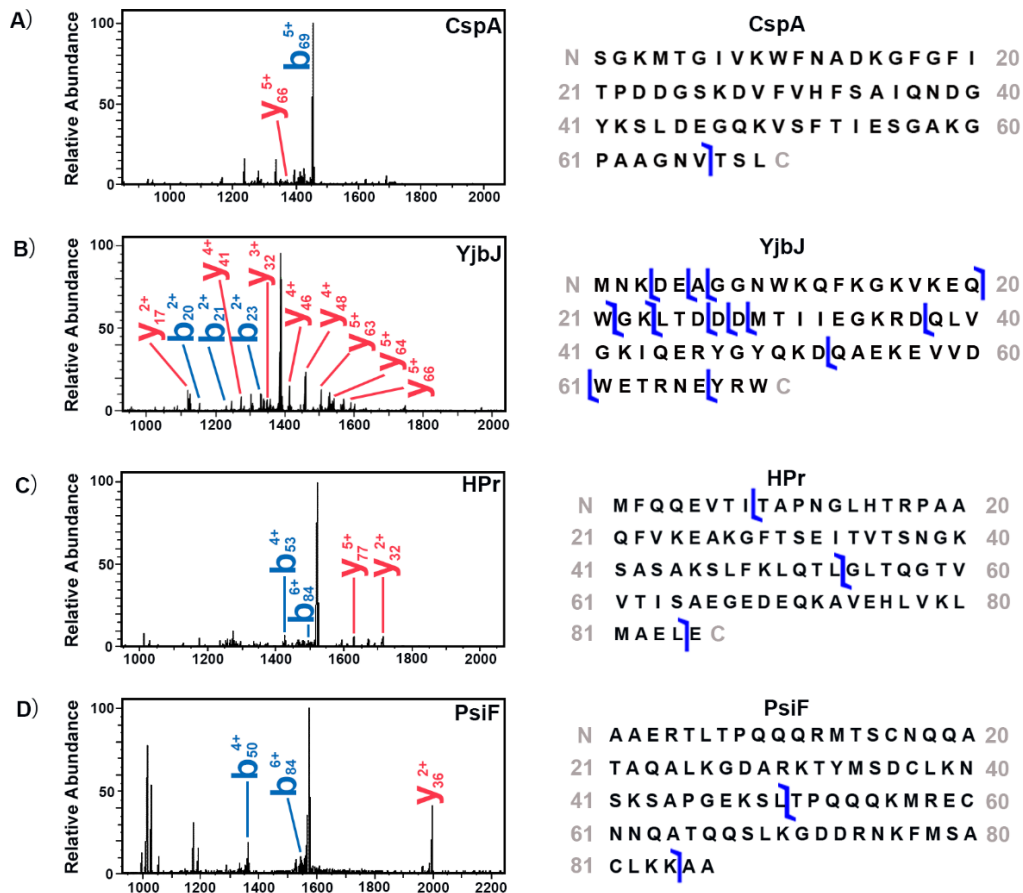


Figure 3.6 The identification of monomeric periplasm proteins; A) (left) HCD MS/MS spectrum of protein CspA 5+ monomer ions ($m/z\ 1454.3 \pm 4$) and (right) summary of sequence fragments observed; B) (left) HCD MS/MS spectrum of protein YjbJ 6+ monomer ions ($m/z\ 1387.7 \pm 4$) and (right) summary of sequence fragments observed; C) (left) HCD MS/MS spectrum of protein Hpr 6+ monomer ions ($m/z\ 1520.0 \pm 4$) and (right) summary of sequence fragments observed; D) (left) HCD MS/MS spectrum of protein PsiF 6+ monomer ions ($m/z\ 1575.0 \pm 4$) and (right) summary of sequence fragments observed.

Table 3.2 Summary of proteins identified following electroporation and native LESA top-down mass spectrometry of *E. coli* K-12 colonies

protein identity	m/z means	m/z calc	Δ ppm	charge state	MWcalc	remarks
Cold shock protein CspA	1454.3318	1454.7190	1.9360	+5	7267.5877	
Cold shock-like protein CspE	1466.5429	1466.5547	0.0590	+5	7327.7735	
Uncharacterized protein YibT	1310.8637	1310.8690	0.0319	+6	7859.2141	
UPF0337 protein YjbJ	1387.6714	1387.6821	0.0640	+6	8320.0923	
Acyl carrier protein	1701.6255	1701.6361	0.0526	+5	8503.1803	no methionine, no acetylation, no O3-phosphopantetheine-L-serine
	1770.4559	1769.6224	0.0958	+5	8843.1122	no methionine, no acetylation, O3-phosphopantetheine-L-serine
	1778.0382	1778.0244	0.0688	+5	8885.1222	no methionine, acetylation, O3-phosphopantetheine-L-serine
Acid stress chaperone HdeB	1812.4694	1812.8781	2.0437	+5	9059.3905	monomer
	2014.7389	2014.1979	-4.8690	+9	18118.7811	dimer
Phosphocarrier protein HPr	1519.9440	1519.9562	0.0731	+6	9113.7373	
Phosphate starvation-inducible protein PsiF	1574.9150	1587.4404	75.1527	+6	9518.6425	
Uncharacterized protein YbgS	1745.0939	1744.1006	-5.9596	+6	10458.6036	
Protein YgiW	1710.8410	1710.8553	0.1005	+7	11968.9873	
Acid stress chaperone HdeA	1947.4906	1947.9657	2.0430	+5	9734.8287	monomer
	2163.8416	2164.2953	4.0833	+9	19469.6575	dimer
Antigen 43 α chain	3557.1320	3557.1426	0.1487	+14	49758.7680	
Superoxide dismutase	3288.5097	3287.4381	-15.0024	+14	46023.1376	dimer with 2 Mn

HdeA (monomer), HdeB (monomer), CspE and Hpr have previously been detected using MALDI top-down MS in cell lysates from different strains of *E. coli* [272]. In a proteomics study using bottom-up LC-MS, HdeA, HdeB, YibT, and YgiW were successfully identified within outer membrane vesicles sourced from various strains of *E. coli* [273]. The Acyl carrier protein was previously detected in a bottom-up MALDI study [274].

Significantly, all of the proteins detected by the current method were native and intact. Protein YjbJ consistently exhibits stable signal intensities across the charge states of 4+, 5+, and 6+ in the mass spectrum, as depicted in Figure 3.4. In earlier work on LESA MS of bacteria with denaturing solvents, YjbJ was identified in a higher charge state of 7+ [77, 78]. Typically, native protein ions are detected in lower charge states [135] than are observed under denaturing conditions.

Protein monomers HdeA and HdeB consistently exhibit stable signal intensities in most full scan spectra, both with charge states of 5+ and 6+, shown in Figure 3.4. Additionally, HdeA and HdeB dimers, observed in charge states 8+ and 9+, also show stable signal intensities in most full scan spectra, illustrated in Figure 3.4. HdeA and HdeB are chaperone proteins which function in protecting bacteria from acidic environments [275, 276]. The primary process in which they are involved and play a crucial role is to assist in protein folding and prevent protein aggregation under low pH conditions [271, 276].

It is notable that the acyl carrier protein exhibits three different post-translational modification (PTM) states under the same conditions within the colony. That is,

a single protein can be detected in multiple proteoforms by this method, suggesting dynamic protein function. This protein serves as a carrier for acyl groups during fatty acid synthesis, facilitating the transfer of these groups between enzyme active sites to support fatty acid chain elongation and modification [277-281]. These processes are crucial for cellular functions such as membrane formation and energy production. The three observed PTM states of the acyl carrier protein include (1) no methionine, no acetylation, and no O3-phosphopantetheine-L-serine; (2) no methionine, no acetylation, with O3-phosphopantetheine-L-serine; and (3) no methionine, with acetylation, and O3-phosphopantetheine-L-serine. These three PTMs corresponds to different m/z values with 5+ charge states: 1701.63, 1770.36, and 1778.04, respectively. The MS/MS mass spectrum for the three proteoforms are presented in Figure 3.7.

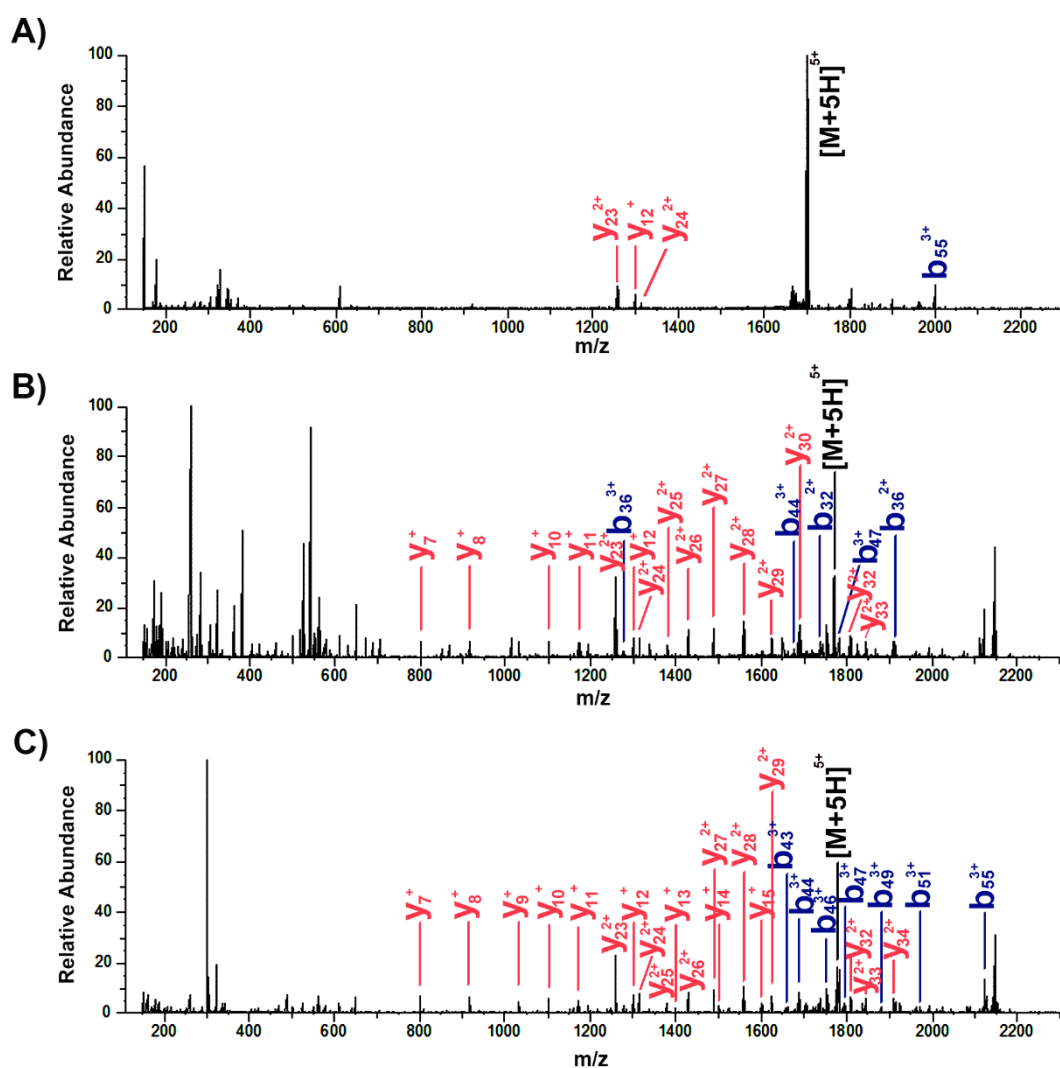


Figure 3.7 Native HCD MS/MS spectrum of three proteoforms of the acyl carrier protein. A) HCD MS/MS of acyl carrier protein with no methionine, no acetylation, and no O3-phosphopantetheine-L-serine (8503.1277 Da). B) HCD MS/MS of acyl carrier protein with no methionine, acetylation, and no O3-phosphopantetheine-L-serine (8843.2080 Da). C) HCD MS/MS of acyl carrier protein with no methionine, acetylation, and O3-phosphopantetheine-L-serine (8885.1910 Da).

3.3.4 Identification of protein assemblies

During the initial experiment, the analysis revealed the presence of two homodimer proteins. These proteins were identified as the acid stress chaperone protein HdeA and HdeB, as described above.

Focusing on the acid stress chaperone protein HdeA, which occurred in two different forms: the monomeric state, which had an approximate molecular weight of 9734.8 Da and exhibited charge states range from 4+ to 6+, and the homodimeric state with a molecular weight of around 19,469.7 Da and showed charge states of 8+ to 9+. Figure 3.8 shows the identification of HdeA intact homodimer.

The 9+ peak of HdeA dimer (m/z 2165.2 $\pm$ 4) was isolated and subjected to HCD (2% normalised collision energy). Two peaks corresponding to the monomeric mass of HdeA were observed in 4+ and 5+ charge states, shown in Figure 3.8 A), confirming the stoichiometry. Increasing the HCD normalised collision energy resulted in a higher signal intensity for the monomeric protein peaks of HdeA, while the signal intensity for the homodimer forms of HdeA decreased. HCD MS/MS of 5+ monomer ions at normalised collision energy to 32% resulted in the detection of sequence fragments, shown in Figure 3.8 B) and C), confirming the identity of the protein. Observed sequence fragments are listed in Table 3.3. All observed sequence cleavages were either C-terminal to aspartic acid or N-terminal to proline. These specific cleavage sites are known to be preferred in native mass spectrometry [282], enhancing the confidence level in protein

identification. The homodimer form of HdeA, as well as the homodimer of Hpr (which was not observed here), have been previously identified in proteomics experiments using *E. coli* lysates, which involved coupling size exclusion chromatography with capillary zone electrophoresis MS [283].

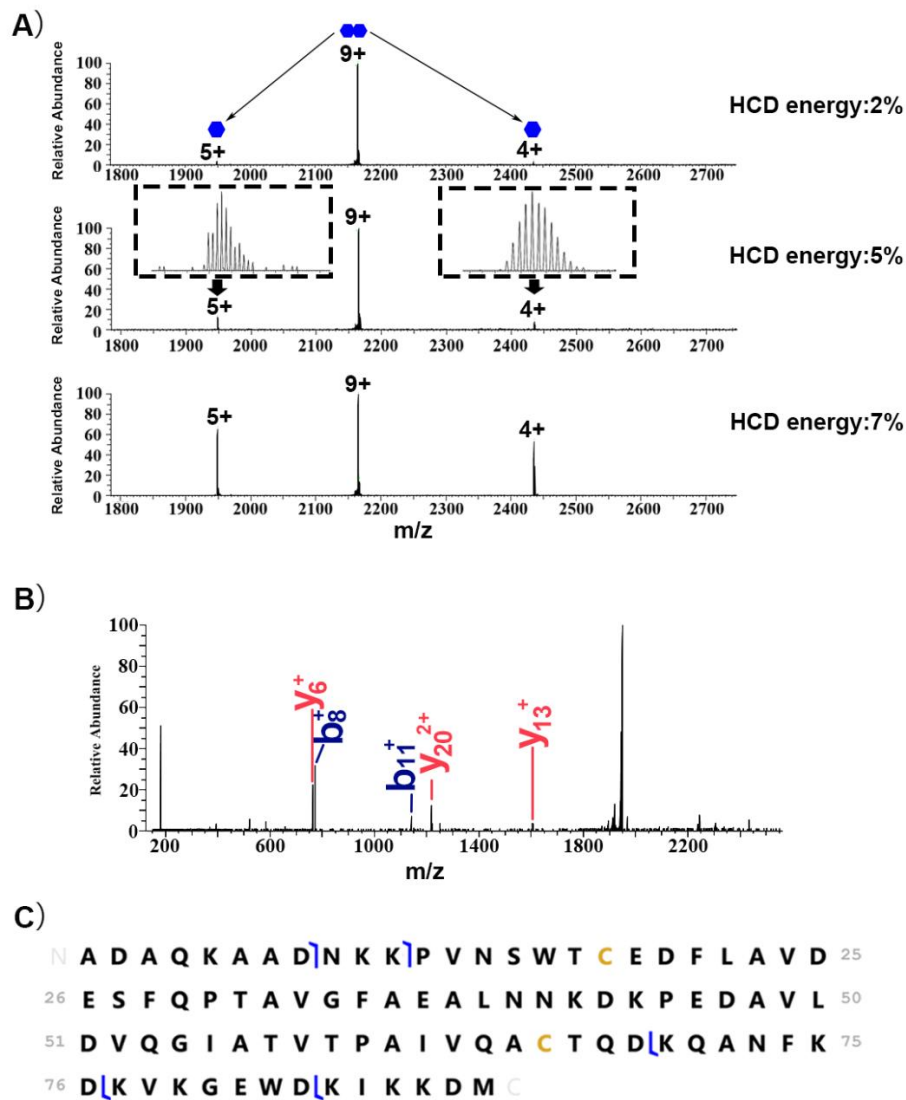


Figure 3.8 Identification of the HdeA homodimer. A) HCD MS/MS of 9+ precursor ions ($m/z\ 2165.2 \pm 4$) results in dissociation of dimer to monomer (5+ and 4+ charge states). B) HCD MS/MS of 5+ monomer ions ($m/z\ 1948.6 \pm 5$). C) Summary of sequence fragments observed.

Table 3.3 Sequence fragments observed following HCD of HdeA 5+ monomer ions

Name	m/z Monoisotopic	Mass Monoisotopic	Theoretical Mass	Mass Shift (Da)
b8	771.3584	770.3511	770.3559	-0.0048
b83	1795.2445	8971.1862	8973.3816	-0.0643
y81	1793.8864	8964.3956	8964.4727	-0.0772
y87	1592.1034	9546.5769	9548.7645	-0.0565

HdeB was successfully identified in two different forms: as the monomer (9059.4 Da, with charge states ranging from 4+ to 6+) and as the homodimer (18,118.80 Da, with charge states of 8+ to 9+). The identification of the homodimer is illustrated in Figure 3.9. HCD of the 8+ precursor ions ($m/z\ 2266.6 \pm 4$) resulted in the observation of monomer subunits across charge states 5+, 4+, and 3+. The 4+ monomer was overlapped with 8+ dimer peak in this spectrum. For further identification and characterisation of HdeB, MS/MS of the monomeric form was conducted, and the sequence fragments obtained are depicted in Figure 3.9. Observed fragments are listed in Table 3.4. These fragments not only confirmed the identity of the protein HdeB but also suggested the presence of a disulfide bond between Cys10 and Cys58, due to the 2 Da mass shift for most of the b ions apart from b₇.

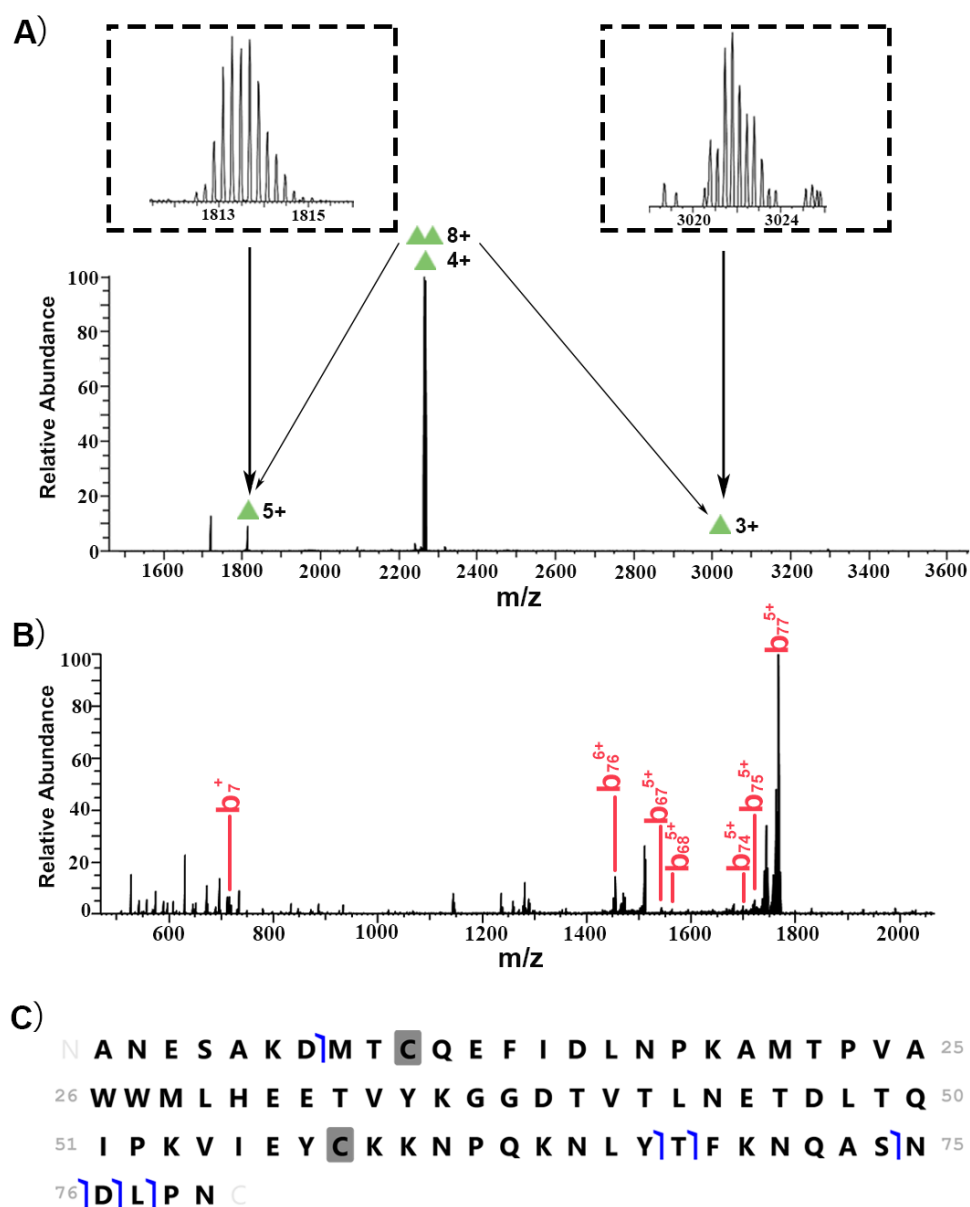


Figure 3.9 Identification of the HdeB homodimer. A) HCD MS/MS (35% NCE) of 8+ precursor ions ($m/z\ 2266.6 \pm 4$) results in dissociation of dimer to monomer (5+ and 3+ charge states). B) HCD MS/MS of 5+ monomer ions ($m/z\ 1813.5 \pm 5$). C) Summary of sequence fragments observed.

Table 3.4 Sequence fragments observed following HCD of HdeB 5+ monomer ions

Name	m/z Monoisotopic	Mass Monoisotopic	Theoretical Mass	Mass Shift (Da)
b7	716.3205	715.3132	715.3137	-0.0005
b67	1542.9328	7709.6276	7711.7485	-2.1209
b68	1563.1545	7810.7363	7812.7962	-2.0509
b74	1698.2065	8485.9963	8488.1302	-2.1339
b75	1721.0329	8600.1280	8602.1731	-2.0451
b76	1744.0288	8715.1075	8717.2001	-2.0926
b76	1453.5261	8715.1130	8717.2001	-2.0969
b77	1766.6602	8828.2647	8830.2841	-2.0194

To validate the presence of the disulfide bond, EThcD was performed on the 5+ monomer ions (m/z 1813.5 ± 5), as detailed in Table 3.5. EThcD is a hybrid fragmentation technology that combines ETD and HCD. In this process, ETD is first applied by making multiple charged protein ions interact with radical anions, producing c- and z- ions from peptide ions via cleavage of N-C α bonds [284-287], and cleaving disulfide bonds [287, 288]. Following ETD, HCD was applied to generate b- and y- ions through peptide backbone fragmentation. EThcD allows for the comparison of different types of fragments and provides additional information on protein structure. The fragments observed following EThcD of 5+ HdeB monomer ions are shown in Table 3.5.

Table 3.5 Sequence fragments observed following EThcD of HdeB 5+ monomer ions

Name	Mass Monoisotopic	Theoretical Mass	Mass Shift (Da)
b27	3077.3334	3077.4230	-0.0896
c27	3094.4582	3094.4496	0.0086
c30	3475.6430	3475.6330	0.0100
z30	3518.6194	3518.8252	-0.2058
y24	2854.3316	2856.4010	-2.0694

Based on the information obtained from EThcD MS/MS of HdeB monomer ions, it was confirmed that there is a disulfide bond between Cys10 and Cys58. Most experimentally observed fragment ions matched their theoretical mass to within 60 ppm with the exception of the y₂₄ fragment, which shows a 2 Da mass difference. Possible explanations are that the free radicals generated from the broken disulfide bond may react with the amino acid side chain, leading to the formation of a cyclic structure [289], or the interaction between radicals of different fragmentations resulting in a loss of two H⁺.

3.3.5 Identification of higher mass proteins Protein Metal Bond

The proteins identified so far in this work have a maximum molecular weight of 20,000 Da. In order to target larger proteins, the mass spectrometer was tuned for transmission of higher mass ions as described in Chapter 2, section 2.2.7. In particular, the in-source collision voltage was tuned to 80V, while no voltage was

applied in previous experiments. In addition to the proteins identified as described in Chapter 3.3.3 and 3.3.4, two higher molecular weight proteins were observed with the application of in-source collision energy, shown in Figure 3.10. The peaks at m/z 3319.6 (15+ charge state) and 3557.1 (14+ charge state) were identified as the monomeric antigen 43 α chain (49,757.7672 Da). This protein has not been detected by LESA before, although has been identified in bottom-up studies [290-292]. The 14+ charge state peak of the superoxide dismutase (SOD) [Mn] dimer at m/z 3288.5 was observed in the full scan spectrum.

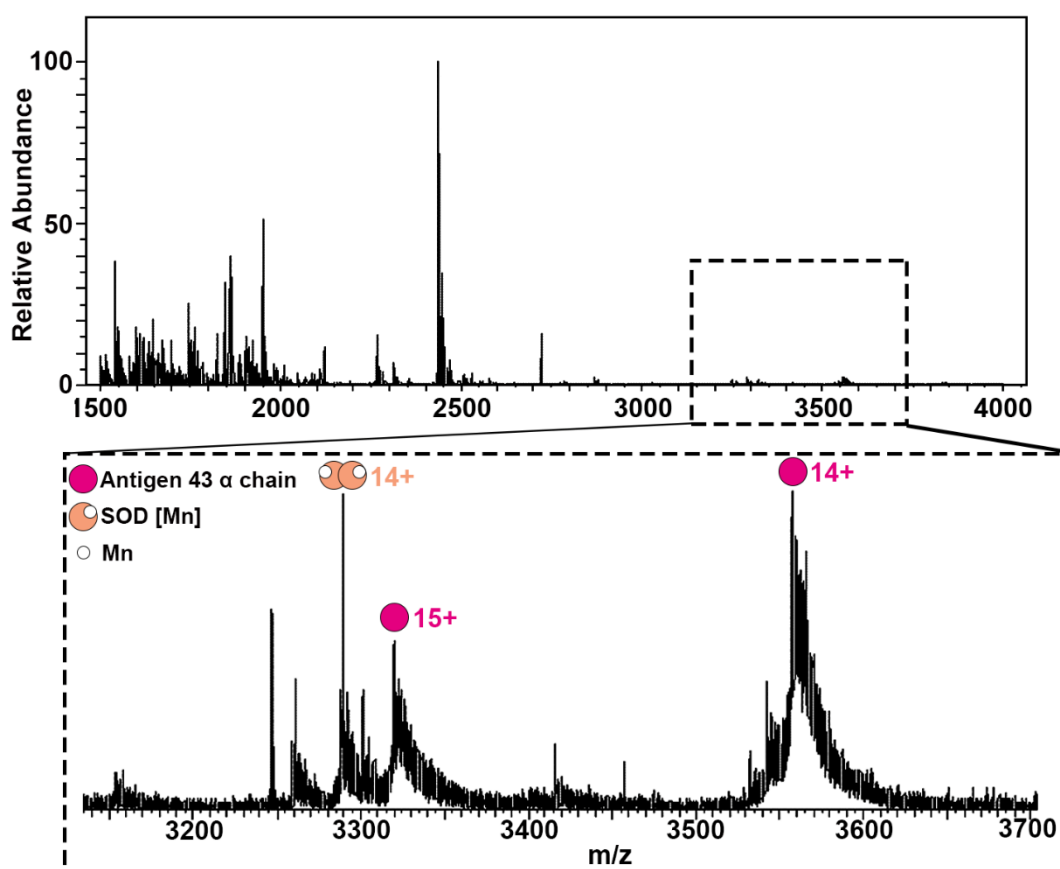


Figure 3.10 Representative full scan mass spectrum (from three replicates) obtained from living *E. coli* K12 colony with 80V insource collision energy.

Antigen 43 α chain has functions related to cell adhesion, biofilm formation, and bacterial aggregation [293]. Accurate determination of the intact mass in the high-resolution full scan mass spectrum proved challenging due to high background noise, relatively low signal intensity and isotope distribution. To address this, PT-CR MS was employed as described in Chapter 2, section 2.2.8. PT-CR involves an ion-ion interaction between protein cations and a reagent anion with resulting proton transfer and reduction in the protein charge state. The PT-CR mass spectrum obtained for the 15+ charge state is shown in Figure 3.11 A) and confirmed the protein mass as 49,758.6 Da, in good agreement with the theoretical mass (49,758.7680 Da). The MS/MS spectrum of antigen 43 α chain is shown in Figure 3.11 B).

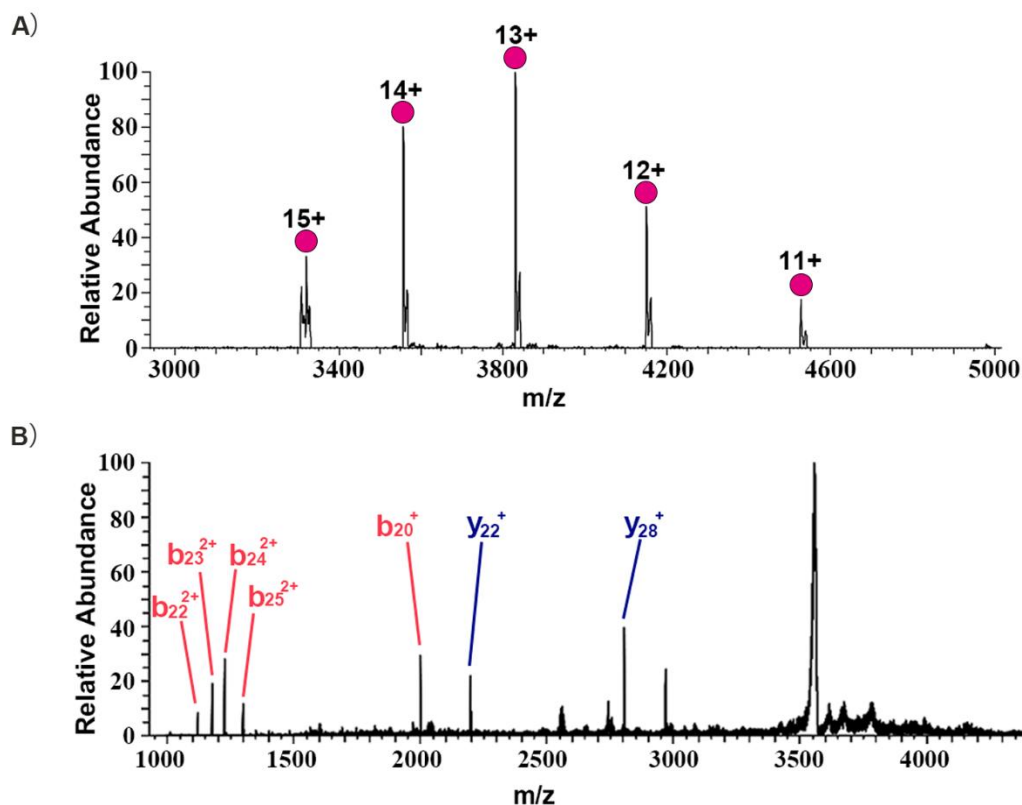


Figure 3.11 Identification of protein antigen 43 alpha chain A) PTMS mass spectrum obtained for precursor ions with m/z 3320 (15+ charge state) with isolation window of 10 m/z. Deconvolution of this spectrum confirms the mass of the proteins to be 49,758.6 Da; B) HCD MS/MS spectrum of ions centred at m/z 3556.0 ± 6 (14+ charge state), identified as antigen 43 alpha chain.

The second protein, identified as SOD [Mn], was observed as a homodimer with a mass of 46,038.0158 Da. SOD [Mn] is the largest protein assembly successfully detected using native LESA MS of bacteria. Significantly, each of the protein subunits binds a Mn²⁺ ion. The protein SOD [Mn] plays a crucial role in getting rid of harmful and toxic superoxide anion radicals inside the bacterial cell to maintain cellular redox balance and to protect cells from oxidative damage [294]. The complex stoichiometry was verified using MS/MS with HCD at 28% normalised collision energy, leading to the dissociation of the dimer into its monomeric

subunits observed in charge states 5+ and 9+, as illustrated in Figure 3.12.

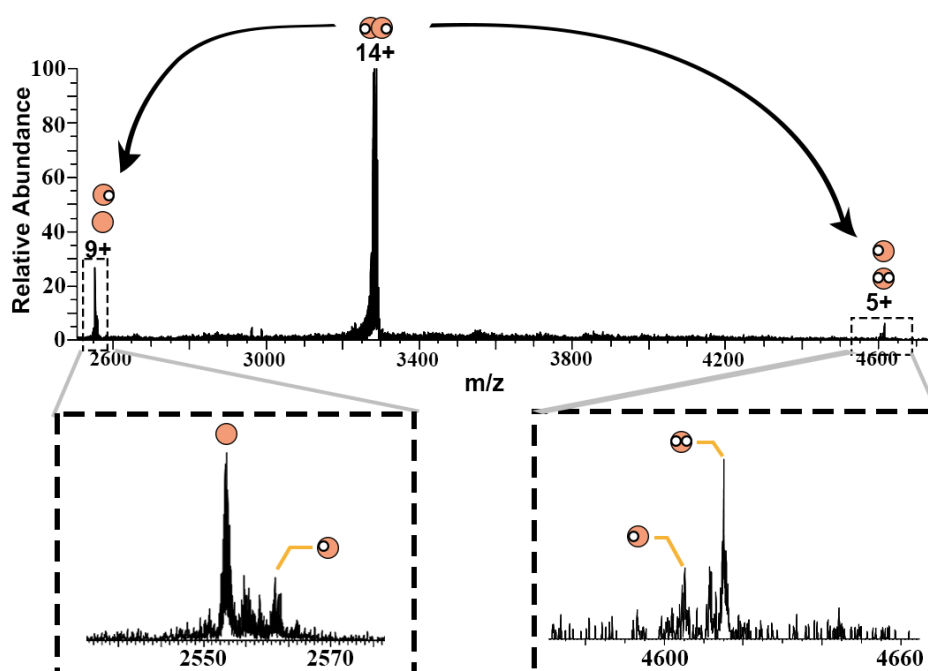


Figure 3.12 Native LESA mass spectrum of Mn-bound superoxide dismutase dimer. HCD of the 14+ precursor ions ($m/z\ 3289.5 \pm 6$) results in dissociation of the dimer to monomer (9+ and 5+ charge state) with differing numbers of Mn^{2+} bound.

After subjecting the sample to HCD fragmentation, an interesting asymmetric distribution of metal ions among the subunits was observed. Specifically, for the 9+ charge state monomer, two distinct peaks were observed: one peak corresponded to the monomer with no metal bound, while the other corresponded to the monomer with one Mn^{2+} bound, (see Figure 3.12. Similarly, in the monomer with a 5+ charge state, two distinct forms were detected: one with a single Mn^{2+} ion bound and the other with two Mn^{2+} ions bound. This observation is indicative of a complex connection between metal coordination, charge states and MS

fragmentation. Similar metal ion asymmetric distribution of SOD [Mn] homologous protein SOD 1 were observed in previous research [295]. Each SOD1 dimer can bind up to three metal ions, ranging from none to three, after redistribution, as shown by native MS/MS [295]. Here, the results indicate that after native HCD MS/MS, each SOD [Mn] subunit can bind between zero and two Mn^{2+} ions due to ion redistribution. Moreover, as the HCD energy increased further, we were able to detect additional sequence fragments, that provided crucial information which allowed us to confirm the identity of the protein.

3.3.6 Gram Positive Bacteria Native Protein Detection

Following the experiments with Gram-negative *E. coli*, the workflow was applied to the Gram-positive bacteria *Staphylococcus epidermidis*. Two strains of *Staphylococcus epidermidis*, *RP62a* and *HB* were used, as described above. First, the Gram-positive bacterial colonies were cultured on TSB medium in 6 cm diameter Petri dishes and incubated overnight at 37°C. Once the colonies reached an appropriate thickness of approximately 0.2-0.3 mm, samples were collected. After applied 2 μ l of 200 mM ammonium acetate, electroporation was performed on the Gram-positive bacteria using a voltage of 3 kV (corresponding to a field strength of 15 kV/cm), with 35 pulses and a pulse length of 20 μ s. Sample extraction was then carried out using 3 μ L of a solvent containing 200 mM ammonium acetate, 2 x CMC C8E4. The extraction process was consistent with the method previously described for *E. coli* K12. Full scan MS detection of

three replicates was applied in IRM standard pressure mode with m/z range from 1500 to 6000. Unfortunately, this method which combines electroporation and native LESA MS showed a negative result for detecting native intact proteins from *Staphylococcus epidermidis* by Figure 3.13.

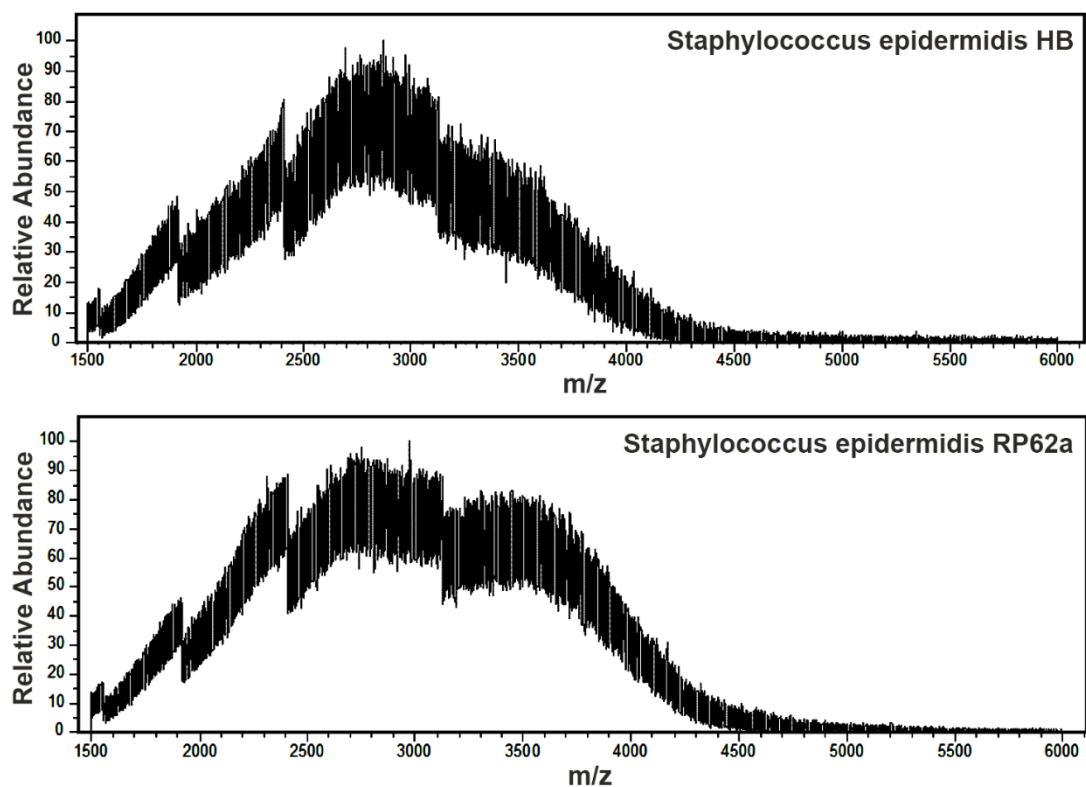


Figure 3.13 Representative LESA mass spectrum of three replicates obtained following electroporation on *Staphylococcus epidermidis* HB and RP62a of three replicates.

Previous research suggests that there is no method of electroporation which is suitable for all classes of bacteria [296], likely due to the structural differences between gram-positive and gram-negative bacteria. Gram-positive bacteria, with their distinct cell wall structure and composition compared to gram-negative

bacteria, may respond differently to electroporation conditions [297]. Specifically, the cell wall of gram-positive bacteria is mainly composed of peptidoglycans [296, 298, 299]. The peptidoglycan layer is thick and contributes significantly to the cell shape and strength [298-301]. In gram-negative bacteria, the cell is covered by a thinner peptidoglycan layer, located between the inner cytoplasmic membrane and the outer membrane [218, 302, 303]. Furthermore, Gram-negative bacteria have an outer membrane outside the peptidoglycan layer, which is important for structural integrity and protection [304]. This structural difference would imply potential variations in susceptibility to electrical fields during electroporation, resulting in different cell disruption efficiency by electroporation for both type of the bacteria. The thicker cell wall of gram-positive bacteria will make the cell particularly resistant to disruption [305], which might limit the release of intact proteins for subsequent analysis by native LESA MS. Multiple investigations have previously demonstrated that gram-negative bacteria could be rapidly disrupted by an electrical field in order to either introduce or eliminate molecules [270, 306-308]. Due to the non-efficient disruption of gram-positive bacteria, the efficiency of protein extraction of gram-positive bacteria would be lower compared to gram-negative bacteria.

Previous research on electroporation has mostly focused on gram-negative bacteria strains [270, 306-308]. The electroporation conditions for the current method (such as voltage, pulse duration, and number of pulses) which were established based on gram-negative bacteria are not certain to be efficient and useful for gram-positive bacteria.

3.4 Conclusions

In summary, an approach was established which integrates electroporation with native LESA MS. This method is suitable for identification of intact proteins in their native, i.e., folded, state, providing valuable structural information, which offers a more comprehensive understanding of protein function, interactions, and conformational states within biological systems. The experimental results also verified the importance of electroporation in enhancing the repeatability and reliability of protein detection using native LESA MS. While it was possible to detect proteins without electroporation in some cases, the application of this technology consistently yielded higher repeatability in protein identification. Moreover, optimising experimental conditions, such as adjusting the pipette tip to the proper height to increase protein extraction efficiency, or using a small volume of 200 mM ammonium acetate, was crucial in preventing air breakdown and ensuring the stability of the electroporation process.

By this method several proteins were observed, out of which thirteen were successfully identified using MS/MS. It is possible to identify multiple proteoforms, i.e., the same protein with different PTMs. Among the proteins identified, three protein assemblies were detected, including the homodimers HdeA, HdeB, and the manganese-bound dimer of superoxide dismutase SOD [Mn]. The confirmation of these protein stoichiometries and their identification were achieved through MS/MS analysis, which led to the dissociation of the dimers into their monomeric subunits across different charge states. In comparison to

previous studies focusing on denaturing intact protein identification using LESA MS in bacteria, two larger proteins were observed with a mass approximately around 50,000 Da, one of which was metal bound. An interesting aspect of the findings was the observed asymmetric distribution of metal ions among the subunits of SOD [Mn] protein complexes when it was fragmented with HCD. This observation matched with similar behaviours observed in other metal-binding superoxide dismutase protein assemblies studied in previous research [75, 309].

This research shows the potential applications of monitoring the changes of proteins and protein structures, such as cold shock proteins, in living bacteria. By tracking these molecular variations in real time, this research could provide a deeper understanding of different biological processes in bacteria, for example, stress response, adaptation mechanism, or antibiotic resistance process. Monitoring these processes in living bacteria enhances the understanding of protein dynamics, which has implications for various fields. Further work would involve extending this method to gram-positive bacteria and yeast, which could significantly broaden its applications across related fields.

Chapter 4

Native LESA MS detection of native intact proteins directly from yeast colonies

4.1 Introduction

Detecting native intact proteins from a yeast colony presents a significant challenge due to the relatively thick yeast cell walls (50-500 nm) [310] compared to bacterial cell walls (1-80 nm) [214, 311]. However, unlike the double-layer membrane structure which exists in Gram-negative bacteria, yeast cells possess a single-layer membrane. This structural difference suggests that once the barrier of the cell wall is overcome, a greater number of cytoplasm proteins should be detected.

Based on the successful application of native LESA MS in detecting native intact bacterial proteins (described in Chapter 3), the exploration of different methodologies was conducted to adapt this technique for detecting proteins in yeast, specifically *S. cerevisiae*. Due to a higher sensitivity of yeast cells to electric fields [254, 312], the electroporation parameters were optimised to enhance cell wall permeability, and a solvent with optimised composition was

employed to facilitate protein extraction. Additionally, FAIMS was employed in the identification of some proteins. The gas-phase ion separation before MS analysis afforded by FAIMS, enabling ions to be separated before mass analysis based on their differential mobilities in variable electric fields, leads to a less noisy mass spectrum and reduced overlap of protein peaks with similar m/z values [144, 313-315].

These combined efforts led to the development of an effective method for detecting native intact yeast proteins by native LESA MS.

4.2 Experimental Section

4.2.1 Sample Preparation

S. cerevisiae (strain ATCC 204508 / S288c) colonies were cultured as described in Chapter 2, section 2.2.1.

4.2.2 Method Optimisation

A method was designed (shown in Figure 4.1) based on those presented in Chapter 3 with the aim of detecting native proteins from yeast colonies.

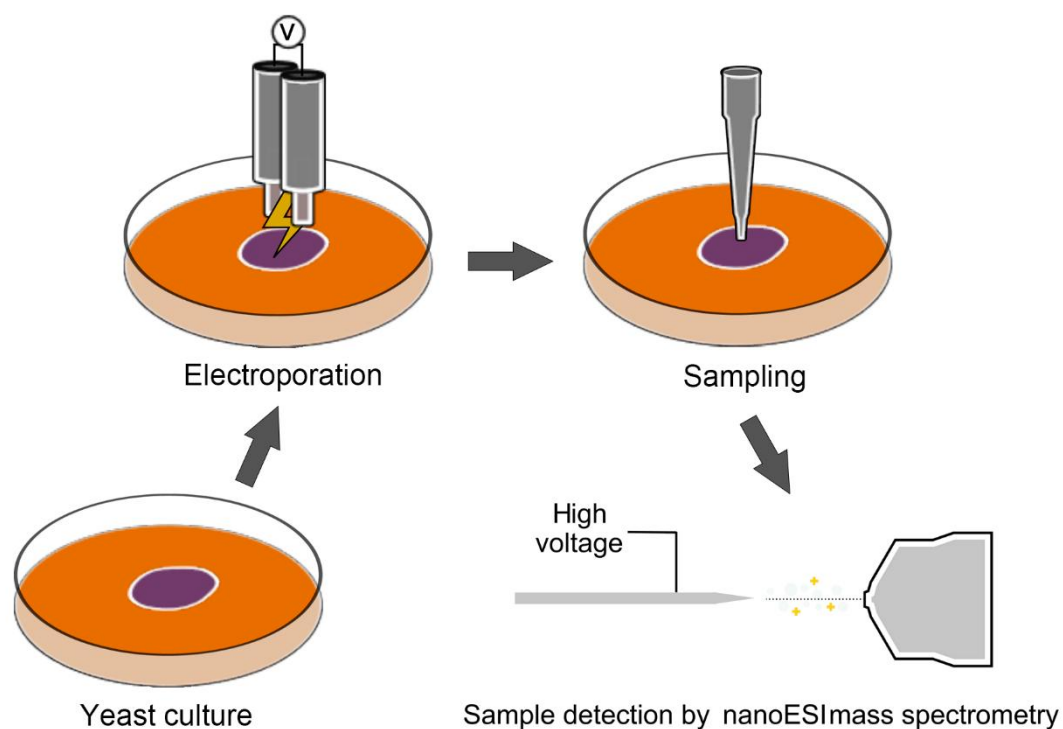


Figure 4.1 Workflow for native LESA mass spectrometry detection of proteins in yeast colonies. Optimised extraction solvents and MS parameters were applied.

4.2.3 Protein Identification

Protein identification was achieved by different fragmentation technologies including HCD and PTMR, deconvolution and database searching, as described in Chapter 2, section 2.2.8, section 2.2.9 and section 2.2.10.

4.3 Results and Discussion

4.3.1 Native LESA MS of *S. cerevisiae*

A new method was developed to analyse native proteins from living yeast colonies by combining electroporation with native LESA MS. In the initial experiment, the yeast colony was exposed to an electric field of 10 kV/cm, which is equivalent to 2 kV across a 2 mm gap. A representative mass spectrum of three replicates obtained following LESA with an extraction solvent comprising 200 mM ammonium acetate (workflow 1) is shown in Figure 4.2 A). Since the results showed that proteins were barely detected, 2 x CMC of the detergent C8E4 (CMC=0.5% w/w), a non-ionic and relatively mild MS-compatible detergent, were added to the LESA extraction solvent of 200 mM ammonium acetate (workflow 2). This detergent can help disrupt the lipid environment of the cell membrane [316, 317]. A representative mass spectrum of three replicates is shown in Figure 4.2 B).

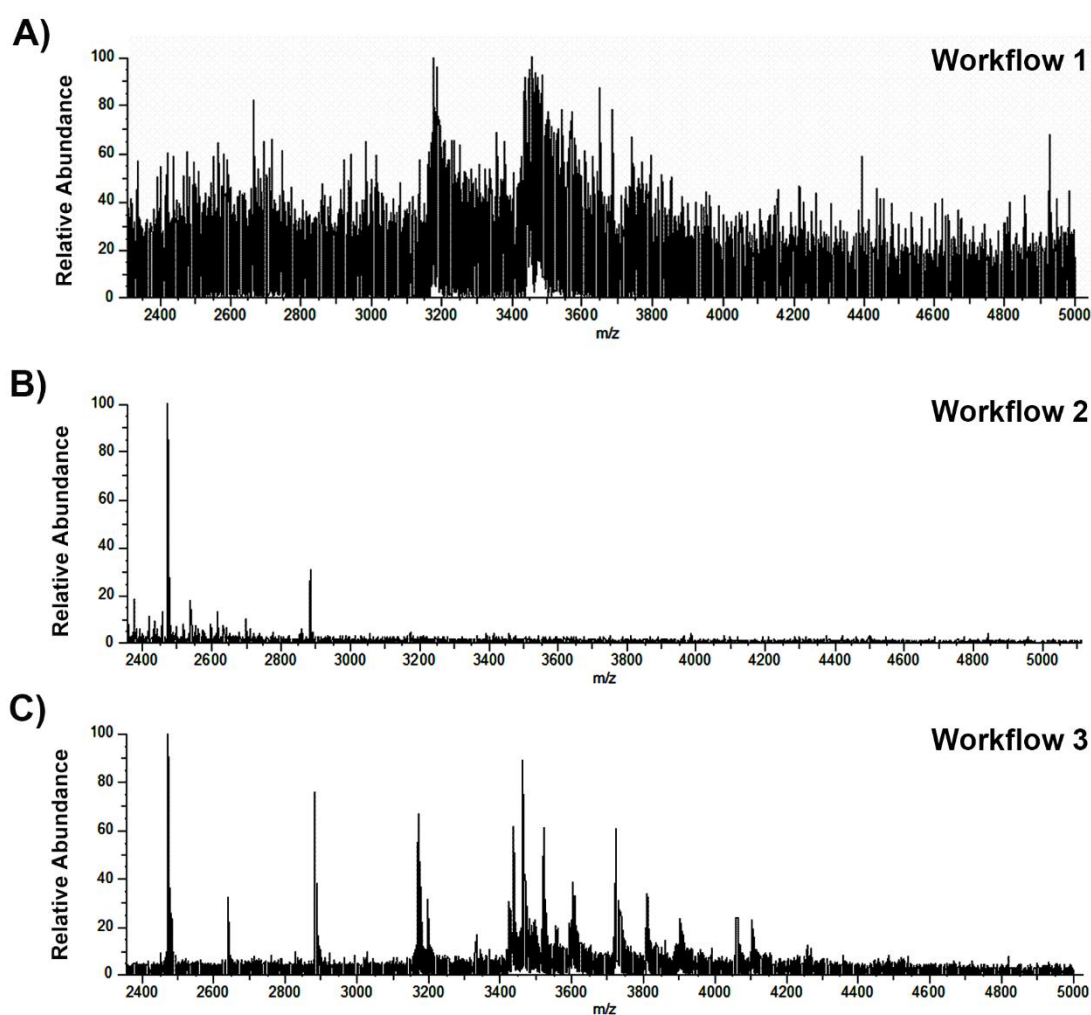


Figure 4.2 Representative LESA mass spectrum obtained following the three workflows described in Chapter 4.3.1. A) LESA mass spectrum obtained following electroporation after extraction of *S. cerevisiae* (strain ATCC 204508 / S288c) colony using 200 mM ammonium acetate. B) LESA mass spectrum obtained following electroporation after extraction of yeast colony using 200 mM ammonium acetate with 2 x CMC C8E4. C) LESA mass spectrum obtained following electroporation after extraction of yeast colony using 200 mM ammonium acetate with 2 x CMC C8E4 and 3% of methanol.

As only a few proteins were observed, a low concentration (3%) of methanol was added to the solvent containing 200 mM ammonium acetate and 2 x CMC C8E4 (workflow 3). This adjustment increases cell permeability while preserving protein complex integrity [317]. A representative mass spectrum is shown in Figure 4.2 C).

As demonstrated by the mass spectrum in Figure 4.2, the workflow that involved sampling the yeast colony with 200 mM ammonium acetate, 2 x CMC C8E4, and 3% methanol after electroporation exhibited optimal signal-to-noise ratio for detecting proteins from yeast colonies by native LESA MS.

4.3.2 Identification of proteins

Figure 4.3 shows the annotated spectrum of Figure 4.2 C).

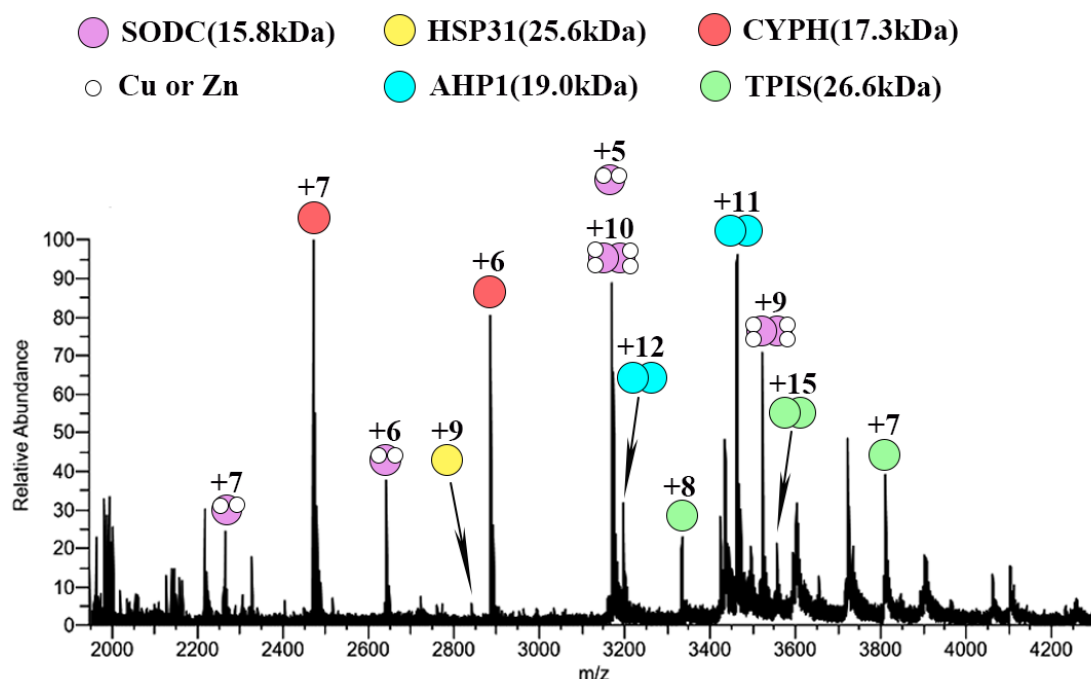


Figure 4.3 Representative full scan native LESA mass spectrum obtained from living *S. cerevisiae* colony.

Most of the proteins were detected within the m/z range of 2200-4500. The methodology for protein identification is detailed in Chapter 2, section 2.2.8 and section 2.2.10. In comparison to the detection method used for *E. coli* colonies, in-source collision energy of 80V was employed to minimise the impact of detergent micelles, thereby enhancing the signal-to-noise ratio.

The initial experiment was conducted in IRM standard pressure mode, resulting in the identification of 10 proteins, as detailed in Table 4.1. Notably, all identified proteins were cytoplasmic, suggesting that the current approach could detect intracellular proteins in yeast cells.

Table 4.1. Summary of proteins from *S. cerevisiae* identified following electroporation and native LESA top-down mass spectrometry in IRM standard pressure mode.

ID (UniProt)	Protein mass calc (Da)	Protein mass observed (Da)	m/z average	Charge state	mass diff (Da)
P22943(HSP12_YEAST)	11596.6288	11596.5888	1934	6	0.04
			1290	9	
P0CG63(UBI4P_YEAST)	8551.5872	8551.5861	1712	5	0.011
			1426	6	
P14832(CYPH_YEAST)	17290.6690	17290.592	2884	6	0.077
			2472	7	
P53912(YNN4_YEAST)	41049.5388	41050.3085	3424	12	0.7697
			4103	10	
P31787(ACBP_YEAST)	9924.0450	9925.0186	1978	5	0.9736
			2768	4	
P22803(TRX2_YEAST)	11063.5450	11079.4902	1846	6	15.9452
			2842	9	
Q04432(HSP31_YEAST)	25554.97	25554.85	3810	7	0.121
			3333	8	
P00942(TPIS_YEAST) monomer/dimer	26647.9230(monome r)	26647.7358(monomer)	3556	15	0.3744
	53295.8460(dimer)	53295.4716(dimer)	3462	11	
P38013(AHP1_YEAST) monomer/dimer	38027.0612(dimer)	38053.8294(dimer)	3174	12	26.7682
			2930	13	
P00445(SODC_YEAST) monomer/dimer	15871.7327(monome r)	15835.6357(monomer)	3170	5	36.097
			2642	6	
			2264	7	
	31743.4654(dimer)	31671.2714(dimer)	3522	9	72.194

The 12 kDa heat shock protein (HSP12) was detected by LESA mass spectrometry under denaturing conditions with observed molecular weight of 11,596.59 Da in previous research [145]. Under denaturing conditions, the charge state distribution of the protein HSP12, which lacked the initiator methionine and had an acetylation on the N-terminal serine, was observed to include 725.93 (16+), 774.19 (15+), 829.34 (14+), 893.06 (13+), 967.57 (12+), 1055.44 (11+), and 1161.28 (10+) [145]. Under the native-like conditions used here, a lower charge state distribution was observed, including two prominent peaks at 1932.94 (6+) and 1289.64 (9+). The peak at 1932.94 (6+) was isolated and fragmented by HCD for identification, as shown in Figure 4.4.

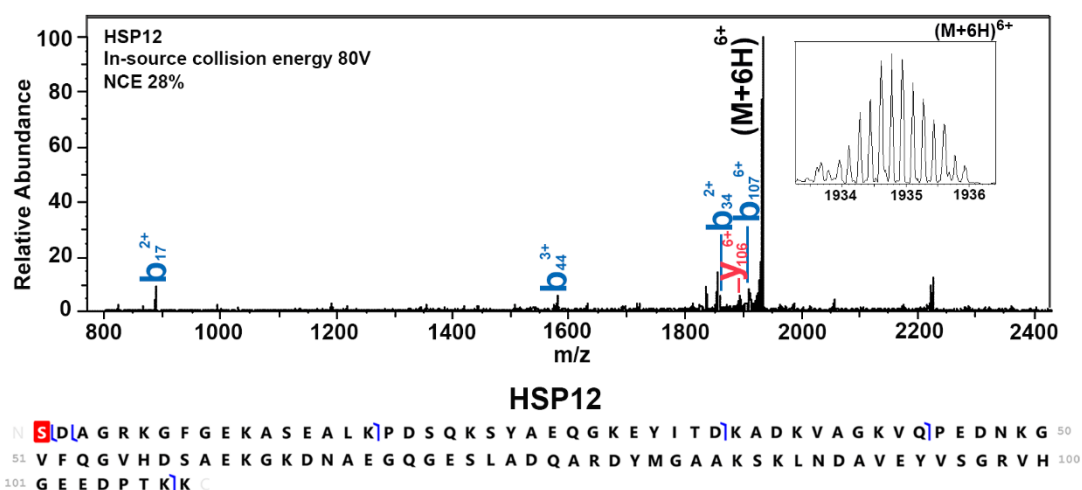


Figure 4.4 Protein identification of HSP12. (Top) MS/MS mass spectrum of HSP12 and (bottom) summary of sequence fragments observed.

With the exception of the HSP12, the other nine proteins summarised in Table 4.1 had never been detected using LESA mass spectrometry under denaturing conditions in previous research. These proteins include Superoxide dismutase [Cu-Zn] (SODC), Triosephosphate isomerase (TPIS), Peroxiredoxin (AHP1),

Polyubiquitin (UBI4P), Thioredoxin-2 (TRX2), Peptidyl-prolyl cis-trans isomerase (CYPH), Glutathione-independent glyoxalase (HSP31), Uncharacterized protein YNL134C (YNN4) and Acyl-CoA-binding protein (ACBP). Among the nine proteins detected by native LESA MS but not by denaturing LESA MS, SODC, TPIS and AHP1 were observed in their dimeric forms.

The monomeric protein UBI4P has been investigated in previous studies using bottom-up LC-MS in yeast [318] and for its role in protein-protein interactions via affinity-purification mass spectrometry (AP-MS) [319, 320]. However, it has never been detected in its native intact state by mass spectrometry. In the native LESA mass spectrum, UBI4P displayed a charge state distribution that included m/z 1711.31 (5+) and m/z 1426.66 (6+). The fragmentation and identification of the 5+ ions of UBI4P by native LESA MS is shown in Figure 4.5.

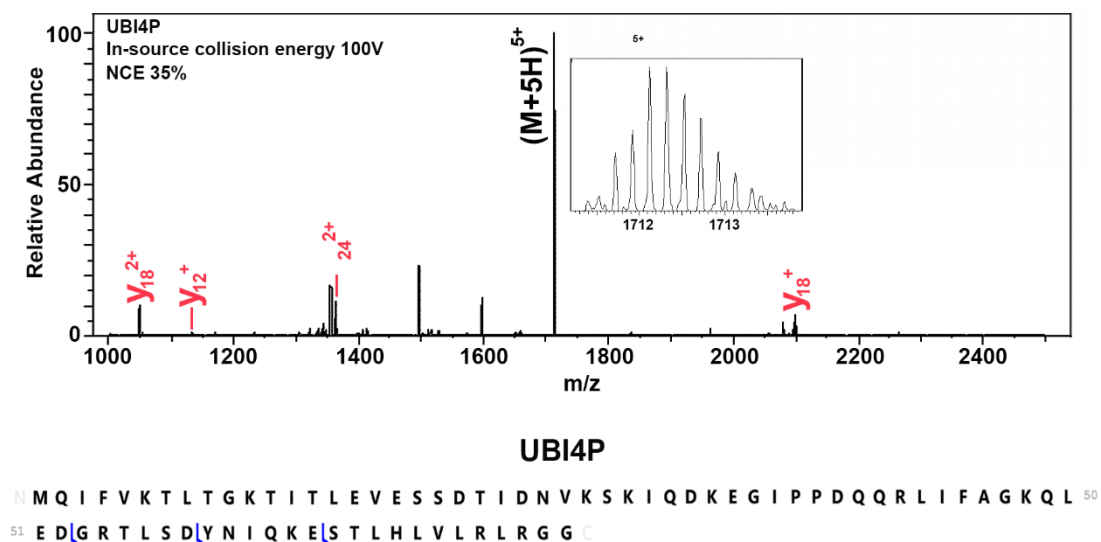


Figure 4.5 Protein identification of UBI4P. (Top) MS/MS mass spectrum of UBI4P and (bottom) summary of sequence fragments observed.

Only three y ions of UBI4P were detected; however, two of them matched the characteristic pattern of native top-down fragmentation, which tends to occur predominantly at the C-terminal to aspartic acid or N-terminal to proline [282]. This provides convincing evidence for protein identification.

CYPH, which catalyses the cis/trans isomerization of the peptide bond N-terminal to a proline residue [321], was previously identified by top-down MS coupled with high-field asymmetric waveform ion mobility spectrometry and capillary zone electrophoresis [322]. Here, the CYPH protein chain, with the N-terminal methionine removed, exhibited a charge state distribution featuring peaks at 2471.51 (7+) and 2883.61 (6+). Figure 4.6 provides the identification details of CYPH.

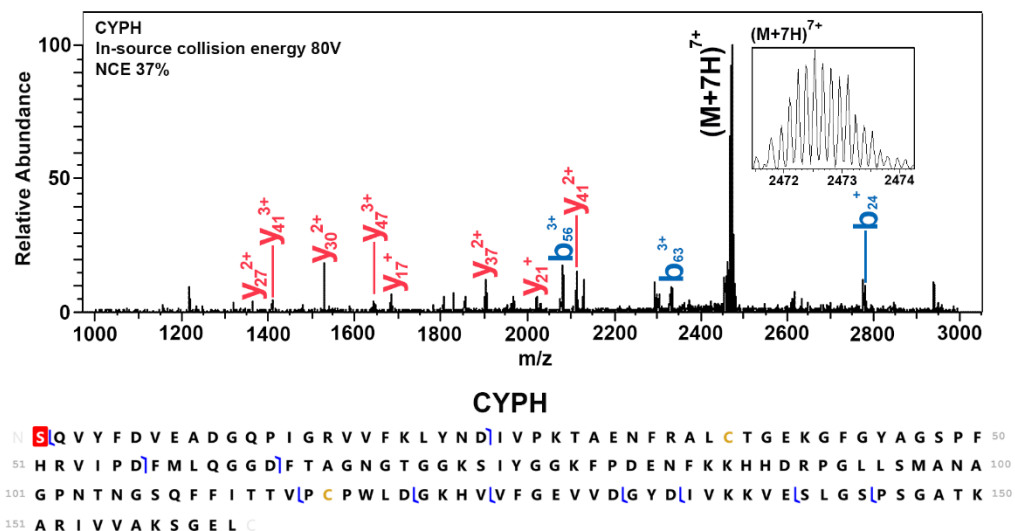


Figure 4.6 Protein identification of CYPH. (Top) MS/MS mass spectrum of CYPH and (bottom) summary of sequence fragments observed.

In total, 11 fragments of CYPH were identified including three b ions and eight y

ions. Among those fragments, six of them occur at the C-terminal to aspartic acid while two of them occur at the N-terminal to proline. The fragments observed confirm the presence of acetylation at the N-terminus.

YNN4, an uncharacterized protein, was previously identified using bottom-up MALDI-MS [323, 324] and LC-MS [325]. Although the removal of the N-terminal methionine is not indicated in the UniProt database for YNN4, MS/MS detection suggests that this modification has occurred. As a result, the YNN4 protein chain, consisting of amino acids 2-376, exhibits peaks at m/z 3423.87 (12+) and m/z 4103.13 (10+). The identification of YNN4 following fragmentation of the 12+ ions is illustrated in Figure 4.7. The results confirm the presence of N-terminal acetylation. In total, 31 fragments were observed with a sequence coverage of 7%.

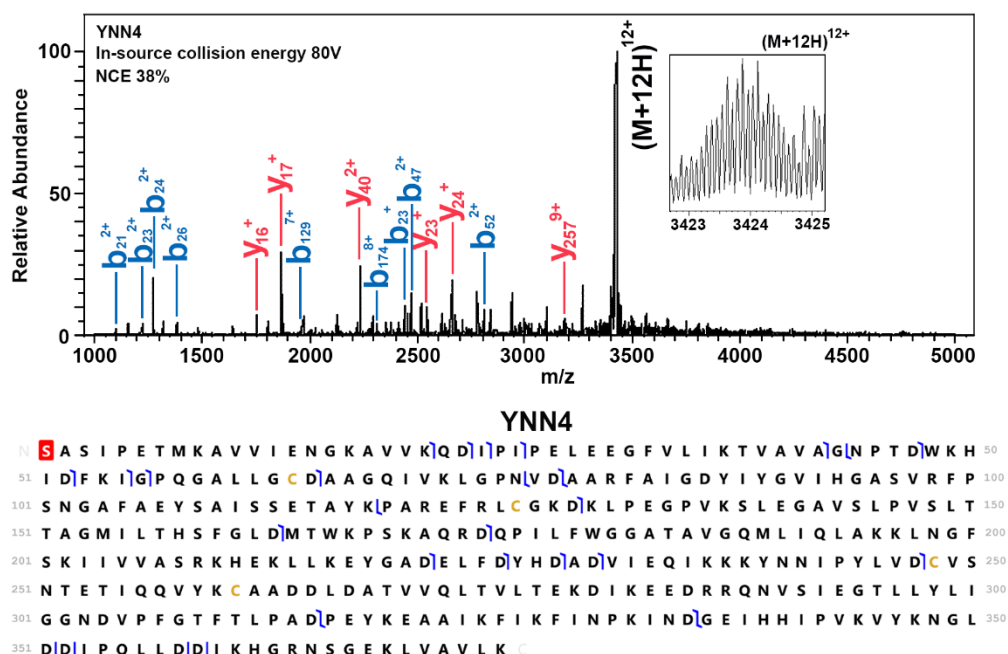


Figure 4.7 Protein identification of YNN4. (Top) MS/MS mass spectrum of YNN4 and (bottom) summary of sequence fragments observed.

ACBP, known for its role in transporting acyl-CoA molecules and interacting with cellular and plasma membranes [326, 327], was analysed by plasma-desorption time-of-flight mass spectrometer [328] and LC-MS [329, 330]. With the N-terminal methionine removed, the ACBP protein chain, consisting of amino acids 2-87 and having a theoretical mass of 9924.05 Da, was observed at m/z 1987.21 (5+). The identification of ACBP is shown in Figure 4.8.

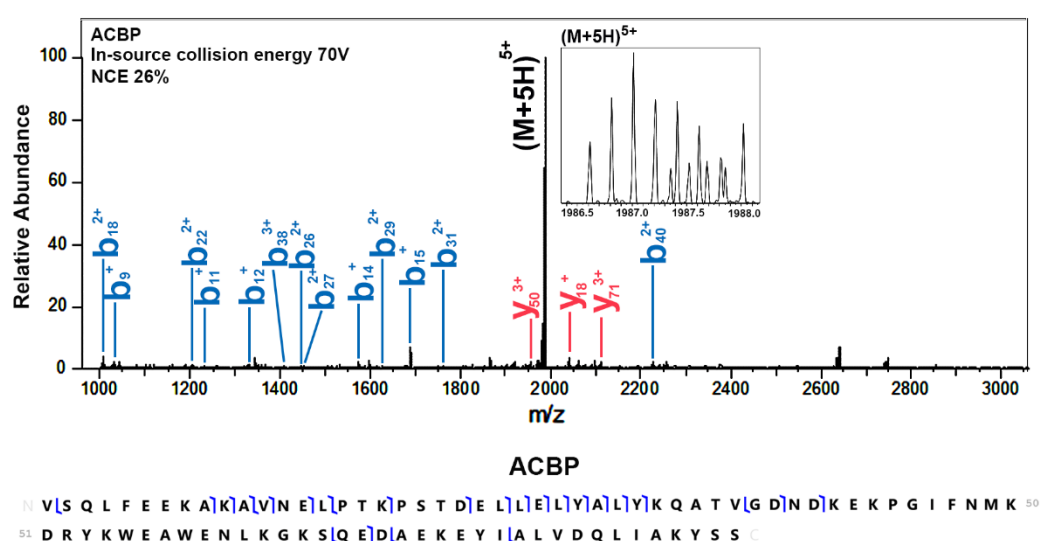


Figure 4.8 Protein identification of ACBP. (Top) MS/MS mass spectrum of ACBP and (bottom) summary of sequence fragments observed.

In total, 26 fragments were detected, including 19 b ions and 7 y ions. The sequence coverage is 28%.

Most of the proteins in this study were identified using HCD MS/MS. However, certain proteins, such as TRX2 and HSP31, were identified through the combination of FAIMS with HCD MS/MS. The FAIMS CV was set to -35V. Protein TRX2 was identified by bottom-up MALDI MS [331] and inductively coupled

plasma-high resolution mass spectrometry (ICP-MS) [332] in previous studies, but there is still a lack of mass spectrometry-related research on this protein. Here, using FAIMS combined with native LESA MS, the TRX2 protein chain, consisting of amino acids 2-104 without the N-terminal methionine, was observed at m/z 1848.59 (6+). The identification details are provided in Figure 4.9.

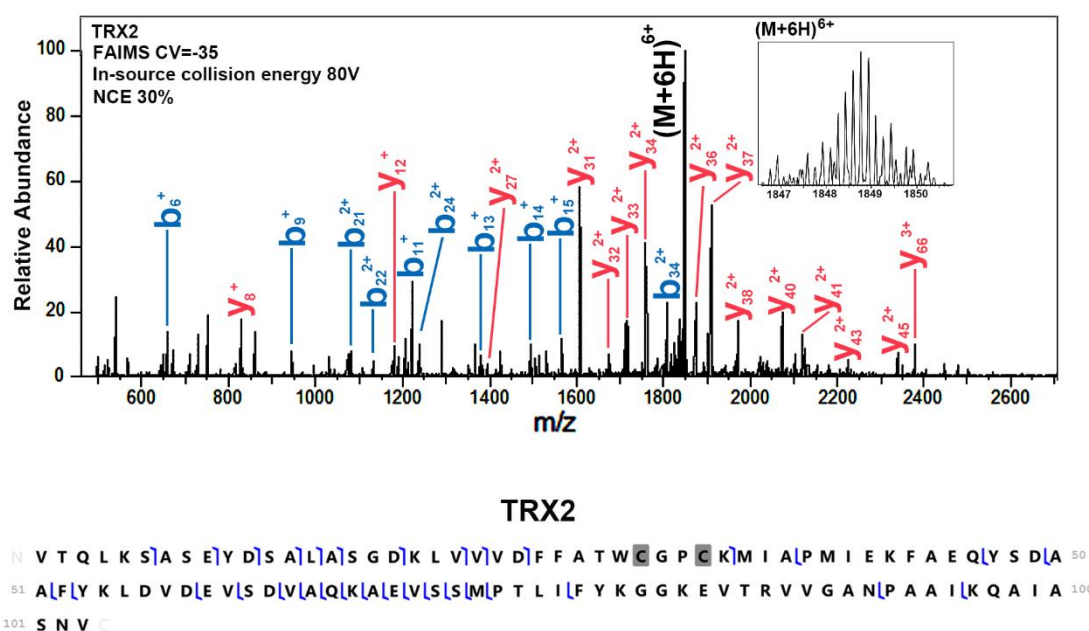


Figure 4.9 Protein identification of TRX2 (Top) MS/MS mass spectrum of TRX2 and (bottom) summary of sequence fragments observed.

A total of 32 b and y ions were detected, resulting in a sequence coverage of 31%. These results suggest the removal of N-terminal methionine and a presence of a disulfide bond between cysteine 31 and cysteine 34. Moreover, the observed mass of TRX2 exhibited a discrepancy of approximately 16 Da compared to the theoretical mass. This difference could potentially be attributed to an undetected post-translational modification, such as hydroxylation or methionine oxidation.

The use of FAIMS was critical for identification of some proteins, as it enhanced the signal-to-noise ratio. By selectively filtering out background ions and reducing noise, FAIMS enabled the detection of low-abundance proteins, which might have otherwise been undetectable using HCD MS/MS alone. For instance, the protein HSP31 detected here was only observed after employing FAIMS. Figure 4.10 presents the identification details of the HSP31 protein chain, comprising of amino acids 2-237 without the N-terminal methionine, observed at m/z 2842.10 (9+).

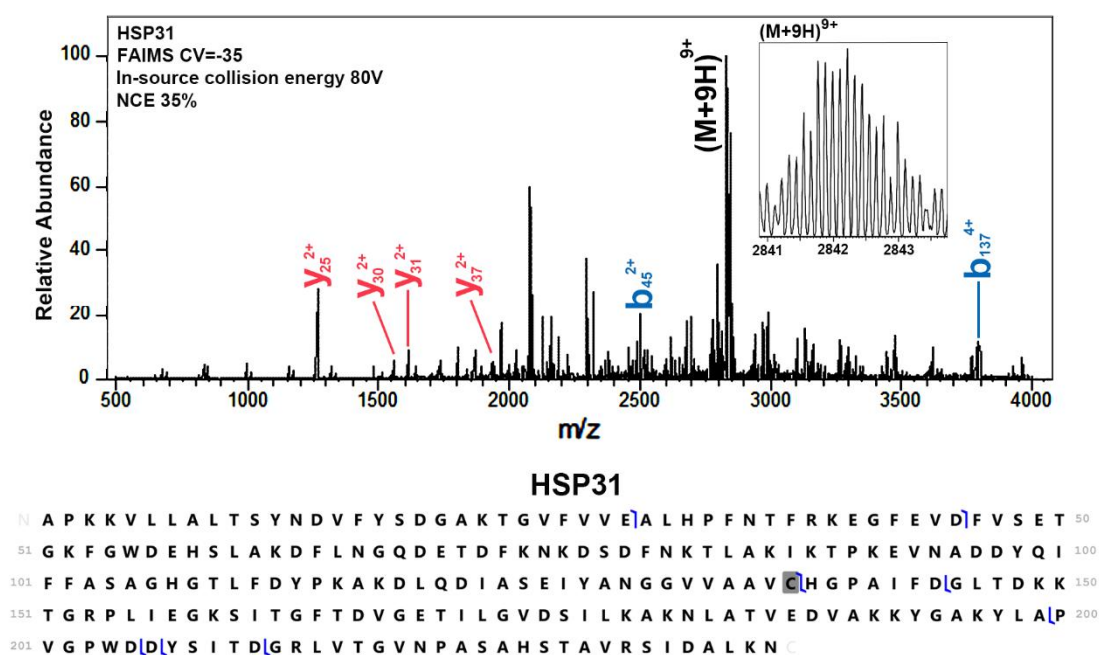


Figure 4.10 Protein identification of HSP31 (Top) MS/MS mass spectrum of HSP31 and (bottom) summary of sequence fragments observed.

Though the previous research [333] using native MS has detected the HSP31 homodimer. Here, only the monomer of this dimeric protein was observed and identified using FAIMS coupled with native LESA MS. A potential reason could be

the selective transmission and separation of protein monomers by FAIMS rather than the influence of field heating, as previous research has shown that FAIMS in native MS can hardly disrupt protein structure due to field heating [313, 334].

4.3.3 Identification of protein assemblies

In addition to monomeric proteins, protein assemblies were also detected using this method. Notable examples include TPIS, AHP1 and SODC.

TPIS monomer was previously identified by top-down MS coupled with high-field asymmetric waveform ion mobility spectrometry and capillary zone electrophoresis [322]. Here, the monomer of TPIS was observed in the 8+ (m/z 3808.7) and 9+ (m/z 3332.7) charge states and the 15+ charge state of the dimer of TPIS were observed in the same spectrum, shown in Figure 4.3. Figure 4.11 shows the MS/MS of the dimer.

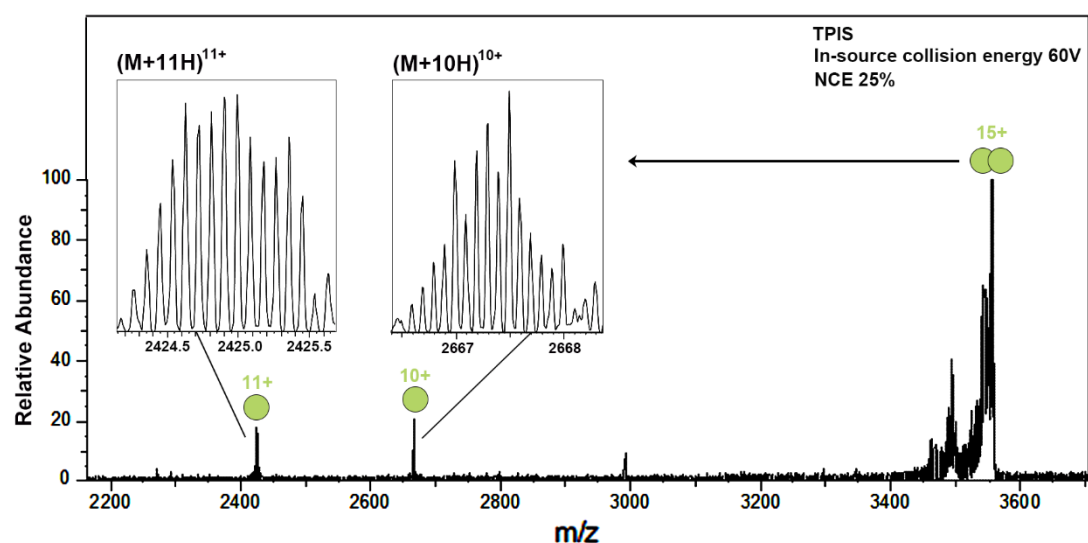


Figure 4.11 HCD MS/MS mass spectrum of 15+ ions of TPIS protein dimer.

The TPIS dimer was observed only in the absence of FAIMS, at an m/z of 3555.2439 with a 15+ charge state, as shown in Figure 4.11. The mass of the dimer was accurately determined after deconvolution, confirming the identity and structural integrity of the dimeric complex.

To further investigate the structural details of TPIS, a HCD NCE of 25% was applied to the m/z 3555.2439 ion with a 15+ charge state. This resulted in the dissociation of the dimer and the subsequent detection of the TPIS protein monomer. The monomer, observed lacking the N-terminal methionine, was detected at m/z 2424.9013 (11+ charge state) and at m/z 2267.1881 (10+ charge state).

While using FAIMS, the TPIS protein monomer chain without the N-terminal methionine was detected at an m/z of 3332.7243 with an 8+ charge state and an m/z of 3808.6781 with a 7+ charge state. The identification of this precursor was

carried out with the FAIMS CV set to -35V, as shown in Figure 4.12. Several b ions and one y ion were detected, with a sequence coverage of 7%. While using FAIMS in the identification of monomeric protein TPIS, the existing monomer with 8+ charge state was detected, but the dimer was not. The reason could be that the FAIMS device allowed the separation of the monomer and dimer, without disruption of its native structure by field heating [313, 334] .

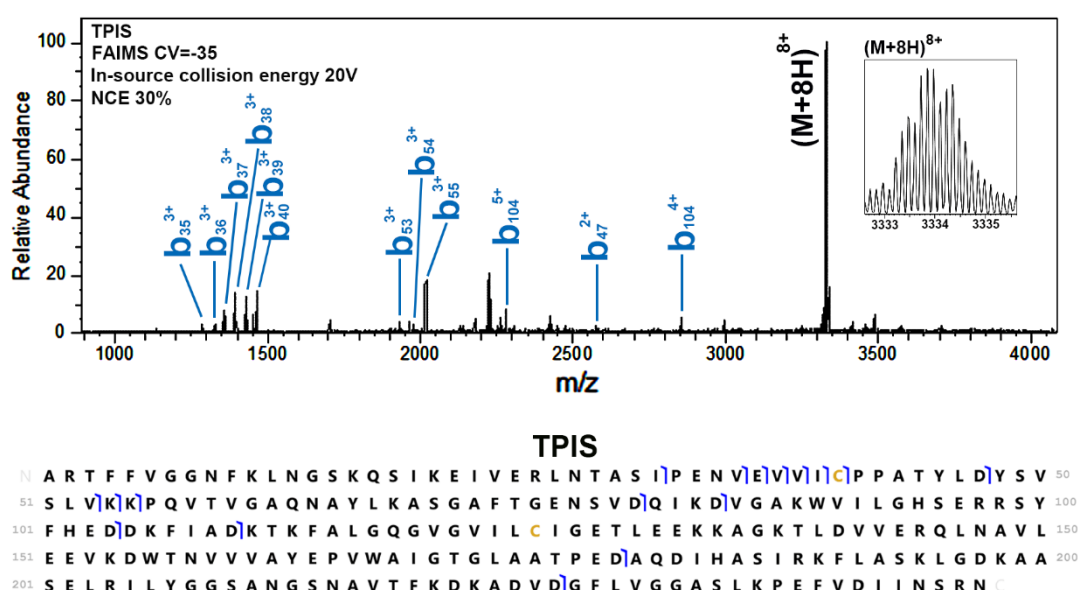


Figure 4.12 Protein identification of TPIS (Top) HCD MS/MS mass spectrum of TPIS and (bottom) summary of sequence fragments observed.

The detection of AHP1 protein by mass spectrometry has been reported [335]. In this study, the AHP1 dimer was successfully observed using native LESA MS, with an m/z of 3460.2458 and an 11+ charge state determined after deconvolution.

To further explore the structure, an in-source collision energy of 80V and an HCD NCE of 30% were applied, leading to the dissociation of the dimer and the

subsequent detection of the AHP1 monomer at an m/z of 2715.9277 with a 7+ charge state, as illustrated in Figure 4.13.

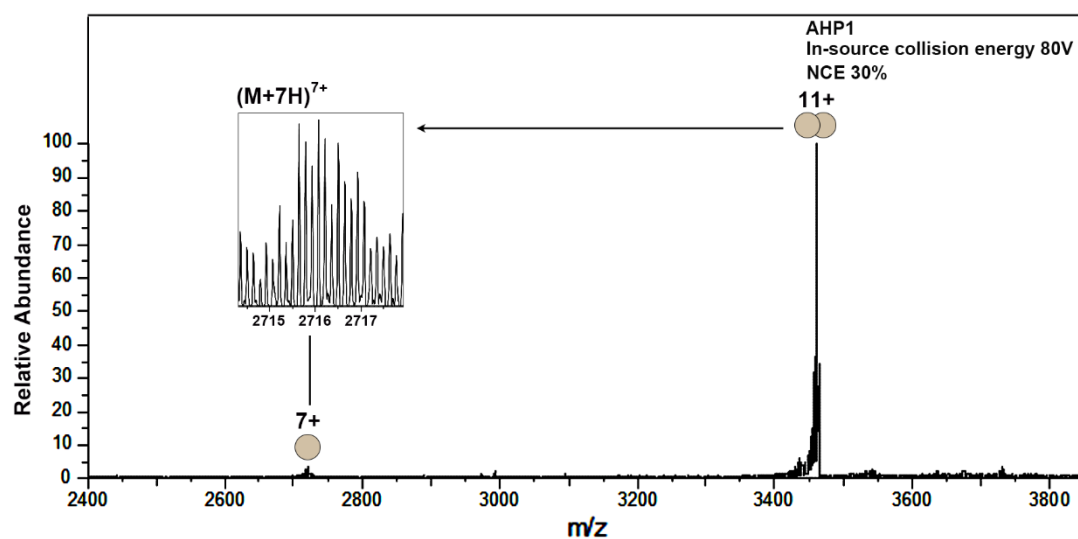


Figure 4.13 HCD MS/MS mass spectrum of 11+ ions of AHP1 protein dimer.

The following MS/MS detection was carried out with the 11+ dimer precursor with an insource collision energy of 80V and a HCD NCE of 37%. The details are shown in Figure 4.14.

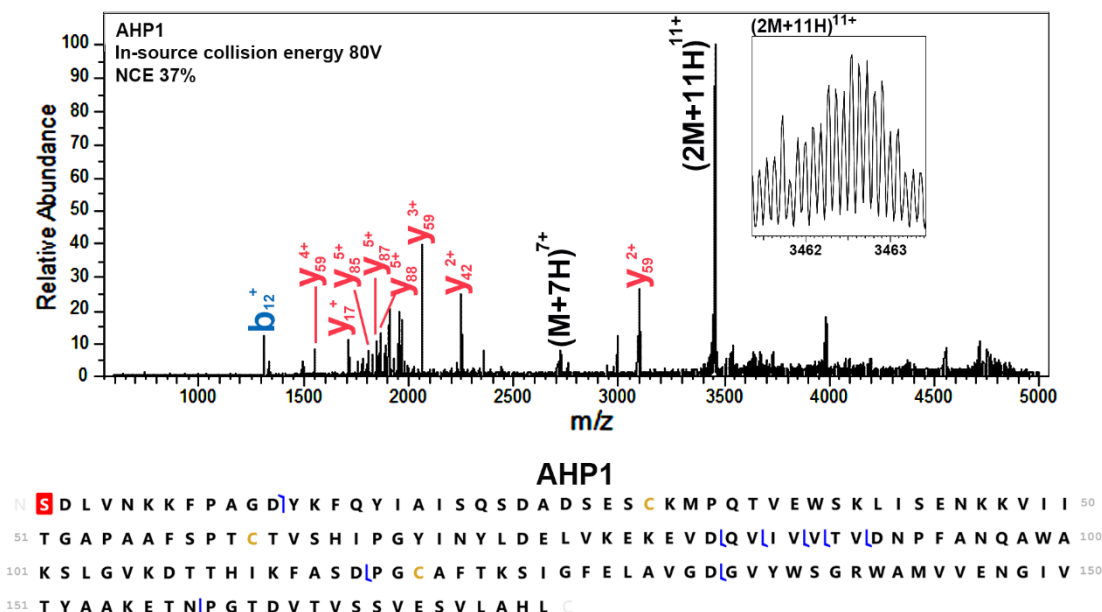


Figure 4.14 Protein identification of AHP1 (Top) MS/MS mass spectrum of AHP1 and (bottom) summary of sequence fragments observed.

There were in total 9 ions detected from AHP1. The results indicated that the N-terminal methionine was removed, and that the subsequent N-terminal serine was acetylated. According to previous research, a disulfide bond exists between cysteine 31 and cysteine 62 [336]. No fragmentation was observed between cysteine 31 and cysteine 62, suggesting that this region likely contains the disulfide bond.

The SODC dimer is with each subunit bound to one Cu^{2+} ion and one Zn^{2+} ion. The identification of SODC has been achieved previously by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry [337], ICP-MS and electrospray ionization mass spectrometry (ESI-MS) [338]. The peak at m/z 3522.2435 with 9+ charge state was identified as the SODC dimer. This intact protein assembly has not been detected by LESA before.

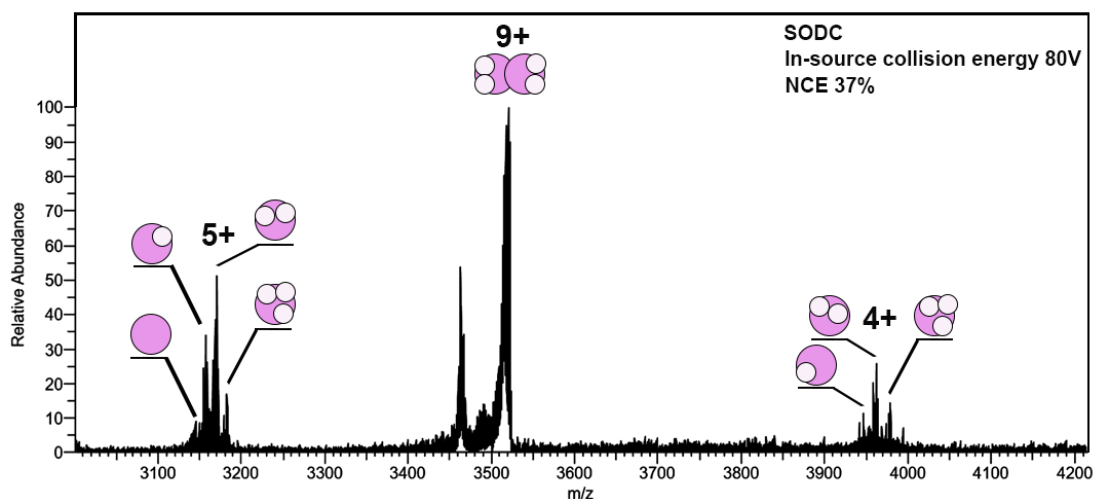


Figure 4.15 MS/MS mass spectrum of SODC protein dimer.

A HCD NCE of 37% was applied to this protein precursor to determine its structure, resulting in the detection of the protein monomer chain without the N-terminal methionine. The spectrum shown in Figure 4.15 displays the protein monomer peaks with associated metal ion distribution. The dimer, with each subunit containing two metal bonds, exhibited a notable feature: after applying HCD energy, the resulting monomer displayed a range of metal bound distributions, varying from no metal bonds to three metal bonds.

In the absence of FAIMS, while the SODC dimer was detectable, the signal intensity of the MS/MS spectrum was insufficient to confidently identify SODC. However, after applying FAIMS with a CV of -35V, although only the monomeric form of SODC was detected, the signal intensity improved, allowing for the identification of a greater number of fragments. This enhancement is illustrated in Figure 4.16, demonstrating the effectiveness of FAIMS in improving the quality of the spectrum data of SODC protein.

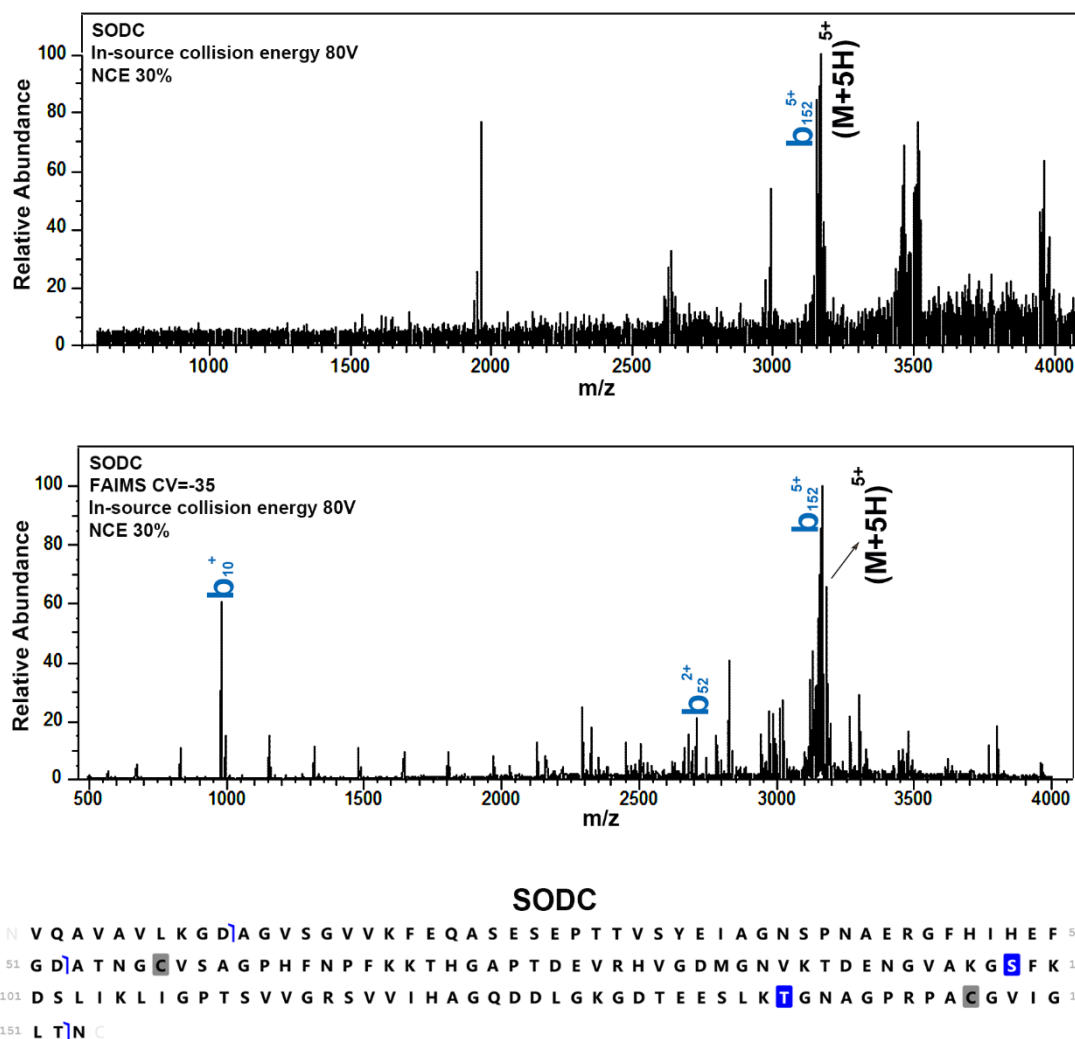


Figure 4.16 Protein identification of SODC. (Top) MS/MS mass spectrum of SODC monomer 5+ precursor without FAIMS, (middle) MS/MS mass spectrum of SODC monomer 5+ precursor with FAIMS and (bottom) summary of sequence fragments observed.

Three b ions were detected, suggesting the possible presence of phosphoserine at serine 99 and phosphothreonine at threonine 138, along with a disulfide bond between cysteine 58 and cysteine 147.

4.3.4 Identification of larger proteins

The following experiment was conducted in IRM high-pressure mode, enabling the identification of four additional larger proteins with masses ranging from 44,000 Da to 93,000 Da, as summarised in Table 4.2. These proteins included Phosphoglycerate kinase (PGK), Enolase 1 (ENO1), Enolase 2 (ENO2), and Superoxide dismutase [Mn] (SOD [Mn]). Among them, the protein assemblies ENO1 dimer and SOD [Mn] tetramer were observed in their native states. ENO2 which exists as dimer was only identified in its monomeric form.

Table 4.2. Summary of proteins from *S. cerevisiae* identified following electroporation and native LESA top-down mass spectrometry in IRM high pressure mode

ID (UniProt)	Protein mass calc (Da)	Protein mass observed (Da)	m/z mean	Charge state	mass diff (Da)
P00560(PGK_YEAST)	44621.7899	44660.00	3721	12	38.2101
P00924(ENO1_YEAST) monomer/dimer	93952.1896(dimer)	93926.00(dimer)	4937	19	26.1896
P00925(ENO2_YEAST)	46993.8710	46786.00	4679	10	207.871
P00447 (SOD[Mn]_Yeast) monomer/tetramer	23068.93(monomer) 92275.72(tetramer)	23060.00(monomer) 92524.00(tetramer)	2307 5442	10 17	8.93 248.28

Protein PGK has previously been analysed by bottom-up MALDI mass spectrometry [339] and ESI-MS [340]. Due to the high mass and low charge state typical of native proteins, in high-pressure mode, proteins are typically detected at lower resolution to improve signal intensity. However, the mass of the protein is difficult to be determined by low resolution detection. Here, PTCR was utilised to determine the protein mass and to separate overlapping protein peaks [61, 82, 341-343]. The PTCR MS/MS of PGK is shown in the bottom of Figure 4.17.

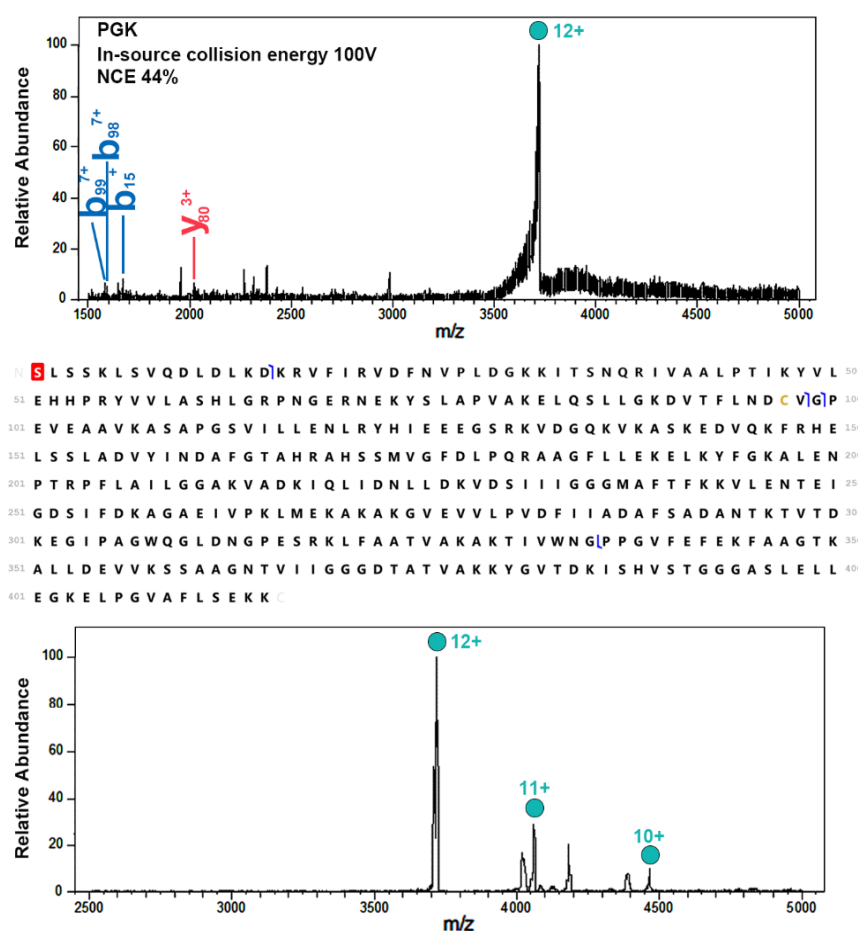


Figure 4.17 Protein identification of PGK. (Top) HCD MS/MS mass spectrum of 12+ ions of PGK, (middle) summary of sequence fragments observed and (bottom) PTCR mass spectrum of 12+ ions of PGK.

The identification of the protein precursor without N-terminal methionine with 12+ charge state was carried out by HCD, shown in the top of Figure 4.17. The fragmentation indicated that there is an acetylation on the serine at the second position of the protein chain. A mass difference of approximately 38 Da between the observed and theoretical protein masses may be due to inaccuracies in PTCR determination of the intact protein mass. This is because, to minimise the impact of isotopic peaks, PTCR in this experiment was typically conducted at a low resolution of 7500 at m/z 200. As a result, during deconvolution, the average mass of the peak was more likely to be selected instead of the monoisotopic peak mass to determine the protein theoretical mass. ENO2, a natively dimeric protein extensively studied using mass spectrometry in its monomeric form, was observed in as a monomer in this study using native LESA MS. With this method, the intact protein dimer was not detected. It is possible that the weak non-covalent bonds of the protein dimer may dissociate under the in-source collision energy of 120V. The MS/MS spectrum by HCD and PTCR were shown in Figure 4.18.

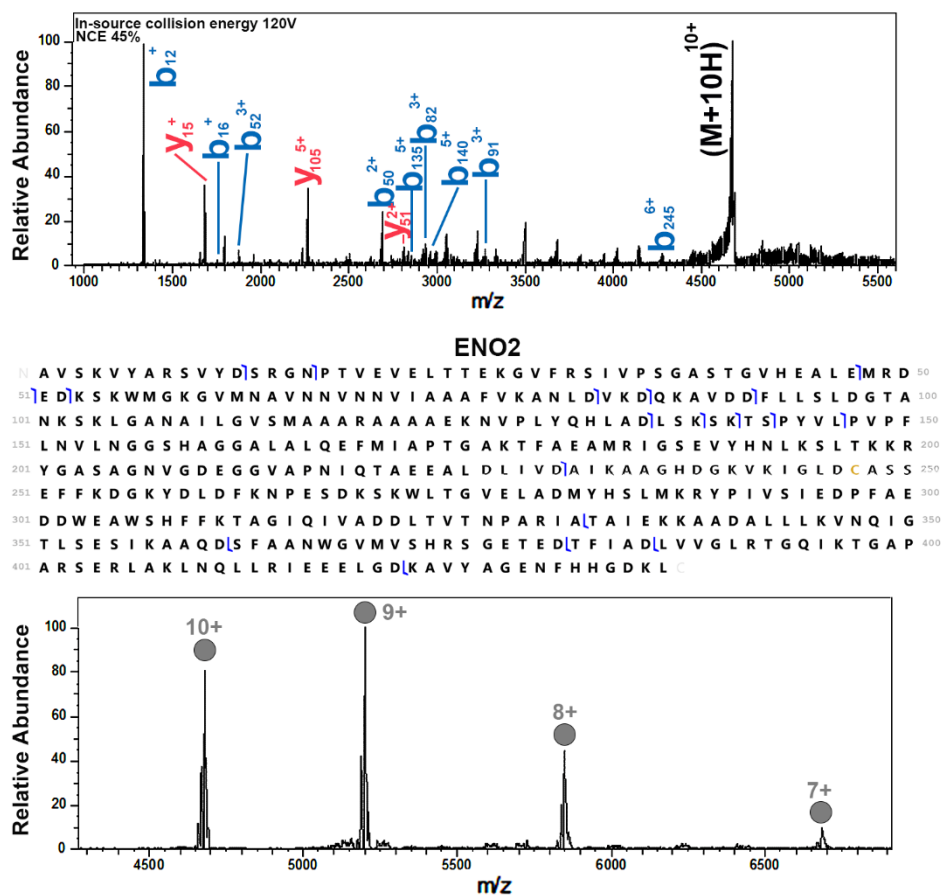


Figure 4.18 Protein identification of ENO2 (Top) HCD MS/MS mass spectrum of 10+ ions of ENO2, (middle) summary of sequence fragments observed and (bottom) PTMR mass spectrum of 10+ ions of ENO2.

ENO2 monomer was detected without N-terminal methionine. Several protein fragments were observed at the C-terminal of aspartic acid or the N-terminal of proline. The mass difference of approximately 208 Da between the observed and theoretical masses could potentially be caused by an unknown PTM or adduct. In the case of the ENO2 dimer, two Mg²⁺ ions may interact with each subunit to stabilise its dimeric form [344]. Although there is insufficient evidence to confirm

the presence of the characteristic metal bond when detecting ENO2 using the current method, it is possible that this metal bond is redistributed upon the protein dimer dissociating into monomers [295], described in Chapter 4.3.3.

ENO1 was observed here as a protein dimer in a 19+ charge state with a mass of 93,312.48 Da. The HCD MS/MS spectrum (NCE 53%) for 19+ precursor ions of the ENO1 dimer is shown in Figure 4.19. Monomer product ions in charge states 13+ and 14+ were observed.

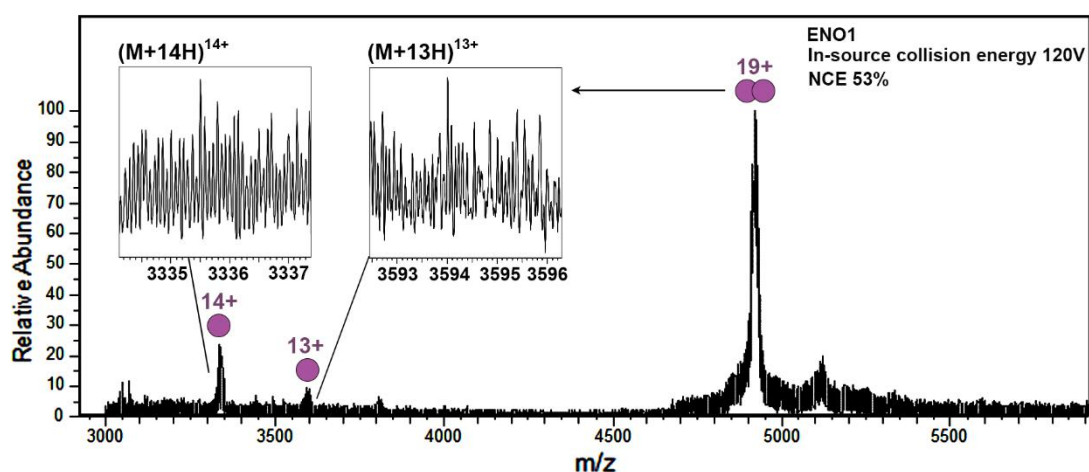


Figure 4.19 HCD MS/MS mass spectrum of 19+ ions of ENO1 protein dimer.

Because of its high molecular weight and low charge state, PTCR was used to determine the ENO1 protein dimer mass, shown in Figure 4.20. The observed mass of ENO1 differs from the theoretical mass by approximately 26 Da, likely due to the binding Mg^{2+} to stabilise the protein dimer. While the presence of a metal ion is possible, it could not be definitively determined in this analysis. The

HCD MS/MS of on the 19+ precursor ions (NCE 64%), shown in Figure 4.20, reveals that most of the fragments occurred at the C-terminal of aspartic acid or the N-terminal of proline.

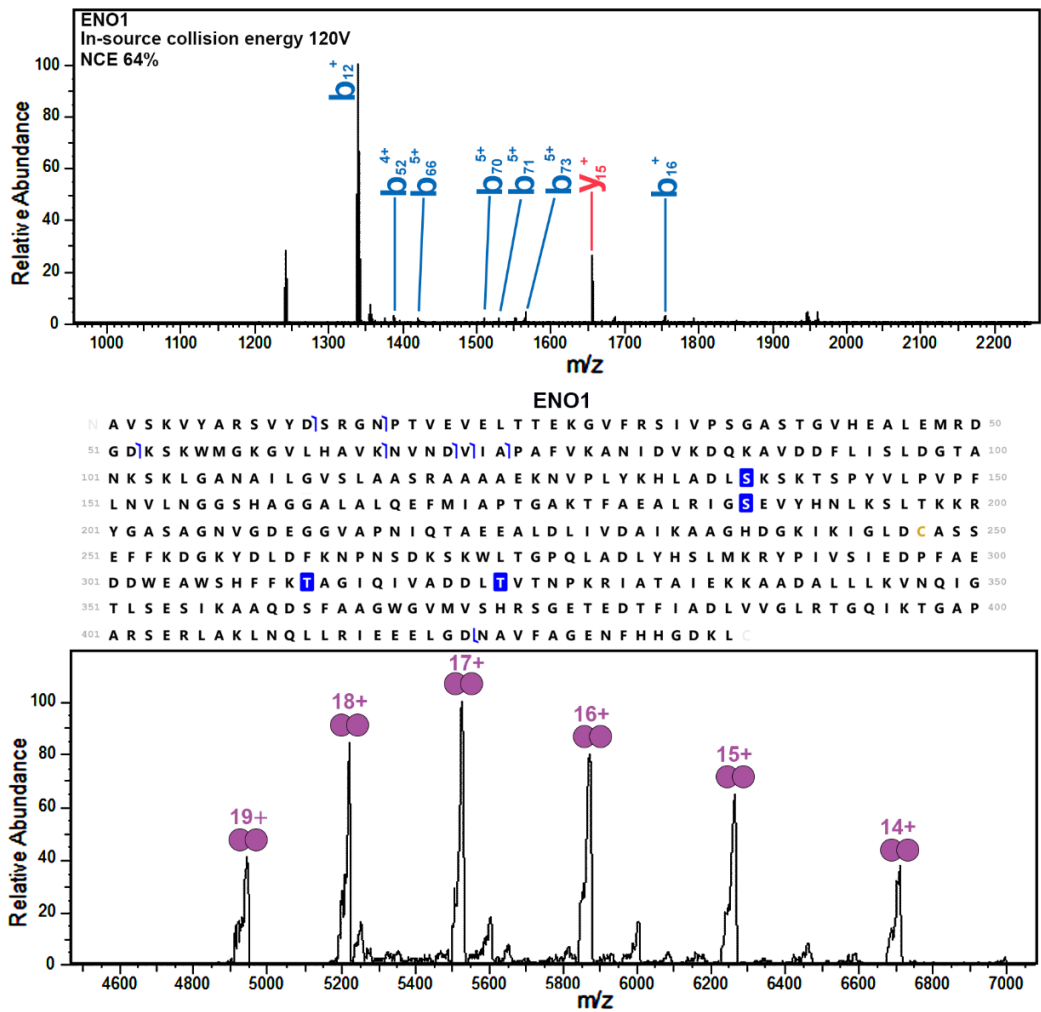


Figure 4.20 Protein identification of ENO1. (Top) MS/MS mass spectrum of ENO1, (middle) summary of sequence fragments observed and (bottom) PTCL mass spectrum of ENO1.

The protein tetramer SOD [Mn] was detected as an intact assembly, with one

Mn²⁺ ion bound to each subunit. The 17+ tetramer precursor, containing four Mn²⁺ ions, was observed at an m/z 5442 using an in-source collision energy of 120V. To further investigate the protein structure, an HCD NCE of 35% was applied, resulting in the detection of two peaks corresponding to the protein monomer chain lacking the 1-26 transit peptide, with 9+ and 10+ charge states, as illustrated in Figure 4.21. The mass of the 17+ precursor was determined by PTCR, as shown at the bottom of Figure 4.22. Though the SOD [Mn] tetramer shows about 248 Da mass shift due to 4 X Mn²⁺ binding the protein complex, the SOD [Mn] monomer mass observed following HCD did not exhibit a significant mass difference with its theoretical mass.

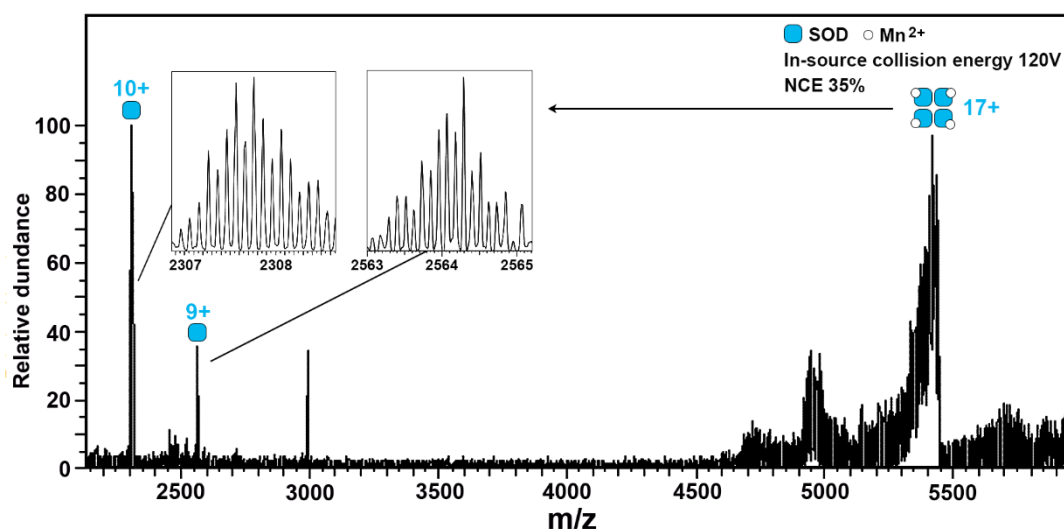


Figure 4.21 HCD MS/MS mass spectrum of 17+ ions of SOD [Mn] protein tetramer.

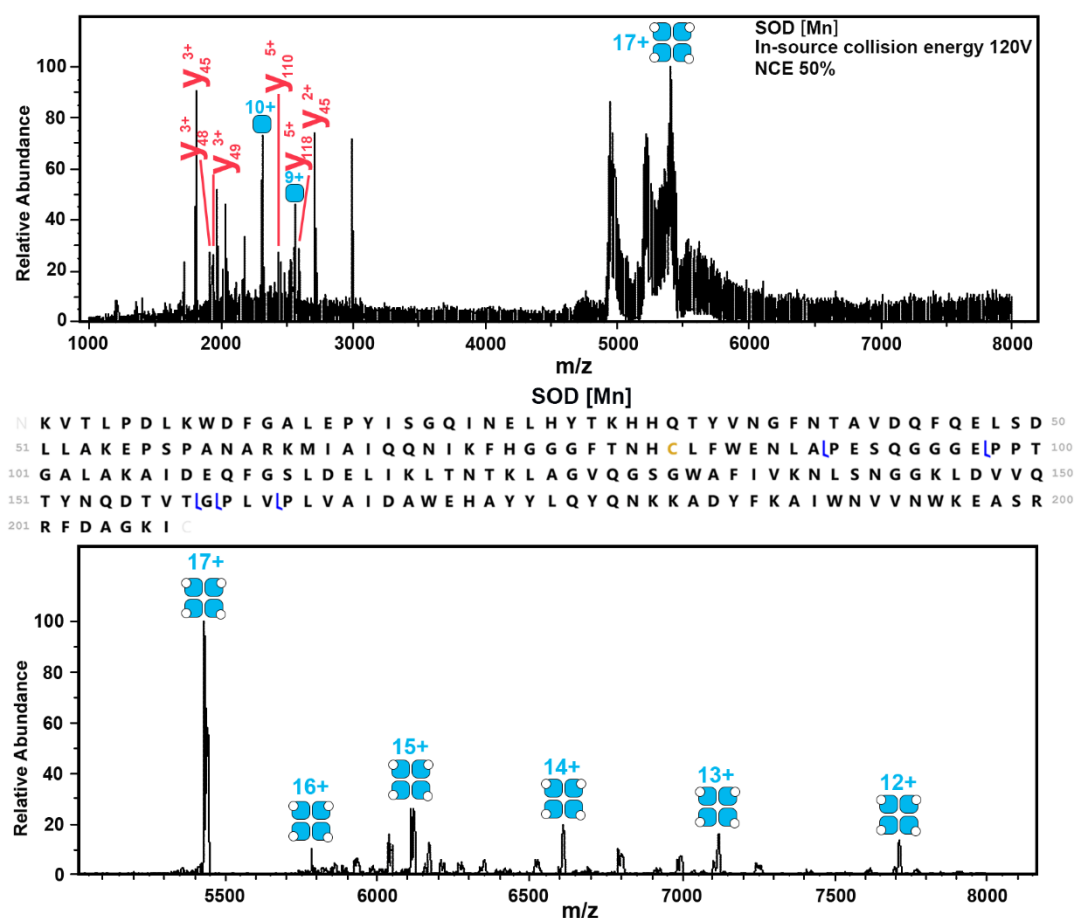


Figure 4.22 Protein identification of SOD [Mn]. (Top) HCD MS/MS mass spectrum of 17+ ions of SOD [Mn], (middle) summary of sequence fragments observed and (bottom) PTCL mass spectrum of 17+ ions of SOD [Mn].

After increasing the HCD NCE energy to 50%, the SOD [Mn] protein chain without 1-26 transit peptide was fragmented. Five y ions were detected, four of which were located at the C-terminal of aspartic acid or the N-terminal of the following residue, confirming the accurate identification of SOD [Mn], Figure 4.22.

4.3.4 Gram Positive Bacteria Native Protein Detection

The following experiment was conducted on the Gram-positive bacterium *Staphylococcus epidermidis*. Two strains, RP62a and HB, were used to assess whether the method established for yeast could be successfully applied to a Gram-positive bacterium.

First, the Gram-positive bacterial colonies were cultured on TSB medium in 6 cm diameter Petri dishes and incubated overnight at 37°C. Once the colonies reached an appropriate thickness of approximately 0.2-0.3 mm, samples were collected. Electroporation was performed on the Gram-positive bacteria using a voltage of 3 kV (corresponding to a field strength of 15 kV/cm), with 35 pulses and a pulse length of 20 μ s. Sample extraction was then carried out using 3 μ L of a solvent containing 200 mM ammonium acetate, 2x CMC C8E4, and 3% methanol. The extraction process was consistent with the method previously described for yeast colonies.

Unfortunately, no protein peak was observed in both types of gram-positive strains. The potential reason could be the structure difference between gram-positive bacteria and yeast. Gram-positive bacteria have a thick peptidoglycan layer embedded by teichoic acids. This rigid layer provides structural support but also makes these cells more resistant to physical disruptions like electroporation.

Although Gram-positive bacteria were electroporated at a higher field strength of 15 kV/cm, compared to 10 kV/cm typically used for yeast, no proteins were observed when using the same extraction solvent with detergent. Further exploration of electroporation and extraction conditions for Gram-positive bacteria is still needed.

4.4 Conclusion

In summary, this chapter describes the development and application of a method to detect native proteins and protein assemblies directly from yeast colonies, building on the method combining electroporation and native LESA MS for analysis of proteins in Gram-negative bacteria described in Chapter 3. This approach has proven effective for identifying and analysing native proteins directly from yeast colonies. The incorporation of electroporation and detergent in the extraction solvent was shown to enhance the efficiency of this detection method.

Using the method demonstrated here, fourteen proteins were successfully identified through tandem mass spectrometry. Some of these experiments incorporated FAIMS to achieve better signal-to-noise ratios for the precursor ions and fragment ions, resulting in a more accurate identification. However, some

protein dimers were only observed in their monomeric form after FAIMS. This could be caused by the selective transmission and separation of protein monomers in the setup or the effect of field heating on protein structure [345, 346]. However, previous research has shown that FAIMS in native MS rarely disrupts protein structure due to field heating [313, 334], suggesting that the selective transmission and separation of protein monomers is the more likely explanation.

The approach of combining electroporation and native LESA MS allowed for the identification of PTMs and bound metal ions in their native states. Among the identified proteins, five protein assemblies were confirmed, including the homodimers APIS, AHP1, SODC, ENO1, and the homotetramer SOD [Mn]. The stoichiometry of these protein assemblies and their identification were confirmed through MS/MS analysis, which dissociated the protein complexes into their monomeric subunits.

Compared to the study on native intact protein identification using native LESA MS in bacteria, this method incorporated a small amount of detergent (2 x CMC) in the extraction process to more effectively disrupt the thicker yeast cell wall. Utilizing IRM high-pressure mode, several larger protein assemblies, with masses ranging from approximately 40,000 to 100,000 Da, were observed. Some of these proteins, including SODC, ENO1, and SOD [Mn], were found to be associated with bound metal ions. The asymmetric distribution of metal ions following HCD, as described in Chapter 3, was also observed here. For example,

the protein SODC, which was bound to two metal ions (Cu^{2+} or Zn^{2+}) per subunit, was fragmented with HCD, resulting in protein monomer peaks with varying numbers of metal ions bound.

Chapter 5

Development of methods for direct analysis of membrane proteins from bacterial colonies

5.1 Introduction

Membrane proteins are important for several key physiological processes including metabolism, apoptosis, and signal transmission [184, 347]. They constitute nearly half of all known therapeutic targets [177]. Therefore, investigating the structure of membrane proteins- which is closely related to their function-is crucial for understanding biological processes as well as developing innovative therapeutic approaches. However, the characterization of membrane protein structure, function, and interactions is complex due to the naturally low abundance of membrane proteins as well as the highly heterogeneous and hydrophobic nature of the membrane environment [184]. To address challenges in membrane protein detection using MS, several techniques have been applied including the use of MS-compatible detergents [348-350], SMALPs [184, 351, 352], nanodiscs [353] and lipid vesicles [199]. MS-compatible detergents,

amphipathic compounds which can solubilise membrane proteins while keeping the non-covalent connections for native MS analysis, are one of the most widely used approaches for solubilising membrane proteins [317, 348].

To date, NAMS has been used exclusively for detection of soluble proteins and their assemblies in bacteria; however, NAMS has been applied to the analysis of membrane proteins in mammalian tissue including the highly abundant aquaporin-0 (Aqp-0) tetramer in eye lens tissue sections [341] and multiple membrane proteins in rat brain and kidney tissue [85]. The latter study employed a tissue washing step to deplete soluble proteins in the tissue.

In this chapter, a workflow for direct analysis of membrane proteins in living bacterial colonies was developed. This workflow combines colony washing with LESA MS and the optimal washing conditions were determined. The inclusion of additional techniques, including SMALP extraction and electroporation, were also explored.

5.2 Experimental Section

Colonies of *E. coli* BL21 (DE3) and *Pseudomonas aeruginosa* PS1054 were cultured as described in Chapter 2, Section 2.2.1. *Staphylococcus epidermidis* HB and *Staphylococcus epidermidis* RP62a were cultured as described in

Chapter 2, Section 2.2.1. Bacterial colonies were subjected to washing as described in the Results section 5.3.2 prior to LESA MS as described in Chapter 2, Section 2.2.2. The effect of integration of electroporation was explored. Electroporation parameters were as described in Chapter 2, Section 2.2.3. The use of SMALPs in this research was described in Chapter 2, Section 2.2.5. Proteins were identified by MS/MS as described in Chapter 2, Section 2.2.8 and 2.2.9. And database searching of the resulting fragments Chapter 2, Section 2.2.10.

5.3 Results and discussion

5.3.1 Optimisation of washing solvent

Previous studies have demonstrated that tissue washing improves the detection of membrane proteins from mammalian tissue using native ambient mass spectrometry [85]. Here, a washing solution consisting of 200mM ammonium acetate containing the detergent C8E4 was employed for washing *E. coli* K12 colonies. As a non-ionic, relatively mild MS-compatible detergent, C8E4 is suitable for transfer of membrane proteins into the gas phase without affecting protein-protein interactions [317]. Different concentrations of C8E4 were tested

in the initial experiment to determine the most effective washing solvent for the washing steps on *E. coli* K12 colonies. Firstly, control experiments were conducted in which LESA MS was performed without prior washing. The LESA sampling solvent was 200mM ammonium acetate with 2 x critical micelle concentration (CMC) C8E4. MS detection was conducted in the range m/z 1500 to 6000, with an in-source collision energy of 80V. Figure 5.1 A) shows a representative LESA mass spectrum of three replicates from the control group of a *E. coli* K12 colony, where only one protein, with a molecular weight of approximately 18,800 Da, was observed.

Subsequently, 20 μ L of 200 mM ammonium acetate was applied to wash a *E. coli* K12 colony cultured under the same conditions as the control group. To minimise the loss of bacterial cells, the washing process was performed by gently pipetting the washing solvent over the colony to ensure full coverage. The solvent was then carefully aspirated while tilting the plate to collect the droplet on one side of the colony. After sampling by LESA with an extraction solvent comprising 200mM ammonium acetate with 2 x CMC C8E4 (0.5%), the sample was then transferred into homemade gold-coated borosilicate tips and sprayed into MS, described in Chapter 2, Section 2.2.4. A representative mass spectrum of three replicates is illustrated in Figure 5.1 B). Two proteins were detected, with masses of approximately 18,800 Da and 9,500 Da.

The subsequent experiments involved washing with solvents containing different

concentrations of the detergent C8E4. Each colony was washed once before sampling and MS detection.

Figure 5.1 C) shows a representative mass spectrum obtained following LESA sampling of an *E. coli* BL21 (DE3) colony after washing once with 20 μ L of 200 mM ammonium acetate containing 0.5 x CMC C8E4 (0.125% v/v). Due to the change in the washing solvent, the in-source collision energy was adjusted from 80V to 100V to mitigate the impact of using detergent, while all other sampling and MS detection parameters remained unchanged. Three proteins were observed.

The mass spectrum shown in Figure 5.1 D) was obtained following LESA sampling of a colony washed once with 20 μ L of 200 mM ammonium acetate containing 2 x CMC C8E4 (0.5% v/v). The in-source collision energy was maintained at 100V to break the micelle and release the proteins. However, the high background noise in this spectrum caused by a high concentration of detergent brought about no clear observation of any protein peaks.

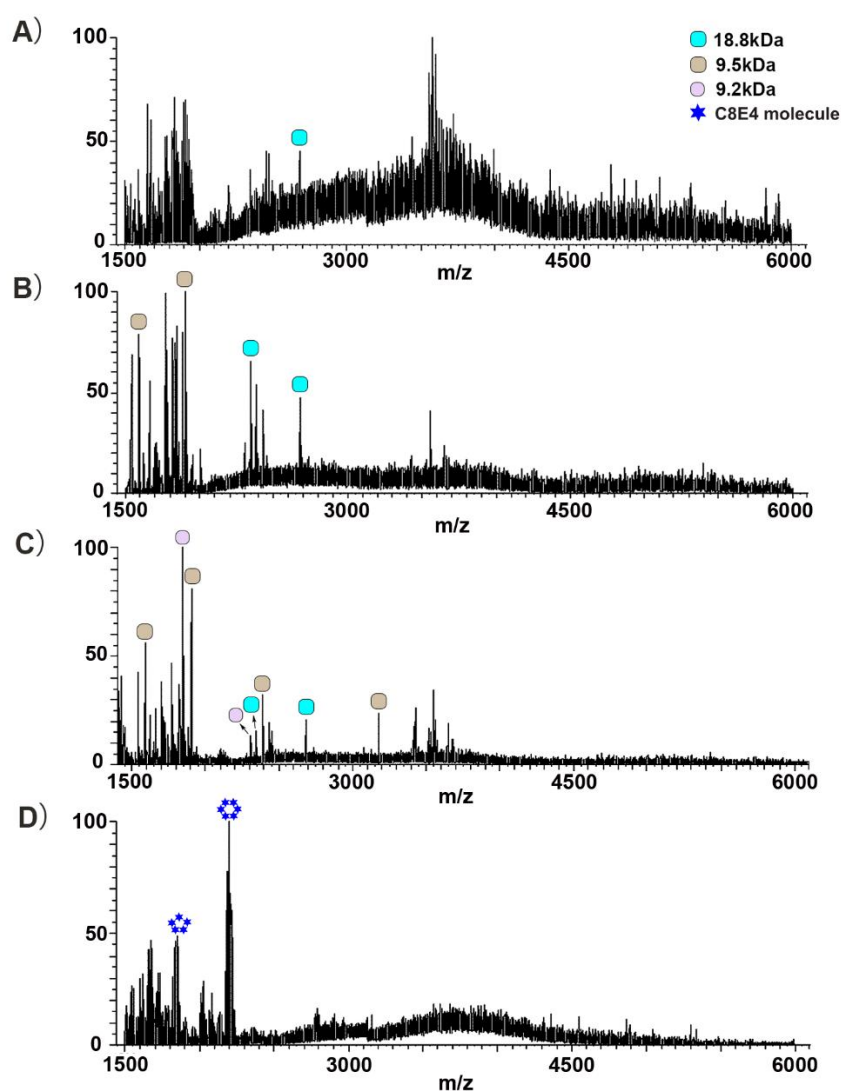


Figure 5.1 Optimisation of washing solvent. The sample *E. coli* BL21 (DE3) colony and LESA sampling solvent was 200mM ammonium acetate with 2 x CMC C8E4. A) Representative mass spectrum following LESA sampling of colony with no prior washing. B) Representative mass spectrum following LESA sampling of colony with 200mM ammonium acetate washing. C) Representative mass spectrum following LESA sampling of colony with 200mM ammonium acetate with 0.5 x CMC C8E4 washing. D) Representative mass spectrum following LESA sampling of colony with 200mM ammonium acetate with 200mM ammonium acetate with 2 x CMC C8E4 washing.

The results above suggest that a washing solvent comprised of 200 mM ammonium acetate containing 0.5 x CMC of C8E4 is optimum for detection of proteins from the bacteria. A potential explanation for this observation could be that a C8E4 concentration below its CMC can disturb protein-lipid interactions without effectively incorporating membrane proteins into detergent micelles [317], thereby contributing to the efficiency of washing process. Drawing from this result, the following workflows of bacterial washing were designed with the solvent composed of 200mM ammonium acetate with 0.5 x CMC C8E4.

5.3.2 Development of washing protocol

To further investigate methods for detecting membrane proteins, multiple washing steps were employed. In the following experiment, the samples firstly underwent three rounds of washing with the solvent comprising 200 mM ammonium acetate with 0.5 x CMC C8E4, followed by a final wash with 200 mM ammonium acetate, for a total of (3+1) washes. A schematic of this method is shown in Figure 5.2.

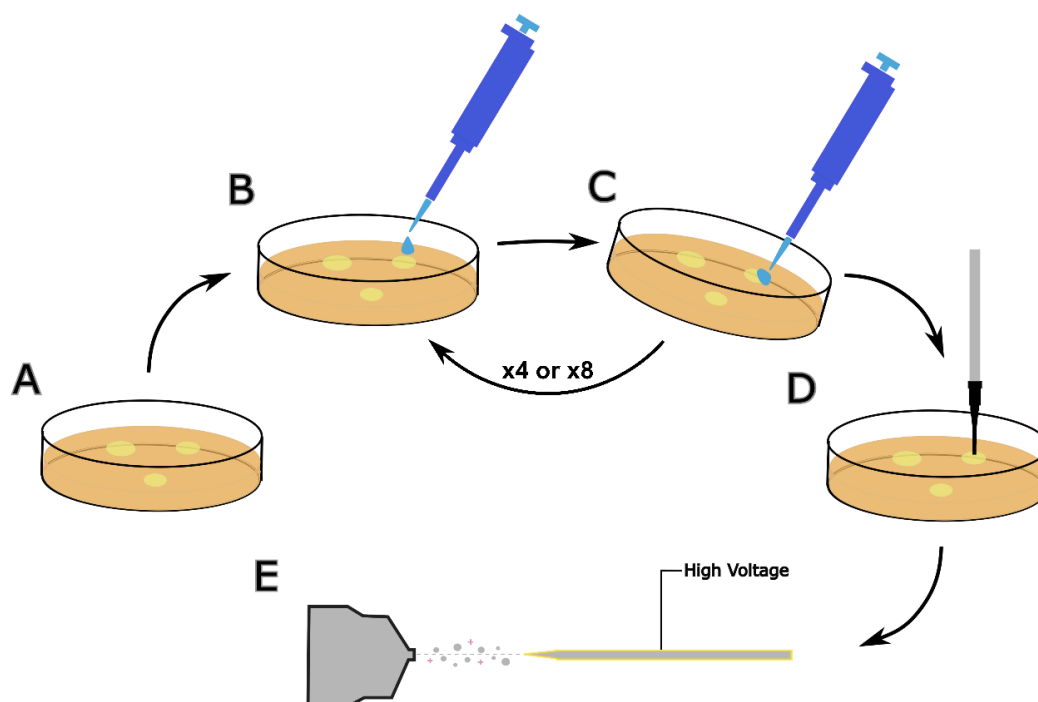


Figure 5.2 Schematic of workflow. A) Bacteria were cultured on agar and grew as single colonies. B) Washing solvent comprising 200mM ammonium acetate containing 0.5 x CMC C8E4 (0.125% v/v) was deposited on the colony. C) The plate was gently tilted, and the washing solvent aspirated and sent to waste. D) The colony was sampled by LESA. Extraction solvent comprised 200 mM ammonium acetate containing 2 x CMC C8E4 (0.5% v/v). E) The sample was transferred to a home-made gold coated borosilicate tip and introduced to the mass spectrometer by electrospray ionisation.

Initially, the mass spectrometer was operated with the IRM in high-pressure mode and in-source collision energy as 110V described in Chapter 2, Section 2.2.7. Under these conditions, however, reproducibility was poor and spectrum noise was high. A possible explanation is that the in-source collision energy was insufficient to effectively dissolve the protein ions (i.e., remove the detergent

molecules). To address this, a two-step wide isolation window was applied (centred at m/z 5000 $\pm$ 2000), with HCD at 30% NCE in the first step and 5% NCE in the second step. For this HCD clean up, NCE was normalised to 10+ charges. Figure 5.3 shows a representative mass spectrum obtained following (3+1) rounds of the washing process. Figure 5.3 A) shows the mass spectrum obtained with a source CID compensation scaling factor (CSF) of 5% and Figure 5.3 B) shows that obtained with source CID CSF of 7%.

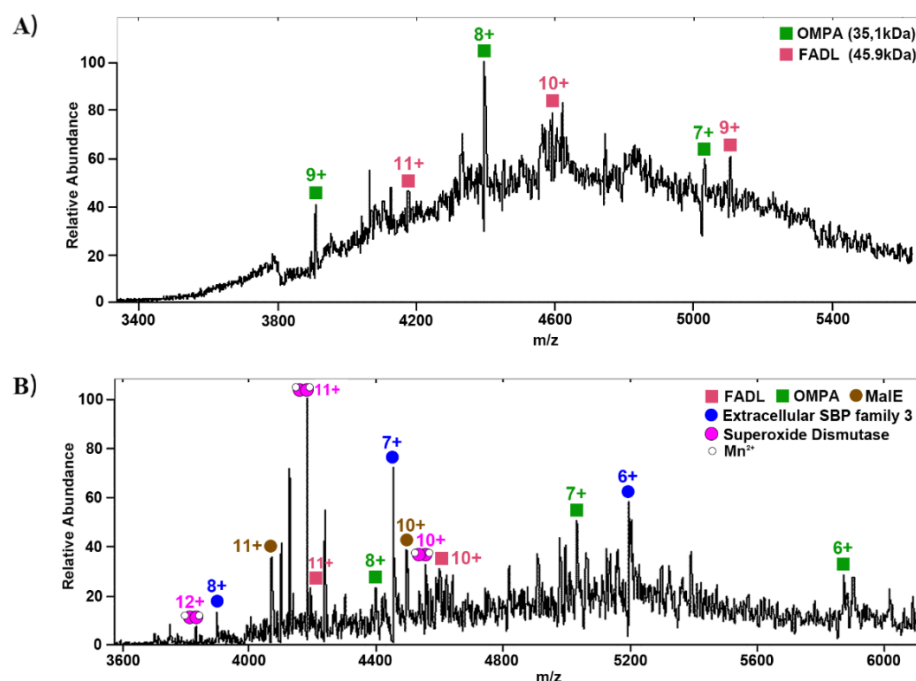


Figure 5.3 Representative mass spectrum obtained from *E. coli* BL21 (DE3) following (3+1) rounds of washing. A) Representative mass spectrum with instrument CSF 5%. Two membrane proteins - OMPA (35,148 Da) and FADL (45,878 Da) - were detected in various charge states. B) Representative mass spectrum with instrument CSF 7%. Several membrane and soluble proteins were detected.

Source compensation ensures proper ion transmission during native protein analysis. It is applied on the ion path for effective transmission through the instrument's front-end, with Source CID enabled for ion declustering while detecting intact protein in IRM high pressure mode. In detail, ions were ejected from the inlet capillary and then experienced in-source collision in a stacked ring ion guide (SRIG). After this process, the kinetic energy of the ions was high, resulting in the loss of larger ions with greater kinetic energy, as they are faster and more challenging to guide with the ion guides. To address this issue, a lower voltage, compared to that of the following bent flatapole (MP0), was applied at the injection flatapole (MP00) and interflatapole lens (L0), allowing ions to slow down and collide with the background gas, thereby reducing their kinetic energy [354]. The relative voltage is shown in Figure 5.4.

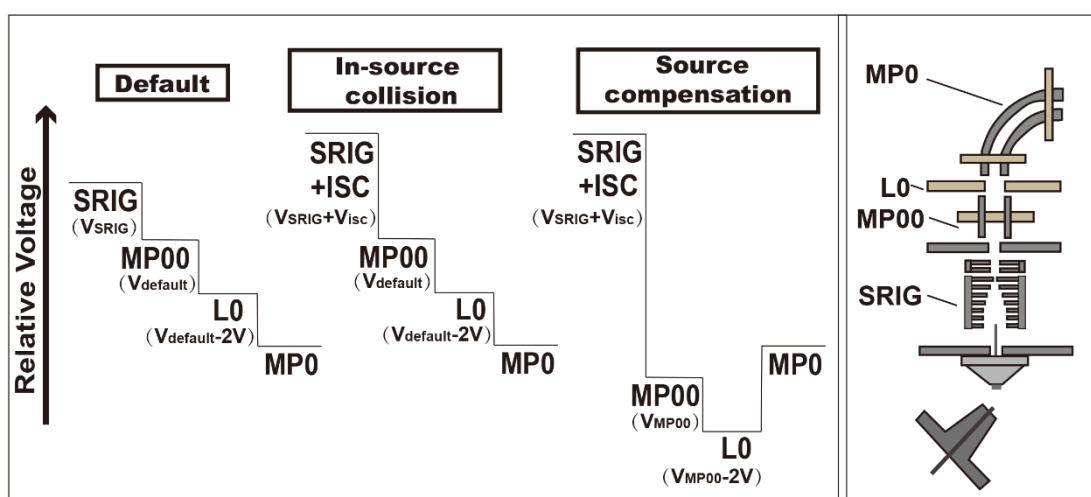


Figure 5.4 Scheme of source compensation and default voltage setting. $V_{default}$ is the default voltage of MP00. V_{isc} is the in-source collision voltage. V_{MP00} is the voltage of MP00 with the application of source CID CSF.

This process effectively slowed down the larger, higher-energy ions to make them easier to detect and removed the smaller, lower-energy ions. The relationship between voltage in MP00 and CID CSF is shown in the following equation:

$$V_{MP00} = V_{default} - (V_{isc} \times CSF) \quad (5.1)$$

V_{MP00} represents the voltage of MP00 with the application of source CID CSF. $V_{default}$ is the default voltage of MP00, which here equals 7.7V. V_{isc} is the in-source collision voltage, and CSF is the percentage of voltage decrease. The voltage of L0 is 2V less than that of MP00. A higher source CID CSF results in a greater rate of voltage decrease in MP00 and L0, leading to a tendency to detect ions with higher m/z values in the mass spectrum.

Under these conditions, several proteins were identified, with the majority being soluble proteins. For protein identification, either MS/MS or MS3 were performed detailed in Table 5.1.

Table 5.1 Summary of proteins identified following colony washing and LESA sampling.

The number of washing	Protein name	m/z	Charge state	In-source collision energy(V)	MS/MS precursor $\pm$ isolation width	Protein mass calc (Da)	Protein mass observed (Da)	Mass difference (Da)	Residue Cleavage	MS/M S HCD NCE (%)	MS3 precursor $\pm$ isolation width	MS3 HCD NCE (%)	IRM pressure mode
3+1	Extracellular solute-binding protein family 3	3118	10+	121	3124 $\pm$ 10	31152	31151	-1.039	2%	45	-	-	Standard
3+1	Maltodextrin-binding protein	3700	11+	121	3705 $\pm$ 5	40682	40662	-19.995	1%	48	-	-	Standard
3+1	Superoxide dismutase	3550	13+	100	4000 $\pm$ 2000	22933	22915	-18.251	4%	15	3550 $\pm$ 10	50	High
3+1	Phosphoenolpyruvate carboxykinase (ATP)	4217	14+	120	5000 $\pm$ 2000	59648	59912	263.983	1%	30	4217 $\pm$ 10	54	High
3+1	Cationic amino acid ABC transporter, periplasmic binding protein	4298	6+	100	4500 $\pm$ 2000	25769	25783	13.993	3%	30	4298 $\pm$ 5	48	High
3+1	Serine hydroxymethyltransferase	5066	18+	120	5000 $\pm$ 2000	45559	45578	19.002	3%	30	5066 $\pm$ 10	50	High
3+1	2,3-bisphosphoglycerate-dependent phosphoglycerate mutase	4406	13+	130	4406 $\pm$ 10	28580	28541	-39.765	2%	38	-	-	High
3+1 or 2x(3+1)	Long-chain fatty acid transport protein	5102	9+	130	5102 $\pm$ 5	45878	45711	-167.104	1% and 3%	38	-	-	High
3+1 or 2x(3+1)	Outer membrane protein A	4397	8+	130	4397 $\pm$ 5	35148	34978	-170.159	1% and 2%	37	-	-	High
2x(3+1)	Enolase	5085	18+	130	5085 $\pm$ 10	45459	45551	55.619	2%	55	-	-	High

5.3.2.1 Soluble Protein identification

Superoxide dismutase (SOD) [Mn], detected here as a homodimer with metal ions bound (see Figure 5.5 for identification), is an enzyme which tends to bind one Mn^{2+} each subunit and protects organisms from oxidation damage [355]. The accurate mass of SOD [Mn] was determined by deconvoluting isotope peaks obtained in high-resolution mode (see inset). This protein was also detected in the work combining electroporation and NAMS described in Chapter 3.

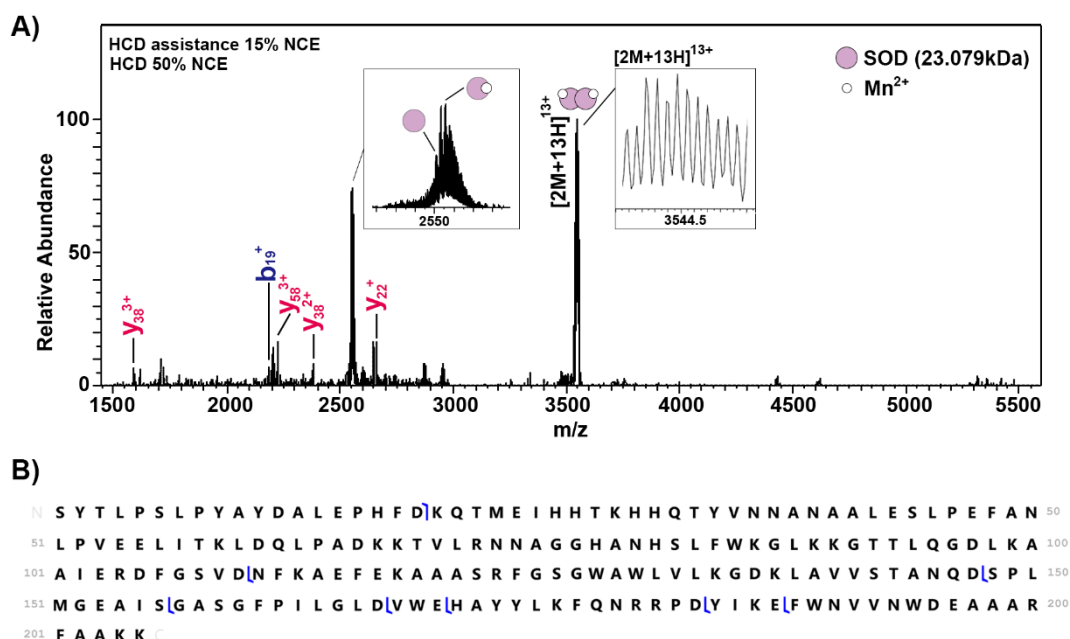


Figure 5.5 Identification of SOD [Mn]. A) HCD mass spectrum of the 13+ charge state (see inset) of SOD [Mn] (m/z 3550 $\pm$ 10) obtained with NCE of 50%; B) sequence coverage obtained.

Phosphoenolpyruvate carboxykinase (ATP) (PCKA) (Figure 5.6) plays a crucial role in gluconeogenesis and glycolytic pathways in *E. coli* and other organisms.

Its function involves catalysing the conversion of oxaloacetate into phosphoenolpyruvate via decarboxylation and mononucleotide-dependent phosphorylation [356]. It has been detected by in-gel digestion and LC-MS in *Entamoeba histolytica* [357] and by size exclusion chromatography MS in *Arabidopsis thaliana* [358, 359]. Mn^{2+} serves as the essential activation cofactor for PCKA. Additionally, a second divalent cation, either Mn^{2+} or Mg^{2+} , is required to form the metal–nucleotide complex, which represents the active form of the substrate [356, 358, 360-362]. Here, the accurate mass of PCKA was determined by deconvoluting isotope peaks observed in high-resolution mode to be 59,912 Da. None of the metal-protein interaction of PCKA was observed.

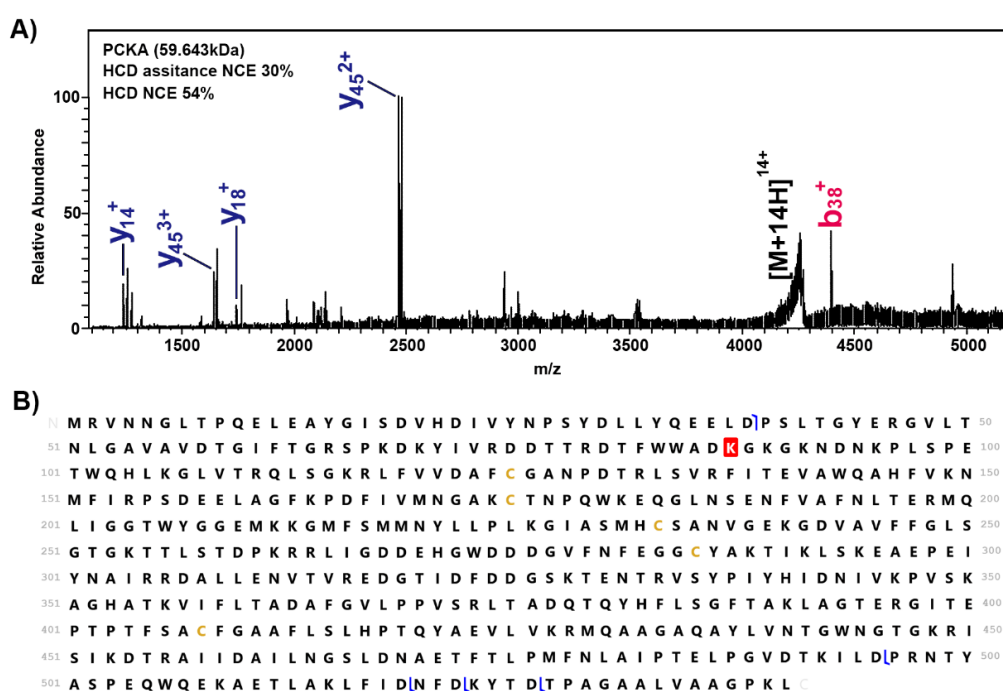


Figure 5.6 Identification of PCKA. A) HCD mass spectrum of the 14+ charge state (see inset) of PCKA (m/z 4217 $\pm$ 10) obtained with NCE of 54%; B) sequence coverage obtained.

Serine hydroxymethyl transferase (SHMT) (Figure 5.7) is an essential enzyme that plays a crucial role in both folate recycling and the synthesis of dTMP [363]. In previous research, it has been analysed by ESI-MS in its intact protein monomeric form with a mass of 45,559 Da, after being extracted from *E. coli* and purified [364, 365]. Here, SHMT is observed as a homodimer with a total mass of 90,634 Da, shown in Figure 5.7. Due to the high m/z and relatively low abundance (range of 10^2 - 10^3 peak intensity) of SHMT, PTCR was applied to determine its protein mass. During the PTCR reaction, the 19+ SHMT precursor

ion was reacted with a high proton affinity reagent, producing a series of precursor peaks with lower charge states, shown in Figure 5.7 A. After deconvolution, the protein accurate mass was determined to be 45,578 Da.

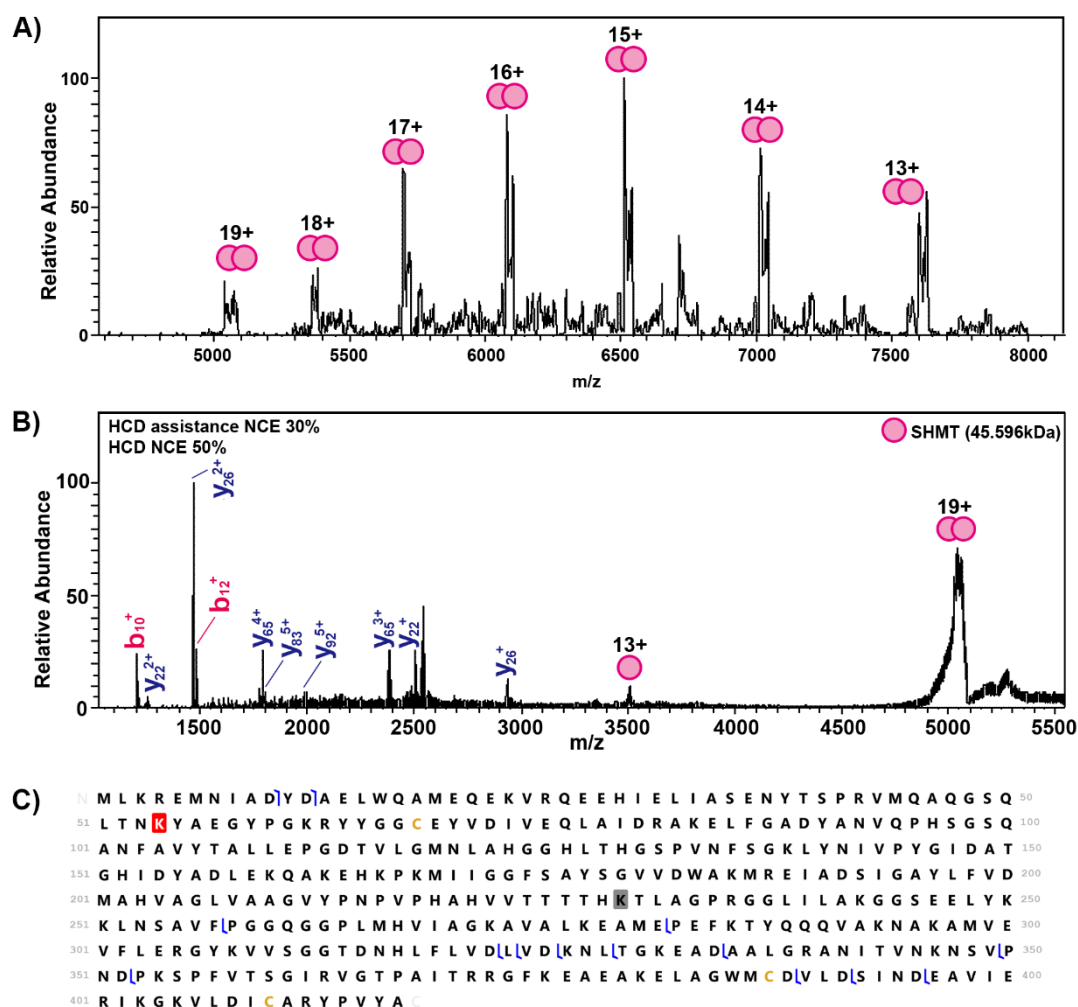


Figure 5.7 Identification of SHMT. A) PTMR mass spectrum obtained for precursor ions with 5066 ± 20 . PTMR reaction time = 7 ms B) HCD mass spectrum of the 19+ charge state (see inset) of SHMT ($m/z 5066 \pm 20$) obtained with NCE of 50% C) sequence coverage obtained.

2,3-bisphosphoglycerate-dependent phosphoglycerate mutase (GPMA) (see Figure 5.8) has been detected by bottom-up LC-MS in *Acidovorax citrulli* [366] and peptide mass fingerprinting with MALDI-TOF MS in *E. coli* [367]. This protein requires the cofactor 2,3-bisphosphoglycerate (2,3-BPGA) to catalyse the interconversion of 2-phosphoglycerate (2-PGA) and 3-phosphoglycerate (3-PGA) in the Embden-Meyerhoff pathway [368]. Here, the cofactor was not observed bound to the protein, likely due to its dissociation caused by the in-source collision energy (here, 130V) which is essential for breaking the detergent micelles. GPMA is observed as a homodimer and its accurate mass was determined by deconvoluting isotope peaks obtained in high-resolution mode to be 28,541 Da.

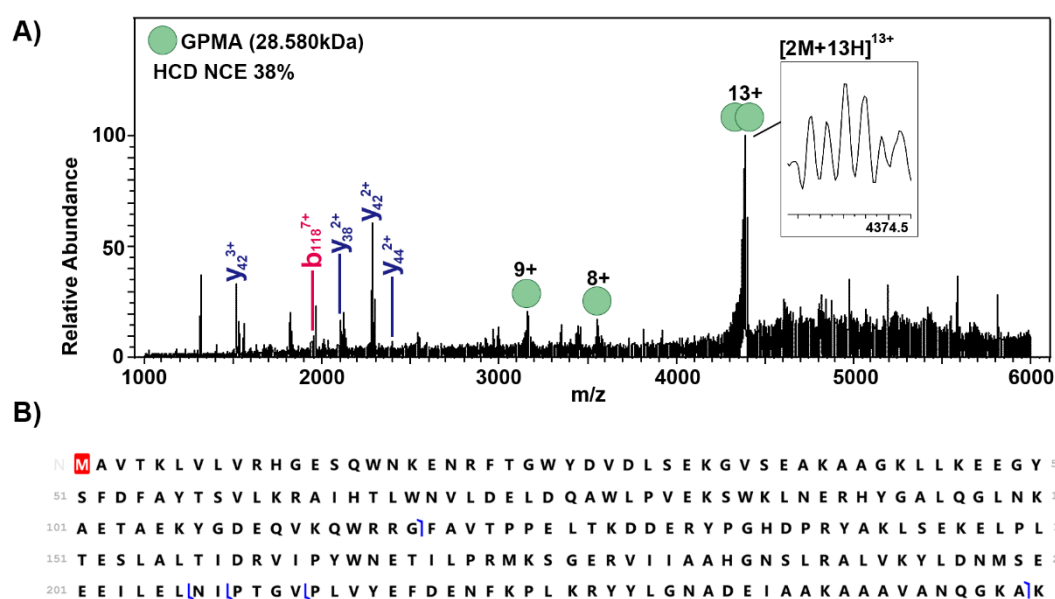


Figure 5.8 Identification of GMPA. A) HCD mass spectrum of the 13+ charge state (see inset) of GMPA ($m/z\ 4406 \pm 20$) obtained with NCE of 38% B) sequence coverage obtained.

5.3.2.2 Membrane-related Protein identification

In addition to the soluble proteins described above, three membrane-related proteins were detected, including extracellular solute-binding protein (SBP) family 3, maltodextrin-binding protein (MalE) and cationic amino acid ABC transporter (CAT)-periplasmic binding protein. Among these proteins, SBP family 3 and MalE were identified in IRM standard pressure mode and CAT-periplasmic binding protein was identified in IRM high pressure mode due to its lower charge state and lower signal intensity. The identification of these three proteins is illustrated in Figure 5.9, Figure 5.10 and Figure 5.11.

Bacterial extracellular SBPs act in several functions in bacterial cells, including as chemoreceptors to recognise specific molecules, sense and respond to cell external environmental changes, and as essential parts of transport systems to assist the absorption of ions and nutrients [369, 370]. Moreover, they can trigger signal transduction pathways, resulting in cellular responses according to specific extracellular binding events [369, 371]. The accurate mass of SBP-family 3 was determined by deconvoluting isotope peaks.

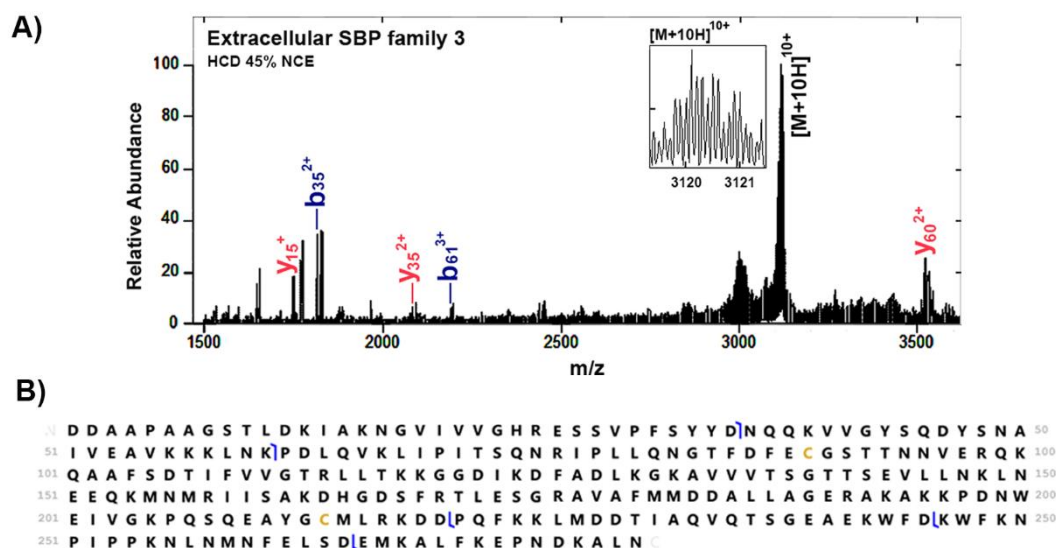


Figure 5.9 Identification of extracellular SBP family 3. A) HCD mass spectrum of the 10+ charge state (see inset) of extracellular SBP family 3 (m/z 3124 $\pm$ 10) obtained with NCE of 45% B) sequence coverage obtained.

MalE, as a periplasmic binding protein, can react with membrane-associated proteins, such as maltodextrin permease, and function in transporting maltodextrins into the bacterial cell [372]. It is an important part of the ATP-binding cassette ABC transporter complex MalEFGK, helping to import

maltose/maltodextrin into the cell [373]. The accurate masses of SBP-family 3 and MalE were determined by deconvoluting isotope peaks.

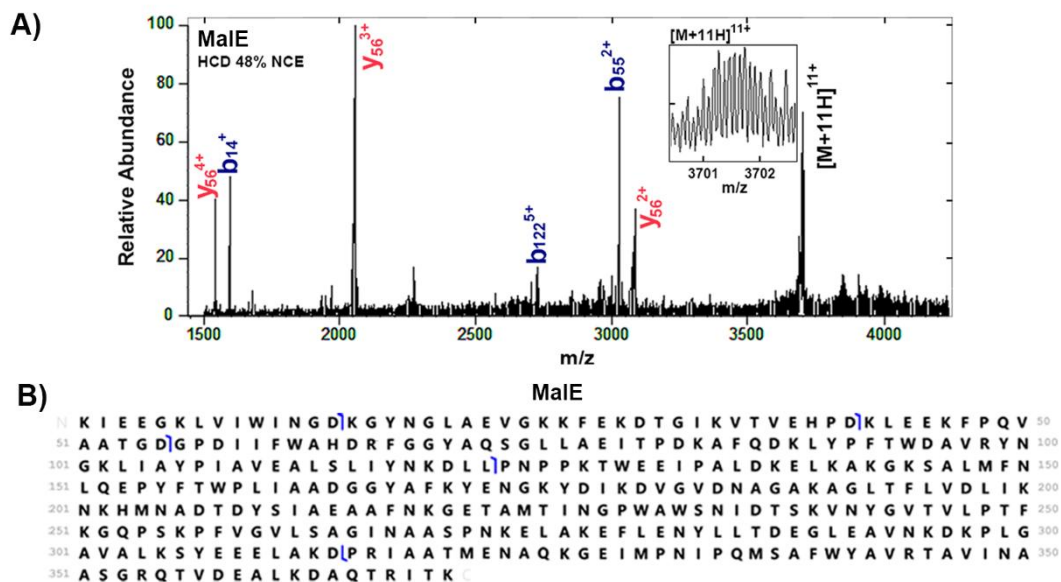


Figure 5.10 Identification of MalE. A) HCD MS/MS mass spectrum of the 11+ charge state (see inset) of MalE (m/z 3705±5) obtained with NCE of 48% B) sequence coverage obtained.

The other membrane-related protein, CAT-periplasmic binding protein, is an essential part of an ABC transporter system named CAT. CAT helps the bacterial cell's absorption of cationic amino acids [374, 375]. In the periplasmic area, the CAT-periplasmic binding protein can bind to cationic amino acids and subsequently experience changes in conformation [375]. Afterward, the transmission process of these conformational changes will be activated, transferring the information about the changes to the transmembrane region of the associated membrane proteins, thereby initiating the transport process.

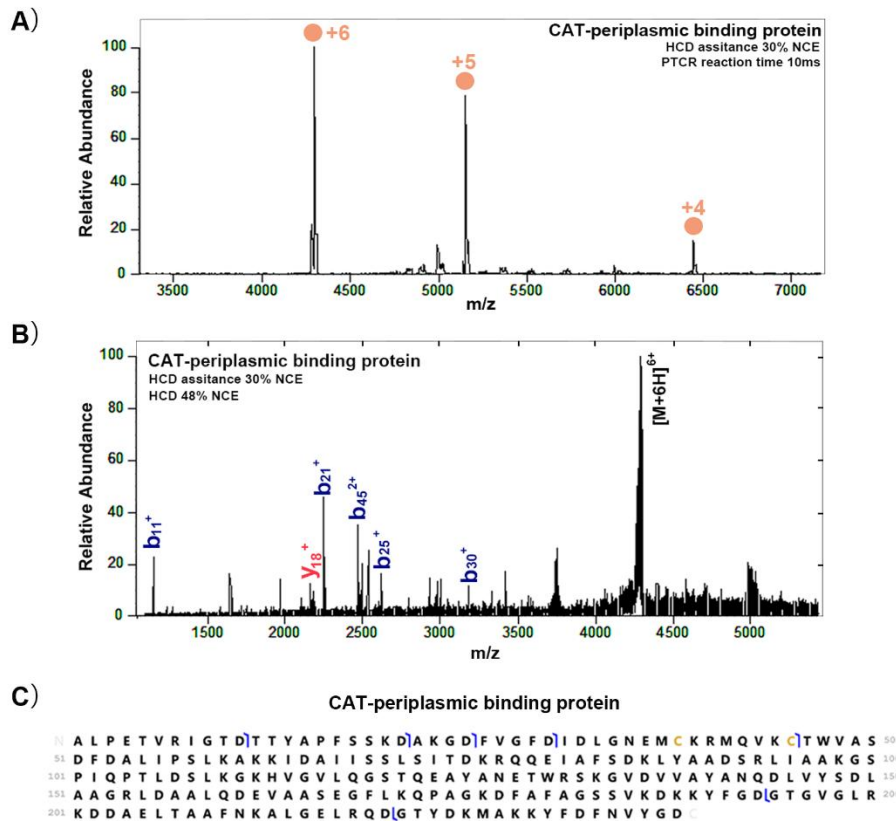


Figure 5.11 Identification of CAT-periplasmic binding protein. A) PTCR mass spectrum obtained for precursor ions with 4298 ± 5 . PTCR reaction time= 10 ms B) HCD mass spectrum of the 6+ charge state (see inset) of CAT-periplasmic binding protein ($m/z 4298 \pm 5$) obtained with NCE of 48% C) sequence coverage obtained.

CAT-periplasmic binding protein was identified with IRM in high-pressure mode. Due to the low signal-to-noise ratio in high-resolution detection, the low-resolution setting of 7500 at m/z 200 was employed, bringing about higher signal intensity of protein peaks. In this condition, protein isotope peaks were unable to be determined, resulting in difficulties in defining the accurate mass of CAT-periplasmic binding protein. Therefore, PTCL was applied to define the mass of

this protein, see Figure 5.11 top.

5.3.2.3 Membrane Protein identification

Additionally, through this washing protocol, two membrane proteins, long-chain fatty acid transport protein (FADL) and outer membrane protein A (OMPA) were successfully detected (Figure 5.3) and identified, using HCD fragmentation (Figure 5.12 and Figure 5.13). All fragmentation sites were observed either in C-terminal to aspartic acid or N-terminal to proline illustrated to be favoured [282] in top-down mass spectrometry, thereby enhancing the confidence in protein assignments. In the analysis of two outer membrane proteins, the reproducibility of detection was unsatisfactory, due to a low signal to noise ratio, resulting in the detection of limited number of fragments, shown in Figure 5.12 and Figure 5.13. This could be caused by the proteins' low abundance (range of 10^2 - 10^3 peak intensity) and spectrum overlap of soluble proteins.

For FADL, only four b ions were detected, resulting in 1% sequence coverage (Figure 5.12). FADL is an outer membrane protein that takes part in cellular uptake and transportation of long-chain fatty acids [376, 377]. It plays an important role in transferring fatty acids via the plasma membrane into the cell and activating them by transforming them into acyl-CoA [376], a form with important functions in different metabolic pathways.

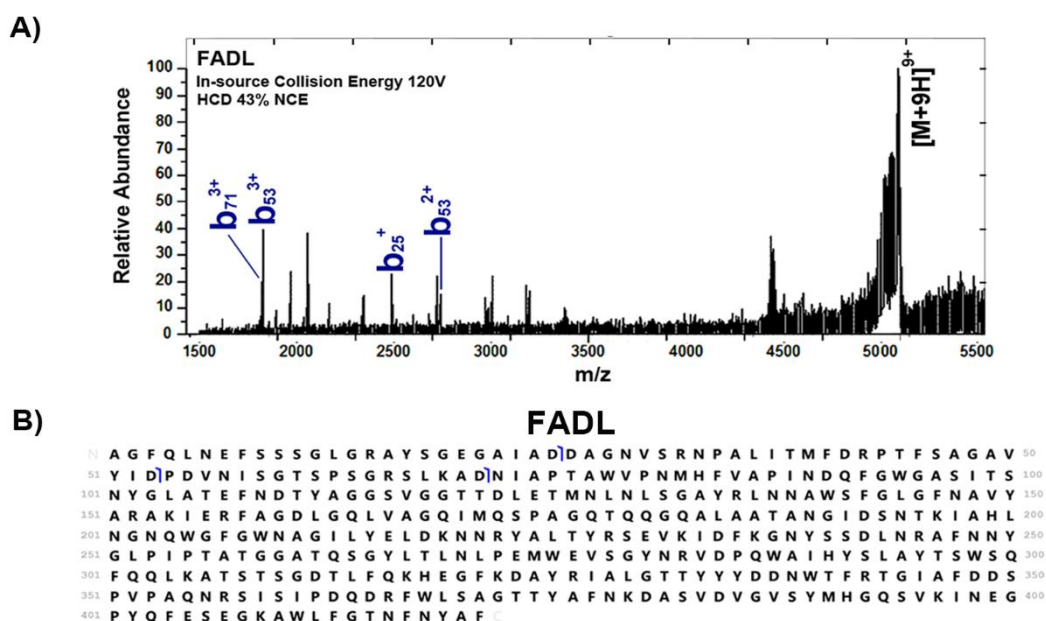


Figure 5.12 Identification of FADL protein. **A)** HCD mass spectrum of the 9+ charge state (see inset) of FADL (m/z 5102 $\pm$ 5) obtained with NCE of 43% **B)** sequence coverage obtained.

While for the other membrane protein OMPA, only three fragments were detected, also providing 1% sequence coverage (Figure 5.13). OMPA in *E. coli* is responsible for a number of crucial processes that influence the structure and functioning of the bacterial cell [378-380]. It is essential for preserving the stability and integrity of the outer membrane as well as helping in binding it to the cell envelope's peptidoglycan layer [379, 380]. It also strengthens the outer membrane's permeability barrier [381] and facilitates the regulation of molecules that enter and exit the bacterial cell, such as ions and nutrients [379].

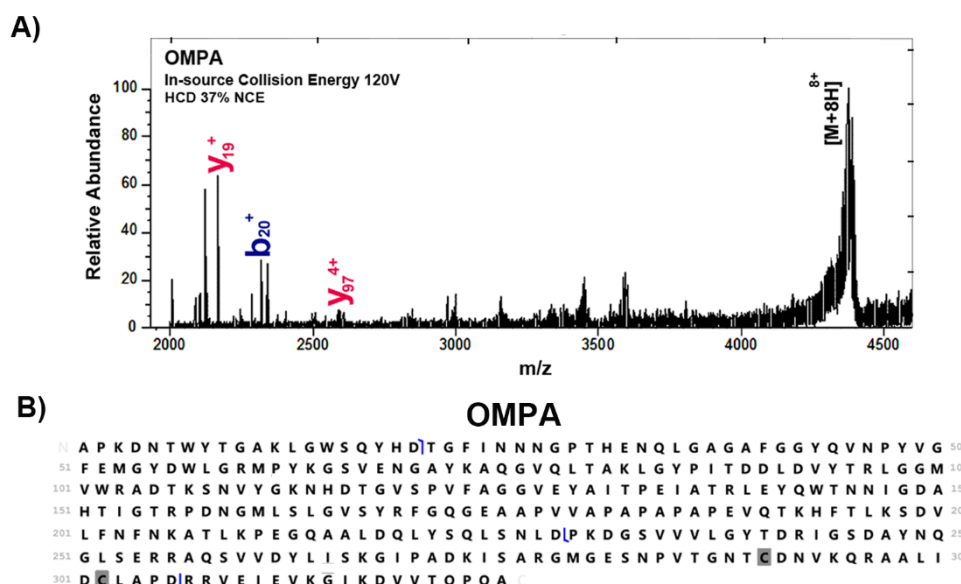


Figure 5.13 Identification of OMPA protein. A) HCD mass spectrum of the 8+ ions (see inset) of OMPA (m/z 4397 $\pm$ 5) obtained with NCE of 37%, B) sequence coverage obtained.

5.3.3 Further development of washing protocol for the analysis of membrane proteins

A potential approach to enhance the signal intensity of membrane protein ions, involves modifying the washing process to remove more soluble proteins. The following washing protocol was trialed: The colonies were initially washed with 200mM ammonium acetate and 0.5 x CMC C8E4 for three rounds, followed by another round of washing using 200mM ammonium acetate solution, as described above. That process was then repeated, resulting in a total of eight

washing steps. Figure 5.14 shows a representative LESA mass spectrum obtained following this washing protocol.

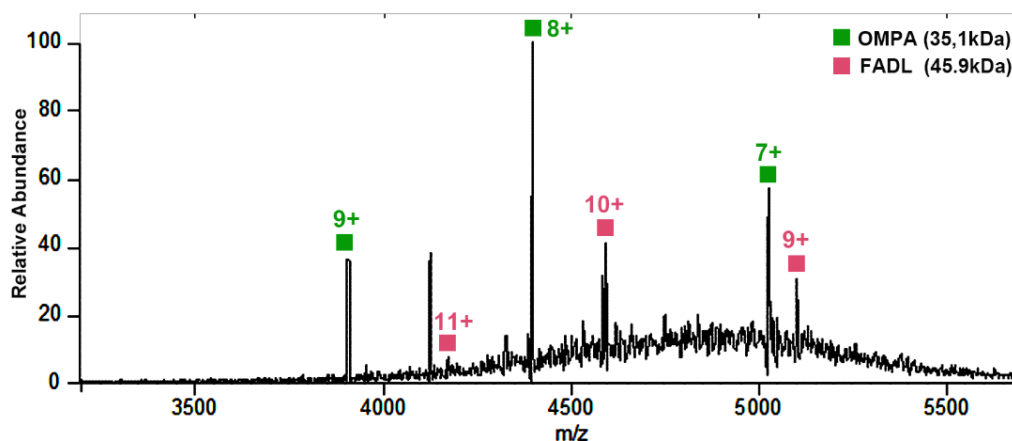


Figure 5.14 Representative full-scan mass spectrum obtained from *E. coli* BL21 (DE3) following 2 x (3+1) washing protocol. The two outer membrane proteins, OMPA and FADL, were observed.

Peaks corresponding to the same outer membrane proteins OMPA and FADL were observed with higher signal-to-noise ratio (OMPA (8+) 4.99:1 and FADL (10+) 2.01:1) than observed following the (3+1) protocol (OMPA (8+) 1.65:1 and FADL (10+) 1.26:1). With the 2 x (3+1) process, the sequence coverages of membrane proteins were increased compared to previous results. The identification of FADL is shown in Figure 5.15 and OMPA is shown in Figure 5.16. For FADL, seven b ions and three y ions were detected with a sequence coverage of 3%. For OMPA, one b ion and four y ions were detected with a sequence coverage of 2%.

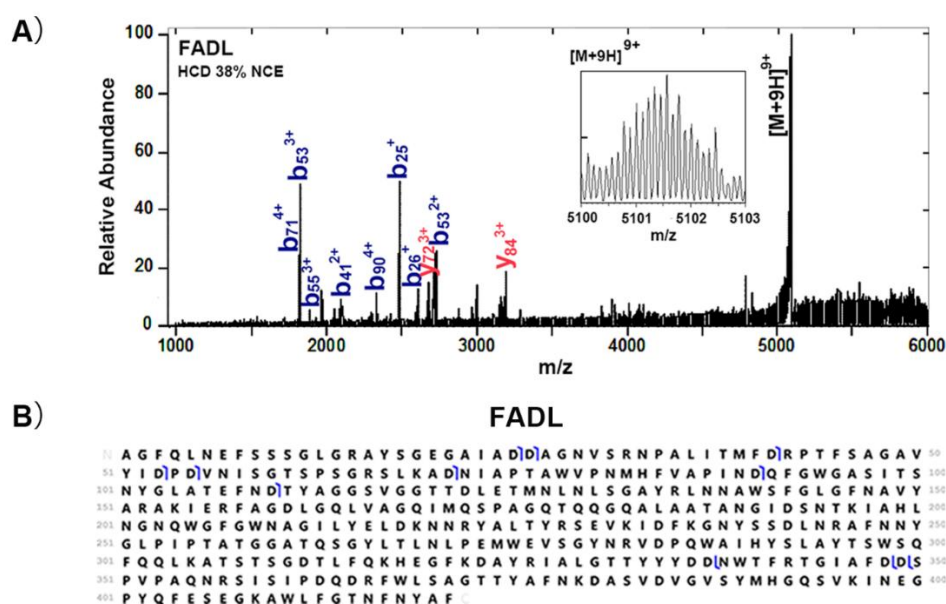


Figure 5.15 Identification of outer membrane proteins FADL. **A)** HCD mass spectrum of the 9+ ions (see inset) of FADL (m/z 5102 $\pm$ 5) obtained with NCE of 38%, **B)** sequence coverage obtained.

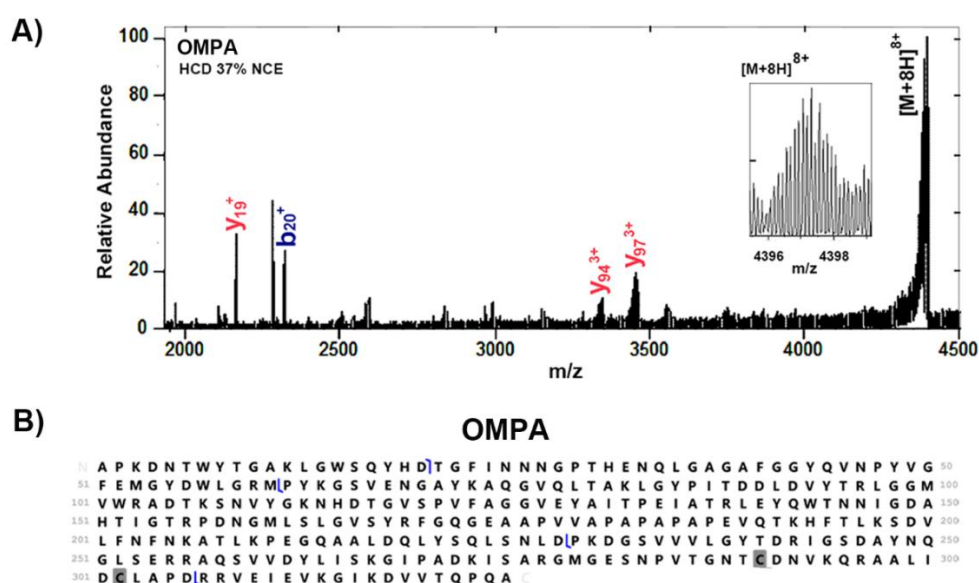


Figure 5.16 Identification of outer membrane proteins OMPA. **A)** HCD mass spectrum of the 8+ ions (see inset) of OMPA (m/z 4397 $\pm$ 5) obtained with NCE of 37%, **B)** sequence coverage obtained.

Interestingly, a previously undetected soluble protein was observed following this harsher washing protocol. Enolase, observed as a protein dimer with a mass of 91,102 Da, was only detected following eight steps of washing. The identification is shown in Figure 5.17. The protein was not observed using the (3+1) protocol. A potential explanation could be that serine hydroxymethyltransferase, detected at m/z 5066 ± 10 in the (3+1) protocol, overlapped with enolase, which exhibited a higher signal intensity and was identified first. After applying the 2 x (3+1) protocol, serine hydroxymethyltransferase was effectively removed. However, due to broad intracellular distribution of enolase [382-385], it is more challenging to remove by washing compared to serine hydroxymethyltransferase.

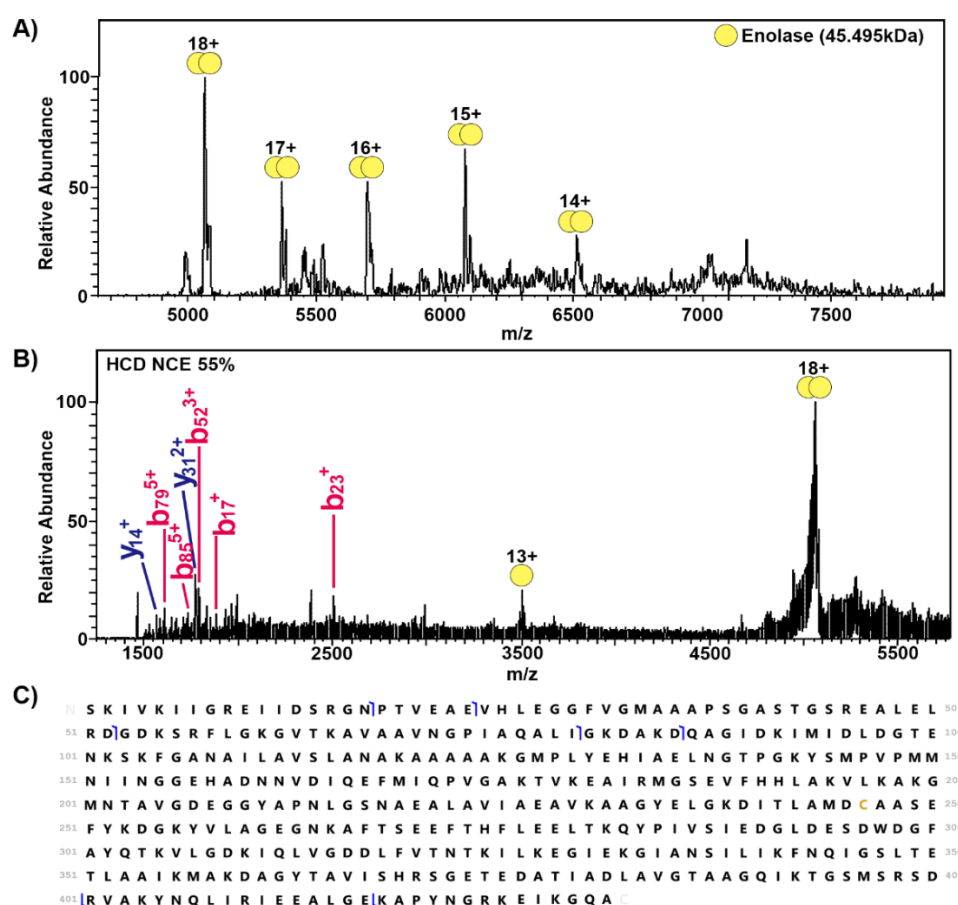


Figure 5.17 Identification of Enolase. A) PTMR mass spectrum obtained for precursor ions with 5085 ± 20 . PTMR reaction time= 3 ms B) HCD mass spectrum of the 18+ charge state (see inset) of Enolase protein (m/z 5085 ± 20) obtained with NCE of 55% C) sequence coverage obtained.

5.3.4 Investigation of washing protocol used in *Pseudomonas aeruginosa*

To investigate whether the method could be more broadly applied to detection of

outer membrane proteins in gram-negative bacteria, the washing protocol involving eight washing steps was applied to *Pseudomonas aeruginosa* PS1054. Figure 5.18 shows a representative LESA mass spectrum of *Pseudomonas aeruginosa* PS1054 obtained following washing.

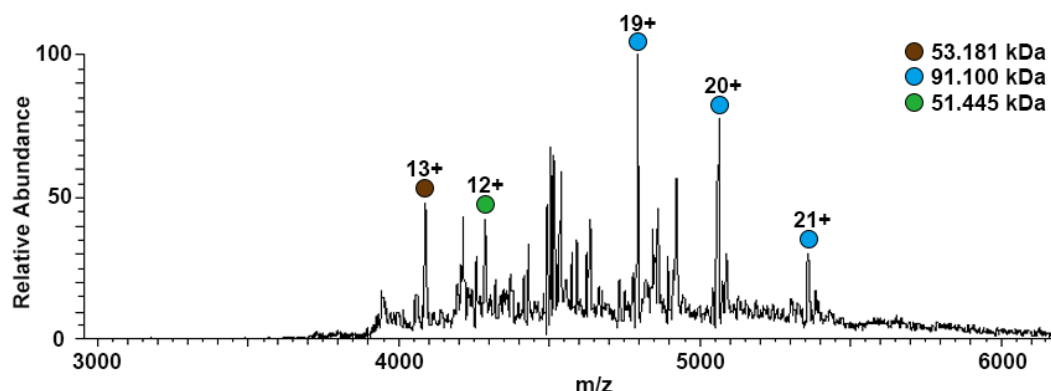


Figure 5.18 Representative full-scan mass spectrum obtained from *Pseudomonas aeruginosa* PS1054 following 2 x (3+1) washing protocol.

Three proteins, as shown in Figure 5.18, were successfully isolated, while other peaks could not be distinguished due to either low signal intensity or excessive overlap with multiple peaks. Subsequently, MS/MS analysis using HCD was employed for protein identification. The three proteins labelled in Figure 5.18 was subjected to MS/MS analysis. However, due to the limited signal intensity and insufficient fragmentation, two of the proteins, with masses of 91,100 Da and 51,445 Da, could not be identified. Ultimately, only one protein chitinase, with a mass of 53,042 Da was successfully identified, shown in Figure 5.19.

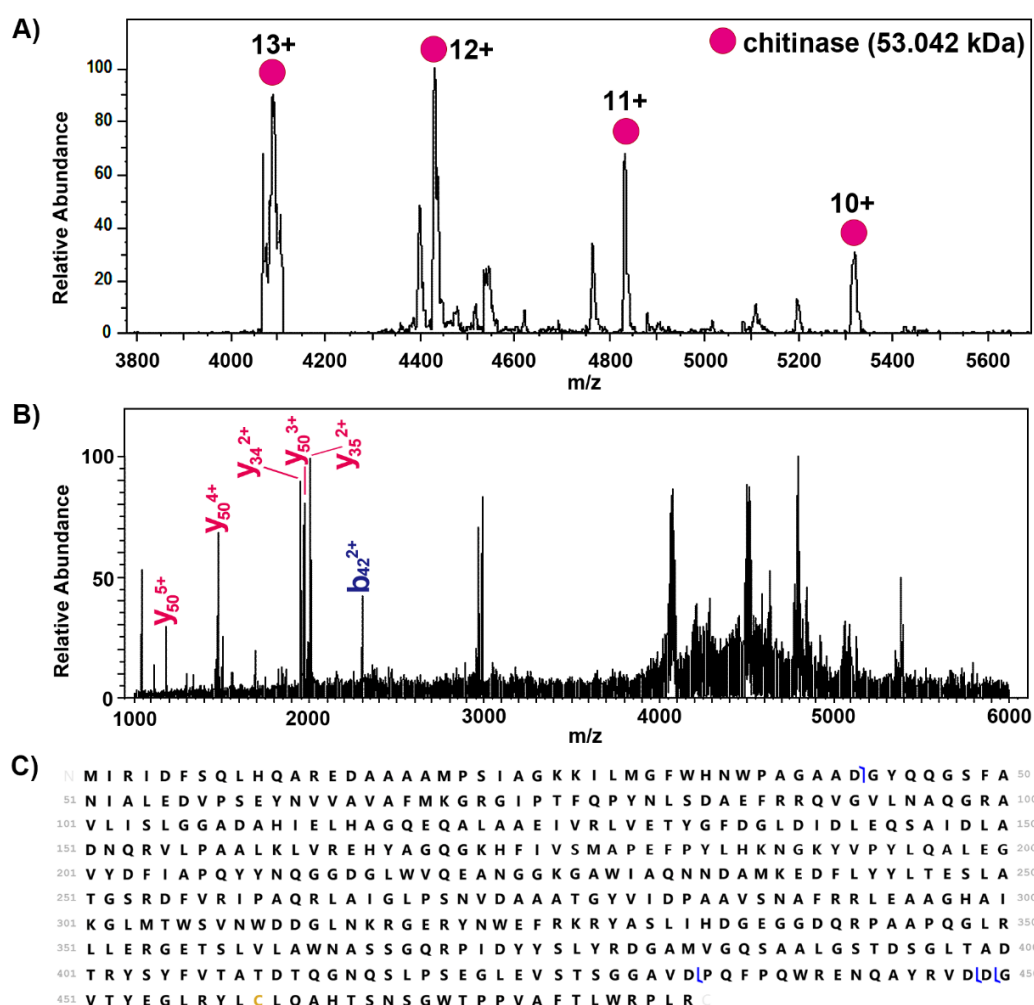


Figure 5.19 Identification of Chitinase. A) PTMR mass spectrum obtained for precursor ions with 4088 ± 20 . PTMR reaction time= 3 ms B) HCD mass spectrum of the 13+ charge state (see inset) of Chitinases (m/z 4088 ± 20) obtained with NCE of 58% C) sequence coverage obtained.

Chitinases facilitate the decomposition of chitin, a common polymer found in the cell walls of fungi, the shells of crustaceans, and the exoskeletons of insects [386]. The identification information of this protein is shown in Figure 5.19. PTMR was applied to determine the accurate mass, shown in Figure 5.19 A), and HCD was

used for fragmentation and identification, shown in Figure 5.19.B). Here, sequence fragmentation sites were observed either at the C-terminal of aspartic acid or the N-terminal of proline, shown in Figure 5.19 C), which complies with the characteristic fragmentation pattern in native MS as described before.

5.3.5 Membrane Protein Detection of Gram-positive Bacteria

To investigate if this method would be suitable for analysis of gram-positive bacteria, *Staphylococcus epidermidis* HB and *Staphylococcus epidermidis* RP62a were employed. The washing protocol eight rounds of washing (2 x (3+1)) was applied before LESA sampling and MS analysis. Unfortunately, the results show that neither of these washing approaches enabled detection of any proteins in gram-positive bacteria, illustrated in Figure 5.20.

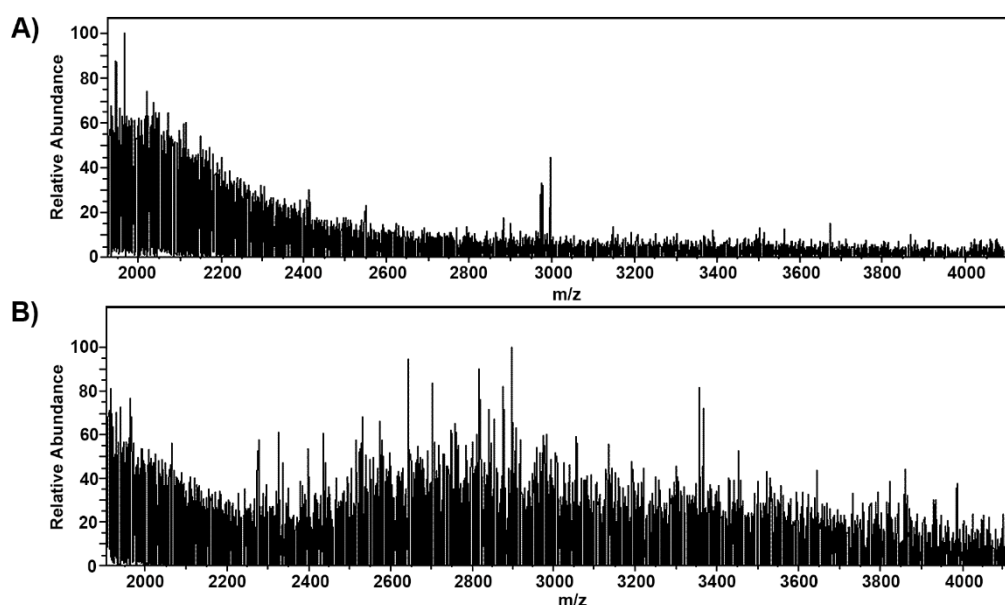


Figure 5.20 Full scan mass spectrum of *Staphylococcus epidermidis* strains after 2 x (3+1) rounds of washing with 80V in-source collision energy and IRM configured as standard pressure. A) mass spectrum of *Staphylococcus epidermidis* HB. B) mass spectrum of *Staphylococcus epidermidis* RP62a.

The potential reason could be that the structure of gram-positive bacterial cell walls and cell membranes is different from gram-negative bacteria, as described before. Since the method of colony washing only allowed the outer membrane proteins to be detected, the gram-positive bacteria with only one layer of plasma membrane covered by a thick layer of peptidoglycan would be difficult to detect by this approach. Furthermore, as described in Chapter 3, the electroporation did not perform well for gram-positive bacteria, therefore the combination of electroporation and colony wash did not work to detect any protein either.

5.3.6 Combination of Electroporation and Colony Washing Process

Further work investigated the combination of electroporation together with colony washing for detection of membrane proteins. The initial investigation combined the 2 x (3+1) washing protocols with electroporation, while the subsequent investigation extended the washing steps to twelve rounds with 2 x (5+1) washing protocol describe in Chapter 2, Section 2.2.2.

Firstly, three workflows were trialled, all of which made use of the 2 x (3+1) washing protocol comprising eight washing steps. The initial workflow involved the application of electroporation which was set up to 3 kV (field strength= 15 kV/cm), detailed at Chapter 2, Section 2.2.3, followed by colony washing and LESA MS. A representative LESA mass spectrum of three replicates is shown in Figure 5.21 A). Protein peaks were barely observed with a low signal intensity and a high spectrum noise. Only three proteins were identified using HCD MS/MS, and these had been previously detected by MS following the (3+1) washing protocol without electroporation. Under these conditions, no membrane proteins were detected while one of the membrane related protein MalE was observed, in contrast to the results from the 2 x (3+1) washing protocol. A potential explanation could be that the initial electroporation before washing may have affected the colony surface environment, causing some of the opened bacterial cells to be

washed away during the harsh 2 x (3+1) washing steps, which affected the subsequent sample extraction and MS detection results. Consequently, the signal intensity of the spectrum was lower, and no membrane proteins were observed. Subsequently, the positioning of the electroporation step was amended such that it occurred immediately after the fourth round of washing (with 200 mM ammonium acetate without detergent). A representative LESA mass spectrum of three replicates is shown in Figure 5.21 B). Six proteins were observed, five of which, including enolase, SBP family 3, GPMA, PCKA and SOD [Mn], were confirmed by HCD MS/MS and had previously been observed using the (3+1) washing process, as described in Chapter 5 Section 5.3.2. Compared to the 2x (3+1) washing protocol without electroporation, more soluble proteins were detected as a result of applying the electroporation step. Since electroporation occurred after the first (3+1) rounds of washing, followed by an additional (3+1) washing steps, the incomplete washing after electroporation allowed some soluble proteins to remain and be detected. Furthermore, the remaining unknown protein with a mass of 32,300 Da could not be identified due to the low signal-to-noise ratio of its fragmentations.

Lastly, electroporation immediately after the completion of the 2 x (3+1) washing process was trialled, as depicted in the representative LESA mass spectrum in Figure 5.21 C). This approach exhibited fewer protein peaks but with a higher signal-to-noise ratio compared to Figure 5.21 A) and B). The same two proteins,

SOD [Mn] and MalE were observed with a higher signal-to-noise ratio compared to Figure 5.21 A) which was acquired without electroporation. Additionally, another membrane-related protein, extracellular soluble-binding protein family 3, was detected. While the technique of combining electroporation with colony washing allows for the observation of protein peaks, the majority of these peaks were found to be soluble proteins.

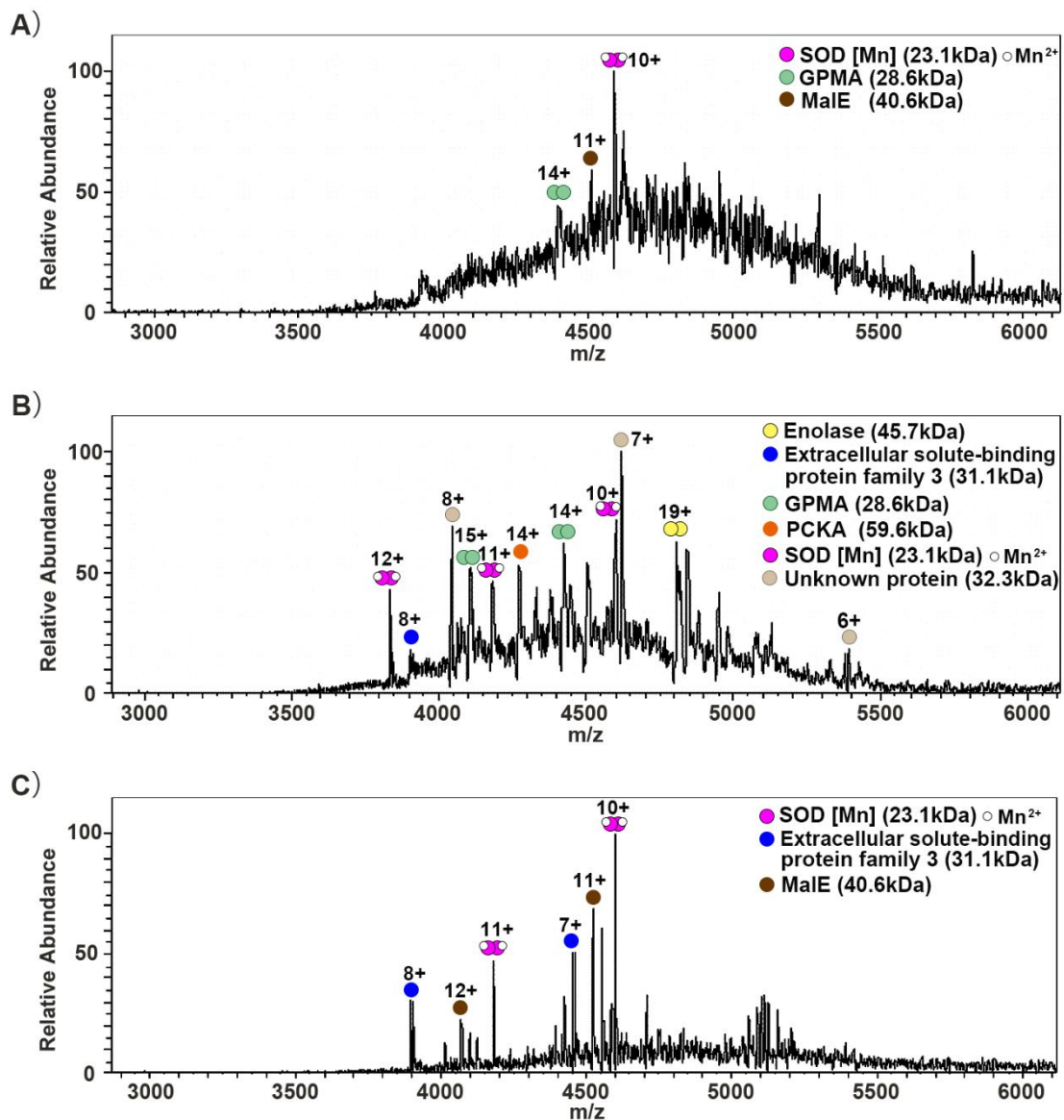


Figure 5.21 Representative mass spectrum of 2 x (3+1) washing protocols with electroporation with 7% CSF. A) Mass spectrum obtained when electroporation was performed before washing. B) Mass spectrum obtained when electroporation was performed after first (3+1) washing, i.e., mid-washing protocol. C) Mass spectrum obtained when electroporation was performed post washing.

However, certain proteins that had not been detected using previous methods were observed at higher masses only when the approach incorporating electroporation into the 2 x (3+1) round washing process was employed, shown in Figure 5.22 and Figure 5.23. The detection of these proteins with higher m/z values is more challenging in a lower m/z range due to their complexity, low abundance (range of 10^2 - 10^3 peak intensity), and interference from smaller, higher-abundance proteins, resulting in their absence in Figure 5.21. To isolate these bigger proteins without overlapping other smaller protein peaks, the CSF was adjusted from 7% to 18%. Increasing the source CSF shifted the mass spectrum to a higher m/z range, enabling the detection of larger proteins. By these settings, several bigger proteins were observed, shown in Figure 21, however only two of which were successfully isolated, with masses of 106,100 Da and 142,370 Da. The remaining protein peaks overlapped in complex patterns, making analysis difficult and preventing clear identification. The 106,100 Da protein mass was determined by PTCL, shown in Figure 5.22, but was not successfully identified by MS/MS. The protein with molecular weight 142,370 Da, shown in Figure 5.22, was identified as the glyceraldehyde-3-phosphate dehydrogenase tetramer, see Figure 5.23.

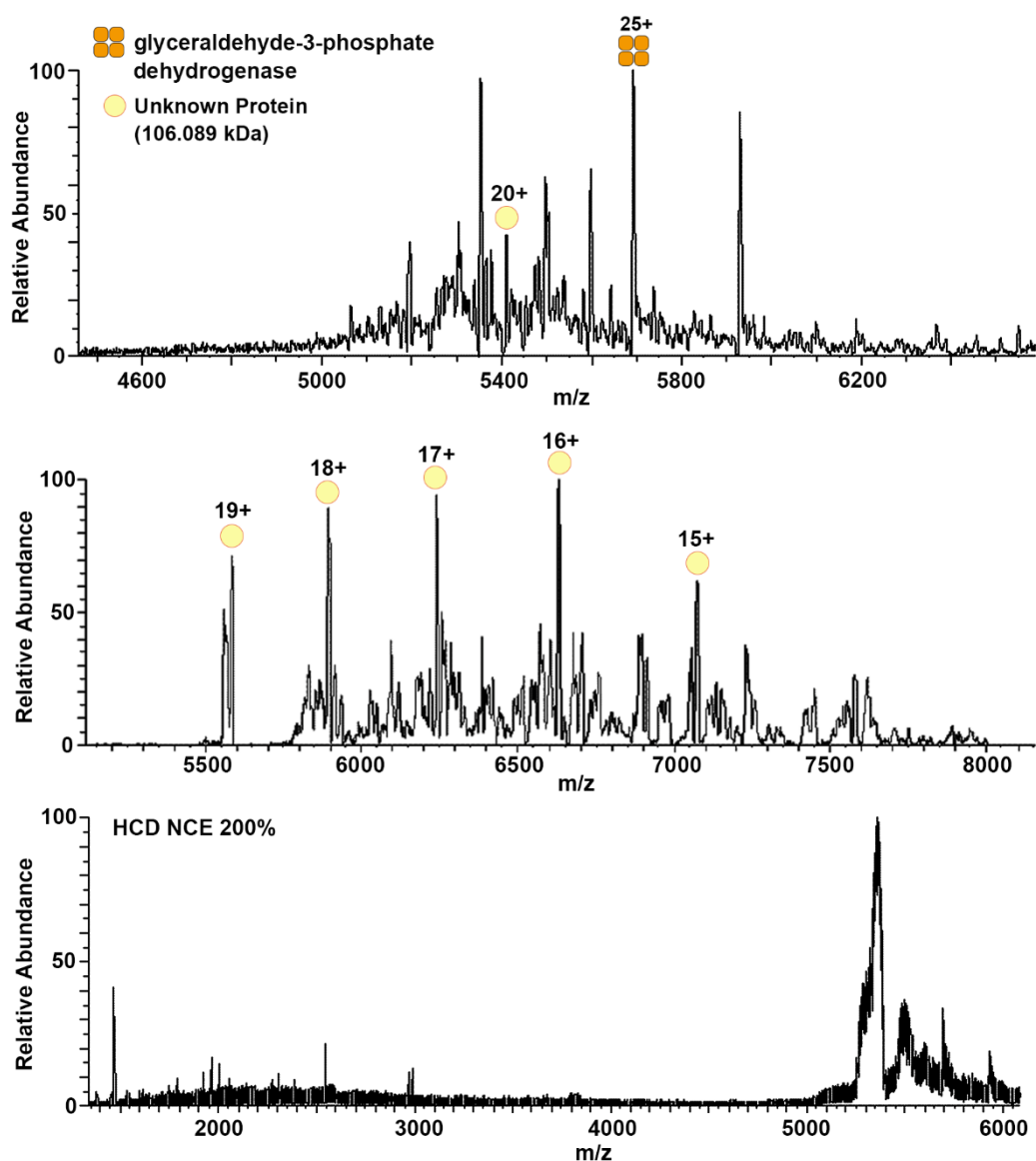


Figure 5.22 (top) Representative mass spectrum of 2 x (3+1) washing protocols with electroporation with 18% CSF; (middle) PTMR mass spectrum obtained for precursor ions with 5413 ± 20 . PTMR reaction time= 3 ms. The protein mass was confirmed as 106,089 Da; (bottom) HCD mass spectrum of the 20+ charge state of precursor ($m/z\ 5413 \pm 20$) obtained with NCE of 200%.

Though the tetramer of glyceraldehyde-3-phosphate dehydrogenase was identified, its subunit was not detected following HCD. A potential explanation is that control by ion guide and dissociation of large protein structures is challenging due to insufficient fragmentation energy and preferential fragmentation [135, 155, 387]. For large proteins and protein assemblies, internal and external molecular interactions may be stabilised by hydrogen bonding and ionic linkages, preventing the dissociation of fragments [388].

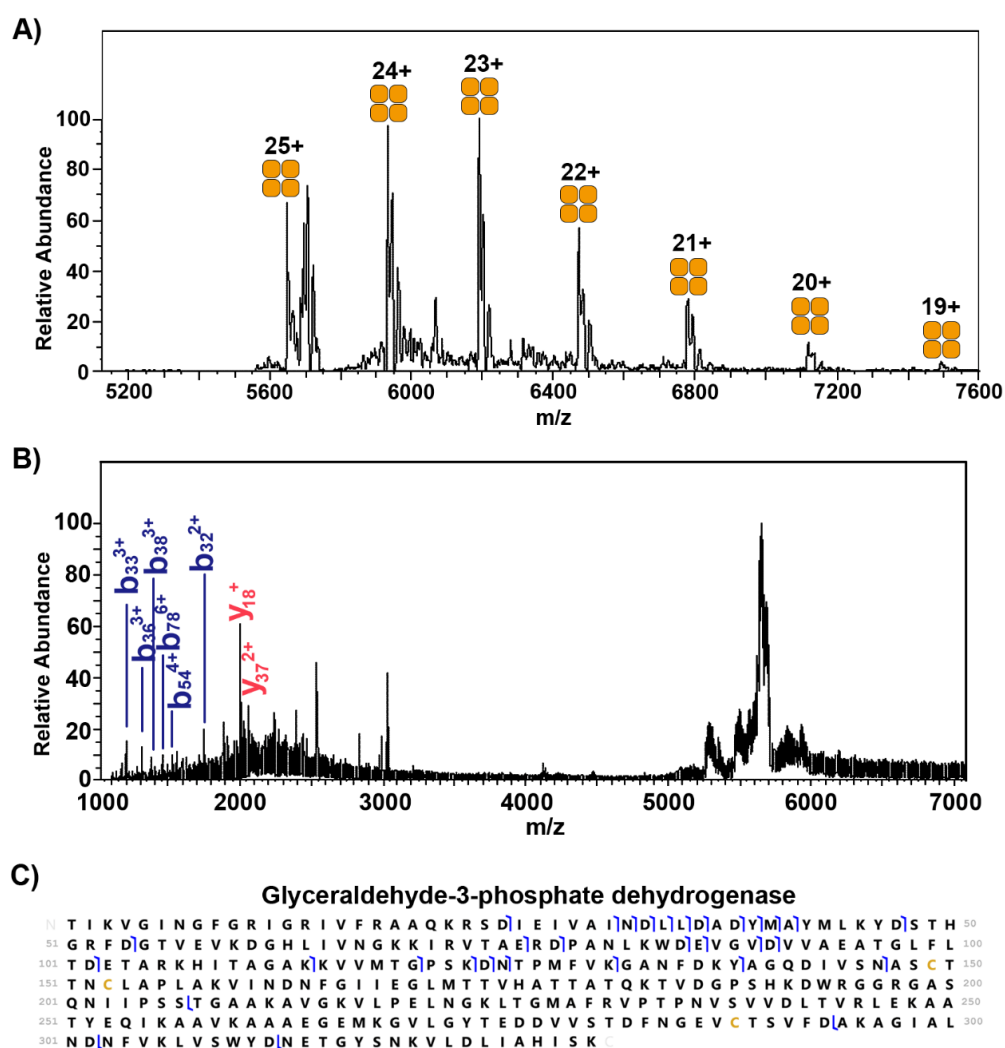


Figure 5.23 Identification of glyceraldehyde-3-phosphate dehydrogenase. A) PTMS mass spectrum obtained for precursor ions with m/z 5694 ± 20 . PTMS reaction time= 2 ms B) HCD mass spectrum of the 25+ charge state of glyceraldehyde-3-phosphate dehydrogenase (m/z 5694 ± 20) obtained with NCE of 180% C) sequence coverage obtained.

Further work explored a 12-step washing approach (described in Chapter 2, Section 2.2.2) in combination with electroporation. In summary, the colony was initially washed five times with 200 mM ammonium acetate containing 0.5 x CMC

C8E4, followed by one additional wash with 200 mM ammonium acetate, completing a (5+1) washing cycle. This (5+1) cycle was then repeated. Electroporation was applied after the first (5+1) cycle, with the settings outlined in Chapter 2, Section 2.2.2. Representative mass spectrum obtained with different compensation scaling factor settings are shown in Figure 5.24.

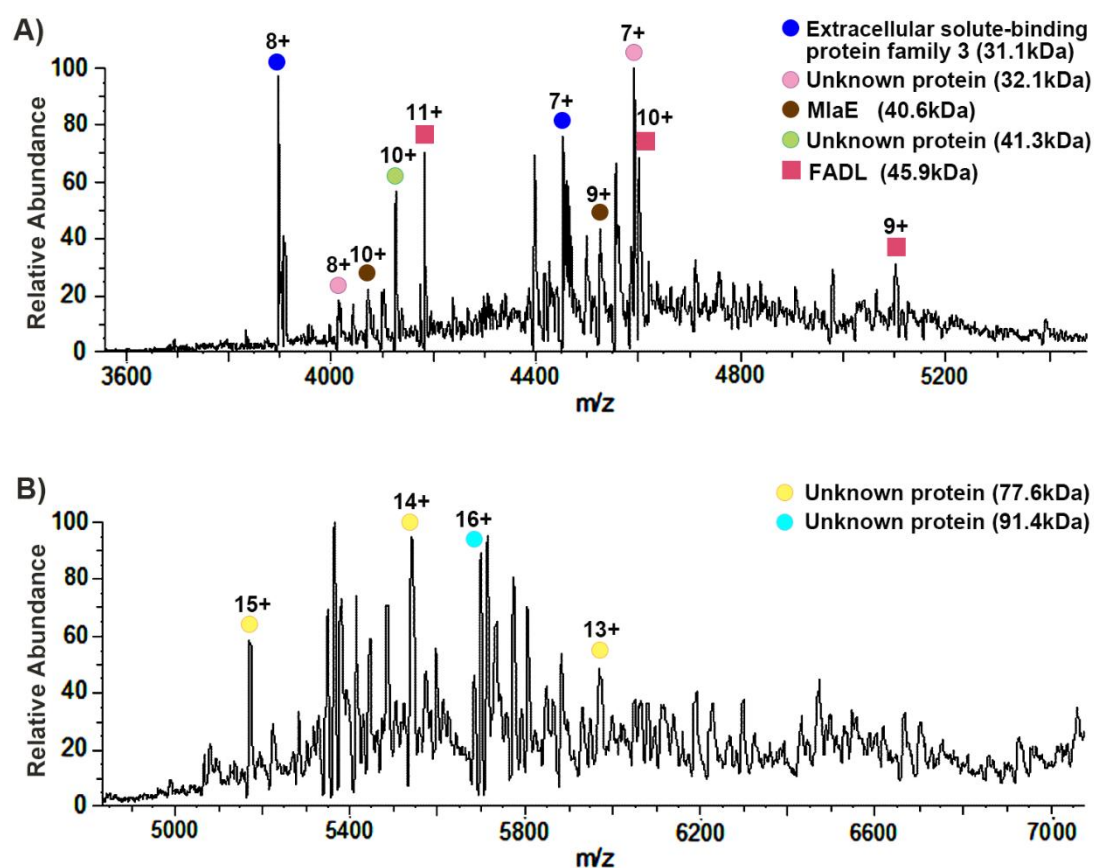


Figure 5.24 Representative mass spectrum obtained following an integrated washing/electroporation protocol ((5+1) – electroporation – (5+1)). A) Representative mass spectrum obtained with compensation scaling factor 7%. B) Representative mass spectrum obtained with compensation scaling factor 14%.

Although several larger proteins were observed with a higher compensation scaling factor (14%) at the full scan level, as shown in Figure 5.24 B), none of them was successfully identified.

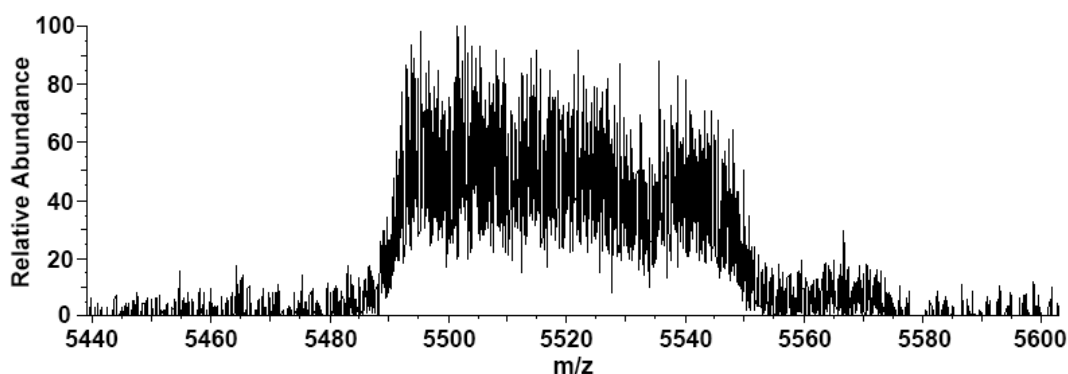


Figure 5.25 HCD MS/MS mass spectrum of the 14+ charge state precursor ion with 5541 ± 50 m/z obtained with NCE of 14%.

The isolation of precursor ion with 5541 ± 50 m/z (the 14+ unknown protein peak shown in yellow at the bottom of Figure 5.24.) is shown in Figure 5.25. Isolation of larger m/z protein peaks is particularly difficult due to their low abundance [389-393], broad isotopic spread [165, 394-397], and the challenges of maintaining isolation accuracy at higher mass-to-charge [170, 398, 399] (over 5500 m/z). Common issues include mass shift [400, 401], low signal intensity [141, 402], and salt adducted peaks around the target mass [402] even after instrument calibration. To address these issues, a wider isolation width of 50–100 m/z is required, however, which results in several overlapping protein peaks [402]. Additionally, as m/z values increase, isolation efficiency and signal intensity

decrease, and proteins with masses over 100,000 Da require much more energy to fragment, complicating identification.

5.3.7 Analysis of SMALPs by Native MS

SMA amphiphilic copolymers have been demonstrated to be useful in extracting membrane proteins directly from their native cell membrane [403, 404]. The long-term aim of the work presented in this section is to translate the SMALP approach into NAMS for direct analysis of membrane proteins from bacteria growing on agar. Two SMALP samples, including the membrane proteins ZipA and Sav 1866, were employed to develop and validate a reliable method for analysis of SMALP samples.

In initial experiments, SMALP samples of inner membrane protein ZipA expressed in *E. coli* were provided by Professor Timothy Dafforn at the University of Birmingham. After buffer exchange into 200 ammonium acetate, the sample was analysed by native MS. Figure 5.26 A) shows a representative full scan mass spectrum of SMALP ZipA (40,016 Da). Two distinct peaks corresponding to the 12+ and 11+ charge states of a single protein were observed. After isolation of the 12+ ions (m/z 3123 $\pm$ 50) in high resolution and deconvolution, the mass of these peaks was determined to be approximately 37,265 Da. The calculated

mass of ZipA is 40,016 Da, a mass shift of 2,751 Da. The mass shift could potentially be attributed to fragmentation occurring at the terminus of the protein's transmembrane segment, shown in Figure 5.26 C). ZipA is a protein possessing a single transmembrane domain [405-407]. To confirm the identity of the 37,265 Da protein, the 12+ precursor ions were isolated and subjected to HCD (45% NCE), see Figure 5.26 B) and D). All of the observed fragments corresponded to y ions of ZipA, indicating that fragmentation predominantly occurred within the non-transmembrane peptide sequence at the C-terminal of ZipA, see figure 5.26 D).

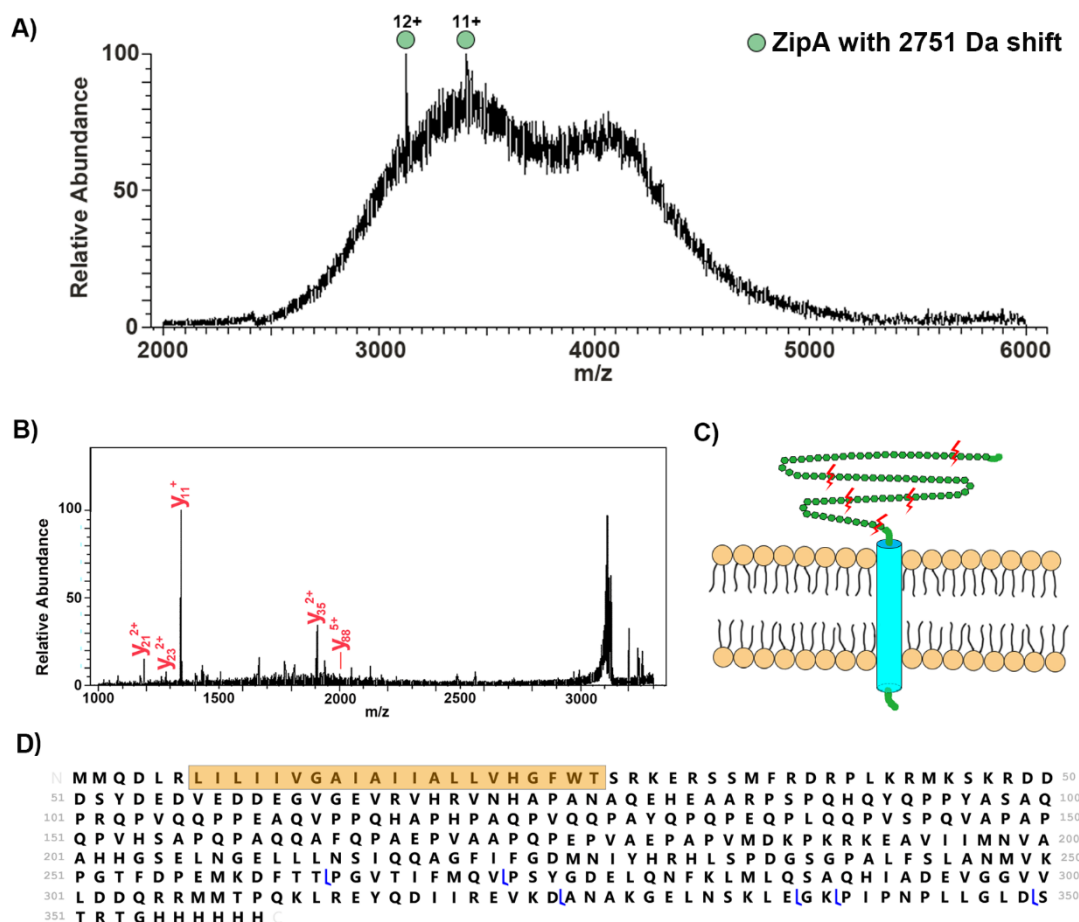


Figure 5.26 The characterisation of SMALPs ZipA. A) Representative mass spectrum of SMALP ZipA sample. B) HCD MS/MS mass spectrum of the 12+ charge state precursor ion with 3123±50 m/z obtained with NCE of 45%. C) The schematic of a possible principle of HCD fragmentation using SMALPs ZipA. The red-labelled fragmentation sites are specific to the non-transmembrane peptide sequence of protein C terminal. D) Observed fragments in the sequence of ZipA. The transmembrane sequence was labelled orange.

Based on the characterization of the SMALP ZipA results shown above, it is possible that the 37,265 Da mass corresponds to the portion of the protein devoid of transmembrane regions. This points to the fact that the in-source collision

energy fragmented the non-transmembrane tail of the protein before fragmenting the SMALPs thereby releasing the intact protein. PTCR MS of the 12+ precursor ions are shown in Figure 5.27, to gain further information on the accurate mass. Three protein peaks with overlapping signals and varying masses were detected at 34,180, 37,280, and 40,390 Da, as illustrated in Figure 5.27.

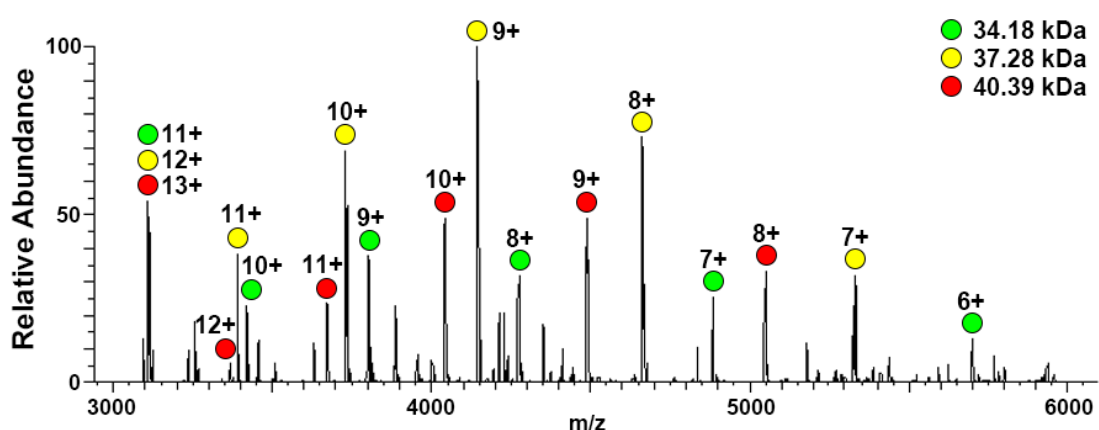


Figure 5.27 PTMS of precursor in 3120 ± 20 m/z with reaction time 5ms and in-source-collision energy 100V.

Interestingly, these three protein peaks exhibit a consistent mass difference of 3,100 Da between each other. However, the precursor ions corresponding to the 40,390 Da and 34,180 Da peaks were not observed in the full scan spectrum but instead were co-isolated with the 37,280 Da peak. Additionally, no b ions were detected by HCD MS/MS, limiting the possibility to obtain information about the transmembrane region of ZipA.

In a separate experiment, a SMALP sample containing the protein Sav 1866 with SMALPs, was analysed. Sav 1866 is a membrane homodimeric protein, with each subunit featuring six transmembrane domains [408] and a theoretical monomer mass of 67,645 Da. It is expected to exist as a homodimer in its natural state. A representative mass spectrum of the SMALP Sav 1866 is shown in Figure 5.28. Due to the high noise of the spectrum, high charge state, and low signal intensity of the protein peaks, it was challenging to determine their accurate mass using high-resolution detection. Therefore, PTCR was employed to determine the mass of the peaks, as shown in Figure 5.28 B). After deconvolution, the protein mass was determined to be 90,620 Da. Considering the theoretical mass of Sav 1866 is 67,645 Da, there is a mass difference of about 22,975 Da. The potential reason for this difference could be that during the fragmentation of Sav 1866, a membrane protein with a theoretical mass of 135,290 Da for its homodimer, the fragmentation occurs by breaking the protein backbone rather than first disrupting the bond between the two monomers. Additionally, it has six transmembrane domains, which could affect the fragmentation and thereby impact the protein structure we are trying to detect.

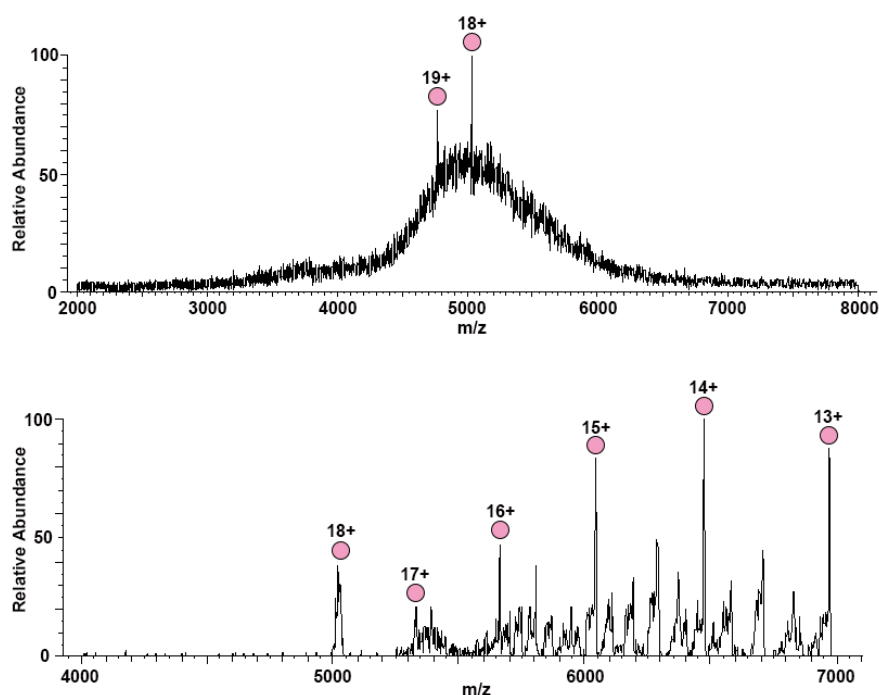


Figure 5.28 (Top) Representative mass spectrum obtained from the SMALP Sav 1866 sample. (Bottom) A mass spectrum obtained in positive mode with in-source collision energy 140V and PTCR MS/MS with a reaction time of 5ms, isolation with m/z 5035 ± 20 .

The HCD MS/MS spectrum of 19+ ions of the 90,620 Da protein, putatively assigned as Sav1886, is shown in Figure 5.29. The fragmentation observed in the spectrum indicates that only y ions without a transmembrane domain were detected, similar to the fragments observed for the SMALP ZipA sample

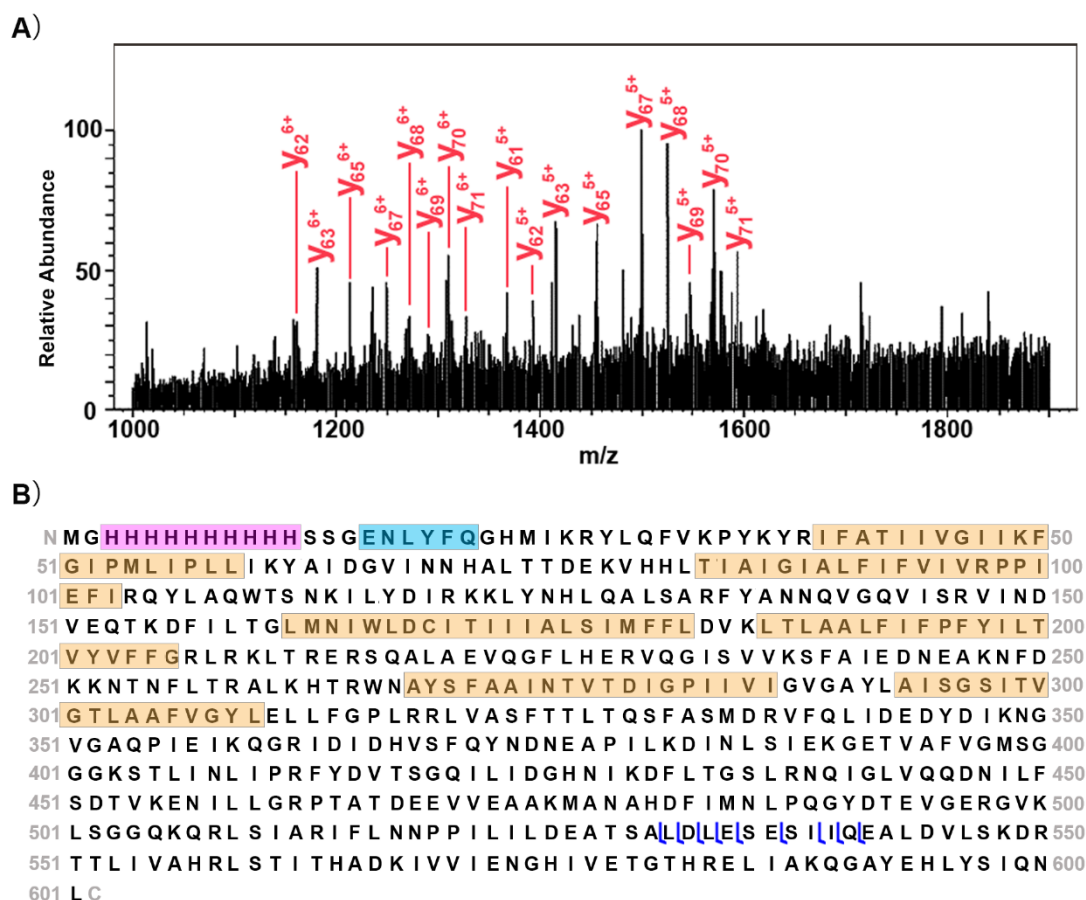


Figure 5.29 The identification mass spectrum of Sav 1866 with SMALPs. A)) HCD MS/MS mass spectrum of the 19+ charge state precursor ions with 4768 ± 50 m/z obtained with NCE of 45%. B) Observed fragments in the sequence of Sav 1866 with SMALPs. The His tag is denoted in purple. Tobacco etch virus (TEV) cleavage site is denoted in blue. Transmembrane domains are denoted in orange.

5.4 Conclusion

The work presented in the chapter shows that by combining LESA MS with a colony washing protocol, it is possible to detect intact outer membrane proteins

and membrane-related proteins directly from living gram-negative bacterial colonies.

Although the results demonstrate that the harsher washing protocol effectively removes more soluble protein peaks and spectrum noise, the membrane protein ZipA, which is overexpressed in the *E. coli* BL21 (DE3) strain used throughout Chapter 5, was not detected with either of the two washing approaches. A possible explanation might be that due to the structural feature of the gram-positive bacterial cell membrane system consisting of two membranes, it is difficult to analyse their inner membrane proteins. This method has only demonstrated the detectability of outer membrane proteins and challenges in detecting inner membrane proteins such as ZipA.

Integration of electroporation into the colony washing protocol extended the approach to larger proteins.

The investigation of membrane protein-containing SMALP samples in this Chapter shows that the current approach for removal of SMA molecules, i.e., by collisional activation through either in-source collision or HCD, is not suitable, as the membrane protein backbone cleaved in preference to removal of the polymer. In addition to this, other potential factors may include sample-related issues such as low concentration after buffer exchange, contamination with other types of protein, or protein degradation, all of which could have affect detection. To

improve detection, optimising sample preparation or adjusting nanoESI parameters may help enhance signal quality and accuracy.

Chapter 6

Conclusions and Future work

Native mass spectrometry of proteins provides information about tertiary and quaternary structures, which are intricately linked to their functions [130]. Additionally, the structure and function of membrane proteins which represent more than 60% of drug targets [177], can be revealed by native mass spectrometry [184, 409, 410].

In the results presented in Chapters 3 and 4 of this thesis, methods for detecting proteins in their native state from microorganisms were developed. Certain factors are particularly important in these chapters, such as living colonies, native protein detection, and direct in-situ analysis. These factors provide information indicating that the analysis occurs in the natural state of the samples, offering first-hand insights into protein structure, protein changes, and even protein function. In Chapter 5, efforts were extended to detect native membrane proteins, which are notoriously challenging to identify using MS.

6.1 Native LESA MS detection of native intact protein directly from bacterial colonies

Chapter 3 presented a LESA MS approach for the identification of native proteins in bacteria. Initially, the method was optimised based on previous research on denatured proteins using LESA MS with a requirement of electroporation to make the cell permeable. During method development, four main aspects were considered: pretreatment, sample extraction, ionization, and MS detection.

The pretreatment involving electroporation was assessed, and the repeatability of the workflows, both with and without electroporation, was evaluated by calculating the success rate across multiple trials. For sample extraction, precise position control was critical. The Advion NanoMate platform was used to adjust the coordinates and heights for sample extraction to minimise human-operated subjective error. The position needed to be in the location of the applied electrical field. Furthermore, the height had to be set so that the tip just touched the colony, avoiding solvent spread on the surface or penetration of the agar by the tip. A chip-based ESI was used for ionization prior to the MS detection.

With the application of MS/MS by HCD, several proteins were identified in *E. coli* K12, including three protein assemblies HdeA, HdeB and SOD[Mn]. The disulfide bond in HdeB was identified by EThcD.

This method could potentially be used for colony imaging. However, the challenge

lies in the fact that electroporation can damage the biofilm of the bacterial colony through contact and the application of an electric field, resulting in the disruption of the colony structure [411-414]. Consequently, more precise designs of electroporation devices are needed, such as smaller sizes and different shapes. As the spatial resolution of bacterial colony imaging by LESA is difficult to control and relatively low, nano-DESI could be considered as an alternative. Challenges for nano-DESI include the uneven surfaces of the colonies and the agar media, resulting in a large amount of sample or agar media entering the capillary and causing blockages. Laskin and co-workers designed an automated height adjustment system coupled with nano-DESI to achieve automatic adjustment of the micro-junction for imaging of small molecules in bacterial colonies [252]. A similar approach could be applied for native nano-DESI imaging of proteins in bacterial colonies.

Another potential application of this method could be the analysis of bacterial co-culture. When two different types of bacteria are cultured on the same plate, there are checks and balances between different species [415, 416]. To reveal the communication between these organisms by analysing protein changes, native LESA MS could be applied by extracting samples from different positions of the co-cultured colony. This could then help us to understand the principles of the different biological processes in the co-cultured system.

This method could also be used to track protein changes during bacterial

mutation and the emergence of bacterial resistance, which could help to provide new methods to combat pathogens.

6.2 Native LESA MS detection of native intact proteins directly from yeast colonies

The work presented in Chapter 4 demonstrated that it is possible to detect native intact proteins from living yeast colonies using LESA MS coupled with electroporation. Due to their thick cell walls, the analysis of yeast proteins by MS poses greater challenges compared to bacteria. Electroporation effectively solved this issue, enabling the identification of several periplasmic and cytoplasmic proteins by applying an electric field on the colony. Additionally, using a solvent containing detergent and a low percentage of organic solvent facilitated the analysis by MS while preserving the protein structure intact, though an in-source collision energy had to be applied to remove the influence of the detergent. Furthermore, the combination of FAIMS and MS unlocked more possibilities for identifying different proteins.

In total, fourteen proteins were identified, five of which are protein assemblies, including four homodimers (APIS, AHP1, SODC, ENO1) and one homotetramer (SOD [Mn]). Four larger proteins were detected in MS with the ion routing multipole in high-pressure mode at 20 mTorr, while the remaining proteins were

identified in standard pressure mode at 8 mTorr. Higher pressure in the ion routing multipole can help slow down larger ions with higher kinetic energy, making them easier to detect in the analyser.

Notably, the metal-binding proteins SOD [Mn], detected in *E. coli* K12 in Chapter 3, and SODC, detected in *S. cerevisiae*, each bind two metal ions per subunit and exhibit a feature of asymmetric distribution of metal ions in MS/MS detection. Specifically, the protein monomer peaks in the spectrum show varying numbers of bound metal ions, ranging from zero to three.

The research in Chapter 4 could be extended to the detection of different fungal strains, including environmental and clinical strains. This is helpful for understanding variations in pathogenicity, drug resistance, and adaptation mechanisms among different yeast strains, aiding both clinical diagnostics and environmental monitoring. Moreover, different strains of yeast may introduce variations in fermentation processes [417-419]. Their protein variations could be associated with these processes, leading to the production of different chemicals and the development of unique flavours [419, 420]. Therefore, the method demonstrated in Chapter 4 could potentially be applied in the food industry.

Notably, both methods described in Chapter 3 and Chapter 4 were not suitable in detecting proteins from gram-positive strains. To verify the method, the electroporation experiment was conducted on Gram-positive bacteria *Staphylococcus epidermidis*, using two strains, RP62a and HB. However, the

combination of electroporation and native LESA MS failed to detect native intact proteins. This may be due to the thicker peptidoglycan layer in Gram-positive bacteria, which provides rigidity, making them more resistant to electroporation and thereby limiting protein release.

6.3 Development of methods for direct analysis of membrane proteins from bacterial colonies

Although several proteins were identified in Chapter 3 and Chapter 4, none of those proteins were membrane proteins. In Chapter 5, methods were developed to analyse membrane proteins.

Colony washing, derived from tissue washing [85], was effectively employed for membrane protein detection on gram-negative bacteria by native LESA MS. Two membrane proteins and several membrane-related proteins were detected through the integration of colony washing and MS. The membrane proteins identified by this method were all outer membrane proteins. The overexpressed inner membrane protein ZipA was not observed, likely due to the double membrane structure of *E. coli* BL21 (DE3) cells, which poses challenges for detecting inner membrane proteins. The inner membrane is covered by the outer membrane and surrounded by the periplasm, and tightly associated to each other

[421]. The application of methods specifically designed to extract the inner membrane, such as density gradient ultracentrifugation [422], could be helpful in dealing with this issue.

By combining electroporation with detergent, larger proteins were identified. This approach could be used to extend the detectable mass range of proteins when analysing proteins in bacterial colonies.

For SMALPs, two types of protein samples, ZipA and Sav1866, were analysed. Both in-source collision energy and HCD were applied to remove the SMA polymer; however, these methods proved unsuitable for this process. To address this issue, infrared multiphoton dissociation (IRMPD), a charge-independent dissociation method, could be potentially used for declustering proteins from SMALPs while preserving the intact structure of membrane proteins [198].

In summary, this thesis described various methods for directly analysing native proteins in different microorganisms, including LESA MS, electroporation, detergent-based extraction, and SMALPs. This exploration contributes to the understanding of native protein detection and has potential applications in revealing different biological processes. However, the native LESA MS method detected 15–25 proteins per strain in Gram-negative bacteria and yeast and only 2 outer membrane proteins, significantly fewer than the thousands identified in bottom-up proteomics. This highlights a key limitation of native ambient mass spectrometry (NAMS) : its lower proteome coverage, likely due to ionization

challenges and the difficulties of intact protein analysis. To improve NAMS and make it a more general and effective method here, several optimisations, such as improving protein extraction efficiency through mild enzymatic or chemical treatments to facilitate better protein release, can be explored. Also, optimising ionisation conditions could improve signal intensity and protein coverage, while pretreatment to reduce sample complexity can enhance detection. By addressing these challenges, NAMS could become a more robust and widely used method, providing more proteome information while retaining its advantages in native protein analysis. Future work could focus on improving the analysis to detect more native proteins by NAMS, analysing native proteins in Gram-positive strains, addressing the challenges posed by their cell walls, detecting inner membrane proteins in Gram-negative bacterial cells, and further developing of SMALPs, which could enhance the detection of native membrane proteins.

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Appendices

Appendix 1. Publication

1. Du, Yuying, Robin C. May, and Helen J. Cooper. "Native ambient mass spectrometry of intact protein assemblies directly from *Escherichia coli* colonies." *Chemical Communications* 58.48 (2022): 6857-6860.
2. Du, Yuying, and Helen J. Cooper. "Native ambient mass spectrometry of membrane proteins directly from bacterial colonies." *Chemical Communications* 61 (2025): 4168-4171.

Cite this: *Chem. Commun.*, 2022, 58, 6857Received 12th April 2022,
Accepted 19th May 2022

DOI: 10.1039/d2cc02085h

rsc.li/chemcomm

Native ambient mass spectrometry of intact protein assemblies directly from *Escherichia coli* colonies†

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Here, we demonstrate that by combining electroporation with native ambient mass spectrometry, it is possible to detect intact non-covalent protein complexes directly from bacterial colonies growing on agar. Homodimers HdeA and HdeB were identified, together with the 50 kDa Mn-bound superoxide dismutase homodimer, in addition to some previously undetected monomeric proteins.

Liquid extraction surface analysis (LESA)¹ mass spectrometry (MS) is an ambient mass spectrometry technique which combines liquid microjunction sampling with nano-electrospray mass spectrometry, and is particularly suitable for the analysis of intact proteins. LESA MS has been demonstrated for the analysis of proteins for a range of substrates, including dried blood spots,² thin tissue sections³ and bacterial colonies growing on agar.^{4–6} Compared with matrix-assisted laser desorption/ionization time-of-flight (MALDI TOF) MS, which is recognized as the gold standard for routine identification of a wide range of microorganisms,^{7,8} LESA MS focuses on the identification of intact bacterial proteins rather than spectral matching for species identification. Other ambient MS approaches that have been applied to bacteria, albeit for the analysis of small molecules, include rapid evaporative ionization mass spectrometry (REIMS),⁹ laser ablation electrospray ionization (LAESI),^{10,11} paperspray mass spectrometry¹² and nanospray desorption electrospray ionization (nano-DESI).¹³

In terms of protein identification, analysis can either be “top-down”, in which the intact protein ion is fragmented in the mass spectrometer, or “bottom-up”, in which proteolytic peptides are subjected to MS analysis. The advantage of top-down MS is that all information relating to primary sequence and post-translational modifications are retained, unlike the bottom-up approach where information relating to the presence of single

nucleotide polymorphisms or connectivity between post-translational modifications may be lost. Our work to date on LESA MS of bacteria has made use of denaturing LESA solvents based on aqueous organic mixtures.^{5,14} The use of these has been necessary to breach the cell wall. A limitation of this approach is that any structural information is lost as the proteins are unfolded. As protein structure and function are intricately linked, there is a drive to develop analytical methods capable of obtaining structural information, *e.g.*, protein assembly stoichiometry, protein-ligand binding, directly from the bacteria.

Another branch of MS, native mass spectrometry,¹⁵ allows the analysis of tertiary and quaternary protein structure. Proteins are electrosprayed from solutions designed to mimic native conditions, and intra- and intermolecular non-covalent interactions present in solution are maintained in the gas-phase. We have demonstrated that native mass spectrometry may be coupled with LESA MS and nano-DESI MS for the analysis of protein assemblies and complexes directly from tissue.^{16,17} Native LESA MS generally offers higher sensitivity but lower spatial resolution for mass spectrometry imaging than nano-DESI MS due to the larger sampling area. Sun and co-workers have demonstrated native mass spectrometry of *E. coli* lysates by integrating size exclusion chromatography and capillary zone electrophoresis.¹⁸ Huang and co-workers introduced an approach termed “in-cell” MS in which suspensions of bacteria are subjected to on-line electroporation, releasing endogenous proteins for introduction to the mass spectrometer.¹⁹ Electroporation is the process by which cell membranes are made permeable, either reversibly or irreversibly, through the application of an electric field,^{20–22} and has found applications in both the delivery of exogenous molecules into cells and the extraction of molecules from cells.²³ For the latter, it has been shown that higher molecular weight molecules such as proteins present a greater challenge than those with lower molecular weight.²⁴ Nevertheless, complexes of the protein calmodulin overexpressed in *E. coli* have been observed by the in-cell MS approach.¹⁹

We recently designed and built an electroporation device for integration with the LESA MS workflow.¹⁴ In that work, our aim

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† Electronic supplementary information (ESI) available: Experimental details, comparison of workflows, protein identification, additional MS/MS spectra. See DOI: <https://doi.org/10.1039/d2cc02085h>



was analysis of intact proteins in yeast colonies. Yeast had proved resistant to LESA MS, even with the use of harsh solvents, due to its rigid chitinous cell wall, but electroporation resulted in rapid release of intact proteins. A feature of electroporation is that it is associated with minimal heating,²² which led us to hypothesise that by combining electroporation with native-like LESA solvents, it would be possible to perform native ambient mass spectrometry, *i.e.*, obtain structural information, directly from living bacterial colonies.

Three workflows were investigated (summarised in Fig. S1, ESI†). In the first, a small volume (2 μ l) of 200 mM ammonium acetate was pipetted onto the bacterial colony, which was then subjected to electroporation followed by LESA sampling. The rationale behind addition of ammonium acetate was avoidance of air breakdown during electroporation. In the second workflow, 2 μ l of 200 mM ammonium acetate was deposited onto the colony before LESA sampling, *i.e.*, the electroporation step was omitted. In the third, the bacterial colony was subjected to LESA sampling only. The success rate, defined as the number of sampling events that led to mass spectra containing peaks corresponding to proteins in relation to the total number of sampling events, was 77% for addition of solvent and electroporation, 14% for addition of solvent with no electroporation and 31% for no solvent addition and no electroporation. See Table S1, ESI†.

Initial mass spectrometry experiments were performed in the mass range m/z 900–3000, with no source collision energy. Fig. 1 shows a representative native LESA mass spectrum obtained following electroporation of an *E. coli* K12 colony. Twenty-two proteins were detected, of which eleven were identified by tandem mass spectrometry (MS/MS). See Table S2, ESI† for a summary of protein assignments. Full details are provided in Protein Identification, ESI†. Eight of the proteins identified (HdeA, HdeB, CspE, Acyl carrier protein, YibT, Hpr, PsiF and YgiW) were previously undetected by LESA MS. Among those proteins, HdeA (monomer), HdeB (monomer), CspE and Hpr have previously been identified by MALDI top-down MS of cell lysates of various strains of *E. coli*.²⁵ HdeA, HdeB, YibT and YgiW were identified in a bottom-up LC-MS proteomics study of outer membrane vesicles obtained from a range of *E. coli* strains.²⁶ Acyl carrier protein has been identified in a bottom-up MALDI study.²⁷

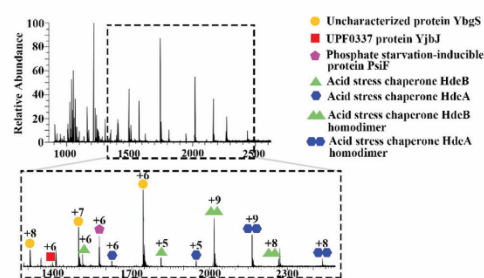


Fig. 1 Representative mass spectrum acquired by native LESA MS following electroporation of *E. coli* K12 colony.

Subsequent experiments were performed in a higher mass range (m/z 1500–4000) with 80% source fragmentation energy, resulting in identification of two further proteins, including the monomeric antigen 43 α chain ($\sim$ 49.8 kDa, see Fig. S2 and S3, ESI†) which has not previously been detected by LESA MS, although has been identified in bottom-up studies.^{28–30}

In addition to monomeric proteins, three protein assemblies were identified, corresponding to the homodimers HdeA, HdeB and Mn-bound superoxide dismutase. HdeA and HdeB are acid stress chaperones which restrict aggregation of denatured periplasmic proteins in acidic environments.³¹ HdeA was detected as both the monomer ($\sim$ 9.7 kDa, charge states 4+ to 6+) and homodimer ($\sim$ 19.5 kDa, charge states 8+ to 9+) as indicated in Fig. 1. Fig. 2 shows the identification of the intact homodimer. Tandem mass spectrometry by higher energy collision dissociation (HCD)³² of the 9+ precursor ions (m/z 2165.2) resulted in detection of monomer subunits in charge states 5+ and 4+. The abundance of the monomer subunits increases with HCD collision energy (Fig. 2a). HCD of the 5+ monomer ions (32% normalised collision energy, NCE) results in detection of sequence fragments allowing the protein identity to be confirmed (Fig. 2b and c). The homodimer of HdeA, along with the homodimer of Hpr (not observed here) have previously been identified from *E. coli* lysates in native proteomics experiments which coupled size exclusion chromatography with capillary zone electrophoresis MS.¹⁸

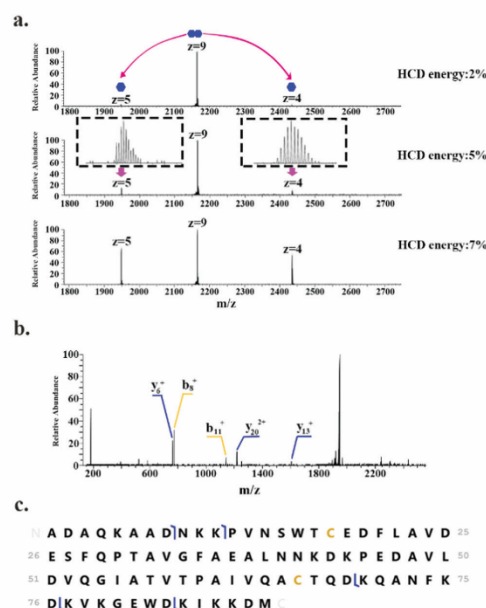


Fig. 2 Identification of the HdeA homodimer. (a) HCD MS/MS of 9+ precursor ions (m/z 2165.2 $\pm$ 4) results in dissociation of dimer to monomer (5+ and 4+ charge states). (b) HCD MS/MS of 5+ monomer ions (m/z 1948.6). (c) Summary of sequence fragments observed.

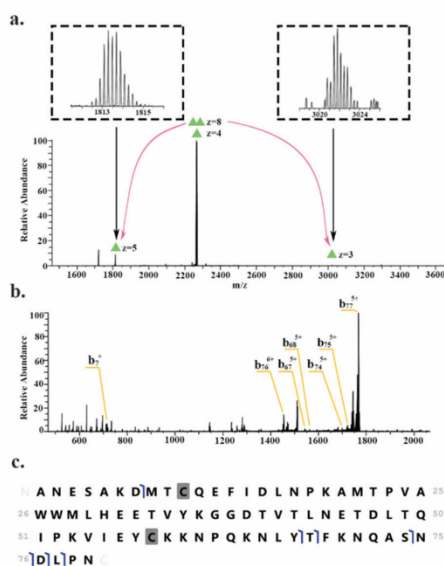


Fig. 3 Identification of the HdeB homodimer. (a) HCD MS/MS (35% NCE) of 8+ precursor ions (m/z 2266.6 $\pm$ 4) results in dissociation of dimer to monomer (5+ and 3+ charge states). (b) HCD MS/MS of 5+ monomer ions (m/z 1813.5). (c) Summary of sequence fragments observed.

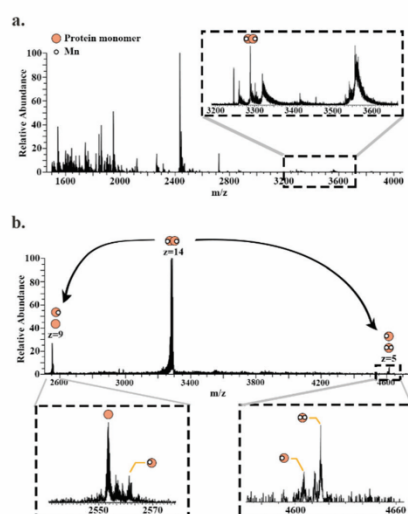


Fig. 4 Native LESA mass spectrum of Mn-bound superoxide dismutase dimer. (a) Full scan mass spectrum with enlarged m/z region showing the peak corresponding to superoxide dismutase dimer. (b) HCD of the 14+ precursor ions (m/z 3289.5 $\pm$ 6) results in dissociation of the dimer to monomer (9+ and 5+ charge state) with differing numbers of Mn^{2+} bound.

HdeB was detected as both the monomer (~ 9.1 kDa, charge states 4+ to 6+) and homodimer (~ 18.1 kDa, charge states 8+ to 9+). Fig. 3 shows the identification of the homodimer. HCD of the 8+ precursor ions (m/z 2266.6) results in the observation of monomer subunits in charge states 5+, 4+ and 3+ (Fig. 3a). MS/MS of the monomer was obtained for identification (Fig. 3b) and sequence fragments are shown in Fig. 3c. The fragments observed confirm the identity of the protein and suggest the presence of a disulfide bond between Cys10 and Cys58. Confirmation of the disulfide bond was obtained by electron transfer dissociation with higher-energy collision dissociation (ETDCD)^{33,34} of the 5+ monomer ions (m/z 1813.5). See Table S3, ESI†

Superoxide dismutase was detected as a dimer (~ 49.8 kDa) in which both subunits are bound to two Mn^{2+} ions. Fig. 4a shows a full scan mass spectrum with the peak corresponding to the 14+ charge state of the dimer indicated. The stoichiometry of the complex was confirmed by MS/MS (HCD at 28% NCE) which resulted in dissociation of the dimer to its monomeric subunits in charge states 5+ and 9+ (Fig. 4b). Asymmetric partitioning of the metal ions amongst the subunits was observed following HCD. Further increase in HCD energy resulted in detection of sequence fragments allowing the protein identity to be confirmed (see ESI†).

In summary, we have demonstrated that by combining electrospray with native LESA MS, it is possible to detect and identify intact protein assemblies up to 50 kDa directly from colonies of *E. coli* growing on a solid substrate. The stoichiometry of the protein assemblies and the identities of the proteins were confirmed by HCD MS. Although it was possible in some cases to detect proteins in the absence of electrospray, this could not be achieved reliably (success rate 14–31%).

YD is funded by a Darwin Studentship. HJC is funded by EPSRC (EP/S002979/1). The authors thank Dr Emma Sisley for helpful discussions. The Orbitrap Eclipse mass spectrometer used in this work was funded by BBSRC (BB/S019456/1). Supplementary data supporting this research is openly available from: <https://doi.org/10.25500/edata.bham.00000841>.

Conflicts of interest

There are no conflicts of interest to declare.

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Native ambient mass spectrometry of membrane proteins directly from bacterial colonies†

Yuying Du  and Helen J. Cooper *Cite this: *Chem. Commun.*, 2025, **61**, 4168Received 2nd August 2024,
Accepted 30th January 2025

DOI: 10.1039/d4cc03881a

rsc.li/chemcomm

Native ambient mass spectrometry (NAMS) enables analysis of protein structure directly from biological substrates by use of liquid junction sampling techniques together with sampling solvents which mimic the proteins' natural environment. Here, we demonstrate detection of membrane and membrane-associated proteins directly from *E. coli* by combining liquid extraction surface analysis (LESA) with a straightforward washing protocol, which attenuates soluble proteins and enables detection of membrane proteins.

Native ambient mass spectrometry (NAMS)^{1,2} combines native mass spectrometry^{3–5} with ambient mass spectrometry⁶ to directly analyse biological substrates such as bacterial colonies⁷ and thin tissue sections² without complicated sample preparation, while maintaining solution-phase noncovalent interactions into the gas phase. We have previously demonstrated NAMS of proteins and their complexes from living colonies of *Escherichia coli* K12 (*E. coli* K12) by use of liquid extraction surface analysis (LESA) mass spectrometry;⁷ however, to date, all of those detected have been soluble proteins. In parallel work, we have shown NAMS detection of membrane proteins in mammalian tissue sections by tailoring the extraction solvent⁸ and use of tissue washing.¹

Membrane proteins play a crucial role in essential cellular functions, such as signal transduction, apoptosis, and metabolism.^{9,10} They constitute 50% of all known drug targets.¹¹ Understanding of the structures of membrane proteins is therefore important for unravelling their biochemical mechanisms and advancing the development of novel therapeutics. Due to the naturally low abundance of membrane proteins and the highly heterogeneous and insoluble nature of the membrane environment, characterization of membrane protein structure, function, and interactions poses a significant challenge.⁹ One of the most commonly-applied methods to address this is to use MS-compatible detergents – amphipathic

molecules that can solubilise membrane proteins while preserving the non-covalent interactions for native MS analysis.^{12–14}

Additional challenges for analysis of membrane proteins in Gram-negative bacteria are posed by the structure of the cell wall, which comprises an inner membrane, a peptidoglycan layer and the outer membrane.¹⁵ To address these challenges presented by the hydrophobic nature and low abundance of membrane proteins, together with the challenge of the inherent complexity presented by direct sampling of bacterial colonies, *i.e.*, the presence of soluble proteins and other molecules which can complicate or even attenuate detection of membrane proteins, we designed and optimised a workflow which incorporates washing steps and NAMS for directly detecting membrane proteins from living *E. coli* colonies.

We have shown previously that washing is beneficial for detection of membrane proteins from mammalian tissue by native ambient mass spectrometry.¹ Here, we employed a washing solution consisting of 200 mM ammonium acetate containing 0.5× the critical micelle concentration (CMC; 0.125% v/v) of the detergent octyl tetraethylene glycol ether (C8E4). (A range of washing solvents were trialled including 200 mM ammonium acetate (without C8E4), 200 mM ammonium acetate with 0.5 × CMC C8E4, and 200 mM ammonium acetate with 2 × CMC C8E4, see Fig. S1, ESI†). C8E4 is a non-ionic and comparatively mild MS-compatible detergent which is suitable for transfer of membrane proteins into the gas phase without affecting protein–protein interactions.^{12,16} For the washing steps, we employed a concentration of C8E4 below its CMC to disrupt protein–lipid interactions without reconstituting membrane proteins into detergent micelles.¹²

Fig. S2 (ESI†) illustrates the workflow employed. Bacterial colonies were cultured on agar plates. Two washing protocols were trialled. In the first, the bacteria were washed three times followed by a wash with 200 mM ammonium acetate (without detergent), making a total of four initial washes which were then repeated (denoted here as 2 × (3 + 1)). In the second washing protocol, the bacteria were washed three times followed by a final wash with 200 mM ammonium acetate (without detergent) (denoted here as (3 + 1)). After washing, the

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† Electronic supplementary information (ESI) available. See DOI: <https://doi.org/10.1039/d4cc03881a>

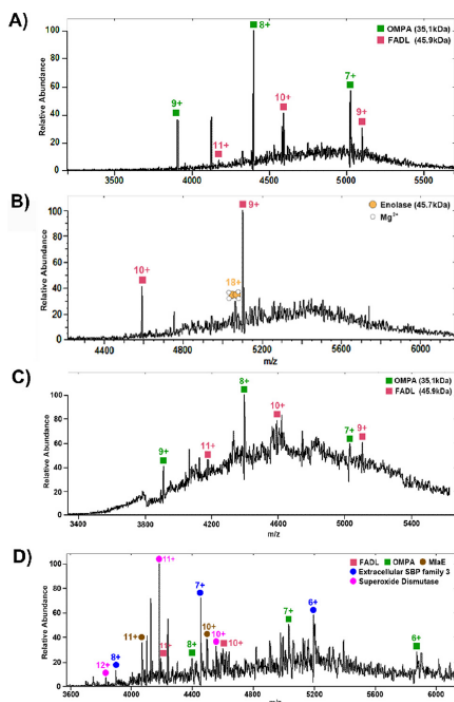


Fig. 1 Representative full-scan mass spectrum obtained following eight rounds of washing (A) and (B) and four rounds (C) and (D) of washing. (A) Mass spectrum with compensation scaling factor 5%. (B) Mass spectrum with CSF 7%. (C) Mass spectrum with CSF 5%. (D) Mass spectrum with CSF 7%.

colonies were sampled by liquid extraction surface analysis (LESA) and the extracts were transferred into home-made gold-coated borosilicate tips and electrosprayed into the mass spectrometer. A total of 10 proteins were identified in this work and are summarised in Tables S1 and S2 (ESI†). Although there is known variability in LESA sampling efficiency, the proteins identified were detected in all replicates following the respective washing protocols.

Fig. 1A shows representative mass spectra obtained following the $2 \times (3 + 1)$ washing protocol. Peaks corresponding to two membrane proteins, outer membrane protein A (OmpA) and long-chain fatty acid transport protein (FadL) were observed. The proteins were identified by higher-energy collision dissociation (HCD), see Fig. 2. Full details of protein identifications are given in the ESI†. Sequence coverages of 3% (FadL) and 2% (OmpA) were obtained; however, all cleavages observed were either C-terminal to aspartic acid or N-terminal to proline. These cleavage sites are known to be favoured¹⁷ in native mass spectrometry and provide additional confidence in the protein assignment. Both of these proteins are outer membrane proteins. FadL is responsible for cellular transport of long-chain

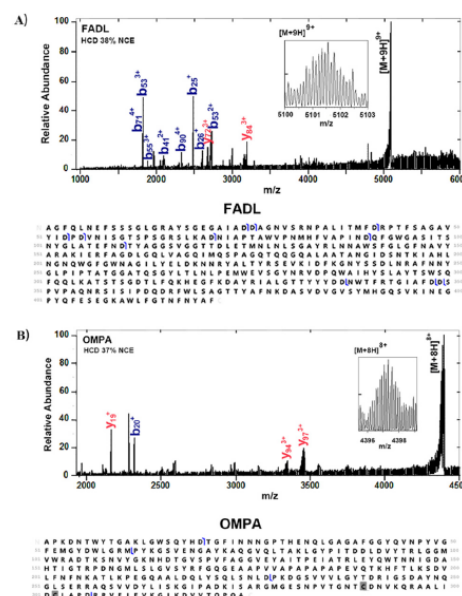


Fig. 2 Identification of FadL and OmpA following the $2 \times (3 + 1)$ washing protocol. (A) HCD mass spectrum of 9+ ions (see inset) of FadL (m/z 5102 $\pm$ 5), NCE38%, and sequence coverage. (B) HCD mass spectrum of 8+ ions (see inset) of OmpA (m/z 4397 $\pm$ 5), NCE 37%, and sequence coverage.

fatty acids^{18,19} and their conversion to acyl-CoA.¹⁸ OmpA serves several important functions including maintaining the stability and integrity of the outer membrane,^{20–22} anchoring it to the peptidoglycan layer,^{21,22} contributing to the permeability barrier²³ and regulating the passage of molecules, including ions and nutrients.²¹ OmpA was detected with 2 Da mass shift, indicating the presence of a disulfide bond between C290 and C302.

The initial washing method resulted in the detection of just two membrane proteins. To improve transmission of higher mass ions, the compensation scaling factor was increased from 5% to 7%, Fig. 1B. Under these conditions, a further protein was detected and identified as the soluble enolase dimer but no further membrane proteins were detected. Presumably, the removal of smaller, higher solubility proteins during the colony washing process enabled the detection of this soluble protein. Enolase is a magnesium-binding protein and the mass difference between the calculated and measured masses can be ascribed in part to the binding of two Mg²⁺ ions to each enolase subunit.

The detection of just two membrane proteins suggests that the protocol may be too harsh. In subsequent experiments, the number of washing steps was reduced. Fig. 1C and D show representative mass spectra obtained following the $3 + 1$ washing protocol. Under these washing conditions and with a



compensation scaling factor of 5%, the membrane proteins OMPA and FADL were again detected. At a compensation scaling factor of 7%, several soluble proteins, all of which were cytoplasmic proteins, were identified (see Table S2, ESI†). Several of these were observed with considerable mass shifts between the measured and calculated masses: superoxide dismutase is a Mn-binding protein and the mass shift can be attributed to binding of one Mn^{2+} ion to each subunit. Phosphoenolpyruvate carboxykinase and serine hydroxymethyl transferase exhibited differences of 324 Da and 578 Da respectively between their calculated and measured masses, suggesting the presence of either ligand binding or large modification. The two membrane proteins FADL and OMPA were also observed, but did not show good reproducibility. In addition, three membrane-related proteins were identified: extracellular solute-binding protein (SBP) family 3, maltodextrin-binding protein (MalE) and cationic amino acid ABC transporter (CAT)-periplasmic binding protein (see Fig. S3, ESI†). Full details of protein identifications are given in the ESI†. For SBP-family 3 and MalE, it was possible to determine the accurate mass directly as high resolution mass spectrometry provided isotopic resolution. For CAT-PBP, however, the low signal-to-noise precluded isotopic resolution and it was necessary to employ proton transfer charge reduction MS to determine the intact mass of the protein. PTICR MS^{24,25} involves an ion-ion reaction between multiply-charged protein ions and the PTICR reagent anion resulting in proton transfer. Deconvolution of the resulting charge-reduced product ion spectrum yields the intact mass.

Bacterial extracellular SBPs have multiple roles in cellular functions,²⁶ including acting as chemoreceptors, recognizing specific molecules, serving as key components in transport systems,^{26,27} and initiating signal transduction pathways to trigger cellular responses.^{26,28} MalE is a periplasmic binding protein and a part of the ATP-binding cassette (ABC) transporter complex MalEFGK that works with maltodextrin permease to transport maltodextrins into the bacterial cell.^{29,30} The CAT-periplasmic binding protein is a part of CAT ABC transporter system that helps bring cationic amino acids into the bacterial cell.^{31,32} Overall, decreasing the number of washing steps resulted in lower signal-to-noise ratios for the membrane protein peaks and lower sequence coverage following MS2 fragmentation (see Tables S1 and S2, ESI†). More soluble proteins were observed with fewer wash steps; however, the larger soluble protein dimer enolase was only observed and identified with the $2 \times (3 + 1)$ wash (see Table S1, ESI†). We hypothesise that the harsher washing protocol removed the overlapping serine hydroxymethyltransferase, but not the enolase, enabling its detection. Interestingly, this strain of *E. coli* is engineered to overexpress the membrane protein ZipA and this was not observed following either of the washing protocols. One potential explanation may stem from the structural features of the bacteria, which commonly possesses two membranes. Although all the membrane-related proteins detected are periplasmic proteins associated with inner membrane proteins, all the membrane proteins observed here are outer membrane. ZipA, however, is an inner membrane protein.

In conclusion, our findings demonstrate that colony washing prior to LESA extraction and mass spectrometry analysis effectively diminishes or eliminates signals of soluble proteins

and spectrum noise. This process facilitates the direct detection of integral membrane and membrane-associated proteins within living bacterial colonies. Notably, the signal-to-noise ratio for outer membrane proteins improved post-washing. Subsequent investigations will focus on exploring membrane proteins in different bacterial strains, yeast, and applications of alternative detergents, with the goal of enhancing the detection of membrane proteins across various microorganisms.

YD was funded by the Darwin Trust. HJC was funded by EPSRC (EP/S002979/1). The Orbitrap Eclipse mass spectrometer used in this work was funded by BBSRC (BB/S019456/1).

Data availability

Data for this article, including mass spectrometry data and MS/MS data are available at the University of Birmingham Institutional Research Archive at <https://doi.org/10.25500/edat.bham.00001240>.

Conflicts of interest

There are no conflicts to declare.

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Appendix 2. Chapter 3

Tandem mass fragment assignments

Protein identification

Protein Identification

All masses listed are monoisotopic.

E. coli K12

Protein name: Cold shock protein CspA (Uniprot accession no. P0A9X9)

Charge state: +5

Observed monoisotopic mass: 7266.6590 Da

ID	Name	m/z Monoisotopic	Mass Monoisotopic	Intensity	Theoretical Mass	Error (Da)	Error (ppm)
10	b69	1454.5079	7267.5032	8.6975e+002	7267.5334	-0.0302	-4.1612
5	y66	1373.6779	6863.3531	2.4105e+002	6995.4391	-0.0711	-10.1681
10	y69	1454.5079	7267.5032	8.6975e+002	7267.5876	-0.0844	-11.6100

Protein name: Cold shock-like protein CspE (Uniprot accession no. P0A972)

Charge state: +5

Observed monoisotopic mass: 7327.7145 Da

ID	Name	m/z	Mass	Intensity	Theoretical Mass	Error (Da)	Error (ppm)
		Monoisotopic	Monoisotopic				
35	b21	1135.1000	2268.1855	9.3829e+001	2399.2460	0.0388	16.1708
55	b68	1466.5446	7327.6864	4.7086e+003	7327.7192	-0.0327	-4.4693
55	y68	1466.5446	7327.6864	4.7086e+003	7327.7733	-0.0869	-11.8569

Protein name: Uncharacterized protein YibT (Uniprot accession no. Q2M7R5)

Charge state: +6

Observed monoisotopic mass: 7859.1822 Da

ID	Name	m/z	Mass	Intensity	Theoretical Mass	Error (Da)	Error (ppm)
		Monoisotopic	Monoisotopic				
9	b7	698.3796	697.3724	2.3303e+002	697.3759	-0.0035	-5.0446
22	y61	1433.3650	7161.7884	2.6473e+003	7161.8381	-0.0497	-6.9457
22	y61	1433.3650	7161.7884	2.6473e+003	7161.8381	-0.0179	-2.5035
11	y66	1280.0070	7673.9982	1.2113e+002	7674.0976	-0.0994	-12.9503
11	y66	1280.0070	7673.9982	1.2113e+002	7674.0976	-0.0676	-8.8046

Protein name: UPF0337 protein YjbJ (Uniprot accession no. P68206)

Charge state: +6

Observed monoisotopic mass: 8320.0283 Da

ID	Name	m/z Monoisotopic	Mass Monoisotopic	Intensity	Theoretical Mass	Error (Da)	Error (ppm)
18	b20	1152.5709	2303.1272	1.0326e+002	2303.1480	-0.0208	-9.0359
19	b21	1245.6104	2489.2062	1.3071e+002	2489.2274	-0.0212	-8.5155
23	b23	1338.1713	2674.3281	1.6043e+002	2674.3438	-0.0157	-5.8627
14	y3	524.2609	523.2536	2.0913e+002	523.2543	-0.0007	-1.3034
16	y9	670.3095	1338.6045	8.0786e+002	1338.6105	-0.0060	-4.5174
17	y17	1119.5201	2237.0256	3.1442e+002	2237.0501	-0.0245	-10.9711
26	y32	1348.6631	4042.9675	2.0521e+002	4042.9925	-0.0250	-6.1860
26	y32	1348.6631	4042.9675	2.0521e+002	4042.9925	0.0389	9.6227
20	y41	1272.6240	5086.4669	2.9557e+002	5086.5359	-0.0689	-13.5522
20	y41	1272.6240	5086.4669	2.9557e+002	5086.5359	-0.0050	-0.9868
21	y42	1301.3805	5201.4930	3.4646e+002	5201.5628	-0.0698	-13.4273
21	y42	1301.3805	5201.4930	3.4646e+002	5201.5628	-0.0059	-1.1397
22	y43	1330.1378	5316.5219	3.7821e+002	5316.5898	-0.0679	-12.7642
22	y43	1330.1378	5316.5219	3.7821e+002	5316.5898	-0.0039	-0.7425
35	y46	1412.4386	5645.7254	6.2970e+002	5645.7484	-0.0230	-4.0778
35	y46	1412.4386	5645.7254	6.2970e+002	5645.7484	0.0409	7.2431
36	y48	1458.7054	5830.7926	9.4101e+002	5830.8649	-0.0722	-12.3862
36	y48	1458.7054	5830.7926	9.4101e+002	5830.8649	-0.0083	-1.4247
38	y63	1527.3470	7631.6984	4.2986e+002	7631.8072	-0.1088	-14.2498
38	y63	1527.3470	7631.6984	4.2986e+002	7631.8072	-0.0448	-5.8751
39	y64	1541.5559	7702.7429	3.1376e+002	7702.8443	-0.1014	-13.1633
39	y64	1541.5559	7702.7429	3.1376e+002	7702.8443	-0.0375	-4.8658
40	y66	1590.3722	7946.8249	2.4096e+002	7946.9138	-0.0890	-11.1953
40	y66	1590.3722	7946.8249	2.4096e+002	7946.9138	-0.0251	-3.1526

Protein name: Acyl carrier protein (Uniprot accession no. P0A6A8)

Charge state: +5

Observed monoisotopic mass: 8503.1277 Da

ID	Name	m/z Monoisotopic	Mass Monoisotopic	Intensity	Theoretical Mass	Error (Da)	Error (ppm)
31	b55	1997.9662	5990.8769	5.0673e+002	6164.0231	-0.0131	-2.1201
14	y12	1300.6133	1299.6060	1.3257e+002	1299.6207	-0.0148	-11.3656
13	y23	1257.0969	2512.1792	3.4712e+002	2512.2081	-0.0289	-11.5098
15	y24	1313.6418	2625.2690	7.1360e+001	2625.2922	-0.0232	-8.8226
26	y77	1701.6246	8503.0865	5.2343e+003	8503.1802	-0.0937	-11.0185

Protein name: Acyl carrier protein (Uniprot accession no. P0A6A8) (no methionine, no acetylation, O3-phosphopantetheine-L-serine)

Charge state: +5

Observed monoisotopic mass: 8843.2080 Da

ID	Name	m/z Monoisotopic	Mass Monoisotopic	Intensity	Theoretical Mass	Error (Da)	Error (ppm)
48	b32	1736.3989	3470.7833	4.1859e+002	3601.8297	0.0521	14.4678
62	b36	1914.4641	3826.9137	3.7173e+002	3957.9993	0.0129	3.2541
34	b36	1276.6561	3826.9466	1.7368e+002	3957.9993	0.0458	11.5601
45	b44	1675.4769	5023.4088	2.7960e+002	5154.5393	-0.0320	-6.2124
49	b46	1742.8315	5225.4726	3.8254e+002	5356.6169	-0.0458	-8.5534
57	b47	1780.5515	5338.6326	6.4276e+002	5469.7009	0.0302	5.5187
24	y7	802.3782	801.3709	2.0104e+002	801.3769	-0.0060	-7.5121
26	y8	917.4032	916.3959	2.1364e+002	916.4039	-0.0080	-8.7189
29	y10	1101.5268	1100.5195	2.2875e+002	1100.5251	-0.0055	-5.0312
30	y11	1172.5614	1171.5541	2.1727e+002	1171.5622	-0.0081	-6.8857
35	y12	1300.6169	1299.6096	3.3006e+002	1299.6207	-0.0111	-8.5687
33	y23	1257.1004	2512.1862	2.1631e+003	2512.2081	-0.0219	-8.7119
36	y24	1313.6455	2625.2764	5.9121e+002	2625.2922	-0.0158	-6.0245
38	y25	1378.1635	2754.3125	2.8590e+002	2754.3348	-0.0223	-8.0785
39	y26	1428.6881	2855.3616	6.9033e+002	2855.3824	-0.0208	-7.2855
40	y27	1486.2029	2970.3912	7.4629e+002	2970.4094	-0.0182	-6.1167
41	y28	1559.7245	3117.4344	1.0461e+003	3117.4778	-0.0434	-13.9128
42	y29	1624.2465	3246.4784	6.1767e+002	3246.5204	-0.0419	-12.9175
46	y30	1688.7662	3375.5178	9.1579e+002	3375.5630	-0.0452	-13.3972
51	y31	1753.2868	3504.5590	8.7104e+002	3504.6056	-0.0465	-13.2808
58	y32	1809.8418	3617.6690	6.5612e+002	3617.6896	-0.0206	-5.6904
60	y33	1845.3636	3688.7127	4.3716e+002	3688.7267	-0.0140	-3.7929
61	y34	1910.8688	3819.7231	5.5098e+002	3819.7672	-0.0441	-11.5455
60	y34	1845.3636	3688.7127	4.3716e+002	3819.7672	0.0440	11.5210
56	y77	1769.6518	8843.2228	1.0048e+003	8843.2660	-0.0432	-4.8856

Protein name: Acyl carrier protein (Uniprot accession no. P0A6A8) (no methionine, acetylation, O3-phosphopantetheine-L-serine)

Charge state: +5

Observed monoisotopic mass: 8885.1910 Da

ID	Name	m/z Monoisotopic	Mass Monoisotopic	Intensity	Theoretical Mass	Error (Da)	Error (ppm)
37	b43	1656.4628	4966.3666	2.7976e+002	5097.4814	0.0112	2.1944
40	b44	1689.4968	5065.4686	5.5448e+002	5196.5498	0.0448	8.6303
46	b46	1756.8459	5267.5158	6.6559e+002	5398.6274	0.0144	2.6673
54	b47	1794.5440	5380.6103	7.1284e+002	5511.7115	0.0248	4.5040
58	b49	1880.5644	5638.6713	4.2504e+002	5769.7967	0.0006	0.1119
63	b51	1972.5999	5914.7778	4.0549e+002	6045.9077	-0.0039	-0.6392
67	b54	2087.6437	6259.9093	3.8359e+002	6391.0249	0.0105	1.6460
70	b55	2125.3496	6373.0270	1.4277e+003	6504.1089	0.0441	6.7858
13	y7	802.3814	801.3741	4.0750e+002	801.3769	-0.0028	-3.5002
14	y8	917.4073	916.4001	4.3266e+002	916.4039	-0.0038	-4.1761
15	y9	1030.4916	1029.4843	3.0225e+002	1029.4879	-0.0036	-3.5173
16	y10	1101.5278	1100.5205	4.3408e+002	1100.5251	-0.0046	-4.1635
19	y11	1172.5679	1171.5607	3.5187e+002	1171.5622	-0.0015	-1.2744
25	y12	1300.6185	1299.6112	6.1409e+002	1299.6207	-0.0095	-7.3091
28	y13	1399.6889	1398.6816	1.4667e+002	1398.6892	-0.0075	-5.3722
31	y14	1500.7403	1499.7330	2.2031e+002	1499.7368	-0.0038	-2.5658
34	y15	1601.7865	1600.7792	3.3676e+002	1600.7845	-0.0053	-3.2959
17	y21	1151.0598	2300.1051	1.2364e+002	2300.1284	-0.0234	-10.1564
23	y23	1257.0959	2512.1772	3.1675e+003	2512.2081	-0.0309	-12.2987
26	y24	1313.6408	2625.2669	9.9421e+002	2625.2922	-0.0252	-9.6100
27	y25	1378.1655	2754.3164	5.3691e+002	2754.3348	-0.0184	-6.6735
29	y26	1428.6902	2855.3659	1.1222e+003	2855.3824	-0.0165	-5.7887
30	y27	1486.2053	2970.3960	1.2642e+003	2970.4094	-0.0134	-4.5169
33	y28	1559.7272	3117.4398	1.4823e+003	3117.4778	-0.0380	-12.1852
36	y29	1624.2583	3246.5020	9.8356e+002	3246.5204	-0.0184	-5.6651

39	y30	1688.7694	3375.5243	1.2550e+003	3375.5630	-0.0387	-11.4499
45	y31	1753.2904	3504.5662	1.5234e+003	3504.6056	-0.0393	-11.2278
55	y32	1809.8250	3617.6354	9.9857e+002	3617.6896	-0.0542	-14.9808
57	y33	1845.3571	3688.6996	8.6278e+002	3688.7267	-0.0272	-7.3651
59	y34	1910.8620	3819.7095	8.7020e+002	3819.7672	-0.0577	-15.1161
57	y34	1845.3571	3688.6996	8.6278e+002	3819.7672	0.0584	15.2878
62	y35	1960.4187	3918.8228	3.0574e+002	3918.8356	-0.0129	-3.2831
42	y75	1705.8080	8524.0035	4.9277e+002	8655.1863	-0.0568	-6.5575

Protein name: Acid stress chaperone HdeB (Uniprot accession no. P0AET2)

Charge state: +5

Observed monoisotopic mass: 9057.3468 Da

ID	Name	m/z Monoisotopic	Mass Monoisotopic	Intensity	Theoretical Mass	Error (Da)	Error (ppm)
10	b7	716.3205	715.3132	1.2522e+002	715.3137	-0.0005	-0.6599
37	b67	1542.9328	7709.6276	1.8229e+002	7711.7485	0.0060	0.7797
38	b68	1563.1545	7810.7363	1.0007e+002	7812.7962	0.0670	8.5722
41	b74	1698.2065	8485.9963	1.8497e+002	8488.1302	-0.0070	-0.8301
43	b75	1721.0329	8600.1280	3.9333e+002	8602.1731	0.0818	9.5097
48	b76	1744.0288	8715.1075	2.6939e+003	8717.2001	0.0343	3.9324
25	b76	1453.5261	8715.1130	1.0166e+003	8717.2001	0.0398	4.5699
54	b77	1766.6602	8828.2647	8.1789e+003	8830.2841	0.1074	12.1683

Protein name: Phosphocarrier protein HPr (Uniprot accession no. P0AA04)

Charge state: +6

Observed monoisotopic mass: 9113.6642 Da

ID	Name	m/z Monoisotopic	Mass Monoisotopic	Intensity	Theoretical Mass	Error (Da)	Error (ppm)
45	b53	1423.4911	5689.9353	7.9261e+002	5689.9930	-0.0577	-10.1417
45	b53	1423.4911	5689.9353	7.9261e+002	5689.9930	0.0153	2.6887
54	b84	1495.4396	8966.5941	5.4121e+002	8966.6840	-0.0899	-10.0289
54	b84	1495.4396	8966.5941	5.4121e+002	8966.6840	-0.0169	-1.8871
75	y32	1712.8609	3423.7073	3.3188e+002	3423.7442	-0.0370	-10.7955
75	y32	1712.8609	3423.7073	3.3188e+002	3423.7442	0.0360	10.5276
73	y77	1628.4404	8137.1658	6.8800e+002	8137.2684	-0.1026	-12.6107
73	y77	1628.4404	8137.1658	6.8800e+002	8137.2684	-0.0296	-3.6391

Protein name: Phosphate starvation-inducible protein PsiF (Uniprot accession no. P0AFM4)

Charge state: +6

Observed monoisotopic mass: 9443.4898 Da

ID	Name	m/z	Mass	Intensity	Theoretical Mass	Error (Da)	Error (ppm)
		Monoisotopic	Monoisotopic				
28	b50	1363.6666	5450.6374	9.8024e+002	5450.6551	-0.0177	-3.2455
30	b84	1548.2440	9283.4203	6.0561e+002	9386.5526	0.0153	1.6308
41	y36	1997.4352	3992.8559	6.7765e+002	4095.9823	0.0212	5.1806
26	y36	1331.9713	3992.8920	2.1292e+002	4095.9823	0.0573	13.9993

Protein name: Uncharacterized protein YbgS (Uniprot accession no. P0AAV6)
Charge state: +7

Observed monoisotopic mass: 10464.5632 Da

ID	Name	m/z Monoisotopic	Mass Monoisotopic	Intensity	Theoretical Mass	Error (Da)	Error (ppm)
19	y63	1340.7579	6698.7530	2.8682e+002	6699.9170	0.0047	0.6970
25	y68	1440.6092	7198.0095	4.8371e+002	7199.1197	0.0585	8.1286
16	y72	1271.3656	7622.1499	1.1990e+003	7623.3267	-0.0082	-1.0700
29	y72	1525.4392	7622.1596	8.7935e+002	7623.3267	0.0015	0.2028
21	y80	1413.4375	8474.5811	1.0801e+003	8475.7357	0.0141	1.6680
24	y81	1425.2749	8545.6058	1.6254e+003	8546.7728	0.0017	0.1938
26	y82	1441.7882	8644.6857	3.3924e+002	8645.8412	0.0132	1.5240
27	y84	1472.6308	8829.7411	1.9328e+002	8830.9213	-0.0115	-1.3006

Protein name: Protein YgiW (Uniprot accession no. P0ADU5)

Charge state: +7

Observed monoisotopic mass: 11968.8868 Da

ID	Name	m/z Monoisotopic	Mass Monoisotopic	Intensity	Theoretical Mass	Error (Da)	Error (ppm)
50	b53	1816.5471	5446.6194	1.8482e+003	5488.6519	0.0784	14.2843
52	b54	1854.8721	5561.5945	1.4333e+003	5603.6788	0.0266	4.7479
51	b86	1833.0904	9160.4155	9.9085e+002	9202.5208	0.0056	0.6124
42	b109	1694.4066	11853.7953	1.6044e+003	11895.9344	-0.0282	-2.3713
13	y8	982.6109	981.6036	3.5968e+002	981.6083	-0.0047	-4.8125
16	y9	1081.6793	1080.6721	1.2077e+003	1080.6767	-0.0047	-4.3362
20	y10	1196.6996	1195.6923	5.6489e+002	1195.7037	-0.0114	-9.5007
31	y12	1438.8373	1437.8300	4.3514e+002	1437.8303	-0.0003	-0.2267
12	y16	963.0268	1924.0390	2.3372e+002	1924.0530	-0.0140	-7.2732
17	y18	1084.5784	2167.1422	8.5722e+002	2167.1749	-0.0327	-15.0874
27	y22	1284.6682	2567.3219	1.8753e+003	2567.3343	-0.0124	-4.8284
29	y23	1348.6936	2695.3727	6.1842e+002	2695.3929	-0.0202	-7.4928
30	y24	1405.2291	2808.4436	6.7897e+002	2808.4770	-0.0334	-11.8890
32	y25	1469.7650	2937.5154	9.7927e+002	2937.5195	-0.0042	-1.4260
34	y28	1627.3230	3252.6314	5.0534e+002	3252.6626	-0.0312	-9.5777
18	y30	1160.2642	3477.7707	3.1478e+002	3477.8103	-0.0396	-11.3755
19	y40	1165.1019	4656.3785	5.2075e+002	4656.4412	-0.0627	-13.4581
33	y40	1553.1375	4656.3907	4.5782e+002	4656.4412	-0.0504	-10.8304
36	y87	1635.6608	9807.9212	3.9908e+003	9808.0411	-0.1199	-12.2243

Protein name: Acid stress chaperone HdeA (Uniprot accession no. P0AES9)

Charge state: +5

Observed monoisotopic mass: 9737.7935 Da

ID	Name	m/z Monoisotopic	Mass Monoisotopic	Intensity	Theoretical Mass	Error (Da)	Error (ppm)
3	b8	771.3584	770.3511	1.6472e+002	770.3559	-0.0048	-6.1984
18	b83	1795.2445	8971.1862	3.5522e+002	8973.3816	-0.0643	-7.1633
17	y81	1793.8864	8964.3956	4.5582e+002	8964.4727	-0.0772	-8.6068
5	y87	1592.1034	9546.5769	1.0901e+003	9548.7645	-0.0565	-5.9144

Protein name: Superoxide dismutase [Mn]

Charge state: +14

Observed monoisotopic mass: 46038.0158 Da (dimer)

ID	Name	m/z	Mass	Intensity	Theoretical Mass	Error (Da)	Error (ppm)
		Monoisotopic	Monoisotopic				
3	y10	1062.6039	1061.5966	8.5366e+001	1061.5981	-0.0015	-1.4280
4	y38	1587.1288	4758.3645	3.8800e+001	4758.4036	-0.0391	-8.2189
6	y58	2230.7496	6689.2271	1.9595e+002	6689.3204	-0.0933	-13.9503
20	y205	2551.1626	22951.3980	2.7072e+002	22951.6094	-0.2114	-9.2094

Antigen 43 a

Charge state: +14

Observed monoisotopic mass: 46038.1400 Da

ID	Name	m/z Monoisotopic	Mass Monoisotopic	Intensity	Theoretical Mass	Error (Da)	Error (ppm)
5	b20	1997.9440	1996.9368	7.5395e+001	1996.9602	-0.0234	-11.7413
1	b22	1120.5307	2239.0468	1.9331e+001	2239.0617	-0.0150	-6.6876
2	b23	1177.0733	2352.1321	4.3135e+001	2352.1458	-0.0137	-5.8274
3	b24	1226.6008	2451.1870	7.1793e+001	2451.2142	-0.0272	-11.0921
4	b25	1300.1306	2598.2467	2.7690e+001	2598.2826	-0.0359	-13.8060
14	b176	2967.8226	17800.8917	2.0446e+002	17800.6580	0.2337	13.1314
6	y22	2198.2352	2197.2279	4.9849e+001	2197.2430	-0.0151	-6.8654
13	y28	2802.5255	2801.5182	1.0687e+002	2801.5134	0.0048	1.7051

Appendix 3. Chapter 4

Tandem mass fragment assignments

S. cerevisiae

The details of precursors are provided in Chapter 4.

1.UBI4P_YEAST, P0CG63; Chain (Ubiquitin) [1-76] in Polyubiquitin.

Name	m/z Monoisotopic	Mass Monoisotopic	Intensity	Theoretical Mass	Error (Da)	Error (ppm)
y12	1321.8105	1320.8033	2.9499e+002	1320.7990	0.0043	3.2427
y18	2097.1755	2096.1683	1.2790e+003	2096.1854	-0.0172	-8.1892
y18	1049.0986	2096.1827	2.1027e+003	2096.1854	-0.0027	-1.3114
y24	1363.7470	2725.4795	2.7062e+003	2725.4987	-0.0192	-7.0438

2. TRX2_YEAST, P22803; Thioredoxin-2.

Name	m/z Monoisotopic	Mass Monoisotopic	Intensity	Theoretical Mass	Error (Da)	Error (ppm)
b6	657.3933	656.3861	2.7266e+002	656.3857	0.0003	0.5195
b9	944.5013	943.4940	1.7286e+002	943.4974	-0.0034	-3.6142
b11	1222.5856	1221.5783	7.9611e+002	1221.5877	-0.0094	-7.7309
b13	1380.6679	1379.6606	1.6750e+002	1379.6569	0.0037	2.6949
b14	1493.7450	1492.7378	3.0781e+002	1492.7409	-0.0032	-2.1156
b15	1564.7830	1563.7757	3.9378e+002	1563.7780	-0.0023	-1.4753
b18	1823.8641	1822.8568	3.3788e+002	1822.8585	-0.0016	-0.8980
b21	1082.5490	2163.0835	2.8121e+002	2163.1059	-0.0224	-10.3504
b22	1132.0987	2262.1828	1.5866e+002	2262.1743	0.0085	3.7398
b24	1239.1310	2476.2474	3.3845e+002	2476.2696	-0.0222	-8.9679
b34	1808.3748	3614.7350	1.8658e+002	3614.7425	-0.0075	-2.0787
y8	830.4745	829.4672	3.7432e+002	829.4658	0.0015	1.7758
y12	1182.6841	1181.6768	2.2526e+002	1181.6768	-0.0000	-0.0118
y27	1394.7652	2787.5158	1.2918e+002	2787.5395	-0.0237	-8.5000
y31	1606.8993	3211.7841	2.8274e+003	3211.8080	-0.0240	-7.4628
y32	1672.4251	3342.8357	2.9147e+002	3342.8485	-0.0128	-3.8297
y33	1715.9303	3429.8460	7.5330e+002	3429.8806	-0.0346	-10.0829
y34	1759.4579	3516.9012	2.1590e+003	3516.9126	-0.0114	-3.2469
y35	1808.9843	3615.9541	1.1793e+003	3615.9810	-0.0269	-7.4334
y36	1873.5144	3745.0143	1.2815e+003	3745.0236	-0.0093	-2.4926
y37	1909.0179	3816.0213	2.8696e+003	3816.0607	-0.0394	-10.3180
y38	1973.0755	3944.1365	9.2513e+002	3944.1557	-0.0192	-4.8705
y39	2037.1094	4072.2042	2.1823e+002	4072.2142	-0.0101	-2.4702
y40	2072.6372	4143.2598	8.6631e+002	4143.2514	0.0085	2.0498
y41	2122.1580	4242.3015	6.0934e+002	4242.3198	-0.0182	-4.2960
y43	2223.1923	4444.3700	2.5625e+002	4444.3787	-0.0087	-1.9611
y45	2337.2484	4672.4823	3.5642e+002	4672.4897	-0.0074	-1.5897
y51	1802.9435	5405.8087	2.0546e+002	5405.8544	-0.0457	-8.4558
y52	1851.9722	5552.8949	2.7228e+002	5552.9228	-0.0279	-5.0215
y54	1899.3197	5694.9372	8.0807e+002	5694.9970	-0.0598	-10.5061
y57	2021.0407	6060.1003	3.2808e+002	6060.1193	-0.0190	-3.1362
y66	2378.8803	7133.6189	6.3887e+002	7133.6409	-0.0219	-3.0736

3. CYPH_YEAST, P14832; Peptidyl-prolyl cis-trans isomerase.

Name	m/z Monoisotopic	Mass Monoisotopic	Intensity	Theoretical Mass	Error (Da)	Error (ppm)
b24	2783.3604	2782.3532	1.0917e+002	2782.3602	-0.0070	-2.5256
b56	2081.3540	6241.0401	6.1546e+002	6241.1159	-0.0758	- 12.1376
b63	2330.8047	6989.3924	3.8545e+002	6989.4373	-0.0449	-6.4244
y17	1683.9703	1682.9630	1.3499e+002	1682.9679	-0.0049	-2.9115
y21	2028.1451	2027.1378	9.1575e+001	2027.1375	0.0004	0.1899
y27	1362.8041	2723.5937	6.9834e+001	2723.5908	0.0029	1.0468
y30	1530.3545	3058.6945	5.7218e+002	3058.7026	-0.0081	-2.6410
y37	1903.0345	3804.0545	3.4062e+002	3804.0672	-0.0127	-3.3477
y41	1409.4280	4225.2622	1.5733e+002	4225.3109	-0.0488	- 11.5428
y41	2113.6452	4225.2759	5.3009e+002	4225.3109	-0.0350	-8.2910
y47	1646.5420	4936.6043	1.1143e+002	4936.6160	-0.0117	-2.3628
y160	2452.6277	17161.3431	2.9465e+002	17161.6269	-0.2838	- 16.5370

4. HSP12_YEAST, P22943; 12 kDa heat shock protein.

Name	m/z	Mass	Intensity	Theoretical	Error	Error
	Monoisotopic	Monoisotopic		Mass	(Da)	(ppm)
b17	887.9586	1773.9026	2.5900e+002	1773.9009	0.0017	0.9409
b34	1857.8955	3713.7764	1.8333e+002	3713.7808	-0.0044	-1.1856
b44	1580.4703	4738.3891	2.1548e+002	4738.3837	0.0054	1.1348
b107	1909.4105	11450.4193	5.0831e+002	11450.5233	-0.1040	-9.0804
y106	1893.0851	11352.4668	4.0149e+002	11352.5593	-0.0925	-8.1486
y107	1912.2605	11467.5193	3.0202e+002	11467.5862	-0.0669	-5.8375

5. YNN4_YEAST, P53912; Uncharacterized protein YNL134C.

Name	m/z Monoisotopic	Mass Monoisotopic	Intensity	Theoretical Mass	Error (Da)	Error (ppm)
b21	1098.0976	2194.1807	3.0698e+002	2194.2031	-0.0224	-10.2023
b23	2438.2706	2437.2634	1.6044e+003	2437.2886	-0.0252	-10.3595
b23	1219.6439	2437.2733	5.7899e+002	2437.2886	-0.0153	-6.2795
b24	1276.1846	2550.3546	3.6472e+003	2550.3727	-0.0181	-7.1037
b26	1381.2559	2760.4973	7.6308e+002	2760.5095	-0.0122	-4.4224
b42	1486.4786	4456.4141	2.5222e+002	4456.4542	-0.0401	-8.9890
b47	2471.3108	4940.6071	3.5917e+003	4940.6459	-0.0388	-7.8615
b52	2810.9685	5619.9225	2.1660e+003	5619.9901	-0.0676	-12.0253
b52	1874.3216	5619.9430	9.3712e+002	5619.9901	-0.0471	-8.3881
b55	2003.7344	6008.1812	3.5705e+002	6008.2375	-0.0563	-9.3716
b56	2022.7454	6065.2145	3.7925e+002	6065.2590	-0.0445	-7.3418
b65	3460.8029	6919.5912	1.4991e+003	6919.6546	-0.0634	-9.1607
b78	4092.1359	8182.2572	5.7417e+002	8182.3529	-0.0957	-11.6953
b129	2739.5982	13692.9548	7.0628e+002	13693.0786	-0.1238	-9.0438
b129	2283.1709	13692.9815	7.7589e+002	13693.0786	-0.0971	-7.0918
b129	1957.1487	13692.9897	1.4168e+003	13693.0786	-0.0889	-6.4927
b163	2449.4161	17138.8621	9.3675e+002	17138.9316	-0.0696	-4.0591
b174	2309.4387	18467.4510	1.9304e+003	18467.5976	-0.1465	-7.9340
b174	2639.2214	18467.4992	6.0272e+002	18467.5976	-0.0984	-5.3262
b221	2624.9224	23615.2358	1.8072e+003	23615.4813	-0.2454	-10.3931
b225	3446.6450	24119.4642	1.5358e+003	24119.7033	-0.2391	-9.9134
b225	3015.9426	24119.4829	2.0487e+003	24119.7033	-0.2204	-9.1362
b225	2680.9500	24119.4847	1.8077e+003	24119.7033	-0.2185	-9.0596
b228	2727.0731	24534.5920	1.5271e+003	24534.8524	-0.2605	-10.6161
b230	3091.0851	24720.6225	7.8114e+002	24720.9165	-0.2940	-11.8913
b230	2747.7452	24720.6416	6.8847e+002	24720.9165	-0.2749	-11.1207
b247	3348.3523	26778.7600	1.4199e+003	26779.0678	-0.3079	-11.4968
b315	3804.6089	34232.4150	2.1891e+003	34232.7903	-0.3753	-10.9621
b335	3660.1889	36591.8164	4.4018e+003	36592.0842	-0.2678	-7.3181
y16	1749.0421	1748.0348	1.1144e+003	1748.0420	-0.0072	-4.1240
y17	1864.0588	1863.0515	4.9109e+003	1863.0690	-0.0174	-9.3652
y23	2543.4473	2542.4400	1.6147e+003	2542.4594	-0.0194	-7.6477
y24	2658.4819	2657.4746	3.3607e+003	2657.4864	-0.0118	-4.4380
y40	2229.7125	4457.4105	5.7850e+003	4457.4546	-0.0441	-9.8917
y60	2273.2384	6816.6935	4.9207e+002	6816.7485	-0.0551	-8.0770
y257	3181.9720	28628.6828	3.4713e+003	28629.0999	-0.4171	-14.5692
y297	3287.6970	32866.8972	9.8190e+002	32867.1859	-0.2887	-8.7853

y299	3309.0989	33080.9158	1.1617e+003	33081.2813	-0.3654	-11.0465
y332	3654.5732	36535.6594	2.0037e+003	36536.0632	-0.4038	-11.0521

6. >ACBP_YEAST, P31787; Acyl-CoA-binding protein.

Name	m/z Monoisotopic	Mass Monoisotopic	Intensity	Theoretical Mass	Error (Da)	Error (ppm)
b9	1032.5355	1031.5283	3.8482e+002	1031.5287	-0.0005	-0.4673
b10	1160.6282	1159.6210	2.6701e+002	1159.6237	-0.0027	-2.3516
b11	1231.6661	1230.6588	1.6830e+002	1230.6608	-0.0020	-1.6170
b12	1330.7300	1329.7227	3.2641e+002	1329.7292	-0.0065	-4.8927
b14	1573.8195	1572.8122	5.2179e+002	1572.8147	-0.0025	-1.6073
b15	1686.9001	1685.8929	2.0909e+003	1685.8988	-0.0059	-3.5222
b18	1007.0513	2012.0880	1.2777e+003	2012.0942	-0.0062	-3.0774
b22	1207.1319	2412.2492	1.7590e+002	2412.2536	-0.0044	-1.8203
b24	1328.1928	2654.3710	8.3005e+001	2654.3803	-0.0093	-3.4916
b25	1384.7304	2767.4462	8.4567e+001	2767.4643	-0.0181	-6.5558
b26	1449.2569	2896.4993	1.3948e+002	2896.5069	-0.0077	-2.6425
b27	1505.7910	3009.5674	2.1538e+002	3009.5910	-0.0236	-7.8409
b28	1587.3352	3172.6558	1.4977e+002	3172.6543	0.0015	0.4822
b29	1622.8454	3243.6763	1.5103e+002	3243.6914	-0.0151	-4.6552
b30	1679.3889	3356.7632	2.5370e+002	3356.7755	-0.0122	-3.6431
b31	1760.9174	3519.8202	2.0829e+002	3519.8388	-0.0186	-5.2872
b38	1407.3942	4219.1608	2.1534e+002	4219.1939	-0.0331	-7.8565
b40	1483.7507	4448.2303	1.9800e+002	4448.2638	-0.0335	-7.5353
b40	2225.1251	4448.2356	9.6152e+002	4448.2638	-0.0282	-6.3420
b67	1943.2386	7768.9252	2.6913e+002	7768.9402	-0.0150	-1.9345
y12	1307.7160	1306.7087	7.9965e+001	1306.7132	-0.0046	-3.4966
y18	2041.0751	2040.0678	1.1766e+003	2040.0779	-0.0101	-4.9322
y21	1207.1319	2412.2492	1.7590e+002	2412.2060	0.0432	17.9189
y50	1959.9653	5876.8740	1.3887e+002	5876.8995	-0.0255	-4.3440
y71	2060.5389	8238.1264	1.8915e+003	8238.1462	-0.0199	-2.4099
y75	2174.3414	8693.3366	2.2552e+002	8693.3842	-0.0476	-5.4804
y85	1965.9961	9824.9443	4.8297e+002	9824.9766	-0.0323	-3.2922

7.P00445; Superoxide dismutase [Cu-Zn].

Name	m/z Monoisotopic	Mass Monoisotopic	Intensity	Theoretical Mass	Error (Da)	Error (ppm)
b10	981.5723	980.5651	5.8481e+002	980.5655	-0.0004	-0.3906
b52	2706.7834	5411.5523	4.8180e+002	5411.6333	-0.0810	-14.9713
b152	3148.9214	15739.5704	2.0565e+003	15739.6792	-0.1088	-6.9151

8. P00942; Triosephosphate isomerase.

Name	m/z Monoisotopic	Mass Monoisotopic	Intensity	Theoretical Mass	Error (Da)	Error (ppm)
b31	1703.9205	3405.8264	1.7458e+002	3405.8521	-0.0257	-7.5391
b35	1282.6760	3845.0063	1.3135e+002	3845.0587	-0.0525	-13.6484
b36	1325.6972	3974.0697	1.3405e+002	3974.1013	-0.0316	-7.9618
b37	1358.7157	4073.1253	3.6231e+002	4073.1697	-0.0444	-10.9035
b38	1391.7337	4172.1793	6.2329e+002	4172.2382	-0.0588	-14.1001
b39	1429.4362	4285.2867	5.5506e+002	4285.3222	-0.0355	-8.2955
b40	1463.7635	4388.2687	6.7865e+002	4388.3314	-0.0627	-14.2927
b47	2573.8207	5145.6269	1.1838e+002	5145.6960	-0.0692	-13.4402
b53	1932.3300	5793.9682	1.6175e+002	5794.0443	-0.0761	-13.1343
b54	1975.0373	5922.0900	1.6932e+002	5922.1393	-0.0493	-8.3203
b55	2017.7261	6050.1564	1.1120e+003	6050.2342	-0.0778	-12.8638
b80	2139.8431	8555.3431	1.3841e+002	8555.4478	-0.1046	-12.2314
b84	2260.9097	9039.6098	3.4664e+002	9039.7123	-0.1025	-11.3393
b104	2852.6844	11406.7084	3.0398e+002	11406.8995	-0.1911	-16.7538
b104	2282.3625	11406.7762	6.6634e+002	11406.8995	-0.1234	-10.8142
b110	2420.2257	12096.0920	2.4761e+002	12096.2379	-0.1460	-12.0661
b179	3248.6673	19485.9604	1.9927e+002	19486.1592	-0.1988	-10.2012
b226	3488.9404	24415.5321	6.3066e+002	24415.7328	-0.2007	-8.2210
y21	2233.1654	2232.1581	4.0811e+002	2232.1902	-0.0321	-14.3836

9. P38013; Peroxiredoxin AHP1.

Name	m/z Monoisotopic	Mass Monoisotopic	Intensity	Theoretical Mass	Error (Da)	Error (ppm)
b12	1314.6560	1313.6487	6.6051e+001	1313.6615	-0.0128	-9.7498
y17	1710.8831	1709.8758	7.2094e+001	1709.8835	-0.0077	-4.5167
y42	2247.1073	4492.2001	2.5645e+002	4492.2372	-0.0371	-8.2658
y59	3093.5060	6184.9975	3.0215e+002	6185.0553	-0.0578	-9.3425
y59	1547.2600	6185.0110	1.0766e+002	6185.0553	-0.0443	-7.1655
y59	2062.6832	6185.0277	5.3196e+002	6185.0553	-0.0277	-4.4734
y85	1806.4829	9027.3781	1.1225e+002	9027.4591	-0.0810	-8.9689
y87	1846.5110	9227.5185	1.5581e+002	9227.5752	-0.0567	-6.1424
y88	1866.3214	9326.5706	1.8128e+002	9326.6436	-0.0731	-7.8340
y90	1908.7459	9538.6932	3.2459e+002	9538.7961	-0.1028	-10.7820
y92	1954.1705	9765.8163	3.0876e+002	9765.9231	-0.1068	-10.9380

10. PGK_YEAST, P00560; Phosphoglycerate kinase.

Name	m/z	Mass	Intensity	Theoretical	Error	Error
	Monoisotopic	Monoisotopic		Mass	(Da)	(ppm)
b15	1671.8680	1670.8608	4.0275e+002	1670.8727	-0.0119	-7.1166
b98	1578.8507	11044.9041	7.4539e+002	11045.0024	-0.0983	-8.9012
b99	1586.9967	11101.9259	6.7400e+002	11102.0239	-0.0979	-8.8195
y80	2023.0678	8088.2421	6.7077e+002	8088.2989	-0.0568	-7.0166

11.ENO1_YEAST, P00924; Enolase 1.

Name	m/z	Mass	Intensity	Theoretical	Error	Error
	Monoisotopic	Monoisotopic		Mass	(Da)	(ppm)
b12	1339.6907	1338.6835	7.1648e+003	1338.6932	-0.0097	-7.2571
b16	1753.8865	1752.8792	2.7845e+002	1752.8907	-0.0115	-6.5509
b52	1387.9342	5547.7077	4.2869e+002	5547.7691	-0.0615	-11.0832
b66	1420.5279	7097.6032	3.3281e+002	7097.6606	-0.0574	-8.0901
b70	1508.9622	7539.7748	3.2232e+002	7539.8418	-0.0670	-8.8914
b71	1528.7746	7638.8365	2.8387e+002	7638.9103	-0.0738	-9.6592
b73	1565.5986	7822.9566	7.0201e+002	7823.0314	-0.0749	-9.5691
y15	1655.7869	1654.7796	2.1275e+003	1654.7852	-0.0056	-3.3714

12. ENO2_YEAST, P00925; Enolase 2.

Name	m/z Monoisotopic	Mass Monoisotopic	Intensity	Theoretical Mass	Error (Da)	Error (ppm)
b12	1339.6942	1338.6870	1.1338e+003	1338.6932	-0.0062	-4.6389
b16	1753.8924	1752.8851	3.1162e+001	1752.8907	-0.0056	-3.1725
b47	2487.7628	4973.5111	6.2054e+001	4973.5522	-0.0411	-8.2730
b50	2688.8441	5375.6737	5.8123e+002	5375.7207	-0.0471	-8.7607
b50	1792.8994	5375.6764	3.0256e+002	5375.7207	-0.0444	-8.2556
b52	1874.2566	5619.7479	1.4326e+002	5619.7903	-0.0424	-7.5457
b52	2810.8871	5619.7596	1.7853e+002	5619.7903	-0.0307	-5.4563
b82	2935.7921	8804.3546	2.7708e+002	8804.4540	-0.0994	-11.2904
b85	3049.8599	9146.5580	4.8388e+002	9146.6443	-0.0863	-9.4396
b91	3268.6211	9802.8414	2.0617e+002	9802.9573	-0.1159	-11.8234
b135	2854.0478	14265.2025	1.6256e+002	14265.3366	-0.1341	-9.4032
b138	2919.6877	14593.4019	1.8686e+002	14593.5477	-0.1458	-9.9891
b140	2962.7117	14808.5223	2.5193e+002	14808.6747	-0.1524	-10.2938
b142	3000.3142	14996.5347	1.3305e+002	14996.7544	-0.2197	-14.6526
b146	3094.7861	15468.8943	1.0235e+002	15469.0230	-0.1286	-8.3158
b245	4276.5218	25653.0874	1.8509e+002	25653.2677	-0.1803	-7.0295
y15	1685.8312	1684.8239	4.6894e+002	1684.8321	-0.0082	-4.8592
y51	2806.0094	5610.0042	9.6896e+001	5610.0545	-0.0503	-8.9653
y56	3079.6403	6157.2661	9.6597e+001	6157.3187	-0.0526	-8.5440
y75	2740.3968	8218.1687	1.0806e+002	8218.2198	-0.0511	-6.2180
y105	2268.7734	11338.8304	1.2154e+003	11338.9591	-0.1287	-11.3472
y105	2835.7326	11338.9013	1.6572e+002	11338.9591	-0.0578	-5.0958

13.SODM_YEAST, P00447; Chain (Superoxide dismutase [Mn], mitochondrial)
[27-233] in Superoxide dismutase [Mn]

Name	m/z Monoisotopic	Mass Monoisotopic	Intensity	Theoretical Mass	Error (Da)	Error (ppm)
y45	2708.3586	5414.7026	1.2005e+002	5414.7781	-0.0754	-13.9282
y45	1805.9094	5414.7065	1.6036e+002	5414.7781	-0.0716	-13.2229
y48	1908.9851	5723.9336	3.6287e+001	5723.9833	-0.0497	-8.6891
y49	1927.9854	5780.9343	3.6358e+001	5781.0048	-0.0704	-12.1837
y110	2439.6530	12193.2286	5.0430e+001	12193.3641	-0.1355	-11.1158
y118	2587.9144	12934.5354	5.8799e+001	12934.6570	-0.1216	-9.4000

Appendix 4. Chapter 5

Tandem mass fragment assignments

All masses listed are monoisotopic.
Escherichia coli BL21 (DE3)

Washing for four rounds

1. Protein name: Extracellular solute-binding protein family 3

Charge state: +10

Observed monoisotopic mass: 31152 Da

Name	m/z Monoisotopic	Mass Monoisotopic	Intensity	Theoretical Mass	Error (Da)	Error (ppm)
B35	1816.4011	3630.7876	2.5148e+002	3630.7954	-0.0078	-2.1381
B61	2188.7990	6563.3752	5.9089e+001	6563.3724	0.0028	0.4263
B277	3084.6594	30836.5209	2.4606e+002	30836.7089	-0.1880	-6.0979
Y15	1747.9087	1746.9014	1.0252e+002	1746.8974	0.0040	2.2898
Y35	2081.0676	4160.1207	4.2772e+001	4160.1227	-0.0020	-0.4755
Y60	3527.7643	7053.5141	1.8735e+002	7053.5183	-0.0041	-0.5848

2. Protein name: Maltodextrin-binding protein

Charge state: +11

Observed monoisotopic mass: 40662 Da

Name	m/z Monoisotopic	Mass Monoisotopic	Intensity	Theoretical Mass	Error (Da)	Error (ppm)
B14	1595.8822	1594.8749	1.2992e+002	1594.8719	0.0031	1.9324
B41	2268.7037	4535.3928	6.6815e+001	4535.4063	-0.0135	-2.9686
B55	3025.5832	6049.1519	3.2965e+002	6049.1839	-0.0320	-5.2918
B122	2724.6179	13618.0530	1.3859e+002	13618.1119	-0.0589	-4.3276
Y56	1540.5435	6158.1447	2.3455e+002	6158.1615	-0.0168	-2.7271
Y56	2053.7234	6158.1484	5.5763e+002	6158.1615	-0.0131	-2.1251
Y56	3080.0977	6158.1808	1.5848e+002	6158.1615	0.0193	3.1362

3. Protein name: Superoxide dismutase Superoxide dismutase

Charge state: +9, +13

Observed monoisotopic mass: 22915 Da, 45997 Da (Dimer)

Name	m/z Monoisotopic	Mass Monoisotopic	Intensity	Theoretical Mass	Error (Da)	Error (ppm)
B19	2180.9919	2179.9846	7.2714e+001	2180.0102	-0.0256	-11.7288
Y18	2123.0582	2122.0510	3.2646e+001	2122.0748	-0.0238	-11.2315
Y22	2656.3486	2655.3413	2.0161e+002	2655.3597	-0.0184	-6.9456
Y35	2173.1069	4344.1993	3.9535e+001	4344.2133	-0.0140	-3.2160
Y38	1587.1325	4758.3756	1.0223e+002	4758.4036	-0.0279	-5.8715
Y38	2380.1962	4758.3779	1.3108e+002	4758.4036	-0.0257	-5.4022
Y49	1929.6421	5785.9046	3.4963e+001	5785.9374	-0.0328	-5.6701
Y58	2224.7719	6671.2940	3.4569e+002	6671.3640	-0.0700	-10.4899
Y95	2667.0856	10664.3134	9.2249e+001	10664.4176	-0.1042	-9.7716

4. Protein name: Phosphoenolpyruvate carboxykinase (ATP)

Charge state: +14

Observed monoisotopic mass: 59912 Da

Name	m/z Monoisotopic	Mass Monoisotopic	Intensity	Theoretical Mass	Error (Da)	Error (ppm)
B38	4395.0618	4394.0545	2.0180e+003	4394.0688	-0.0143	-3.2630
Y14	1236.7274	1235.7202	4.9247e+002	1235.7237	-0.0036	-2.8769
Y18	1743.9605	1742.9532	3.1219e+002	1742.9566	-0.0034	-1.9582
Y21	2120.0904	2119.0831	3.5474e+002	2119.0949	-0.0118	-5.5599
Y45	2468.7459	4935.4772	6.0237e+003	4935.5082	-0.0310	-6.2826
Y45	1646.1678	4935.4817	1.4317e+003	4935.5082	-0.0265	-5.3747

5. Protein name: Cationic amino acid ABC transporter, periplasmic binding protein

Charge state: +6

Observed monoisotopic mass: 27992 Da

Name	m/z Monoisotopic	Mass Monoisotopic	Intensity	Theoretical Mass	Error (Da)	Error (ppm)
B11	1153.6183	1152.6110	2.5632e+002	1152.6139	-0.0028	-2.4579
B21	2251.1052	2250.0979	8.6986e+002	2250.1168	-0.0189	-8.3911
B25	2622.2887	2621.2814	2.8880e+002	2621.2973	-0.0158	-6.0340
B30	3187.5429	3186.5356	2.2662e+002	3186.5509	-0.0153	-4.7873
B45	2468.7381	4935.4616	8.9504e+002	4935.3703	0.0913	18.4943
Y18	2161.9747	2160.9674	1.7835e+002	2160.9826	-0.0152	-7.0213
Y45	2494.7113	4987.4080	4.4490e+002	4987.4450	-0.0369	-7.4046

6. Protein name: Serine hydroxymethyltransferase

Charge state: +18

Observed monoisotopic mass: 90634 Da (Dimer)

Name	m/z Monoisotopic	Mass Monoisotopic	Intensity	Theoretical Mass	Error (Da)	Error (ppm)
B10	1202.5970	1201.5897	1.2934e+003	1201.5947	-0.0051	-4.2161
B12	1480.6857	1479.6784	1.6976e+003	1479.6850	-0.0066	-4.4658
Y22	2506.3729	2505.3656	1.9500e+003	2505.3777	-0.0121	-4.8152
Y22	1253.6909	2505.3672	3.4977e+002	2505.3777	-0.0105	-4.1734
Y26	2935.5481	2934.5408	9.7192e+002	2934.5637	-0.0229	-7.7967
Y26	1468.2780	2934.5414	7.0937e+002	2934.5637	-0.0223	-7.5923
Y29	1631.8695	3261.7244	3.9963e+002	3261.7431	-0.0187	-5.7233
Y65	1788.1832	7148.7036	3.9876e+003	7148.7405	-0.0369	-5.1603
Y65	2383.9086	7148.7040	4.1704e+003	7148.7405	-0.0364	-5.0971
Y68	1869.7118	7474.8180	4.1031e+002	7474.8631	-0.0450	-6.0263
Y83	2246.9205	8983.6529	5.5616e+002	8983.7054	-0.0525	-5.8436
Y83	1797.7381	8983.6542	9.4382e+002	8983.7054	-0.0512	-5.6976
Y89	1917.9916	9584.9214	3.6068e+002	9584.9761	-0.0548	-5.7122
Y92	2486.0415	9940.1369	9.7656e+002	9940.1981	-0.0612	-6.1554
Y92	1989.0371	9940.1493	1.1565e+003	9940.1981	-0.0488	-4.9105
Y94	2031.8536	10154.2316	4.9727e+002	10154.2934	-0.0618	-6.0868
Y95	2054.4712	10267.3195	7.6249e+002	10267.3775	-0.0579	-5.6436
Y136	2485.1177	14904.6623	1.8925e+003	14904.7515	-0.0892	-5.9854
Y160	2156.8637	17246.8512	1.1933e+003	17246.9753	-0.1241	-7.1970
Y160	2464.8466	17246.8751	1.4905e+003	17246.9753	-0.1002	-5.8083

7. Protein name: 2,3-bisphosphoglycerate-dependent phosphoglycerate mutase
 Charge state: +13
 Observed monoisotopic mass: 57082 Da (Dimer)

Name	m/z Monoisotopic	Mass Monoisotopic	Intensity	Theoretical Mass	Error (Da)	Error (ppm)
B118	1958.5830	13703.0300	3.9123e+001	13702.9921	0.0380	2.7696
B249	3160.3843	28434.3935	8.3334e+001	28434.6595	-0.2659	-9.3521
Y38	2107.0962	4212.1779	4.6769e+001	4212.2007	-0.0228	-5.4126
Y42	2284.1861	4566.3577	1.6667e+002	4566.3910	-0.0333	-7.2874
Y42	1523.1344	4566.3814	8.0474e+001	4566.3910	-0.0096	-2.1091
Y44	2397.7550	4793.4954	1.6782e+001	4793.5180	-0.0225	-4.7030

Washing for eight rounds

1. Protein name: Long-chain fatty acid transport protein

Charge state: +9

Observed monoisotopic mass: 45910 Da

Name	m/z Monoisotopic	Mass Monoisotopic	Intensity	Theoretical Mass	Error (Da)	Error (ppm)
B25	2486.1531	2485.1459	1.1287e+002	2485.1509	-0.0051	-2.0389
B26	2601.1772	2600.1700	2.8000e+001	2600.1779	-0.0079	-3.0436
B41	2094.4803	4186.9460	2.9708e+001	4186.9654	-0.0193	-4.6131
B53	2733.2987	5464.5829	9.9930e+001	5464.6058	-0.0229	-4.1880
B53	1822.5378	5464.5917	1.9189e+002	5464.6058	-0.0141	-2.5780
B55	1893.2233	5676.6480	1.8843e+001	5676.6855	-0.0375	-6.6000
B71	1812.6216	7246.4574	4.8399e+001	7246.4965	-0.0392	-5.4061
B90	2334.6295	9334.4890	5.4541e+001	9334.5216	-0.0326	-3.4973
B110	2874.3621	11493.4192	2.2351e+001	11493.4812	-0.0620	-5.3949
Y72	2679.6019	8035.7838	6.9634e+001	8035.8281	-0.0442	-5.5029
Y72	4018.9062	8035.7979	2.7442e+001	8035.8281	-0.0302	-3.7529
Y73	2717.9450	8150.8131	1.0918e+002	8150.8550	-0.0419	-5.1404
Y73	4076.4141	8150.8137	2.3245e+001	8150.8550	-0.0413	-5.0663
Y84	3154.1545	9459.4417	3.4485e+001	9459.4801	-0.0384	-4.0636

2. Protein name: Outer membrane protein A

Charge state: +8

Observed monoisotopic mass: 35170 Da

Name	m/z	Mass	Intensity	Theoretical	Error	Error
	Monoisotopic	Monoisotopic		Mass	(Da)	(ppm)
B20	2320.0690	2319.0617	6.7219e+001	2319.0709	-0.0091	-3.9395
Y19	2165.2337	2164.2265	8.6899e+001	2164.2328	-0.0063	-2.9050
Y94	3347.0385	10038.0937	4.3396e+001	10038.1541	-0.0604	-6.0182
Y97	3460.4298	10378.2675	5.6057e+001	10378.3287	-0.0612	-5.8956
Y324	4385.6516	35077.1547	1.0816e+003	35077.4445	-0.2898	-8.2611

3. Protein name: Enolase

Charge state: +13, +18

Observed monoisotopic mass: 45551 Da, 91102 Da (Dimer)

Name	m/z Monoisotopic	Mass Monoisotopic	Intensity	Theoretical Mass	Error (Da)	Error (ppm)
B17	1880.1031	1879.0958	1.8294e+001	1879.1003	-0.0044	-2.3676
B23	2506.3823	2505.3750	2.8232e+001	2505.3914	-0.0164	-6.5439
B52	1791.5983	5371.7731	4.4942e+001	5371.7945	-0.0214	-3.9910
B79	1607.8670	8034.2988	3.8235e+001	8034.3227	-0.0240	-2.9846
B85	1730.7270	8648.5984	3.6648e+001	8648.6251	-0.0267	-3.0867
Y14	1559.8651	1558.8578	1.5697e+001	1558.8579	-0.0002	-0.0988
Y31	1771.9775	3541.9405	6.5183e+001	3541.9481	-0.0075	-2.1282