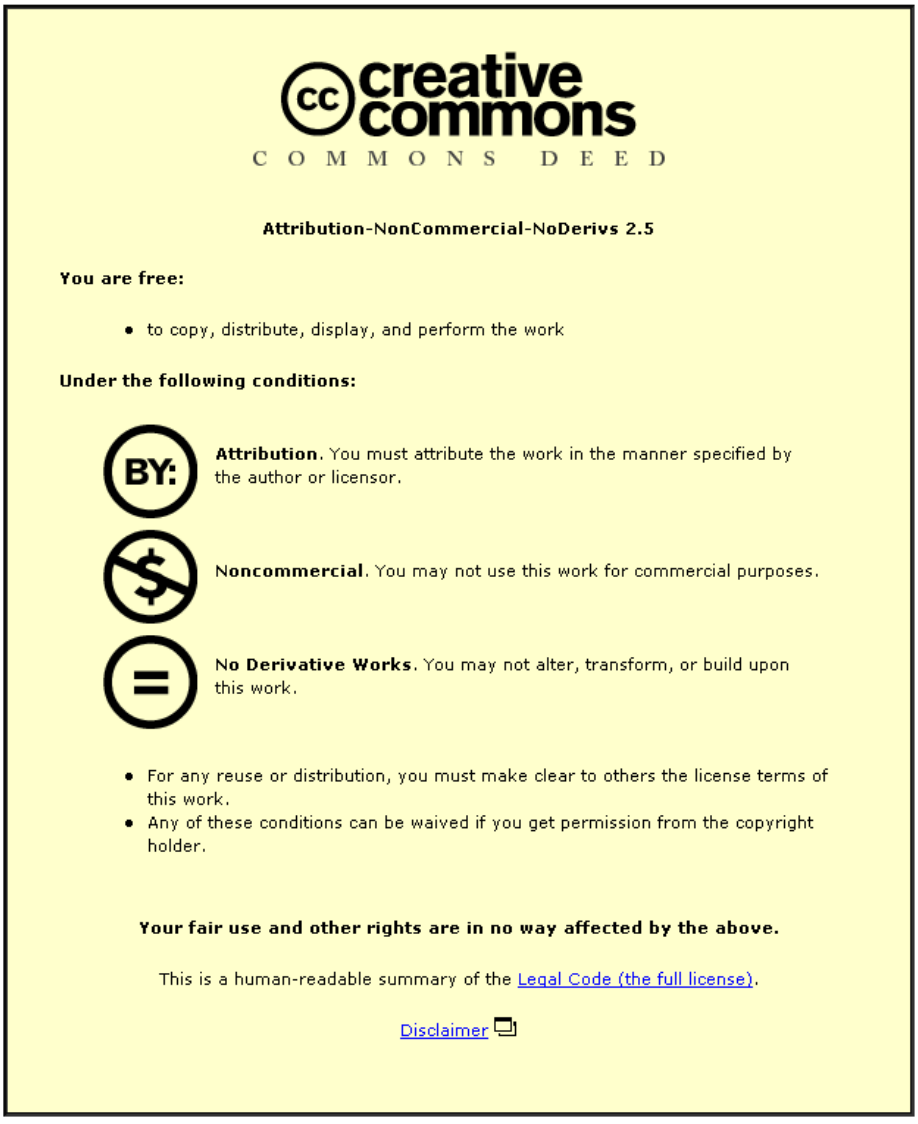

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Enhanced Gel Electrophoresis (GE) and Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) Based Methods for the Identification and Separation of Proteins and Peptides

Syed Razi Haider

A Doctoral Thesis

Submitted in partial fulfilment of the requirements for the award of Degree of Doctor of Philosophy (PhD) of Loughborough University

Abstract

The main focus of the PhD study was to develop new gel electrophoresis and ICP-MS based methods to analyze a wide variety of the bio-molecules such as proteins, phosphoproteins and metalloproteins etc. The tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis (tricine-SDS-PAGE) method is commonly used to resolve low molecular mass proteins, however, it requires a high percentage gel and a very complicated procedure to achieve this separation. This study describes a modification to tricine-SDS-PAGE to make it more effective for the separation of smaller proteins and for coupling to ICP-MS. The modified method employs low percentage PAGE gels and low reagent concentrations that provide efficient separations, good quantitation and low matrix levels that are compatible with ICP-MS. This modified method was applied to analyze phosphopeptides. Phosphopeptides are very small in size and difficult to separate using the other techniques such as Laemmli SDS-PAGE, original tricine-SDS-PAGE, immobilized metal affinity chromatography (IMAC), size exclusion chromatography (SEC) etc. In this study a simplified procedure is described based on modifying the original tricine-SDS-PAGE method. A comparative study showed that this modified method successfully resolved a digest mixture of very low to high molecular mass phosphopeptides/peptides. In off-line coupling of this method with ICP-MS, much better recoveries of the peptides from the gel were obtained as compared to traditional methods which indicate the compatibility of this modified method for quantitative studies. An on-line coupling of the modified system with ICP-MS was also demonstrated and it was applied for the separation, detection and quantification of phosphopeptides.

Another application of this modified system was the separation of serum proteins. Blood serum contains five major protein groups i.e., albumin, alpha-1 globulin, alpha-2 globulin, beta globulin and gamma globulin. The separation of these five major proteins in a single gel is difficult to achieve using traditional methods. The modified system was shown to be superior for the separation of these serum proteins in a 7% (m/v) native-PAGE gel and a cellulose acetate membrane.

A further study was carried out into controlling the factors that cause metal loss and protein fragmentation in SDS-PAGE. Using a reducing sample buffer, and heating to high temperatures (90-100ºC) in alkaline or acidic conditions may cause protein fragmentation and decrease the metal binding affinity. 70ºC was found suitable to

prepare the sample at neutral, alkaline or acidic pH as no fragmentation observed. To prevent metal loss, the binding constant (log *K*) values of metal-amino acids, play the major role. Those metals which have high binding affinities with the amino acids in proteins can also be affected by the variation of the pH so prior information about pH to maintain the binding constant values is essential to minimize metal loss. This was observed in the loss of zinc, and to a lesser extent copper from human serum albumin (HSA) as measured by inductively coupled plasma mass spectrometry (ICP-MS).

The method described above was applied for the separation and quantification of the serum proteins obtained from age-related macular degeneration (AMD) patients (where the AMD patients were from Moorfields Eye Hospital, London). Zn and Cu were quantified employing external calibration. Zn concentration showed variation whilst Cu did not show any significant variations in samples from AMD patients.

A brief study of the interaction of cisplatin and oxaliplatin with HSA and transferrin was also performed. Cisplatin bound much faster than oxaliplatin with HSA. After 24 hours incubation, cisplatin showed a decrease in signal intensity which indicates that cisplatin binding decreases with time. Cisplatin binding with transferrin as compared to HSA was not significant, which could be the result of unstable Pt-transferrin complex formation. Oxaliplatin did not show high binding to either protein, perhaps due to the presence of the bulky, non polar DACH ligand.

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The technical staff at Loughborough University were always supportive and helped me on several occasions, thus I would like to acknowledge especially Mr. David Wilson (late) and Mr. Trevor Brown for their kind support for trouble shooting the instrumental problems. Also thanks to Dr. James Reynolds, Mr. Stuart Pinkney, Mr. Mark Weller and Mr. John Spray (retired) to provide me moral and technical support.

I dedicate my PhD thesis to my loving parents especially my mother who put her every effort in my good upbringing.

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Glossary

1D	One Dimensional
2D	Two Dimensional
β	Beta
ºC	Degree Centigrade
µg	Micro gram
µl	Micro litre
µm	Micro metre
%	Percentage
%C	Percentage of the Cross Linker (Bis-acrylamide)
%T	Percentage of the Acrylamide in Gel.
ACN	Acetonitrile
AMD	Age-Related Macular Degeneration
CE	Capillary Electrophoresis
CRC	Collision Reaction Cell
Da	Dalton
DACH	Diaminocyclohexane
DNA	Deoxyribonucleic Acid
DTT	D, L-Dithiothreitol
RNA	Ribonucleic Acid
EDTA	Ethylenediaminetetraacetic Acid
EM	Electron Multiplier
ESI	Electro Spray Ionization
EXPASY	Expert Protein Analysis System
GE	Gel Electrophoresis
HPLC	High Performance Liquid Chromatography
HSA	Human Serum Albumin

IDA	Isotopic Dilution Analysis
IEF	Iso-electric Focussing
IMAC	Immobilized Metal Affinity Chromatography
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
i.d.	Internal Diameter
kb	Kilo Base Pair
kDa	Kilo Dalton
kV	kilo Voltage
LA	Laser Ablation
LC	Liquid Chromatography
LOD	Limit of Detection
Log	Logarithm
M	Molar Concentration
MALDI-MS	Matrix-Assisted Laser Desorption Ionization Mass Spectrometry
MAPS	3-(trimethylsilyl) propyl methacrylate
ml	Milli litre
mM	Milli Molar Concentration
MS	Mass Spectrometry
NSI	Nano Spray Ionization
PFA	Perfluoroalkoxy
pH	power of hydrogen
PhD	Doctor of Philosophy
PI	Isoelectric Point
PS-DVB	Polystyrene Divinyl Benzene
PTM	Post Translational Modification
Rf	Relative Mobility
RP	Reverse Phase

SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SEC	Size Exclusion Chromatography
SPE	Solid Phase Extraction
SPEP	Serum Protein Electrophoresis
TBE	Tris-borate EDTA
TEMED	*N, N, N', N'*-Tetramethylethylenediamine
Tf	Transferrin
TFA	Trifluoroacetic acid
THF	Tetrahydrofuran
ToF	Time of Flight
TRA	Time Resolved Analysis
Tris-HCl	Tris(hydroxymethyl)aminomethane hydrochloride
UV	Ultra Violet
V	Voltage

Chapter 1: Introduction

1.1 Electrophoresis

Electrophoresis is a terminology which is used for the separation of the charged particles (mostly DNA, RNA and proteins) under the influence of an applied electric field.

1.1.1 Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis (PAGE), either by one dimensional (1D) or two dimensional (2D), is mostly and routinely used for the separation of proteins in proteomics base studies. In this technique polyacrylamide is used as a separating medium because it can withstand a high voltage, also it is highly thermo stable, chemically inert and can be prepared with different pore sizes. There are several advantages of using PAGE for separation of proteins: (a) Slab PAGE remains a bench mark for protein analysis and is the most widely used because of its high reproducibility and resolving power, (b) molecular weight determination of unknown proteins is accomplished easily using mass standards in SDS-PAGE, (c) low to high molecular weight proteins can be separated in a single gel and (d) a number of protein samples, including protein standards, can be run simultaneously in a single gel under identical conditions and can be compared using the band patterns. On the other hand there are some disadvantages, which cannot be ignored: (a) acrylamide is a neurotoxin and suspected carcinogen so extra care is required to handle the PAGE gel, (b) this method is not very useful for metal protein recovery specially SDS-PAGE which has been found to cause metal loss, (c) PAGE gel suffers from contamination from several elements present in the gel and buffer media and is difficult to couple with ICP-MS especially for quantitative studies of several elements such as, ^{31}P, ^{32}S, ^{56}Fe, ^{63}Cu, ^{66}Zn, (d) for immuno assays electrophoretic transfer of analyte from gel to membrane is usually required, which may cause loss of the sample. Protein Separation can be performed by using SDS-PAGE or native-PAGE methods.

1.1.1.1 SDS-PAGE

Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) is a very well established technique which is used to separate a protein of interest from a mixture of different proteins on the basis of their molecular weight. This technique is usually used in genetics, microbiology, cell biology and biochemistry. SDS is an anionic detergent which is wrapped around the molecules and swamps their own molecular charges. Molecular weights are calculated on the basis of electrophoretic mobility or relative mobility. Relative mobility is a term used for the movement of the target protein through a gel relative to the other proteins or dye front (protein mass ladder). Protein separation by SDS-PAGE is usually performed in one dimensional and two dimensional gel electrophoresis.

One-Dimensional SDS-PAGE (1D-SDS-PAGE)

The protein separation in one dimensional page depends on the size of the molecules, field strength and the sieving effect of the gel.

Laemmli SDS-PAGE method

This method is also called Tris-glycine method. This method was first introduced by Laemmli [1] in 1970. In this method two gels are made by using two different concentrations of the buffers and different pHs. This method is very popular and has been used widely successfully. There are three major components of this buffer system, Stacking gel, Resolving gel, and Running Tank Buffer.

Stacking and resolving gel

In Laemmli system the stacking gel is used to create the ion gradients between the separating molecules and is responsible for focussing the protein bands before separation into the separating gel medium. The pH of this gel is set to 6.8 which is very appropriate to create the ion gradients between the separating molecules. This gel is prepared by using 0.5M Tris-HCl buffer with 4% polyacrylamide. Larger pore size of the stacking gel enables fast movement of the proteins. The resolving or separating gel is used to separate the proteins. This gel is prepared by using 1.5M Tris-HCl buffer with the required concentration of the polyacrylamide and the pH of the gel is set to 8.8.

Running tank buffer

The buffer which is used to run the molecules in the gel is called the running tank buffer. This buffer is prepared by using 50 mM Tris-HCl, 192 mM glycine and 0.1% SDS.

Sample preparation

To obtain predictable results through gel electrophoresis and to avoid any possible reaction of protein with the component of the gel, it is necessary to disrupt the three dimensional structure of proteins before loading into the gel. This disruption of the protein structure is achieved by denaturisation and reduction of the protein of interest. In this process equal volume (v/v) of protein is mixed with sample buffer. Sample buffer is composed of a reducing agent like dithiothreitol or β-mercaptoethanol (in Laemmli SDS-PAGE), an anionic detergent SDS, and bromophenol blue, used as tracking dye during the gel run. The protein structures are unfolded by heating for 5-10 minutes at 70-100 ºC, in the similar environment, reduction is performed in the presence of (DTT) or β-mercaptoethanol. Finally, SDS wraps around protein molecules with the ratio of 1.4 gm SDS/gm protein and distributes the uniform negative charge throughout molecules of proteins. This uniform distribution swamps the protein's own charge and allows the uniform movement of the protein molecules in the gel, with separation based purely on molecular mass. Fig. 1.1 shows the protein denaturisation process.

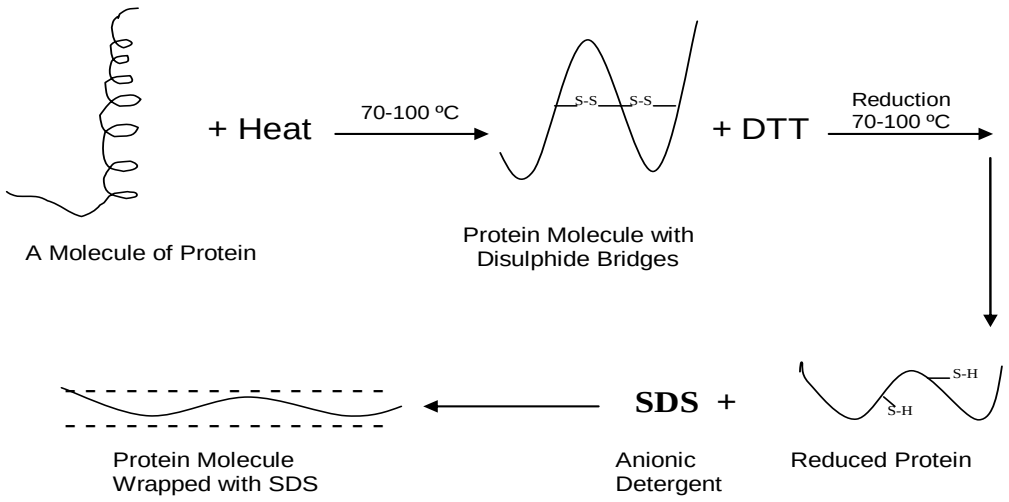


Figure 1.1: Protein denaturisation, reduction and SDS wrapping before loading into the gel

Drawbacks

Although the Laemmli method is a widely used method for protein separation, the highly alkaline separating gel medium can cause band distortion, poor resolution and band artefacts. The most important reasons for poor resolution in the Laemmli system are described below.

- Separating gel medium establishes the pH 9.5 during the run which can cause chemical modifications such as deamination and alkylation of the proteins [2]
- Reoxidation of disulphides of cystein during electrophoresis because redox state of the gel is not constant
- The pH of the Laemmli sample buffer is set at 5.2 which help to cleave the Asp-protein bond at 100ºC during the reduction of the sample prior to loading.
- The polyacrylamide can be hydrolysed at pH 8.8 so the gels have very short shelf life.
- This method is not good for low molecular mass peptides detection [3]

Tricine SDS-PAGE method

In 1987 Schägger and von-Jagow modified the Laemmli method by replacing the glycine with tricine [4]. So this method is called tricine-SDS-PAGE method. As compared to glycine, Tricine shows high ionic strength when combines with Tris-base in running buffer and migrates much faster (than proteins) from cathode to anode and provides a good environment for high resolution of low molecular weight proteins. This method provides very good resolution of proteins in between 1 to 100 kDa.

Novex® NuPAGE® SDS-PAGE

This method is an improved form of the Tris-glycine methods and provides a good solution to the Laemmli's drawbacks by lowering the pH of the stacking and separating gel to 6.5 [5]. The major components of this method are the same as described above but only the running tank buffer tris-glycine which is replaced by tris-Morpholine Ethane Sulfonic acid or (tris-MES) for low molecular weight peptides and tris-Morpholine Propane Sulphonic Acid (tris-MOPS) for high molecular weight peptides [5]

Advantages of Nu-SDS-PAGE over Tris-glycine and Tris-tricine methods

The neutral pH is a revolution in SDS-PAGE discontinuous buffer system and has several advantages over tris-glycine and tris-tricine methods.

- The neutral pH of the system stops alkylation and deamination of proteins during the run and also provides sharp bands and accurate results.
- Addition of Antioxidant (reducing agent) in the tank buffer stops the reoxidation of disulphide bonds during the run.
- Heating the sample at 70ºC in Nu SDS-PAGE prevents the cleavage of Asp-protein bond.
- Longer shelf life of the gels because of the neutral pH.
- It provide a wide range of separation i.e., 1 to 200kDa

Drawbacks of Novex® NuPAGE® SDS-PAGE method

- Neutral pH medium in the separating gel requires very high voltages to run the proteins and this can result in high temperature that break the gel. This method doesn't work very well for gradient gel system because of this reason.
- Protein tracking dye (bromophenol blue) turns yellow or colour less in acidic and neutral medium.

Two-dimensional gel electrophoresis (2-DGE)

This method is also called the Iso-Dalton method because protein separate by Iso-Electric Focussing (IEF) in first dimension and then in slab SDS-PAGE in second dimension on the basis of their molecular weights (Dalton). Isoelectric point (pI) is actually a pH at which there is no net charge on the surface of a molecule.

First dimension

In 2D-GE an immobilized pH gradient (IPG) is required in the first dimension to separate the protein according to their pI value so for this reason, non-linear pH gradient gel strips with an area of 3x180mm are used with the pH range of (3.5 to 10). These strips have the capacity to hold a large amount of proteins and provide excellent resolution.

These strips require overnight rehydration before sample application by placing them in a solution containing 8M Urea, 2% CHAPS, 10 mM DTE, 2% Resolytes (pH 3.5-10) and a trace of Bromophenol Blue. After sample application into these strips they run overnight with a gradient of the voltages and total of 100 kVh of the net voltage is used for overnight run. After first dimension separation, equilibration of these strips is required in order to reduce the disulfide bond and re-solubilize the proteins.

Second dimension

Two- Dimensional PAGE in the second dimension is carried out by the method explained above in the Laemmli or Nu-PAGE methods.

Advantages of 2D-GE

The advantages of 2D-GE are described below.

- By using 2D-GE a large number of proteins can be separated and identified. For example this technique is very useful when a global protein expression of an organism or a tissue is being investigated.
- This technique is also useful when very high resolution of the proteins is required.

Drawbacks

- Because of the protein-gel interaction during IEF in the first dimension, membrane and hydrophobic proteins can be poorly represented during SDS-PAGE in second dimension [6].
- This method is useful to visualize the proteins with molecular mass between 10 to 100 kDa. Mostly phosphopeptides are found in the range of 2 to 6 kDa [7].

1.1.1.2 Native-PAGE

In native-PAGE, separation is based on pI value and size of the separated proteins. It is usually employed when proteins are required to be preserved in their intact form. This separation is performed in the absence of SDS hence no uniform negative charge is

distributed on the molecules. A non-reducing sample buffer (absence of DTT or β-marcaptoethanol) is used to prepare the sample.

Advantages of native-PAGE over SDS-PAGE

Although the resolving efficiency of native-PAGE is poor and it cannot separate several proteins with low MW difference on a single gel, but it still has several benefits over the SDS-PAGE method including:

- In SDS-PAGE use of the reducing sample buffer and SDS require heating the sample at 70-100ºC in the sample preparation step which may cause metal loss from the metal bound proteins.
- Separation of very complex mixture of the proteins such as blood plasma cannot be achieved in 1D-SDS-PAGE because it separates proteins only on the basis of MW and proteins bands either do not properly resolve with each other (if Tris-tricine buffer is employed) or are lost during the separation (if Tris-glycine or any other buffer is employed). Native PAGE can also not separate all proteins present in the complex mixture but it at least separate major plasma proteins in 7% gel using Tris-tricine buffer system (Please see chapter 4)

Disadvantage of native-PAGE over SDS-PAGE

- A washing step or dialysis is required to remove SDS after proteins recovery from the gel which may cause sample loss.

1.1.2 Agarose gel electrophoresis for separation of DNA

Separations of DNA fragments are, in principle, much simpler and easier than separation of proteins and require an agarose gel instead of PAGE. The basic reason for using agarose gel is the size of the DNA molecules. Pore size of the polyacrylamide gel even with very low percentage are not suitable enough to separate the DNA molecules because they are much larger in size thus an agarose gel with different percentages is used to achieve the separation of DNA. Very tiny DNA fragments can be separated using PAGE method.

1.1.2.1 Preparation of the agarose gel

The percentage range of the agarose gels depends on the size of the DNA and it lies between 0.7 to 2.0%. 0.7% gel is used for the separation of 5-10 kilo base pairs (kb) and 2% used for smaller DNA fragments in the range of 0.2 – 1 kb. These gels are prepared by dissolving an amount of the agarose into Tris-borate-EDTA (TBE). Percentage of the gel depends on the amount of the agarose used for example for 1% gel 0.5 g of agarose dissolved in 50 mL of TBE. This agarose solution is then boiled at 100°C. After cooling down to room temperature 1 µL ethidium bromide is added and then poured slowly into the casting apparatus. (Note ethidium bromide is very toxic so extra care is required)

1.1.2.2 Buffer System

Running buffer or tank buffer is the same buffer used in the preparation of the gel for example if 0.5 x TBE is being used in the preparation of the gel then same buffer with same concentration has to be used as a running or tank buffer. 1L of the 10 x TBE running buffer is prepared by dissolving 109 g of Tris base, 55 g of boric acid and 4.6 g of EDTA in deionized water. pH is usually adjusted at 8.3 using NaOH

Sample or loading buffer is required to mix with the sample to visualize the loaded sample in the gel. It is prepared by dissolving 25 mg Bromophenol Blue, 4 g sucrose in 10 mL of deionized water. 2 µL of the loading buffer is enough for 10 µL of the sample.

1.1.2 Serum Protein Electrophoresis (SPEP)

An alternative and commonly used approach in clinical analysis is serum protein electrophoresis (SPEP) or cellulose acetate electrophoresis. This is usually carried out using pure cellulose acetate membranes (Sepraphore® III), or cellulose acetate cast onto a Mylar backing (Super Sepraphore®). This cellulose acetate electrophoresis can be coupled with a densitometer for identification and quantification of the separated proteins and has several benefits over PAGE. The most important are: (a) this method uses a non-toxic membrane for separation, (b) little time is required for the staining and de-staining procedure and (c) it is very effective for immuno-assays because electroblotting is not required, unlike in PAGE. The main disadvantage of using SPEP is the relatively poor separation efficiency of the method compared to PAGE.

1.2 Inductively coupled plasma-mass spectrometry (ICP-MS)

Inductively Coupled Plasma Mass Spectrometry was introduced in early 1980s and since then the large number of the publications clearly indicate its high potential in a wide range of sample analyses. Its high sensitivity and element specificity allow the detection of ultra-trace amount of analytes. It can detect the most of the elements in the periodic table including multiple elemental analysis and isotopic ratios. ICP-MS based methods are highly sensitive, selective and exhibit a large linear range [8, 9]. The high interfacing capabilities with the other detection methodologies such as laser ablation (LA) and separation techniques such as HPLC and GE make ICP-MS a versatile tool of unique quality. The major characteristics of the ICP-MS involve:

- Excellent detection limit (pg g^{-1} to ng g^{-1}) for most elements
- Minimal matrix effect
- Linear dynamic range (capability of up to 8 orders of magnitude)
- Very high analytical throughput
- Very easy coupling with the separation techniques such as HPLC and GE.

The major drawback of this technique is the interferences from the polyatomic ions formed from the atmospheric gases and argon in the plasma.

1.2.1 Instrumentation

A schematic diagram of ICP-MS instrument is shown in Fig. 1.2.

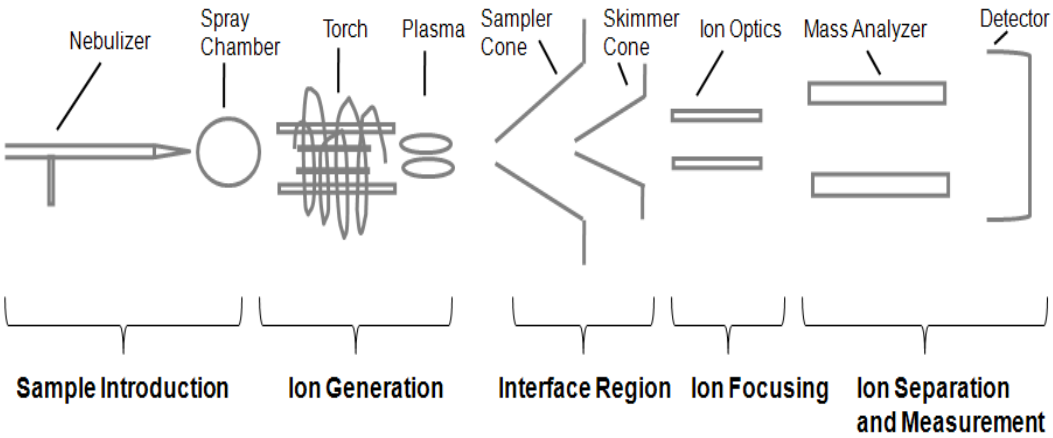


Figure 1.2: A schematic diagram of ICP-MS instrument

1.2.1.1 Sample introduction

The sample introduction system is the most important and major component of the ICP-MS instrument. One of the main functions of the sample introduction system is to convert the liquid sample into aerosols and to transport these aerosols to the plasma. A well designed sample introduction system does not allow the large droplets to enter into the plasma which may destabilize the plasma. The sample is introduced into plasma using the nebulizer, spray chamber and torch.

Normally the sample is introduced to the ICP-MS as an aerosol. To generate these aerosols liquid samples pass through pneumatic nebulizers, large droplets are removed in a spray chamber and only smaller droplets swept towards the plasma using a glass torch where the sample particles are vaporized, atomized and ionized before passing into mass spectrometer.

Sample can be introduced into the ICP-MS using two different methods: (a) Self aspiration and (b) peristaltic or HPLC pump method. In self-aspiration method sample can be nebulised to the ICP-MS without using the pump. For this purpose argon is used to sweep the sample through the nebulizer into the spray chamber. This self-aspiration process is usually used when an auto-sampler is employed for direct analysis of solutions. The HPLC or peristaltic pump is employed when the separation techniques such as GE or HPLC are interfaced with ICP-MS. ICP-MS nebulizers and spray chambers are commercially available with the various flow rates.

Nebulizers

The large droplets of the sample destabilize the plasma and play an important role in mass drifting, so prior to introducing the sample into the ICP-MS instrument, conversion of the liquid sample into fine uniform droplets or aerosols is a necessary requirement for ICP-MS based analysis. Thus commercially available well designed nebulizers are employed to perform this job. The selection of the nebulizer is based on several factors including flow rate required, type of sample and type of pumping to the ICP-MS. Very small volumes may require a low flow rate to provide the long enough time for aspiration. Likewise, some nebulizers are good for the semiconductor application (e.g. PFA microflow) where the high sensitivity is required but for the environmental type

analysis high efficiency nebulizers (e.g. Babbington-type) are preferred. The type of pumping used in the ICP-MS is also very important as several nebulizers cannot perform the self aspiration process and are especially designed for the peristaltic or HPLC pump.

Spray chambers

The liquid sample is sprayed into the spray chamber with the help of the nebulizer where the large droplets are removed and the aerosol of the smaller droplets is swept to the centre of the plasma.

Torch

A well designed torch plays a very important role in diffusing the sample aerosol in the central channel of the plasma. An injector with a large internal diameter (2.5 mm) reduces the aerosols movement and further helps to improve the aerosol's diffusing into the plasma by increasing the plasma loading which provides a proper decomposition of the sample matrix. However, samples containing organic solvents require low internal diameter injectors (e.g. 1-1.5 mm) because organic solvents destabilize the plasma, thus reducing the diameter size reduces the plasma loading and improves its stability. It is important to note that a large injector internal diameter of the injector although, increases the matrix decomposition but decreases the sensitivity, likewise a low internal diameter decreases the matrix decomposition but increases the sensitivity of the system.

1.2.1.2 Ion generation in the ICP

Usually, the stream of the Argon (Ar) generates plasma in a quartz torch which is located into the centre of the copper coil through which a powerful electric current is passed. For plasma generation argon gas, a torch, radio frequency (RF) coil and RF generator are required. The RF generator (operated at 1100-1500W) provides RF power to coil which produces an oscillating current in the coil and in result an electromagnetic field is generated. Argon gas is then ionized through the high voltage spark which is produced from the torch. This releases electrons which are then accelerated in the magnetic field and collide with argon gas which generate further

ions and electrons, until a stable high temperature (10, 000 K) plasma is formed. When sample aerosols are entered into the plasma, at this high temperature, they are quickly dried, decomposed, vaporized, atomized and finally generate positively charged ions by removal of the electrons from each atom.

Plasma

In ICP-MS system, the plasma is used to produce the positively charged ions from the sample aerosols. This is achieved by the removal of a single electron which depends on the ionization potential values of the elements. The first ionization potential (I.P) is defined as “the energy required removing a single electron from the neutral atom”. The very high temperature of the plasma ensures that the sample matrix has been fully decomposed and all the analytes have been ionized whilst retaining the good sensitivity and stability. Efficient decomposition of the matrix prevents the deposition of contaminants on the interface surface and a well optimized plasma at high temperature provides excellent sensitivity of the elements such as Hg, Be and As.

1.2.1.3 Interface between plasma and vacuum

The region between plasma and vacuum is known as the interface. In this region two metal plates (made of Pt or Ni), with precisely drilled orifices, have been placed to transfer the positively charged ions from plasma to higher vacuum region where the ion focussing lenses, mass analyzer and detectors are located. The first cone with the large diameter of the plate is called the sampler cone whilst second one with smaller diameter is referred to as the skimmer cone. Both cones play a very important role in sensitivity, mass response, oxide and doubly charged ion formation and robustness to matrix samples.

The smaller orifices sizes of both cones (normally, 1 mm for sampler and 0.44 mm for skimmer) help prevent large amounts of the un-dissociated sample particles entering the vacuum.

1.2.1.4 Vacuum

High levels of gas molecules may cause a high background signal and scattering of the charged particles, thus to reduce these effects, the high vacuum system is a necessary requirement for the mass spectrometry analyzing system. In order to create a high vacuum in the interface, intermediate and analyzer regions, a commercial ICP-MS instrument uses a 3 stage pump system. A rotary pump is used to create the vacuum in the interface stage. This pump is switched off when ICP-MS is on the standby position which helps to perform the maintenance of the interface cones and ion lenses. To build up the high vacuum in the intermediate and analyzer region two turbo molecular pumps or two stage single pumps are used. A backing rotary pump is also used to remove the exhaust from the turbo pumps. To minimize the starting time these two turbo pumps do not switch off even in the standby position of the ICP-MS instrument, however the intermediate and analyzer vacuum regions are separated from the interface region using a gate valve.

1.2.1.5 Ion focussing

A compact ion beam passes through the vacuum via electrostatic lenses which keep the ion focussed until they reach to the mass spectrometer. These electrostatic lenses also separate the ions from the photons and neutral particles which would otherwise cause the background signals.

Collision /Reaction Cell (CRC)

CRC is a device which has been used in ICP-MS instruments since the 1990's to remove polyatomic interferences. This CRC is located in the intermediate region of ICP-MS instrument after the focussing lenses. In this device a gas interacts with the ion beams in one of the two different ways:

- Reaction Mode: Gas reacts with the interfering ions and converts them into different species.
- Collision Mode: In this mode, polyatomic interferences ions can be removed by collision of the gas molecules which decrease the interference ions energies. Interference ions undergo more collisions because they are large in size and

thus lose more energy. The lower energy interference ions are finally separated by energy difference.

1.2.1.6 Mass spectrometer

In ICP-MS, Mass spectrometer separate and detect the ions based on mass to charge ratio (m/z). It is composed of mass analyzer and detector. There are three types of mass analyzers have been used; these are quadrupole, time of flight (TOF) and magnetic sector analyzers..

After the separation these ions reach the detector. The commonly used detectors are electron multiplier (EM) for the low dynamic range and Faraday cup for the extended dynamic range.

Mass Analyzers

The most commonly used analyzers in ICP-MS is the quadrupole analyzer due to its ease of use, low cost, high scanning speed and mass range, however several other analyzers have also been used such as magnetic sector and time of flight (TOF). Of all of these above mentioned analyzers, magnetic sector offers the best sensitivity and can separate most polyatomic interferences by providing a resolution M/ΔM of 10,000 and thus does not require a CRC. The quadrupole analyzer provides a resolution of 400 and needs to be coupled with CRC to remove the polyatomic interferences. TOF also offers high speed scanning but its sensitivity is low and it cannot separate the interference ions.

1.2.1.7 Detector

In an ICP-MS instrument the detector used is called “electron multiplier”. The sensitivity of this detector is very high because it can generate a measurable signal pulses from a single ion, however it is necessary that the ions arriving at the detector should be distinguished from the background noise arising from vacuum, mass spectrometer or electronics.

Principles of an Electron Multiplier Detector

When a positive ion reaches the detector it deflects towards the negative charged dynode. This ion impact releases several free electrons from the dynode surface and each electron because of the negative charge repel and strikes with the next dynode and further produces several electrons. Finally, electron cascade reaches to the final dynode, where a multiplication factor builds up a pulse which is measured as a count. These electron multiplier detectors have a wide dynamic range and can detect the analytes at very low concentration (e.g. pg/L) to a very high concentration (e.g. 100 x mg/L).

Count and Analog Mode

An electron multiplier detector requires to be switched into different modes for low to high concentration of the analytes. An ion counting detector is not suitable for very high concentration as it can be saturated and fail to detect some of the ions and show non-linear response. The dynamic range in an ICP-MS system works in “dual mode” to overcome this problem. When the detector receives lower counts per second (e.g. few millions counts) it works with a count mode but when it starts to receive bulk of the ions usually at very high concentration it switches into analog mode. An analog mode detector measures the current which is generated by the electron stream, rather than the pulses, which derives from the individual ion impact. A problem encountered sometime, when measurement of low and high concentration analytes is required in one analyses, especially where short duration peaks are required to be measured such as in chromatography and laser ablation, is the poor response time of the analog mode which results in compromised scan speed. A detector with poor electronics spends several milliseconds in an analog dwell time as compared to dwell time of the counting mode (0.1ms). However, a well-designed detector spends short dwell time for both of the counting and analog modes and overcomes the problem.

1.2.2 Handling of the data

1.2.2.1 Acquisition and interpretation of the data

Two most common ways are usually employed for data acquisition in ICP-MS.

(a) Time resolved analysis (TRA)

If the signal of interest for one or more elements changes with time then TRA method is used. TRA is applied in chromatography and laser ablation coupling with ICP-MS.

(b) Isotopic ratio measurement

This is usually employed for isotopic analysis and isotopic dilution analysis. In Isotopic dilution analysis (IDA) an unknown sample is spiked with a know amount of isotope and elements can be quantified based on change in the ratio.

1.2.3 Calibration and quantification methods

Using an ICP-MS instrument the elements can be quantified by adapting one of the following methods:

1.2.3.1 External calibration with or without internal standard

This is the most commonly employed quantification method. In this method normally the signals of 5-6 standards of known concentration are measured and because the signal intensity in ICP-MS shows a linear response to the concentration of the analytes hence a calibration lines is drawn. Finally signals of the analyte elements in the unknown samples are measured and with the help of the calibration their concentrations are determined. Factors such as variation in the composition of the standard, nebulisation efficiency and changes in sample transportation lead to errors which can be corrected using an element of similar characteristics as an internal standard. If an internal standard is used then normally the ratio of the analyte to the internal standard is used for the calibration.

1.2.3.2 Standard addition method

It is usually employed when the high purity material need to be analyzed such as semiconductors. This method is almost the same as external calibration method but the only difference is that in this method the unknown samples are spiked with external

standards with different concentrations which give the calibration response vs. added concentrations rather than the absolute concentrations.

1.2.3.3 Semi quantitative analysis

ICP-MS has been used widely for semi quantitative or relative quantitative analysis. In this type of quantitation an estimation of the concentration of the unknown elements are determined using the calibration curve of the known concentrations of the reference element.

1.2.3.4 Isotope ratio measurement

This type of the quantitation involves the determination of the relative concentration of two or more isotopes of the same element. This is usually performed for those elements whose isotopic composition varies in nature. This applies for the IDA as well and depends on the accurate isotopic ratio determination in a sample after addition of a purified spike of one of the isotopes of the analytes.

1.2.4 Problems associated with ICP-MS analyses

The two major problems associated with ICP-MS analysis are the spectral interferences and type of the solvent used.

1.2.4.1 Spectral interferences

Although the magnetic sector and quadrupole ICP-MS instruments are very powerful techniques for multi-element, they still face several spectral interferences. For example:

- Isobaric interferences: They are formed from the direct overlap of the isotopes of the similar masses such as ^{114}Sn and ^{114}Cd
- Polyatomic interferences: The combination of the species extracted from the plasma gas, sample solvent or sample matrix generate polyatomic interferences, e.g. $^{40}Ca^{16}O$ overlap on ^{56}Fe.
- Doubly charged interferences: Sometime plasma removes more than one electron from the analytical species and because analyzer separate analytes

based on m/z thus doubly charged ions appear at m/2 and overlap singly charged ions, e.g. $^{136}Ba^{+2}$ with $^{68}Zn^{+}$.

Corrections of the spectral interferences

The spectral interferences can be corrected in several ways:

Selection of an interference free isotope

Isobaric interferences can be avoided by choosing another interference free isotope of the same element. For example if ^{114}Cd is interfered by ^{114}Sn then ^{111}Cd can be selected for analysis. However, by adapting this way the detection limit can be compromised because ^{111}Cd isotope has low abundance (12.8%) than ^{114}Cd (28.73%).

Proper tuning and plasma conditions

One way of removing the polyatomic and doubly charged interferences is the proper tuning of the plasma and torch. Comparing doubly and polyatomic interferences the latter are more problematic because there are only a few elements which are ionized as the doubly charged ions. Polyatomic interferences are mostly based on the formation of oxides (MO^{+}) with the species generated from the plasma, sample solvent or sample matrix. Some of the polyatomic interferences for P and S are $^{14}N^{16}O^{1}H^{+}$, $^{15}N^{16}O^{+}$, $^{14}N^{17}O^{+}$, $^{13}C^{18}O^{+}$, $^{12}C^{18}O^{1}H^{+}$ and $^{16}O_{2}^{+}$, $^{14}N^{18}O^{+}$, $^{14}N^{18}O^{1}H^{+}$, $^{15}N^{17}O^{+}$, $^{15}N^{16}O^{1}H^{+}$ respectively. The efficient setting of the plasma with very high temperature decomposes these interferences and thus MO^{+} level significantly reduce.

Collision reaction cell (CRC)

As described in above paragraph collision reaction cell is a device used to remove polyatomic interferences. This device is used in quadrupole and TOF ICP-MS instrument because these two analyzers cannot remove these polyatomic interferences. To avoid the polyatomic interferences some of the major reactions in CRC are described below:

Hydrogen atom transfer

$Ar^+ + H_2 = ArH^+ + H$

Charge transfer

$O_2^+ + Xe = Xe^+ + O_2$

Proton transfer

$ArH^+ + H_2 = H_3^+ + Ar$

Condensation

$CeO^+ + O_2 = CeO_2^+ + O$

Association

$Zr^+ + nO_2 = ZrO_n^+$

Collision dissociation

$Ar_2^+ + He = Ar + Ar^+ + He$

Magnetic sector analyzer

Magnetic sector analyzers are well suited for corrections of the polyatomic interferences due to their high resolution, as noted in section 1.2.1.6.

Cool plasma

This is another way of decreasing the polyatomic interferences. The ICP-MS works with two plasma conditions one is called hot plasma, and second is called cool plasma. Analysing the sample through ICP-MS requires hot plasma (very high temperature). This hot plasma achieve at about 1100-1500 W forward power. The cool plasma conditions are achieved by decreasing the forward power down to 800 W. This reducing the plasma temperature helps to overcome polyatomic interferences problem, however, at low temperature sample is susceptible for matrix effect especially when HPLC or GE is coupled with ICP-MS. For example HPLC organic solvents may cause the signal suppression at low temperature.

1.3 Online Coupling of gel electrophoresis and inductively coupled plasma-mass spectrometry (GE-ICP-MS)

1.3.1 Introduction

The rapid development of ICP-MS based techniques has opened new and exciting opportunities for the characterization of metal and non-metal associated bio-molecules [10]. However, there is still relatively little information about the role of the elements in biochemical processes which has prompted the development of the new field of metallomics. The combination of suitable separation techniques with ICP-MS detection has been a focus of research in recent years and several on-line and off-line techniques have been used for the separation and detection of bio-molecules such as immobilized metal affinity chromatography (IMAC) [11], reversed phase chromatography [12], size exclusion chromatography (SEC) [13], and capillary zone electrophoresis (CZE) [14] etc. However, for macromolecules, gel electrophoresis (GE) is still widely considered to be the definitive method of separation [15]. The combination of GE with detection methods such as: ICP-MS (GE-ICP-MS) and Laser Ablation (LA) ICP-MS (GE-LA-ICP-MS) [16-21] has enabled gel-based methodologies to obtain more detailed information about the elemental composition of bio-molecules. The recently introduced on-line coupling of GE and ICP-MS is a very effective way of obtaining such information. It was first described by Brüchert and Bettmer in 2005 [22] for the determination of double stranded DNA fragments. More recently, it has been reported for several applications including: size characterization of gold nano particles [23], detection of iron in metalloproteins [24], determination of the degree of phosphorylation in casein [25], iodide and iodate determination in aerosols [26], detection of phosphorus in phosphoproteins [27], cisplatin-oligonuleotide interaction [28] and for the detection and quantification of phosphorus in plasmid DNA [29].

Coupled GE-ICP-MS enjoys a some benefits over the conventional reverse phase (RP)-LC-ICP-MS technique [27] such as, the cost of the gel is much cheaper than the cost of the column used in LC-ICP-MS and is readily replaceable in case of damage; the flow rate is flexible (μL min^{-1} to mL min^{-1}) and conventional nebulizers are usually satisfactory; the non-volatile buffers used are more tolerable than the volatile buffers used in most RP-HPLC-ICP-MS separations and a wider range of sizes of bio-molecules can be analyzed.

In micro (μ)-LC-ICP-MS and capillary electrophoresis (CE)-ICP-MS, low flow rates limit the choice of the nebulizer and spray chamber; miniaturization may reduce robustness of the system [30]; μ-LC may not be very useful in analyzing large bio-molecules and limited sensitivity and poor migration time are considered the major disadvantages of

CE-ICP-MS [31]; similarly in some cases interaction of the analytes with the capillary wall may cause sample loss in CE [31]. However, in µ-LC and CE smaller ID columns require low flow rates (µL min^{-1} or nL min^{-1}) which do provide positive environmental and economic benefits. The combination of CE with an ICP-MS detector potentially offers a tool of unique quality and enables both charged and neutral analytes to be separated in a single run [32-33]. Thus, a range of metalloproteins analysis was reported using CE-ICP-MS [31], however, this trend has now been decreasing slowly for the last few years and it has recently been more popular for non-metalloproteins analysis [31].

GE-ICP-MS also has some apparent advantages over SEC-ICP-MS. The commercially available SEC columns are very expensive and separating media are mostly made up of Sepharose or Superdex which cannot tolerate very high pressure whilst in GE-ICP-MS elution buffer is taken from a separate buffer reservoir or an embedded capillary inside the gel so high flow rate can easily pass through and does not damage the gel. To prevent drying out these columns also need some extra care. The most important disadvantage of SEC-ICP-MS is the use of very high buffer concentration in mobile phase which may increase the level of contaminants. In the majority of SEC based analyses (such as serum proteins analysis) the best separation achieved by employing phosphate buffers which limit the detection of P in ICP-MS analysis.

LA-ICP-MS is a powerful way of detecting analytes on solid substrates, however when we compare GE-ICP-MS with GE-LA-ICP-MS then GE-ICP-MS has several benefits over GE-LA-ICP-MS. The clear cut benefit of former is that it is a single step analysis, but latter requires two stages i.e., separation and detection. If electroblotting is required to transfer the proteins onto the membrane before ablation then this may cause the loss of the analytes. Curling of the gel during LA analysis is also a severe issue in GE-LA-ICP-MS normally requiring transfer of the analytes on a membrane which may cause further sample loss. Moreover, poor detection limit, loss of the weakly bound metals and staining and de-staining of the analytes (in some cases) further drop its efficacy in comparison with GE-ICP-MS.

On the other hand, GE-ICP-MS also has several limitations and requires further work to overcome the problems. These limitations include: broader peaks; possibility of fragmentation of the proteins in the gel [27]; contamination from the gel materials [27]

and a time consuming separation in comparison with CE [34] and μ-LC [35]. For metalloproteins analysis by GE-ICP-MS, native-polyacrylamide gel electrophoresis (native-PAGE) is preferred to SDS-PAGE as significant loss of non-covalently bound metals has been reported in SDS-PAGE [19, 36-38].

1.3.2 Coupled on-line GE-ICP-MS

As described in the above paragraphs, gel electrophoresis is a unique and powerful way of separating large molecules, and its online coupling with ICP-MS detection has opened the doors of huge opportunities to get more detailed information about bio-molecules. For example: the exact number of the tryptically digested phosphorylated peptides can be obtained quickly after their separation in the gel [27]. There is very little chance of losing the sample and much less analyte is required (micro to nano grams), as compared to offline separation performed in the gel, where a significant quantity of analytes is required for detection using staining procedures.

1.3.3 Some important components of the GE-ICP-MS system

1.3.3.1 Power supply for GE

Electrical power for the separation is usually supplied by using one of the commercially available power supplies such as: PowerPac™ HC, high current power supply system (Bio-Rad Laboratories, Hemel Hampstead, UK); PowerPac™ 3000, with high voltage supply (Bio-Rad Laboratories, Munich, Germany); Phero-stab 300 power supply (Biotec-Fischer, Reiskirchen, Germany); or 250/250 power supply system (Desaga, Heidelberg, Germany)

1.3.3.2 Separating medium

Separation of the molecules is usually performed by casting the gel into glass tubes of various sizes and internal diameters. These gels are made up of acrylamide or agarose at different percentages depending on the molecular sizes of the molecules being separated. The diameter of the tubes and percentage of the gels significantly affect the separation. Smaller diameter tube gels provide sharper peaks with better resolution of the analytes [27]. Similarly, high percentage gels increase the elution time and slow

down the analytes' migration to the ICP-MS and may cause broader peaks [27]. In SDS-PAGE a stacking gel is usually cast on the top of the separating gel.

1.3.3.3 Electrode buffer

For the separation of the analytes in the gel, cathode and anode buffers are necessary. These electrode buffers are mostly prepared using Tris in combination with any other suitable chemical such as glycine, tricine, boric acid etc. Although it is not necessary to have both buffers with different concentration and pH, in some cases they can be different for example the original tricine-SDS-PAGE method has 100 mM Tris-tricine and 0.1% (w/v) SDS with pH 8.25 at the cathode and 100 mM Tris with pH of 8.9 at the anode [4, 38-39].

1.3.3.4 Elution buffer

The elution buffer required to elute the analytes into the ICP-MS can be taken from the cathode or anode buffer chamber using a capillary connected directly with the ICP-MS nebulizer. However, it is equally possible to employ a separate buffer reservoir for this elution buffer.

1.3.3.5 Dialysis membrane

A dialysis membrane or a plug gel [24] is necessary to connect both electrode chambers in GE-ICP-MS. The dialysis membranes must have a lower molecular weight cut-off value than the molecular weight of the separating molecules, otherwise these molecules will pass through the membrane and will go into the lower buffer chamber instead of the ICP-MS. However, in some cases the molecules which are passed through the dialysis membrane can be sent to the ICP-MS using elution buffer [22]. These membranes are mostly made up of cellulose ester and are commercially available in a wide range of MW cut-off values. To obtain a high recovery of the separating molecules the size of the analytes should be at least double the cut-off sizes of the membranes.

1.3.3.6 Connecting tubings

PEEK (polyetheretherketone) or Teflon tubes are typically used to connect the GE system with ICP-MS. In most cases one end of this tubing system connects with the elution buffer and the other with the ICP-MS nebulizer. The internal diameter of these tubings depends on the flow rate required. When electrode buffer is used as an elution buffer then a small diameter (e.g. 1-2 mm) glass tube is set inside the gel for the continuous flow of the electrode/elution buffer [27].

1.3.3.7 ICP-MS nebulizers and spray chamber

Many different nebulizers, with various flow rates, are commercially available to nebulise the samples into the ICP. Various nebulizers and spray chambers are listed in Table 1.1. In contrast with the µLC-ICP-MS and CE-ICP-MS, the GE-ICP-MS system imposes minimal restriction on nebulizer and spray chamber type and the selection is largely based on the flow rate.

1.3.4 Instrumental configuration

In GE-ICP-MS coupling, when self-aspiration is used, the gel electrophoretic apparatus is connected directly with the nebulizer of the ICP-MS using a smaller diameter tube (e.g. 0.05-0.5 mm) but if a peristaltic pump is required then it connects with the ICP-MS nebulizer via peristaltic pump tubings.

The gel tube, containing the gel for separation, is usually mounted between two buffer reservoirs, one acting as a cathode and the second acting as an anode. If the analytes are separated on the gel from cathode to anode then it is called anodal GE or if they are separated from anode to cathode then it is called cathodal GE [24]. An elution buffer is required to elute the analytes into the ICP-MS. This elution buffer may be one of the electrode buffers or a separate buffer reservoir can be employed. A peristaltic pump or self-aspiration methods can be used to transfer the analytes and buffers to the ICP-MS. Use of the peristaltic pump is sometimes necessary, especially if a detergent such as SDS is used, because SDS generates bubbles which may hinder the self-aspiration. However, if possible self-aspiration should be adopted because reduced transport tube volume provides well resolved and better peak shapes than when a peristaltic pump is used [27].

Table 1.1: Some of the commercially available widely used nebulizers and spray chambers

Nebulizers

- PFA micro-flow nebulizer (Elemental Scientific, Omaha, USA) with flow rate range of 20 to 400 µL min^{-1}
- PFA-ST nebulizer (Elemental Scientific, Omaha, USA) with self-aspiration rate of 20 to 700 µL min^{-1}
- Micromist nebulizer (Glass Expansion, Victoria, Australia), with flow rate range of 0.05 to 0.4 mL min^{-1}
- U5000AT^{+} ultrasonic nebulizer (CETAC Technologies, Omaha, Nebraska, USA) with uptake rate of 0.5 to 2.5 mL min^{-1}
- Aspire^{TM} PFA Microconcentric Nebulizer (CETAC Technologies, Omaha, Nebraska, USA) with flow rate of 50 to 400 µL min^{-1}
- DS-5 Microflow concentric nebulizer (CETAC Technologies, Omaha, Nebraska, USA) for the flow of 3 to 10 µL min^{-1}

Spray Chambers

- Quartz and PFA cyclonic spray chamber (Elemental Scientific Omaha, USA) with higher aerosol transport efficiency.
- Scott type spray chamber (Glass Expansion Victoria, Australia)
- Cinnabar spray chamber with helix (Glass Expansion, Victoria, Australia)
- Tracey PFA44 spray chamber with helix (Glass Expansion, Victoria, Australia)
- Stable sample introduction (SSI) dual Quartz cyclonic spray chamber (Elemental Scientifc, Omaha, USA)
- Laser ablation dual quartz cyclonic spray chamber (Elemental Scientific, Omaha, USA)

A schematic diagram of GE-ICP-MS is shown in Fig. 1.3 with: (a) using the same buffer reservoir (Fig. 1.3a), and (b) using a separate buffer reservoir (Fig. 1.3b). In (a), the elution buffer is one of the electrode buffers. In (b) an additional buffer reservoir is placed in the system to elute the analytes to the ICP-MS instrument. Both instrumental methodologies are useful, however, set-up (a) has a few advantages over set-up (b) such as: most of the commercially available GE instruments contain only two reservoirs i.e., cathode and anode, hence there must be a detailed modification to use a separate buffer reservoir; pumping the buffer from a separate buffer reservoir builds up the pressure and thus displacement of the dialysis membrane may occur [27]. One disadvantage of using the same buffer reservoir system is that ICP-MS does not tolerate high buffer concentrations hence low buffer concentrations are required at the cathode or anode which may adversely affect the separation, however the modified method provides a very good separation at very low concentration of buffers used [3]. i.e., 25 mM Tris-tricine and 0.5% SDS. The low concentration of the buffers is also very helpful in decreasing the background signals of the analyte by increasing the sensitivity. As shown in Fig. 1.4 that ICP-MS signals of ^{31}P can be decreased almost 3 times by using the modified buffer system. Thus modified tricine-SDS-PAGE method described in chapter 3 is perfectly suited for GE-ICP-MS analysis of proteins.

1.3.5 Problems associated with GE-ICP-MS online coupling

GE-ICP-MS is not without its problems and further work has been reported in the literature to address a number of issues, as described below.

1.3.5.1 Gel material

Gels made using polyacrylamide or agarose contain several potential contaminants and it is necessary to remove these from the gels before running the samples. Thus the suggested procedure is the electrophoretic washing of the blank gel prior to running the mixture of analytes [25]. However, longer washing may disturb the sieving properties of the gel, especially in PAGE and this may result in poorer resolution of analytes and hence fewer peaks might be observed than expected. To resolve this problem use of a low percentage gel of smaller diameter and shorter length is recommended because this reduces both the level of the contaminants and the washing time.

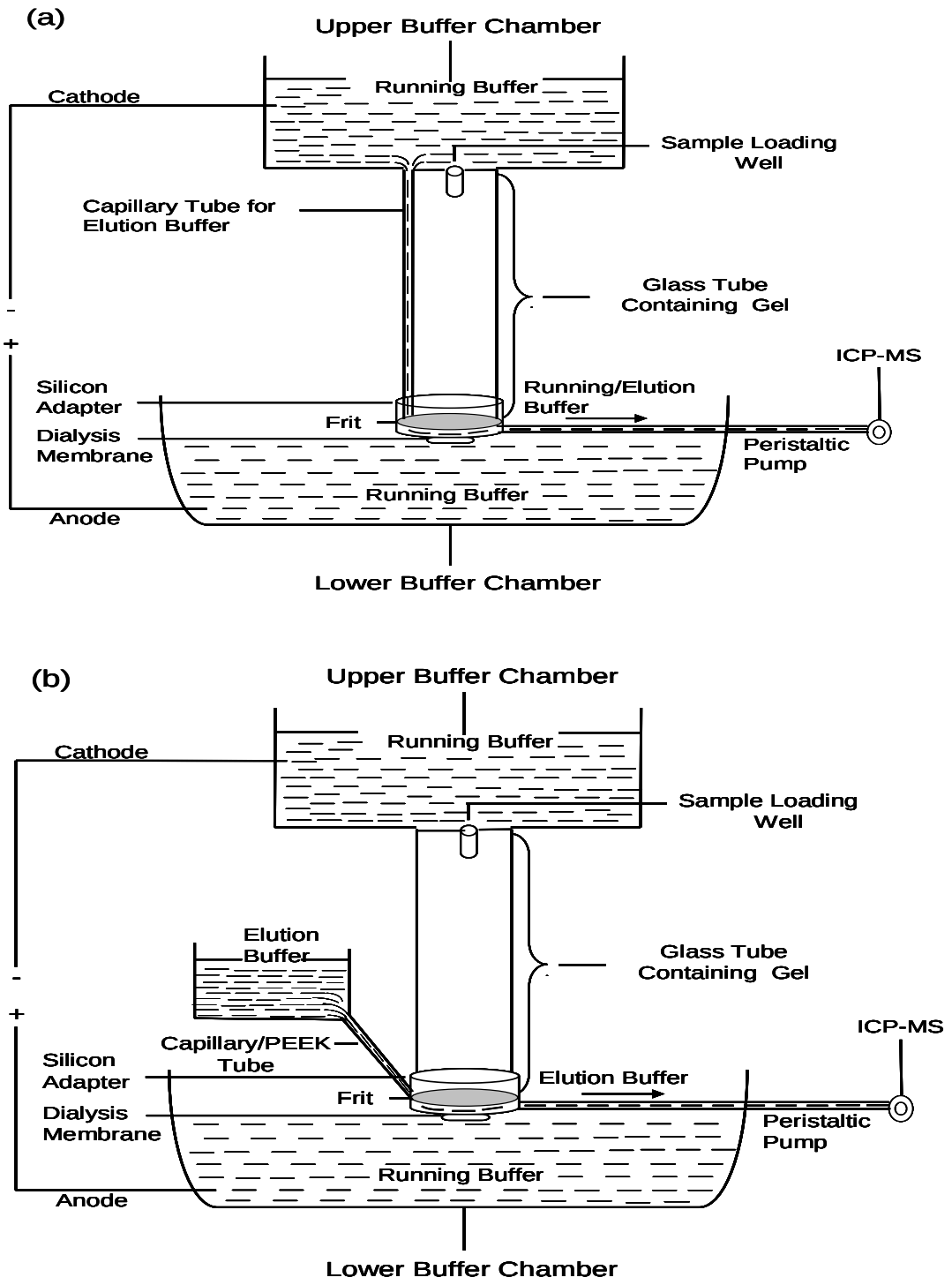


Figure 1.3: A typical Instrumentation of GE-ICP-MS online system, (a) shows same buffer reservoir (b) different buffer reservoir for the elution of the analyte on ICP-MS. (Note: Dialysis membrane can be replaced by a plug gel in some cases).

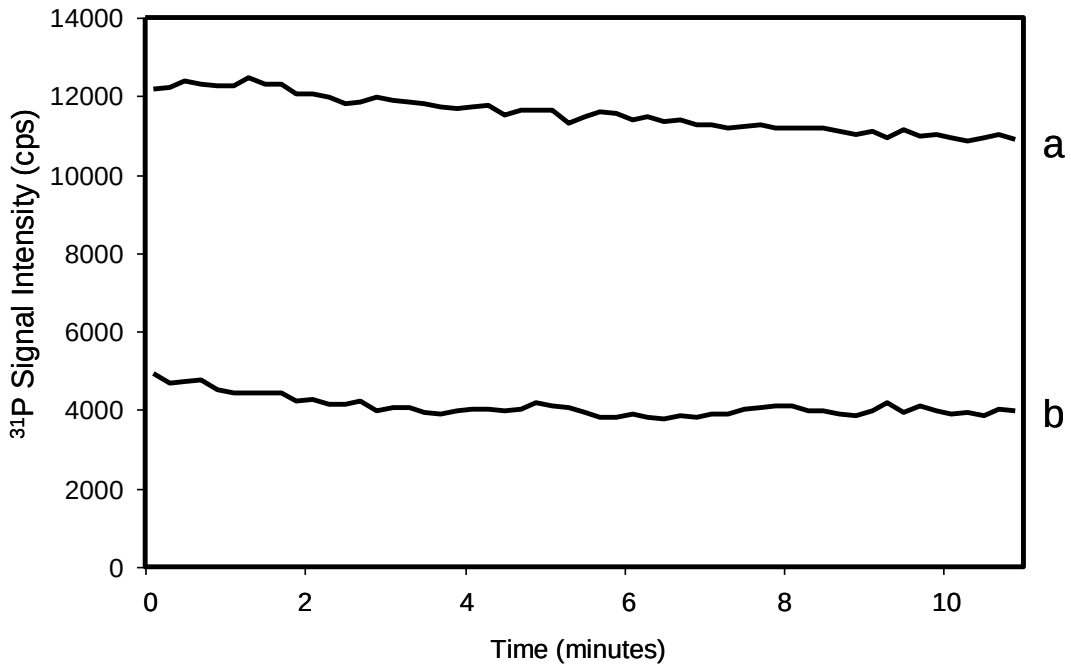


Fig. 1.4 Comparison of the buffer background signals: (a) original Tris-tricine buffer, 100 mM Tris-tricine and (b) modified Tris-tricine buffer, 25 mM Tris-tricine. The ^{31}P background signal intensity in modified system was approximately 3 times lower than the original system

1.3.5.2 Choice of the buffers

An important issue in LC-ICP-MS hyphenated techniques is the optimization of the composition and flow rate of the mobile phase to the ICP-MS because ICP-MS does not tolerate high buffer concentrations.

Organic buffers, which contain a high concentration of carbon have several adverse effects on ICP-MS such as: (a) a build up of carbon deposition leads to clogging of the interface cones and possibly the torch injector and, as a result, decreases the sensitivity of the ICP-MS instrument [40-41]; (b) a high level of carbon generates a number of polyatomic carbon based interferences e.g., C_2^{2+}, CNH^+, CO_2^+ and $^{38}Ar^{13}C^+$ which interfere with Mg^+, Al^+, Ca^+ and V^+ respectively and (c) volatile buffers increase vapour loading and cause destabilization of the plasma. In order to use a high carbon concentration, additional optimization is necessary and for example the addition of oxygen to the nebulizer gas [40-41]. However, the addition of oxygen gas in the nebulizer flow may reduce cone life [10]. Consequently, use of a minimum concentration of non-volatile buffer is recommended in LC-ICP-MS analysis. Lobinski

et al. [42] reported that up to 30 mM Tris-HCl is well tolerated by ICP-MS. However, using a very low concentration of the running buffer in GE significantly affects the separation efficiency of the gel and increases the elution time. To reduce the elution time very high currents need to be applied, but this may produce several negative effects such as an increased temperature in the system which can change the pH, fragmentation of the separated proteins, and worsening of the detection limit by increasing the release of contaminants which start to elute from the gel material at elevated temperature [27, 43]. Similarly, using high buffer concentrations at the electrodes, or in the elution buffer, increases the background signal intensity and also worsens detection limits. For example: tricine-SDS-PAGE traditionally requires 100 mM Tris, 100 mM tricine and 0.1% (w/v) SDS at the cathode and 100 mM Tris only at the anode [4, 38-39].

1.3.6 Applications

Coupled GE-ICP-MS has been used for several applications, including:

- Determination of dsDNA fragments
- α- and β-casein separation
- Phosphopeptides separation
- Determination of iodide and iodate in aerosols
- Size characterization of gold nanoparticles
- Detection of iron in metalloproteins
- Study of oligonuleotide-cisplatin interaction

For detail about each of these applications, a review article entitled “online coupling of gel electrophoresis and inductively coupled plasma mass spectrometry” is attached in appendix section (Appendix 2). The parameters of each application are listed in Table 1.2

1.4 GE-LA-ICP-MS

LA-ICP-MS was introduced in 1985 [44]. Besides the advantages of analysing the samples though ICP-MS, LA does not require a time consuming sample preparation, it reduces the spectral interferences and contamination because of no dilution steps and no digestions [45]. LA-ICP-MS can be used for all kind of solid samples [45]. LA-ICP-MS use a high power laser to ablate the solid samples and pass sample aerosols to

ICP-MS using a carrier gas such as argon or helium. In most of the research an Nd:YAG nano second pulse laser is used [45-46]. Although, the LA-ICP-MS is an attractive technique but still calibration remains a challenge. Many calibration strategies have been proposed such as an external calibration using standard reference material, internal calibration with a specific element or a naturally existing element in the sample and calibration with the solution in which a dual flow system allows a simultaneous introduction of the ablation material and standard solutions. [44, 47].

The first use of GE-LA-ICP-MS, for the detection of the metal after separation of the proteins on the gel, was reported by Neilson *et al.* [48] and since then GE-LA-ICP-MS has been employed widely for the detection and quantification of metal and non-metal bound proteins using both one dimensional and two dimensional PAGE [16-18, 19, 36-37, 44-58]. In GE-LA-ICP-MS analyte can be directly detected on the gel using the laser beam without using any solution based mobile phase or electro-elution. This is also an offline coupling of gel electrophoresis with ICP-MS, however it requires two steps. First step is the separation and second detection via LA.

During the separation of proteins in PAGE some sample loss occurs if high percentage gel is employed to separate smaller proteins or peptides. These smaller proteins or peptides do not separate into the gel and jump out into the lower (cathode) buffer chamber. Similarly if very low percentage gel is employed for the separation of very high MW proteins then these high MW proteins shoot out in the upper (anode) buffer reservoir or remain in the stacking gel and do not migrate towards cathode. Similarly, if proteins are required to be transferred on the blotting membrane then some proteins loss occurs during the electroblotting. Thus in GE-LA-ICP-MS there are some chances of sample loss. However, in comparison with GE offline coupling with ICP-MS this strategy has little sample lost because former require to electro-elution of the proteins of interest after PAGE separation which may cause further sample loss.

Despite its importance GE suffers several problems when coupled with LA-ICP-MS such as it is very difficult to use because agarose gel melts much quicker and cannot tolerate the high energy used in LA process thus PAGE is mostly employed (because it is highly thermo stable and can tolerate the LA energy), but PAGE is not very useful for very large size proteins or DNA which reflects the limitation of GE-LA-ICP-MS technique. Similarly, curling of the polyacrylamide gel during the ablation process is

also an important issue which can be resolved either by transfer of the proteins on the membrane using the electroblotting which may cause sample loss or using the gel drying procedure which is a time consuming procedure. Moreover, poor detection limit, loss of the weakly bound metals and staining and de-staining of the analytes (in some cases) further drop its efficacy.

During the last few years GE-LA-ICP-MS has especially been focussed on speciation of the metalloproteins. For the separation of the analytes prior to LA detection only PAGE methods are used. However, the loss of the weakly bound metals in metal binding proteins during the SDS-PAGE separation and high background signals are the issues which still need to be resolved.

1.4.1 Applications

A selection of relevant papers is discussed below.

1.4.1.1 GE-LA-ICP-MS for phosphorus

GE-LA-ICP-MS has been used for the detection and quantification of ^{31}P on the gel, gel blot and filter papers [16-18, 49].

(a) The Determination of protein phosphorylation on electrophoresis gel blots by LA-ICP-MS

Marshall *et al.* [17] reported the GE-LA-ICP-MS for the detection of ^{31}P. The focus of the study was to determine the lower limit of detection for ^{31}P and it was reported 16 pmol using the gel blots. Briefly, original method of tricine-SDS-PAGE was employed for the separation of β-casein and myoglobin. The analytes were separated on 10-20% gradient PAGE gel. After the separation bands were transferred onto polyvinylidene difluoride (PVDF) using electroblotting procedure. The blots were stained using 0.1% Coomassie R250 and then destained. Finally, they were washed with deionized water and air dried. The UP213 Nd:YAG LA system (New Wave Research Ramsey, Cambridgeshire, UK) was employed for the analysis of the gel blots.

ICP-MS System	Nebulizer	Spray Chamber	Gel Material	Injection Volume	Elution Buffer	Flow Rate	Analyte(s)	Elements Detected	Internal Standard	Reference/ Year
Element 2, Thermo Scientific, Bremen, Germany operated at 1300 Watt	PFA μ-Flow, Elemental Scientific, Omaha, NE	Scott Type Glass Expansion Pocasset, MA	Agarose, Polyacrylamide	5-50-μL	0.09 mol L^{-1} TBE (pH 8.0), 10 μg L^{-1} Rh	100-μL min^{-1}	dsDNA fragments	^{31}P	^{103}Rh	22 / 2005
Element 2, Thermo Scientific, Bremen, Germany, operated at 1300 Watt	Micromist AHF, Feuerbacher Tubingen, Germany	Scott-Type AHF, Feuerbacher Tubingen, Germany	Agarose	2-5 μL	10 mmol L^{-1} Tris-borate. 5 mmol, NaCl (pH 9.1), 10 μg L^{-1} Rh	100-μL min^{-1}	α- and β-casein	^{31}P, ^{32}S	^{103}Rh	25 / 2007
Element 2XR, Thermo Scientific, Bremen, Germany, operated at 1300 Watt	PFA μ-Flow, Elemental Scientific, Omaha, NE	Cyclonic, Glass Expansion Victoria, Australia	Polyacrylamide	10-μL	25 mM Tris-tricine (pH 8.2), 0.05% (w/v) SDS	130-μL min^{-1}	mono and tetra phosphopeptides α- and β-casein	^{31}P,	-	27 /2010
Element 2, Thermo Scientific, Bremen, Germany, operated at 1300 Watt	Micro-Flow, AHF, Feuerbacher Tubingen, Germany	Scott-Type AHF, Feuerbacher Tubingen, Germany	Agarose	2-20-μL	0.05 mol L^{-1} boric-acid (pH 8.0), 10 μg L^{-1} Te	100-μL min^{-1}	iodide and iodate	^{127}I, ^{129}I	^{126}Te, ^{131}Xe	26 / 2007
ELAN 5000 (Perkin-Elmer SCIEX), Operated at 1300 Watt	Cross-Flow AHF, Feuerbacher Tubingen, Germany	Scott-Type AHF, Feuerbacher Tubingen, Germany	Agarose	1- μL	10 mmol L^{-1} SDS, 1 mmol L^{-1} NaH_2PO_4 1 mmol L^{-1} Na_2HPO_4, 10 μg g^{-1} Rh	300-μL min^{-1}	gold nanoparticles	^{197}Au	^{103}Rh,	23 / 2006
Element 2, Thermo Scientific Bremen, Germany, operated at 1300 Watt	Micromist AHF, Feuerbacher Tubingen, Germany	Cyclonic, Cinnabar Glass Expansion, Melbourne, Australia	Polyacrylamide	15 and 35-μL	0.02 mol L^{-1} Tris pH (8.0)	140-μL min^{-1}	transferrin, haemoglobin , ferritin, cytochrome C	^{32}S, ^{56}Fe	-	24 / 2007
Element 2 , Thermo Scientific Bremen, Germany, Operated at 1350 Watt	Concentric (CETAC, Omaha Nebraska, USA)	Not mentioned in original paper	Agarose	10-μL	50 mmol L^{-1} ammonium acetate	700-μL min^{-1}	cisplatinum-oligonucleotide	^{195}Pt ^{31}P,	^{103}Rh	28 / 2008
Element 2, Thermo Scientific Bremen, Germany, Operated at 1350 Watt	Concentric (CETAC, Omaha Nebraska, USA)	Not mentioned in original paper	Agarose	10-μL	50 mmol L^{-1} ammonium acetate	700-μL min^{-1}	plasmid DNA	^{31}P	^{103}Rh	29 /2011

Table 1.2: Parameters used in GE-ICP-MS online coupling

The concentrations of β-casein loaded on the gel were, 1.32 nmole, 440 pmole, 146 pmole, 48 pmole and 16 pmole. Thus 16 pmole was detected as a lower limit of detection. This study suffered from high ^{31}P background signals and authors suggested that a better detection limit could be achieved if further work be carried out to decrease the blank/backgrounds signals. Marshall et al. reported the high background signal and it is likely that these backgrounds signals are generated due to the use of high percentage 10-20% gels and use of the high concentration of the buffers. The research work described in Chapter 3 of this thesis is focussed on the development of much improved methods for the detection of phosphoproteins, to potentially alleviate such problems.

(b) Combination of gel electrophoresis and ICP mass spectrometry-novel strategy for phosphoproteins measurement

This study [16] was focussed mostly on investigating the source of ^{31}P background noise in GE-LA-ICP-MS. In preliminary investigation LA-ICP-MS was carried out on SDS-PAGE gel and filter paper but because of the high background signals authors investigated the source of the ^{31}P background. Thus after purifying the electroeluted or whole gel eluted (WGE) β-casein, using an acidified activated alumina column (particle size, 150μm, 3 cm x 3 mm ID), authors concluded the contamination comes from the gel materials and GE buffers and recommended not to interrogate the gels directly into the LA. However, it was not described how the ^{31}P background can be reduced during the LA process. Briefly, precast Novex Nu-SDS-PAGE (4-12%) gels with Bis-Tris running buffers were purchased from Invitrogen Corporation, UK. For WGE β-casein was run on native-PAGE using Tris-glycine running buffer. WGE elution was performed using an eletroeluter device. 14 fractions were harvested from WGE. These 14 fractions were washed through alumina column and spotted on the gel plate for LA. They were also analysed via flow injection-ICP-MS. For LA an Nd:YAG system (UP266) was employed (Macro New Wave Instruments, St. Neots, Cambridgeshire, UK). The LA was coupled with a quadrupole ICP-MS system (Perkin Elmer Elan DRC^{Plus}, Seer Green Bucks, UK).

1.4.1.2 GE-LA-ICP-MS for metalloproteins

Several publications reported the use of GE-LA-ICP-MS for the speciation of metalloproteins mostly from the biological samples [19, 36-38, 48, 50].

(c) Some Pitfalls in PAGE-LA-ICP-MS for quantitative elemental speciation

In this paper [36] some pitfalls due to metal losses are described. Two different metallo-proteins i.e., superoxide dismutase (SOD) (from bovine erythrocytes) and thyroglobulin with high affinity for metals were studied using native-PAGE-LA-ICP-MS and SDS-PAGE-LA-ICP-MS. Cu and Zn were detected in SOD after tricine SDS-PAGE separation of the proteins. Cu and Zn losses occurred after use of glycine as a running buffer. Thus tricine-SDS-PAGE could be preferred over tris-glycine method for the study of metals however still the quantitative study was not suggested by the authors. The authors further demonstrated that, as compared to tricine, glycine has high affinity towards Cu and Zn ions and they form Cu-glycine and Zn-glycine complexes even if metal is firmly bound with the proteins. Tris-tricine buffer is Thyroglobulin was employed only for the detection of iodine. An Nd:YAg LA system operating at 213 nm (UP-213 New Wave Research, Huntington, UK) was employed for this work. This LA system was coupled with an ICP-MS instrument (Elan 6000, Perkin Elmers SCIEX, Toronto, Canada).

1.5 Phosphoproteins

Phosphoproteins are those proteins which are covalently bound to phosphorus. In all the phosphoproteins so far investigated phosphorus has been found to be covalently bound only to serine, threonine, histidine, lysine, aspartic acid, glutamic acid and tyrosine residues. The structures of these phosphorylated amino acids are shown in Fig 1.5

Phosphoserine is by far the most commonly found protein-bound phosphorylated amino acid, though phosphothreonine is also frequently detected [59]. Tyrosine phosphorylation [60-63] is very rare however, it is very well understood because it can easily be modified and purified by using antibodies [64-65]. Histidine and aspartic acid

phosphorylation occurs in prokaryotes as a part of two component signalling and in some cases eukaryotes in some signal transduction pathways [66].

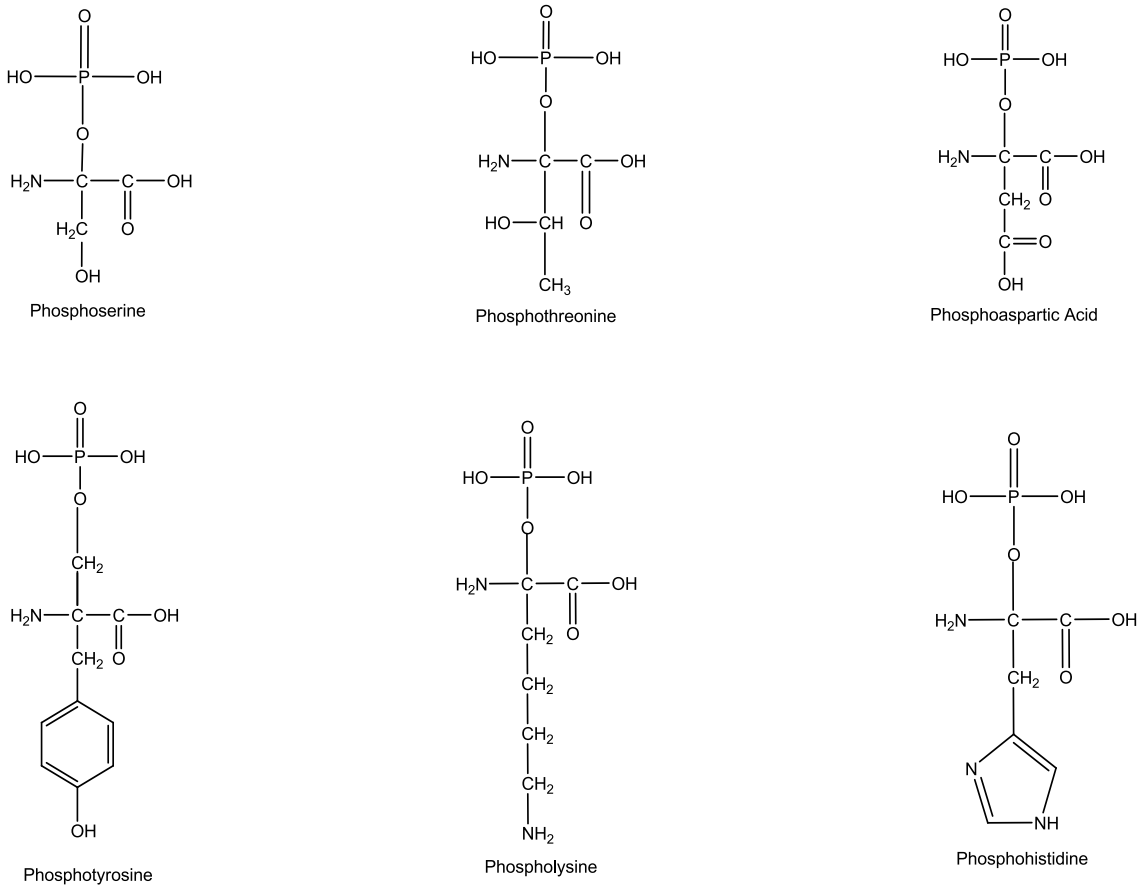


Figure 1.5: Structures of phosphorylated amino acids

1.5.1 Function of phosphoproteins

Post Translational Modification PTM is the process of the addition of groups such as sugar (glycosylation) or phosphate (phosphorylation) to proteins in a cell. Reversible protein phosphorylation is one of the most important PTM's that plays a very important role in regulatory mechanisms that occurs in both prokaryotic and eukaryotic organisms [67-70]. There are two enzymes involved in this process, the kinases, which are involved in phosphorylation and the phosphatases which are involved in dephosphorylation. These kinases and phosphatases constitute about 2% of the human genome [71-73]. Many enzymes and receptors are switched "on" or "off" by phosphorylation and dephosphorylation. Phosphorylation turns an R group, which is a

hydrophobic portion of a protein, into a hydrophilic portion because of the addition of phosphate group (PO_4^{-3}). In this way conformational changes occur in the structure of the protein via interaction with other hydrophobic and hydrophilic residues in the protein.

1.5.2 Phosphopeptides analysis

As described above (section 1.5.1) phosphorylation plays an important role in regulatory mechanism. Its perturbation affects a number of cellular processes and is associated with several neurodegenerative disorders such as Alzheimer's, Parkinson's and Huntington's diseases [74]. Therefore phosphopeptide analysis has attracted great interest towards phosphoproteome research in the biological and medical fields. Phosphopeptide analysis is not very straightforward because of the following reasons [75].

- Phosphorus covalently binds only with specific amino acids (as shown in Fig. 1.5) thus the ratio of the phosphopeptides as compared to the non-phosphorylated peptides is always smaller in the tryptically digested mixture of the peptides.
- Variable phosphorylation sites on protein
- Kinases and phosphatases are enzymes which are involved in phosphorylation and dephosphorylation of the amino acids sites thus precautions must be taken to inhibit their activity during preparation and purification of cell lysates.
- The enrichment methods such as IMAC, chemical modification immunoprecipitations etc have limitations.
- Phosphopeptides analyses have a limit of detection which means that major phosphorylated site could be analysed but minor sites might be difficult to identify.

1.5.3 Identification of phosphopeptides

The identification of phosphopeptides can be performed by using the following techniques.

1.5.3.1 Molecular mass spectrometry

Molecular mass spectrometry is one of the most common techniques which have been used for last few years for phosphopeptide identification. The two most common ways to achieve this are Matrix Assisted Laser Desorption Ionization (MALDI) or Electro Spray Ionization (ESI) mass spectrometry. Identification by using molecular mass spectrometry is based on the following strategy.

The most common strategy used for identification of phosphoproteins in complex mixtures involved tryptic digestion of the protein sample followed by on-line MS detection using electrospray in negative precursor ion or neutral loss scanning has been used to detect specific phosphopeptides [76].

In a complex mixture, the ionization of phosphopeptides is often suppressed by other non-phosphorylated peptides present during the ionization time window. The presence of m/z 63 (PO_2^-) and m/z 79 (PO_3^-) in negative precursor ion mode and the loss of 80 or 98 Da in positive neutral loss mode suggest the presence of peptides carrying the phosphorylated amino acids residues. Subsequent sequencing of the corresponding phosphorylated peptides by mass spectrometry enables the determination of the specific sites of phosphorylation. The other commonly used online method is the shotgun approach described by McCormack [77] that combines rapid scanning in the MS full-scan mode followed by data dependent MS/MS scans and post-acquisition data processing to identify phosphopeptides and localize the site of phosphorylation.

This method of identification is relatively simple and straightforward but unfortunately it is unable to identify all phosphopeptides because of the following reasons.

- The increased acidity of the phosphate group generally results in decreased ionization efficiencies of a peptide in positive ions mode [78].

- Phosphopeptides have low stoichiometry and there is a competition for ionization in a complex mixture so signal suppression occurs for phosphopeptides [78, 79].

However, signal suppression can be overcome by using a nano-electrospray ionization source. Nanospray holds a very small capillary emitter which can spray 1µl for up to 10-30 minutes which can maintain a constant signal during this time [79]. Another approach to overcome this problem is the use of HPLC coupled with MS (LC-MS) to reduce the salt level from the complex mixture and increase the concentration of the peptides before analysis.

1.5.3.2 Two- Dimensional Phosphopeptides Mapping

This technique for phosphopeptide identification is described by Boyle *et al.* [7]. In this method peptides are labelled by using radioactive isotopes after trypsinization and separated according to their isoelectric point (pI) in first dimension of Two-Dimensional Gel Electrophoresis (2D-GE). Afterwards spots are examined and phosphopeptides are detected under X-ray film.

Drawback of this method is that it is time consuming and very expensive because of the use of radioactive isotopes.

1.5.4 Enrichment techniques

During the last few years several methods have been proposed for phosphopeptides enrichment from the complex digest mixtures.

1.5.4.1 Immobilized metal affinity chromatography (IMAC)

Immobilized metal affinity chromatography IMAC has been used widely for the enrichment of phosphopeptides in the past either in off-line or on-line format followed by MS [80-82]. In this method the protein of interest forms a co-ordinate covalent bond with the immobilized metal ions such as Fe^{3+}, Cu^{2+}, Zn^{2+} and retains in the column. After removing the impurities, the protein of interest is finally eluted by changing the pH or by adding the imidazole. The ions Ga^{3+}, Zn^{2+}, or Fe^{3+} have been used in phosphopeptide enrichment because of their great tendency to attract

phosphopeptides [80-83]. Although this approach is useful, it does have several problems as described below.

Drawbacks

- This method is not absolutely specific because non-phosphopeptides, including those containing multiple acidic residues such as histidine and cysteine also elute with the phosphopeptides [84].
- This method works better for the enrichment of multiply phosphorylated peptides [85].
- Recovery of the phosphopeptides depends on the type of the metal ion used, column material and the elution procedure used.

Recently, this method has been enhanced by the methyl esterification of acidic residues prior to enrichment [86]. This methyl esterification increased the specificity but the other problems still remain.

1.5.4.2 Chemical modification

One more approach for enrichment of phosphorylated peptides is a chemical modification. Two methods have been reported for chemical modification of phosphoprotein.

First Method

β-elimination

In this reaction a π-bond formed at β- carbon by elimination of a nucleophile and a proton in the presence of a base. This can be explained by the elimination of phosphate group in the reaction shown in Fig. 1.6 where a nucleophile (PO_4^{3-}) is removed in the presence of a base.

In phosphoproteins this reaction occurs when phosphoserine and phosphothreonine residues are exposed to strong alkaline conditions [87-88]. The resulting dehydroalanine or dehydroaminobutyric acid can be detected directly by MS/MS or they can be modified by Michael addition of ethanedithiol (EDT) before tandem mass spectrometry [89-90]. In the Michael addition reaction EDT acts as a nucleophile and

provides a new reactive thiol group which play a linker role for biotin affinity tagging [88]. The biotin affinity tagging is performed for purification of phosphopeptides. The overall reaction is described in Fig. 1.7.

Drawbacks

This method has not been used widely because of the following drawbacks:

- This method is not applicable for tyrosine Phosphorylation
- Undesired side reactions also occur at cysteine and methionine residues

This problem was overcome by treating the sample first with performic acid leading to the oxidation of these residues, which inactivated them [91]**.**

Alternative Method

An alternate method to isolate phosphotyrosine containing peptides with the other phosphoserine and phosphothreonine residues has been reported [92].

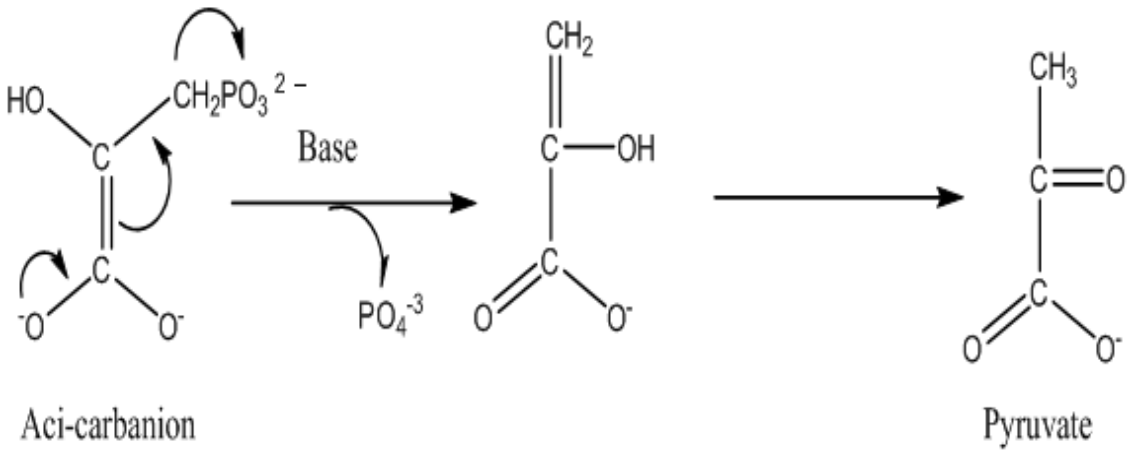


Figure 1.6: β-elimination of aci-carbanion ion containing a phosphate group

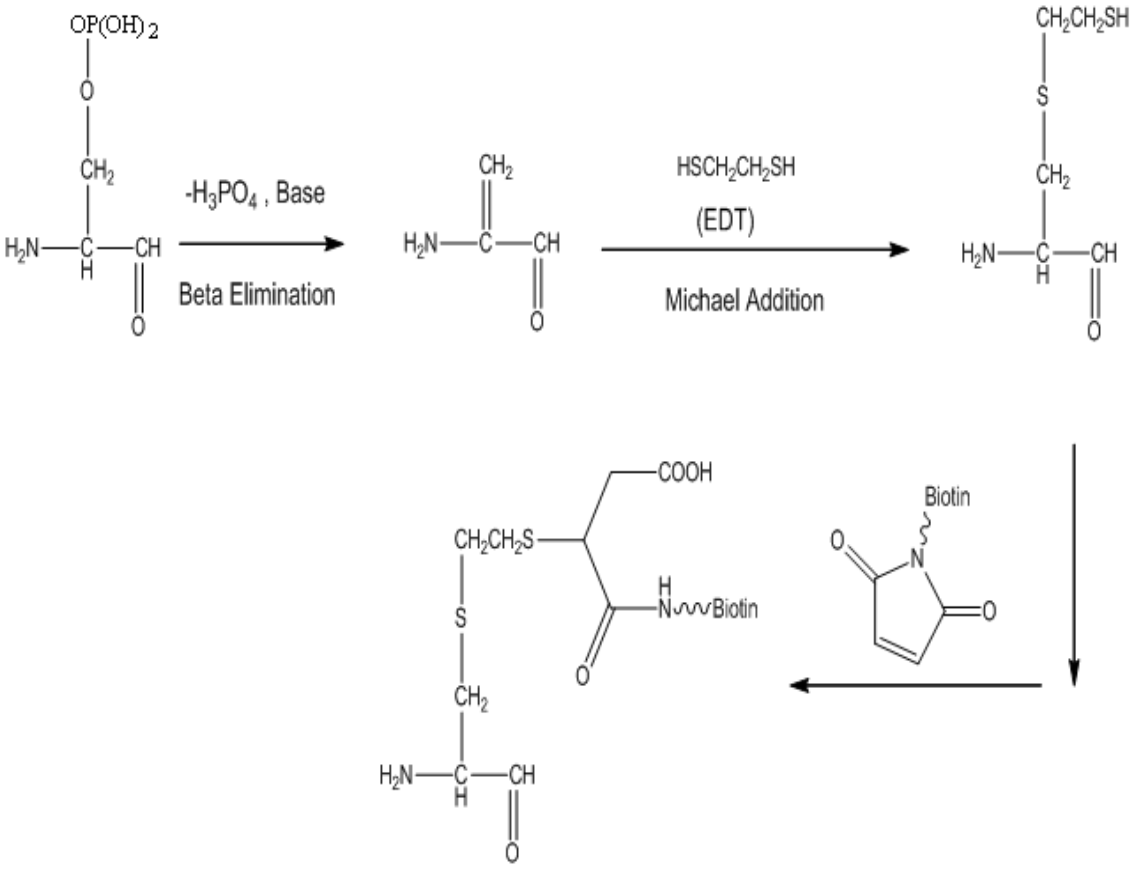


Figure 1.7: β-elimination and Michael Addition Reaction

This method allows the addition of cystamine on phosphate moieties in the presence of ethyl carbodiimide (EDC) as a catalyst and later purification of phosphopeptides on glass beads containing iodoacetyl groups.

Drawbacks

- The main drawback is that both methods require a large amount of sample for identification by MS.
- The selectivity of both methods has not been confirmed yet.

1.5.4.3 Immunoprecipitation

Immunoprecipitation is a method in which an antibody specific to phosphopeptides is used to enrich them from the solution. This method is relatively effective for enriching the low-abundance tyrosine phosphopeptides [93-94] but selectivity is compromised

[95], also serine-phosphate and threonine-phosphate residues cannot be enriched by this method because there is no antibody available specific to these phosphopeptides.

1.5.4.4 Titanium Dioxide Precolumn

Pinkse *et al.* [96] introduced for the first time an alternate method for IMAC by use of titanium dioxide for the enrichment of phosphopeptides. They used a pre-column filled with 5µm spherical TiO_2 particles coupled directly with a reverse-phase capillary column prior to ESI-MS/MS.

Drawback

- This method has not been used widely because non phosphorylated acidic impurities also stick to the column and come out with the phosphopeptides.

1.5.4.5 Titanium dioxide with 2, 5-dihydroxybenzoic acid

Recently, Larsen and co-workers further optimized the use of TiO_2 and reported a highly selective method for the enrichment of phosphopeptides [97]. This method is more selective for binding of phosphopeptides than pre-column and IMAC methods. In this method Titanium dioxide (TiO_2) beads were loaded in to a microcolumn in the presence and absence of 2, 5- dihydroxy benzoic acid. The experimental results show that the TiO_2 established great interaction with phosphopeptides but in the presence of 2, 5-dihydroxybenzoic acid this interaction showed enhanced selectivity.

1.5.4.6 Monolithic column

In this column solid phase is made in one piece instead from small parts. The use of a monolithic column was first introduced by Tanaka *et al.* [98] in 1996. They prepared a column of 7 mm diameter and 83 mm length by using a new sol-gel process developed by Nakanishi *et. al.* [99] based on the hydrolysis and polycondensation of tetramethoxyline (TMOS) in the presence of polyethylene oxide (PEO) used as a template. By using this monolithic column Tanaka and co-workers demonstrated the unique properties of the monolithic column for chromatographic separation. In 1998 Ishizuka *et al.* [100] prepared a monolithic capillary column with internal diameter of 100 µm by adopting the sol-gel methods developed by Nakanishi [99].

After that several capillary monolithic columns were prepared by using different polymerization and cross linking methods. Overall a monolithic column has following advantages over the other columns.

Advantages of the monolithic column

- With the particle packed columns the performance and permeability cannot be controlled independently because the plate number N is inversely proportional to the particle diameter but in monolithic it is possible to control both permeability and performance independently.

- A capillary monolithic column has an efficiency of operation of 100,000 theoretical plates per meter.

- A capillary monolithic column has very low internal diameter which enhances the mass transfer and creates low back pressure.

- The major advantage of this capillary column is high mass sensitivity for ESI-MS analysis of bio-molecules at low attomole level using the ion trap mass spectrometer.

- A capillary monolithic column provides a very low back pressure even at very low internal diameter which makes it possible to operate with LC and tolerable elution delay time.

Classification of monolithic column

Monolithic columns are classified as organic and inorganic based polymers. There are three types of organic based monolithic columns i.e., polymethacrylates, polystyrene and polyacrylamide have been prepared by using different cross linkers. Similarly, silicate columns have been made for inorganic based polymers.

Monolithic capillary column and phosphopeptide enrichment

Several workers have reported the use of capillary monolithic columns for enrichment of phosphopeptides, because of the high resolution and low back pressure, it has been suggested to provide a good enrichment of phosphopeptides. Phosphopeptides enrichment by using a monolithic column also depends on the type of polymerization and cross linker used in the column. Recently, Dong *et al.* [101] reported the use of a monolithic column for enrichment of phosphopeptides by using a self made column. They prepared the capillary column by copolymerization of phosphate containing organic molecules (ethylene glycol methacylates) and immobilized Zr^{4+} on the phosphate groups. They used bis-acrylamide as a cross linker in a porogenic solvent.

The use of Polystyrene Divinyl Benzene (PS-DVB) as a column material has also been reported [102].

The use of capillary monolithic column is a good step towards the enrichment, but several publications indicate the limitation of monolithic capillary column for enrichment of phosphopeptides as described below [103-105]

.

Drawbacks

- Organic based monolithic columns have shown poor mechanical strength and excessive swelling when typical HPLC solvents are used.
- Those monolithic columns which have narrower than 50 µm i. d. have shown the appearance of gaps by dramatic shrinkage of the polymers during polymerization. However, the use of high positive pressure during polymerization reduces the structure irregularities.

1.5.4.7 SDS-PAGE

SDS-PAGE has been described in detail in section 1.1. Both ID and 2D-PAGE have been used for phosphopeptides enrichment, as discussed below.

1D-SDS-PAGE

For direct quantification of phosphopeptides by Laser Ablation ICP-MS (LA-ICP-MS) Wind and co-workers performed the enrichment of phosphopeptides by running the

digested mixture of a protein of interest on a Nu-PAGE SDS system. Sachon *et. al.* [103] and Wind *et al*. [18] used the Laemmli method [1] for enrichment prior to quantifying them.

2D-GE

Two dimensional gel electrophoresis is a pre-dominant method used to enrich phosphopeptides. Usually, enzymatically digested proteins of interest are labelled by ^{32}P (a radioactive isotope) and then resolved by 2D-GE. Finally, relative spot intensities are compared and enrichment obtained by electroelution [106]. However, the use of a radioactive isotope doesn't allow this technique to be run with high throughput analysis of phosphopeptides and also contamination of analytical instruments with radioisotope is possible [7].

Chapter 2: Tris Glycine SDS-PAGE Method

2.1 Introduction

As described in chapter 1 (section 1.1.1.1), Tris-glycine or Laemmli SDS-PAGE [1] is one of the most famous methods for the separation and identification of the proteins and peptides. However, this method is widely employed for the separation of high molecular mass proteins and peptides. For the separation of smaller proteins/peptides, using this method, gradient gels are normally adopted. In order to confirm the potential of Laemmli SDS-PAGE method for such separations this method was employed for the separation of β-casein and its digest mixture, as described in this chapter. The molecular mass of β-casein is approximately 25 kDa and phosphopeptides obtained after its tryptic digestion, are mono and tetra-phosphopeptides [97] with the approximate molecular mass of 2061.8 and 2966. 4 Da (Swiss-Prot).

2.2 Experimental

2.2.1 Chemicals

The following chemicals were purchased from Sigma-Aldrich (Poole, Dorset, United Kingdom): anionic detergent SDS and ultra-pure glycine as the components of the running buffer; β-casein as a model protein for mass balance study; 30 % (m/v) solution of acrylamide/bis-acrylamide (29:1) for the preparation of the polyacrylmaide gel; ultra-pure tris(hydroxymethyl)-amino methane (Tris) for the preparation of the buffer solutions; *N,N,N',N'*- tetramethylethylenediamine (TEMED) to catalyse the polymerization reaction; ultra-pure ammonium persulphate (APS) to initiate the polymerization reaction; 2 x sample buffer to prepare the protein sample before loading into the gel; Coomassie brilliant blue (CBB) to prepare the staining solution and as a protein tracking dye.

All the solutions were prepared in ultra-pure water (Milli-Q water purification system, Millipore, Bedford, MA).

A protein standard with a molecular mass range of 2.5 - 200 kDa was purchased from Invitrogen Corporation UK.

2.2.2 Sample preparation for the detection of β-casein band

The protein sample was prepared by dissolving 2 mg of β-casein in 1 mL of deionised water and in order to denaturize and reduce the protein of interest before loading into the gel, boiling was performed for 5 minutes after mixing β-casein solution with Laemmli sample buffer (purchased from Sigma Aldrich UK) in equal volume as recommended by manufacturer. Sample buffer contained the following chemicals: SDS (4%), Glycerol (20%), 2-mercaptoethanol (10%), Bromophenol blue (0.004%) and 0.125M Tris-HCl pH 6.8.

To prepare the sample for digest mixture, 10 µL of the digest mixture (as described in section 2.2.6) was mixed with 10 µL of the above sample buffer. This mixture was heated up to 5 minutes at 100ºC.

2.2.3 Gel and running buffer

A 0.5 M stacking gel buffer (pH 6.8) was prepared by dissolving 1.2 g Tris-base in 20 mL of deionized water. 1.5 M buffer for resolving gel (pH 8.8) was prepared by dissolving 3.6 g of Tris-base in 20 mL of deionized water. pH of the both gel buffers were adjusted using HCl. Running buffer was prepared with concentration of 192 mM glycine and 50 mM Tris and 0.1% (m/v) SDS. Briefly, 6 g Tris, 14.4 g glycine and 1 g SDS was dissolved in 1 Litre of deionized water. pH of the running buffer was not adjusted.

2.2.4 Gel casting

A 12.5% Laemmli gel was cast for the detection of beta-casein protein band by injecting the 5 mL of the resolving gel solution (see Table 2.1). A 4% stacking gel was cast on the top of the 12.5% resolving gel by injecting 1 mL of stacking gel solution (Table 2.1). Finally, the gel was run at [125 V] by injecting 10 µL of β-casein sample [20 µg] per well.

An efficient separation has been described previously by using gradient gel electrophoresis method [107]. Here this technique was utilized for the separation of β-casein digest mixture. A Laemmli gradient gel (made up of 15% and 20% resolving gel) was cast for the separation of the peptides from the digest mixture (Table 2.1).

Briefly, a 2.5 mL of 20% resolving gel solution was injected first into the gel plates and quick after this 2.5 mL of 15% gel was injected over this gel. After 15 minutes a 4% stacking gel was cast on the top of this gradient gel. The gel was run at [125 V] by injecting 10 µL of sample for digest mixture per well.

The molecular masses of the obtained β-casein peptide bands were determined by using the protein mass ladder.

Table 2.1: Tris-glycine Laemmli SDS-PAGE gels were prepared according to following table

Percentage of the Acrylamide/Bis-Acrylamide in the Gel		Acrylamide/ Bis-Acryl. 29:1	10% SDS (m/v)	Tris-Base	Tris-Base	Deionised Water	TEMED	ammonium persulfate	Total volume
		30% Solution (m/v)		1.5M pH 8.8	0.5M pH 6.8			20 mg/mL	
		(mL)	(mL)	(mL)	(mL)	(mL)	(mL)		(mL)
Stacking Gel	4.0	0.50	0.06	------	1.00	4.16	0.005	0.25	6.00
Resolving Gel	12.5	4.20	0.10	2.00	------	3.33	0.010	0.40	10.00
Resolving Gel	15.0	5.00	0.10	2.00	------	2.50	0.010	0.40	10.00
Resolving Gel	20.0	6.67	0.10	2.00	------	0.83	0.010	0.40	10.00

2.2.5 Staining and destaining

Proteins and peptide bands were stained on the gel for 30 minutes using the CBB staining solution. This solution was prepared by dissolving 2.5 g of CBB in the mixture of 500 mL methanol, 400 mL deionized water and 100 mL of acetic acid solution. After washing the gel with deionized water destaining was performed for 30 minutes. This destaining solution was prepared by mixing 150 mL of methanol, 100 mL acetic acid solution and 750 mL of deionized water.

2.2.6 On gel protein digestion of β-casein

After the separation, as described above, the tryptic digestion of β-casein was carried out to obtain the peptide fragments of β-casein from the gel. In order to avoid self autolysis of the trypsin, the Promega grade methylated trypsin gold was purchased

from Promega Corporation (Delta House, Southampton, UK) and the following procedure was followed as provided by Promega.

After staining with CBB and destaining by deionized water, the β-casein band from the gel was cut into small pieces by a sharp and clean razor. The gel slices were placed into 0.5 ml of a microcentrifuge tube.

In order to avoid contamination, from the centrifuge tube and razor were prewashed twice with 50% acetonitrile (ACN)/ 0.1% trifluoroacetic acid (TFA). The protein gel slices were destained with 0.2 ml of 100 mM ammonium bicarbonate (NH_4HCO_3)/ 50% ACN. The solution was left in the tube for 45 minutes at 37ºC for complete destaining of the slices. The pale blue solution was obtained by dehydrating the gel with 100-µl of 100 % ACN for 5 minutes. In order to remove this pale blue solution the gel slices were then dried for 30 minutes. Trypsin gold [100-µg lyophilized powder] was re-suspended into 100 µL of 50 mM acetic acid and 10 µL of this trypsin solution was then dissolved in 50 µL of 40 mM (NH_4HCO_3)/ 10% ACN (digestion buffer) and incubated at room temperature for an hour. After that, this digestion buffer was added into centrifuge tube containing the gel slices just to cover them. The tube was capped tightly and incubated at 37ºC overnight.

Next day, a further 150-µL of deionized water was added and incubated at room temperature for 10 minutes with frequent vortexing. This digested solution was then transferred in to a new centrifuge tube. The remaining gel slices digest was extracted twice by adding 50-µL of 50% ACN/5% TFA with gentle vortexing and incubating for 60 minutes each time. All extracts were added together and dried to concentrate at room temperature for 2-4 hours.

2.3 Results and discussion

Fig. 2.1 shows the beta-casein band in 12.5 % Laemmli gel. The thickest unknown protein band was calculated as a β-casein (Table 2.2). The molecular mass of β-casein was found 25.7 kDa, which was very close to the published molecular weight (25.5 kDa) [108]. Some other less intense unknown protein bands appeared below the β-casein which suggests that the β-casein was contaminated with traces of some other proteins.

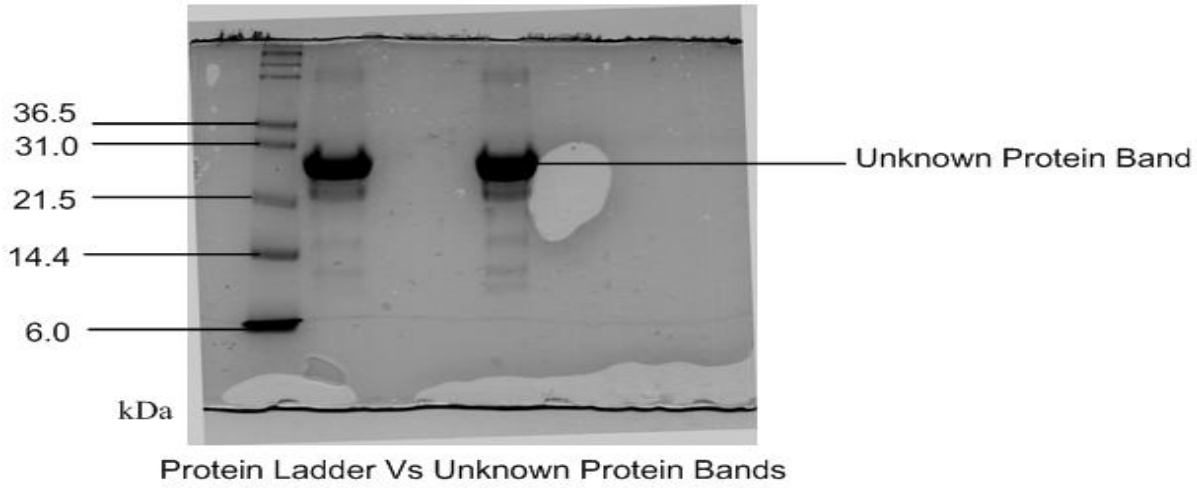


Figure 2.1: Protein ladder vs. β-casein in Laemmli SDS-PAGE

2.3.1 Calculation of unknown protein band

The thickest band was expected to be β-casein. For confirmation the relative mobilities (Rf) with the help of standard (protein ladder) were calculated.

Rf of protein X = (Distance of X from origin) ***/*** **(**Distance of reference from origin)
Where, X is an unknown protein.

Fig. 2.2 shows the graph of the relative mobilities (Rf) vs. molecular weights of the protein mass ladder, which was drawn using the calculated Rf values as shown in Table 2.2 and used to calculate the molecular mass of unknown protein (β-casein).

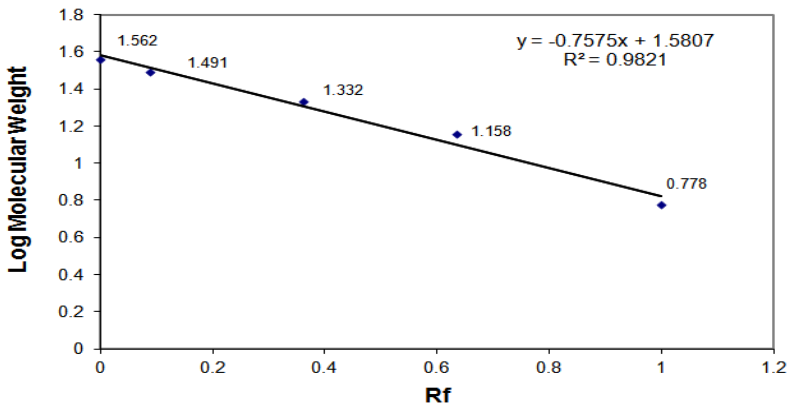


Figure 2.2: Plot of relative mobility (Rf) vs. logarithm molecular weight of a protein standard mixture

Table 2.2: Calculated relative mobilities (Rf) values (R^2 =0.9821)

No	Molecular Weight **kDa**	Molecular Weight **Log**	Relative Mobility **Rf**
1	6.0	0.778	1.00
2	14.4	1.158	0.636
3	21.5	1.332	0.363
4	31.0	1.491	0.090
5	36.5	1.562	0.000
6	Unknown **25.8**	1.411	0.224

2.3.2 Peptides separation

Fig. 2.3 shows result obtained after running the gradient gel. The molecular mass of the unknown protein bands was calculated and it was found that molecular mass of the unknown protein band 1 (3.07 kDa) was very close to the published molecular mass of one of the phosphopeptides i.e., 2.966 kDa [97, 101, 103, 18]. It is important to note that the other expected phosphopeptide with a molecular mass of 2.061 kDa, was not observed in the gel. A possible reason could be the inability of the Laemmli method to separate very low molecular mass proteins. It is also important to note that the resolution was not very good; this was not surprising, as a very high percent gel does not provide good resolution of the separated peptides, as was mentioned above.

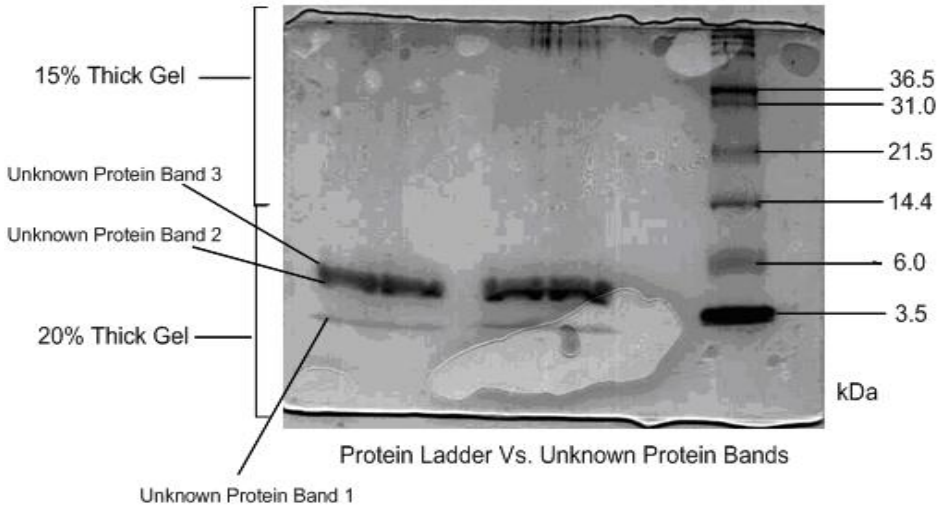


Figure 2.3: peptides separation after tryptic digestion of β-casein by Laemmli gradient gel electrophoresis.

2.3.4 Calculation for unknown band

As shown in Fig. 2.4, the graph of the relative mobilities (Rf) vs. molecular mass of the protein mass ladder was drawn by using the calculated Rf values (Table 2.3). This graph was used to calculate the molecular weight of phosphoproteins.

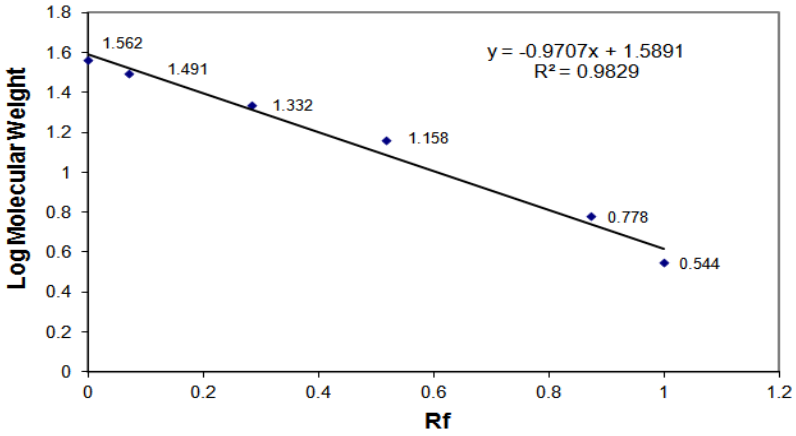


Figure 2.4: Plot of relative mobility (Rf) vs. logarithm molecular weight of protein standard

Table 2.3: Calculated relative mobilities values (R^2 = 0.9829)

No	Molecular Mass kDa	Molecular Mass **Log**	Relative Mobility **Rf**
1	3.5	0.544	1.00
2	6.0	0.778	0.8750
3	14.4	1.158	0.5178
4	21.5	1.332	0.2857
5	31.0	1.491	0.0714
6	36.5	1.562	0.0000
7	Unknown **3.1**	0.487	1.1352

2.4 Conclusion

The Tris-glycine method was employed here to detect the low molecular mass proteins by using gradient gel electrophoresis. By using a high percentage gradient gel Laemmli SDS-PAGE system was able to detect approximately 3 kDa molecular mass proteins, however the use of a very high percentage gradient gel has not been encouraged

because of several reasons as described in chapter 3 (section 3.1). It is important to note that despite using the high percentage gradient gel Laemmli system is unable to detect very low molecular mass peptides (< 3 kDa). So, the modification of the Laemmli SDS-PAGE system was needed to detect ultra-low molecular mass peptides and phosphopeptides using a simplified procedure.

Chapter 3: Modification of Tricine-SDS-PAGE for Online and Off-line Analysis of Phosphoproteins by ICP-MS

3.1 Introduction

Reversible protein phosphorylation is a key event in the post-translational regulation of cells that alters the shape and functions of proteins and involves a wide range of cellular activities such as cell cycle, differentiation, apoptosis, metabolism and the cell growth division [109]. An abnormal phosphorylation may be a factor in several neurodegenerative disorders leading to Alzheimer's, Parkinson's and Huntington's diseases [110-111]. Thus there is much interest in obtaining qualitative and quantitative information about phosphorylation.

ICP-MS, when coupled to an appropriate separation technique, is a very sensitive and selective detector for identifying and quantifying low and high molecular mass bio-molecules. The detail information about the coupling of separation strategies with ICP-MS is described in chapter 1 (section 1.3.1). Quantitation performed by ICP-MS has several advantages: (a) ICP-MS can detect the analyte of interest at very low concentration, (b) mass balance can be established because the response is independent of structure and (c) calibration is straightforward usually being carried out with inorganic salts. In quantitative phosphoproteomics, the use of ICP-MS provides a different approach compared to more traditional methods. The phosphorus to sulphur (P/S) ratio can be experimentally determined and converted into the degree of phosphorylation using the information available from the sequence [112-113, 114], but a major drawback is that it only applies to those phosphoproteins containing methionine (Met) and cysteine (Cys) residues (sulphur containing amino acids) [114]. Wind *et al.* reported that any peptide sequence exceeding 20 amino acids in length can be expected to have at least one Met or Cys residue [115]. However, several phosphorylated peptides, such as the tetra-phosphopeptides obtained from the tryptic digestion of β-casein, have a polypeptide sequence of more than 20 amino acids, but do not contain these residues [97] and are therefore outside the scope of this methodology.

The classical strategy for the determination of the specific sites of phosphorylation is the enzymatic digestion of the proteins followed by ESI-MS or MALDI-TOF analysis

of the protein fractions. ICP-MS has several advantages over molecular MS, but the separation of the phosphopeptides from other phosphorylated or non-phosphorylated peptides, prior to their identification either by molecular MS or ICP-MS, is essential and has been discussed in detail in several literature reviews [116-118].

For obtaining enhanced quantitative information, a low percent gel is ideal because (a) the analyte can be transferred more effectively onto a membrane during the electroblotting procedure, particularly those proteins with a high molecular mass and hydrophobic character which do not properly transfer using high percentage acrylamide gels and show poor recovery [39]; (b) Laser Ablation (LA)-ICP-MS can be performed effectively directly on the gel; (c) a lower voltage and shorter time is required for separation and electroelution so there is less chance of degrading the phosphorus-containing molecules with consequent band distortion [43].

Tris-glycine or Laemmli [1] SDS-PAGE is one of the most widely used methods for the electrophoretic separation of proteins. However, this method has very poor resolving efficiency for separating 1-30 kDa proteins in a low percentage polyacrylamide gel [12]. Therefore, in order to resolve low mass proteins in a low percentage gel, several modifications of this system have been reported. One way is decreasing the pore size of the gel [119-120] by increasing the cross-linking (%C) or by adding urea to the gel [43, 121]. However by adding a greater quantity of the cross-linker, the gel becomes more brittle. The addition of urea is useful for the analysis of low mass proteins, but it crystallizes at low temperature and sometimes decomposes during sample preparation [122]. Urea can also modify proteins by forming a carbamoylate derivative of lysine and other residues [123]. Another possible modification is the use of gradient gels [4], which provide good separation of low mass proteins, but they also have several deficiencies including irreproducibility of separation (especially in mini-gels), a time consuming cast, a tendency to break during the run, and they are difficult to store [124]. Moreover, they are not good for resolving peptides lower than 10 kDa [43].

In another development, Schägger and von Jagow [4] introduced the Tricine-SDS-PAGE method. Compared with glycine, tricine migrates much faster in a stacking gel at usual pH values and shifts the stacking limit from high to low molecular mass range proteins, which allows good separation of the smaller SDS-peptide complexes [43]. Tricine-SDS-PAGE is a further improvement because it enables the separation of

low mass proteins in a low-percentage polyacrylamide gel. Usually, a 10% gel (with pH 8.45) in Tricine-SDS-PAGE can separate proteins in the range of 1-100 kDa using two running buffers: a cathode buffer e.g. 100 mM Tris, 100 mM tricine and 0.1% (w/v) SDS, pH 8.25, in the cathode chamber and an anode buffer e.g.100 mM Tris, pH 8.9, in the anode chamber. However, to obtain highly resolved bands, this method needs three gels (stacking, spacer and resolving gel) [39, 4, 124] and addition of urea [39] in the resolving gel. The use of three gels is tedious, potentially troublesome and requires a freshly prepared gel mixture [124]. Addition of the urea may also create problems in amino acid sequencing [43].

The main objectives of this study were (a) to develop a simplified GE method employing low percentage gels and low reagent concentration Tricine-SDS-PAGE system for the separation of peptides and proteins (b) to use this modified system to enhance the quantitation of phosphopeptides/phosphoproteins in off-line and on-line coupling analysis with ICP-MS. In GE-ICP-MS on-line coupling, the protein of interest can be eluted for detection by ICP-MS by adopting one of two strategies: (a) using running buffer as the elution buffer or (b) using an elution buffer from a separate buffer chamber. Both strategies are effective and purely depend on the instrumentation used. Here, the first strategy was employed as it is simple and required less modification to the original gel separation unit.

3.2 Experimental

3.2.1 Chemicals

All the solutions were prepared in ultra-pure water (18 MΩ. cm, from a Milli-Q water purification system, Millipore Corporation, Bedford, MA).

Chemicals for this work were as described in Chapter 2 (section 2.2.1) with the addition of the following, purchased from Sigma Aldrich unless otherwise stated: α-casein as a further model protein; 1, 4 dithio-DL-threitol (DTT) as a reducing agent iodoacetamide (IAA) as an alkylating agent; ammonium bicarbonate (NH_4HCO_3) as a buffer for protein digestion; and 25% (v/v) glutaraldehyde solution for fixing the proteins in the gel. Sequencing grade modified trypsin for the digestion of β-casein was purchased from Promega Corporation, UK.

3.2.2 Protein standards

A protein standard (1) with a mass range of 2.5-200 kDa was purchased from Invitrogen Corporation UK. Ultra-Low Range Molecular Weight Marker™ (2) with a range of 1-26.6 kDa was purchased from Sigma Aldrich UK. The detail of these protein standards is described in Table 3.1. Mono- [T6 (1P)] and tetra- [T1-2 (4P)] phosphopeptide standards [(M+H) = 2061.8 and 3122.2 respectively] were also purchased from Sigma Aldrich UK.

Table 3.1: Detail of protein standards 1 and 2

Protein standard 1	Protein standard 2	Molecular mass (kDa)
Myosin	———	200.0
β-galactosidase	———	116.3
Phosphorylase B	———	97.4
Bovine serum albumin (BSA)	———	66.3
Glutamic dehydrogenase	———	55.4
Lactate dehydrogenase	———	36.5
Carbonic anhydrase	———	31.0
———	Triosephospate isomerase	26.6
Trypsin inhibitor	———	21.5
———	Myoglobin (from horse heart)	17.0
Lysozyme	———	14.4
———	α-lactalbumin (from bovine milk)	14.2
———	Aprotinin (from bovine lung)	6.5
Aprotinin		6.0
Insulin B chain	Insulin B chain (oxidised bovine)	3.5
Insulin A chain	———	2.5
———	Bradykinin	1.06

3.2.3 Protein digestion

Protein digestion in solution for a comparative study was performed according to the following method. 10 µL of 1 mg/mL β-casein solution was dissolved in 15 µL of 50 mM NH_4HCO_3 and 1.5 µL of 100 mM DTT solution and incubated at 90 ºC for 5-10 min. After cooling to room temperature, 3 µL of 100 mM IAA was added to this solution and incubated for 25 minutes in the dark. 1 µL of 0.1 µg/µL sequencing grade modified

trypsin was then added and incubated at 37 ºC for 4 hours. Finally, an additional 1 µL of this trypsin was added and incubated overnight at 30 ºC. Next day, the digest mixture was dried to 10 µL using vacuum.

3.2.4 Tricine-SDS-PAGE

3.2.4.1 Buffer system

To prepare the stacking and resolving gels, 2.5 M Tris-buffer with a pH of 8.8 for the modified system and with a pH of 8.45 for the conventional system was prepared. The pH of the gel buffers were adjusted using HCl. Running buffers with 25 mM concentration of Tris-tricine and 0.05% SDS for the modified method and 100 mM Tris-tricine and 0.1% SDS for the original method were prepared. (Note: only one running buffer was employed in both methods).

2 x sample buffer was purchased from Sigma Aldrich, containing 100 mM Tris-HCl (pH 6.8), 1% (w/v) SDS, 4% (v/v) 2-mercaptoethanol, 0.02% (w/v) Coomassie Brilliant Blue and 24% (w/v) glycerol.

3.2.4.2 Sample preparation

For the slab gels, 10 µL of the sample buffer were mixed with (a) 10 µL of 1 mg/mL β-casein and (b) 10 µL of the digest mixture. For on-line coupling, 10 µL of the sample buffer were mixed with one of the following: (a) 10 µL of the digest mixture, (b) 10 µL α- and β-casein (final concentration 0.10 nM each), or (c) 10 µL mixture of mono and tetra-phosphopeptide standards giving final concentrations of 20.2 µM of [T6 (1P)] and 16.3 µM of [T1-2 (4P)].

3.2.4.3 Gel casting

Stacking and resolving gels were cast for both the modified and original method as described in Table 3.2. Urea, SDS and glycerol were not included in any of the gels. For slab gels, a mini-gel casting apparatus was purchased from Bio-Rad UK (Mini-PROTEAN® 3 Cell) and all the gels were cast at 7.3 cm height x 8 cm width and

0.75 mm thickness. (*Note*: acrylamide is recognised as a neurotoxin, so gloves must be worn all the time and the work must be done in a properly ventilated area). For on-line coupling (see Instrumentation section), 10, 10.5 and 15% tube gels were also cast in a similar manner within a length of 7.3 cm and 1 cm internal diameter (ID) in a borosilicate glass tube.

Table 3.2: Tricine-SDS-PAGE protocol for the preparation of the polyacrylamide gels.

Percentage of Acrylamide Bis/Acrylamide in the gel		Acrylamide/bis-Acrylamide 29:1 30% solution (m/v) mL	Tris-Buffer pH 8.8 mL	Deionized Water mL	TEMED µL	APS 30 mg/mL µL	Total volume mL
Stacking gel	4	0.66	0.76	3.46	5.0	100	~5
Resolving gel	7	2.33	5.60	1.91	7.0	150	~10
	9	3.00	5.60	1.24	6.0	150	~10
	10	3.33	5.60	0.96	6.0	100	~10
	10.5	3.50	5.60	0.79	6.0	100	~10
	15	5.00	4.60	0.29	6.0	100	~10
	16	5.33	4.33	0.22	6.0	100	~10

3.2.4.4 Protein loading

7 µL of protein standard (1) and 5 µL of protein standard (2) were injected onto the slab gels. Similarly, 10 µL of β-casein solution for the mass balance study (by off-line analysis), and 10 µL of the digest mixture were also loaded onto the slab gels. 10 µL of each of the mixtures of: 0.10 nM α-casein and β-casein, the digest mixture of β-casein, and 20.2 and 16.3 µM mixture of [T6 (1P)] and [T1-2 (4P)] respectively were injected into the tube gel for on-line coupling.

3.2.4.5 Gel fixing, staining and destaining

A fixing solution of 5% (v/v) glutaraldehyde was prepared and each slab gel was fixed

for 25 minutes. A 0.025% (w/v) Coomassie Brilliant Blue solution was prepared in 10% (v/v) acetic acid and each gel was stained for 20 minutes after washing with deionized water. For sharp bands, the staining was repeated by replenishing the staining solution. Gels were washed with deionized water and destaining was performed using 10% acetic acid for 15-20 minutes.

3.2.5 Whole Gel Elution (WGE) for off-line analysis

β-casein (10 µg) was electrophoretically run through 9% and 16% modified slab gels. The tracks containing the protein bands were then cut from the gels using a sharp blade. These individual tracks (9% and 16% gels) were loaded into the electroeluter glass tubes separately and electroeluted using the Bio-Rad 422 electroeluter. Tricine-SDS-PAGE modified running buffer was used as an elution buffer for 2 hours at 0.01-0.02 A current applied to each tube. A blank gel of each of the 9 and 16% gels was also electroeluted following the same procedure to subtract the background signal intensity of the ^{31}P. WGE was also performed for phosphorylated peptide band detection. The β-casein digest (10 µL) was injected into a 10% slab gel. After Coomassie staining each band was cut to equal size and electroeluted for 2.5 hours by applying a 0.01-0.02 A current per tube. A blank gel was also electroeluted for the control.

3.2.6 Instrumentation

3.2.6.1 GE-ICP-MS On-line Coupling

A schematic diagram of the GE-ICP-MS on-line coupling is shown in chapter 1 Fig. 1.3a. For this coupling, a Model 422 Electroeluter GE system was purchased from Bio-Rad UK. This electroeluter included a borosilicate glass tube of 1 cm ID x 6 cm length, but here this original elution tube was replaced by a glass tube of 1 cm ID x 7.3 cm length into which a capillary tube of the same length, with an ID of 1 mm was placed. Gels were cast into the outer tube (1 cm ID) and the capillary was used for pumping the buffer and eluted proteins to the ICP-MS nebulizer. (It is important to note that this inset capillary did not show any negative effect on separation. As stated above, a focus in this work was to minimize modification of the original device. Thus the capillary was

taken from the upper reservoir, rather than a separate one, and this helped to balance the pressure and prevented displacement of the dialysis membrane).

At the bottom end of the tube, a porous polypropylene frit was placed to filter the running buffer and reduce further the SDS concentration before introduction to the ICP-MS instrument. This tube was then placed into a silicone adapter with the outlet connected directly to a peristaltic pump via Teflon tubing. The other end of the pump was connected to an ICP-MS nebuliser. A dialysis membrane with the molecular mass cut-off 1.5 kDa was purchased from Sigma Aldrich. This membrane was adjusted into the cap-type dialysis membrane to reduce its cut-off molecular mass which was 3.5 kDa. This membrane now placed at the bottom end of the adapter to connect the electrodes to the buffer reservoirs. The total inner dead volume between polypropylene frit and dialysis membrane was only about 20 µL. The upper (cathode) and lower (anode) buffer chambers were filled with 200 mL and 600 mL of the running buffer respectively.

The separation was carried out at 0.03 A current at a voltage of 80-90 V. When the dye front reached the bottom of the tube, the running buffer (from upper buffer chamber) was then pumped towards the nebulizer with a flow rate of 130 µL min^{-1} via the inset capillary tube until the proteins of interest were eluted. The current applied during this time was 0.04-0.05 A (150-200 V). The GE parameters are listed in Table 3.3.

3.2.6.2 ICP-MS

ICP-MS analysis for ^{31}P was performed on an Element 2 XR Sector Field ICP-MS instrument (Thermo Scientific, Bremen, Germany). The instrument was operated at medium resolution (R=4,000) without CRC to avoid interferences on the ^{31}P signal at m/z=30.973 u from $^{15}N^{16}O$ (m/z=30.999 u) and $^{14}N^{16}O^{1}H$ (m/z=31.005 u). ICP-MS parameters are given in Table 3.4.

Table 3.3: GE parameters for on-line coupling of GE-ICP-MS

Voltage	80-90 V for initial separation and 135-150 V for elution
Running/Elution buffer	25 mM Tris-base, 25 mM tricine and 0.05% SDS
Flow rate	130 µL min^{-1}
Gel length	7.3 cm
Gel ID	1.0 cm
pH of the gel	8.8
Gel material	Polyacrylamide
Percentage of acrylamide in separating gel	10, 10.5 and 15%
Percentage of acrylamide in stacking gel	4%

Table 3.4: ICP-MS parameters

Instrument (ICP-MS)	Thermo Finnigan Element 2 XR, Bremen, Germany
Cones	Ni sampler and Ni Skimmer
Nebulizer	PFA micro flow LC (Elemental Scientific Omaha, USA)
Flow rate	130 µL min^{-1}
Spray chamber	Cyclonic (Glass Expansion, Victoria Australia
Analyte	^{31}P
Magnet mass	30.973
Mass range	30. 968-30.978
Resolution	Medium
Gas flows	Cool = 15.5 $Lmin^{-1}$ Auxiliary = 0.88 $Lmin^{-1}$ Nebulizer = 1.102 $Lmin^{-1}$
Forward Power	1300 W

3.3 Results and discussion

3.3.1 Modified tricine-SDS-PAGE method

To increase the compatibility of the Tricine-SDS-PAGE system with ICP-MS, changes were made to the original method, decreasing the component concentrations of the running buffer and altering the pH of the gel buffer. Separations achieved on the slab gel by employing two different protein mass ladders are shown in Fig. 3.1. The effects of the modification are discussed in detail below.

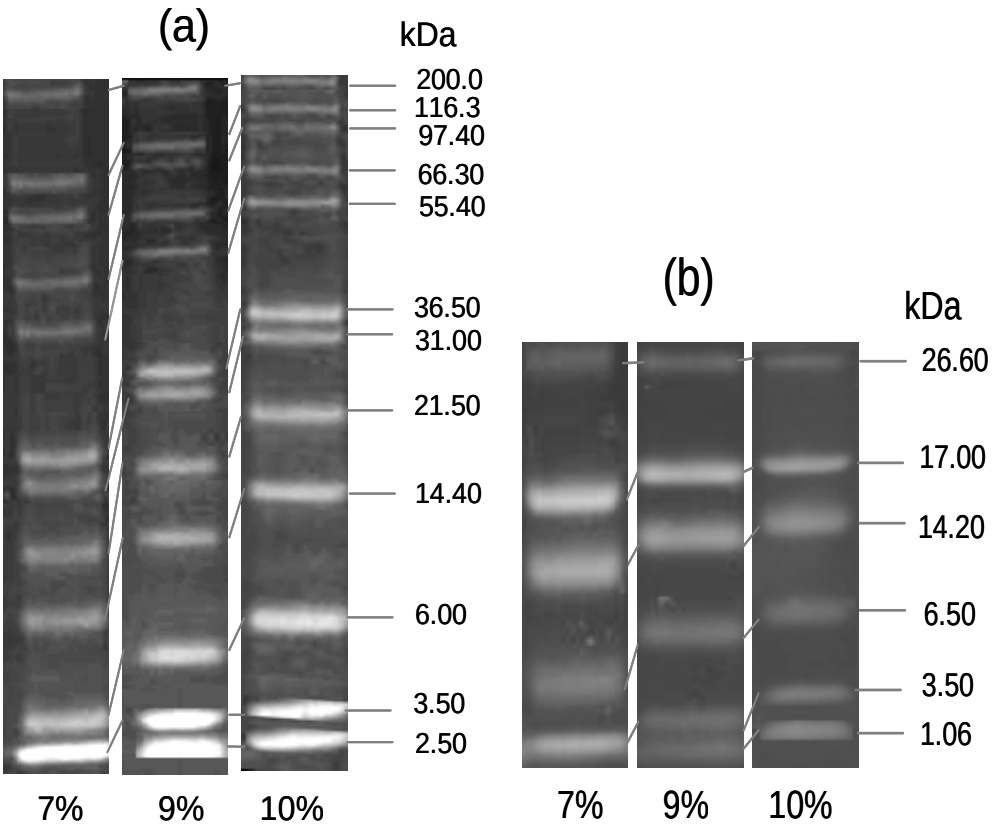


Figure 3.1: Modified Tricine SDS-PAGE separation of low mass proteins in the range of (a) 2.5 to 200 kDa (b) 1.0 to 26.6 kDa in 7, 9 and 10 % gels. For proteins corresponding to the listed masses, see table 3.1.

3.3.1.1 Effect of changing the running buffer concentration

An important step in coupling ICP-MS to separation techniques is to optimize mobile phase composition. For example, organic buffers, which contain a high concentration of carbon have several adverse effects on ICP-MS such as: (a) build up of carbon deposition leads to clogging of the interface cones and injector of the torch and, as a result, decreases the sensitivity of the ICP-MS instrument [22-24]; (b) a high level of carbon generates a number of polyatomic carbon based interferences for e.g., C_2^+, CNH^+, CO_2^+ and $^{38}Ar^{13}C^+$ for Mg^+, Al^+, Ca^+ and V^+ respectively; and (c) volatile buffers increase plasma loading and may cause destabilization of the plasma. To use a high carbon concentration, it is usual to add oxygen to the nebulizer gas [40-41, 125], however, the addition of oxygen may react with Ni cones [10]. Non-volatile buffers are better tolerated than volatile ones. However, where possible, low buffer concentrations are preferable, there are no negative effects and contamination is reduced which is important for ubiquitous elements such as phosphorus. For Tris-HCl Lobinski *et al.* reported up to 30 mM concentration is well tolerated by ICP-MS [42]. Tricine-SDS-PAGE running buffer in the original method contains 100 mM Tris, 100 mM tricine and 0.1% SDS with the gel buffer pH at 8.45. It was found that by reducing this running buffer concentration down to 25 mM Tris, 25 mM tricine and 0.05% SDS and increasing the pH of the gel buffer to 8.8 there was no negative effect on the band resolution.

Comparative results for the digest mixture of the β-casein were obtained after running the tricine original and modified SDS-PAGE method with the 10% gels as shown in Fig. 3.2. The molecular mass values of the bands were calculated using the protein ladder. The serine (S) residues of the amino acid sequence of β-casein are phosphorylated at positions 30, 32-34 and 50 (Swiss Prot). Trypsin cleaves the amino acid sequence from arginine (R) and lysine (K) residues and gives the two phosphorylated fragments i.e., a mono-phosphopeptide [(M+H) = 2061.8] and a tetra-phosphopeptide [(M+H) = 2966.16] with the sequences FQ-Sp-EEQQQTEDELQDK and ELEELNVPGEIVE-Sp-L-SpSpSp-EESITR respectively. Two further tetra-phosphopeptides from β-casein digest i.e., [M+H = 2352.85] NVPGEIVE-Sp-L-SpSpSp-EESITR and [M+H = 3122.27] RELEELNVPGEIVE-Sp-L-SpSpSp-EESITR were also reported [97, 101]. These phosphopeptides are usually obtained because of the mis-cleavages or chemotryptic activities of the trypsin enzyme [97].

It was previously reported that the molecular masses of phosphorylated peptides could not be determined in SDS-PAGE [43] as they do not properly form micelles with the SDS and therefore migrate slower. For the identification of the phosphorylated bands, WGE of each digested band was performed and 10 µL of each fraction was introduced to the ICP-MS instrument by flow injection using the modified running buffer stream. The ^{31}P area of each peak was calculated and the bands containing the phosphopeptides were identified in the gel as the only peptide bands with a ^{31}P peak area above the blank (Fig. 3.2). However, these bands were not well resolved, most likely due to several peptide impurities. The ^{31}P peak area of each band is listed in Table 3.5.

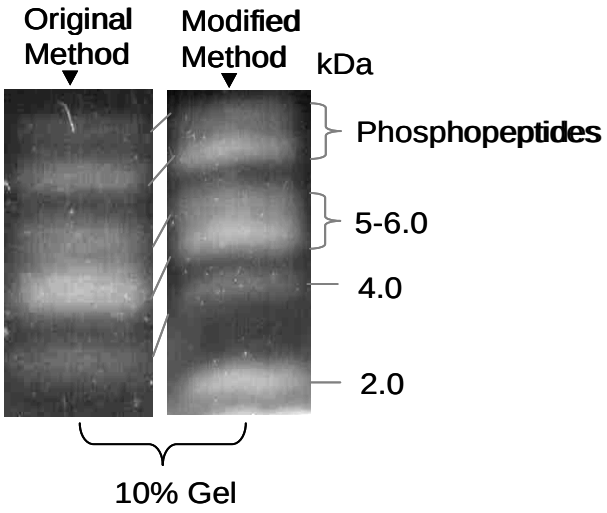


Figure 3.2: Head to head comparative results for the β-casein digest using a 10% polyacrylamide slab gel with the original and modified Tricine-SDS-PAGE system.

Table 3.5: ^{31}P peak area of the β-casein digested peptide bands using WGE-ICP-MS flow injection.

Peptide Band	^{31}P Peak Area (10^3) (N=5)
Blank gel	50 ± 2.0
2.0 kDa	51 ± 4.0
4.0 kDa	49 ± 2.3
5-6.0 kDa	50 ± 2.2
Phosphopeptides	83 ± 5.4

3.3.1.2 Effect of changing the pH of the gel buffer

In a gel electrophoresis procedure, the pH of the resolving gel plays an important role in protein separation. The effect of changing pH in a classical Laemmli system has been reported previously [126] and it was stated that by increasing the pH of the resolving gel, a slightly lower molecular mass band could be observed in a 10% gel. In an SDS-PAGE system a high pH in the separating gel is not only helpful in resolving the proteins, but it also increases the protein (wrapped with SDS) mobility in the gel and results in a low current that reduces heat generation. For quantitation, a low temperature in the gel is more likely to maximise the recovery of the proteins. The pH of the stacking and resolving gels is adjusted to 8.45 in conventional Tricine SDS-PAGE system. Here, it was shown that by increasing the pH of the resolving and stacking gels up to 8.8, the band resolution and separation of peptides/proteins was not degraded. By increasing the pH an additional band was observed in the 7, 9 and 10% gels (Fig. 3.3) compared to the original method. In previous studies 1-100 kDa protein separation has been claimed in a 10% gel using the original method [39, 43, 4]. This necessitated the use of two running buffers, urea, a three gel system or an increase in the cross linking to achieve such level of separation employing the original method. Hence increasing the pH up to 8.8 makes the system much easier and low molecular mass peptides separation was observed in 7, 9 and 10% polyacrylamide gels using a simplified procedure.

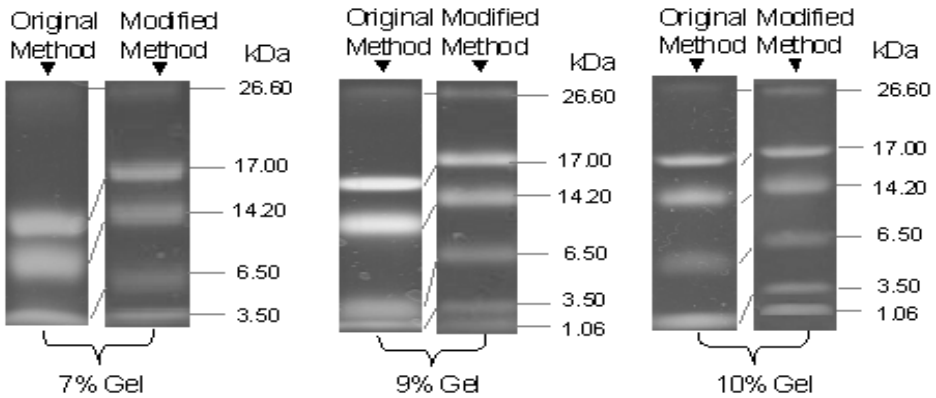


Figure 3.3: By increasing the pH of the gel buffer an additional band was observed in 7, 9 and 10% modified gel.

3.3.2 Off-line analysis for recovery studies

In order to check the efficiency of the low and high percentage gels for protein recovery a mass balance study was carried out using WGE followed by ICP-MS. The total percentage recovery of the ^{31}P was calculated as 73 ± 2.1% and 55 ± 1.6% (*N=3*) (after 2 hours electroelution, section 3.2.5) from the 9% and 16% gels respectively (Fig. 3.4). Thus the quantitative recovery of the β-casein in the low percentage gel was shown to be higher.

The major factors that may affect the recovery using high percentage gels are: (a) a high percentage gel increases the electrical resistance leading to more heat generation, this heat develops a temperature gradient from the centre of the gel to the gel surface due to the uneven heat loss and causes distorted bands [43]; and (b) a high percentage polyacrylamide gel increases the run time and running the sample for too long may cause protein fragmentation. These factors show that a high percentage gel is not an ideal choice to recover the maximum quantity of the protein from the gel.

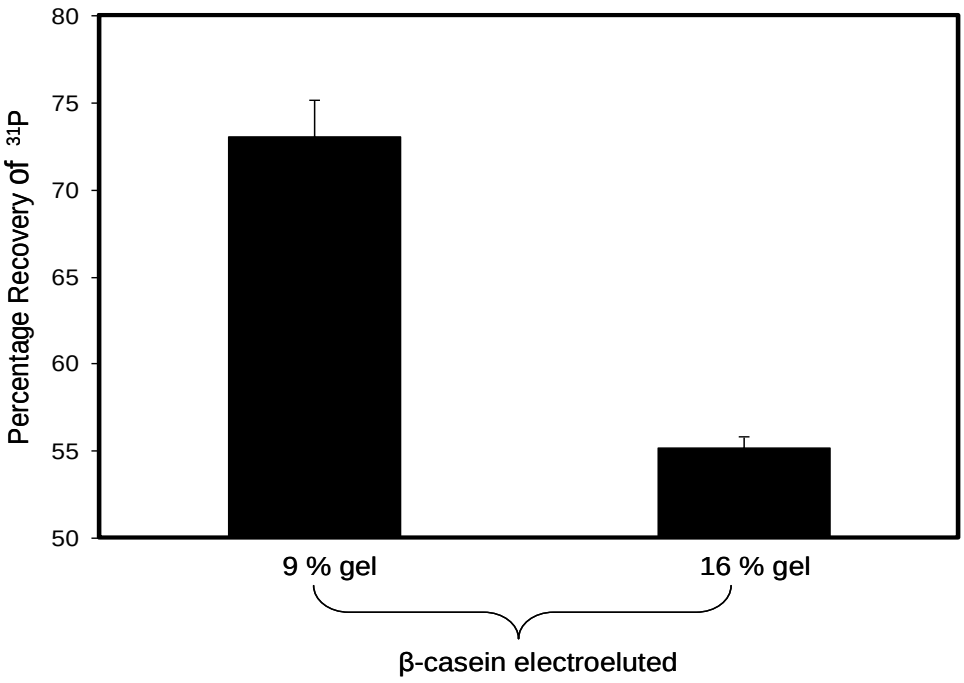


Figure 3.4: Relative recovered percentages of the electro-eluted β-casein. The recovery of 73.1 ± 2.1 and 55.2 ± 0.64 (after 2.00 hours electroelution) was achieved in 9 and 16 % gel respectively (n=3).

3.3.3 GE-ICP-MS on-line coupling

A focus of this study was to investigate the efficiency of the modified Tricine-SDS-PAGE method for on-line coupling to ICP-MS. Previously described GE-ICP-MS on-line systems used a separate elution buffer from a separate buffer chamber and therefore require detailed modification of the system. The decrease in the running buffer concentration provides an additional benefit as it can be used as an elution buffer in the modified system. 200 mL of the running buffer from the upper buffer chamber (coupled to the outlet chamber by the embedded capillary) is enough for many hours running with a flow rate of 130 µL $min.^{-1}$

The initial testing of the effectiveness of this on-line system was performed by the successful online separation of a mixture of α-casein (22.97 kDa) and β-casein (23.58 kDa), as shown in Fig. 3.5. Very good signals were recorded at 0.10 nM concentration of each of α- and β-casein. The degrees of phosphorylation on α-casein and β-casein were reported previously as 9.1 and 4.9 respectively [25, 112]. Therefore, enhanced ^{31}P signal intensity of the first peak was observed compared to the second one, however the ^{31}P signal intensity of β-casein was lower than expected as it was found to be contaminated with several proteins including α-casein, which was further enhancing the α-casein signal intensity (Fig. 3.5). Normally the SEC based methods require more than an hour to separate the analytes especially for smaller proteins and peptides. Here the runtime was below 30 minutes which indicates the good efficiency of GE-ICP-MS using modified tricine-SDS-PAGE method for the separation of the proteins/peptides below 30 kDa.

Resolution (Rs) is a measurement used to quantify the space between 2 peaks in LC. The most common formula for measuring Rs is

$Rs = 2 (t_2 – t_1) / w_1 + w_2$

Where, t_1 and t_2 are retention times of the two peaks of interest whilst w_1 and w_2 are peak width measured at baseline.

If the valley between the two peaks just touches the baseline then Rs approximately equal to 1.5 and this is minimum Rs acceptable value in chromatography. The resolution will enhance if this value increases.

As shown in Fig. 3.5, Rs was sufficient (> 1.5) and after elution of the protein the ^{31}P signals quickly reached the baseline, which is an indication of a very low memory effect, although a slight tailing was still observed.

The role of the running buffer is important not only in protein separation, but also in GE-ICP-MS coupling for peak shape, elution time, detection and quantification limit of the analytes, especially when it is also used as an elution buffer.

The results show good peak shape and very low background signals. In a comparative study it was observed that the ^{31}P buffer background signals of the modified system were 2 to 3 times lower than the original method. This indicates that an enhanced phosphopeptides detection limit can be achieved using this modified system. A blank sample was run using a 10% gel for the control measurement in the modified system and no ^{31}P peak was observed. The use of SDS limits this system to identifying elements other than S, thus the P/S ratio for α- and β-casein could not be determined here.

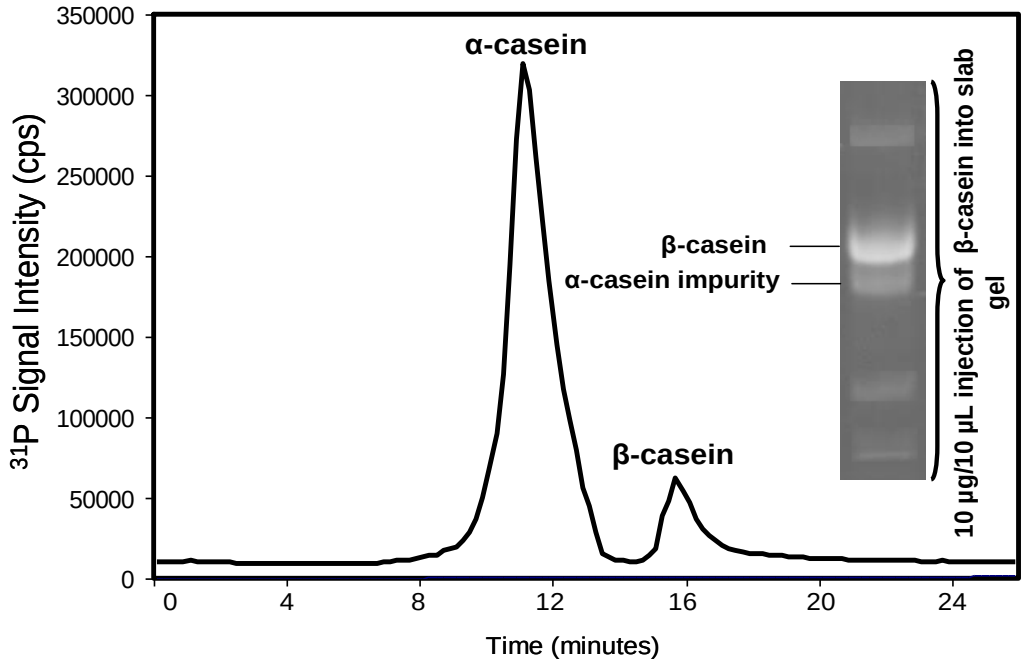


Figure 3.5: GE-ICP-MS on-line separation for α- and β-casein. 10 µL of the mixture of 0.10 nM α- and β-casein was injected into a 10% tube gel. β-casein was contaminated with α-casein and some other proteins causing a decrease in its signal intensity. β-casein impurities were also shown by injecting 10 µg/10 µL in a 10% slab gel.

For the separation of mono- and tetra-phosphopeptides from the β-casein digest, initially a 10% gel was employed to resolve the phosphorylated peaks (Fig. 3.6), but, as there was only one peak observed with comparatively high signal intensity, the gel percentage was gradually increased and a 15% gel was found to resolve the two ^{31}P peaks (Fig. 3.7). Further increasing the gel percentage did not show any additional peaks. It was concluded that the mono and tetra-phosphopeptide peaks were not fully resolved (even in the 15% gel) due to the presence of several phosphorylated and non-phosphorylated peptide impurities in the digest. A low percentage (10%) tube gel was not able to separate these impurities and as a result all the peptides were eluting as a single unresolved band and one peak was observed in the electropherogram. This was confirmed by running the mixture of [T6 (1P)] and [T1-2 (4P)] respectively (Fig. 3.8), which were resolved in the 10.5% gel. As shown in Fig. 3.8, despite the separation of mono- and tetra-phosphopeptides, valley between the both peaks does not touch the baseline which means the Rs value is less than 1.5. However it is not far behind and further work may improve the Rs. The runtime was less than 20 minutes which further confirms the sufficiency of modified tricine-SDS-PAGE online coupling with ICP-MS (GE-ICP-MS) for the separation of smaller peptides.

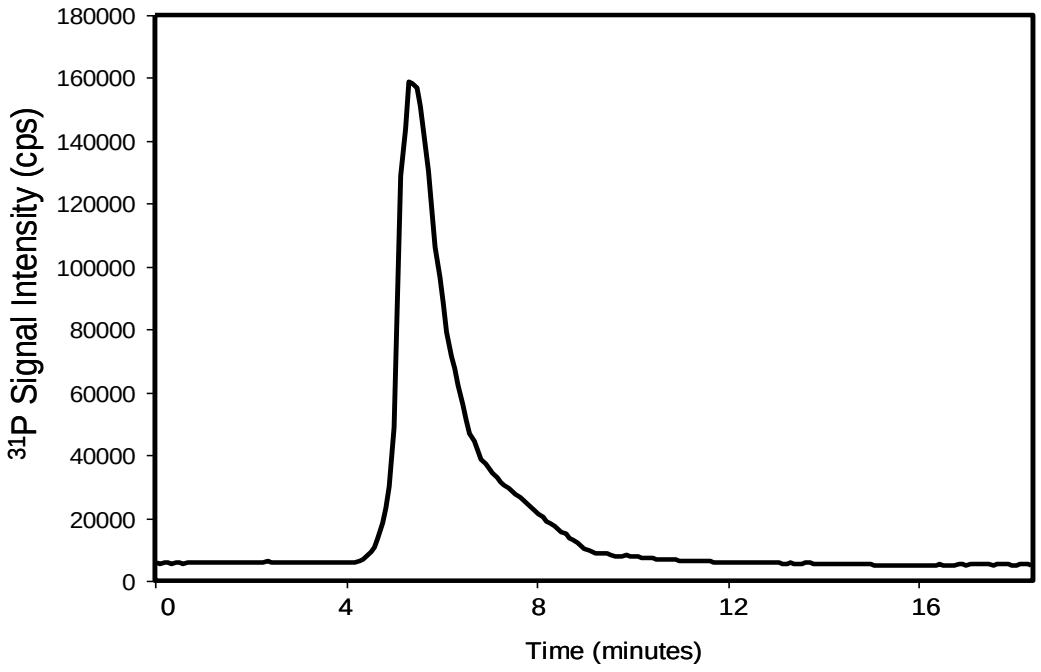


Figure 3.6: Electropherogram for the β-casein digest. 10 µL of the digest mixture of β-casein was injected into the 10% tube gel and only one peak was observed with a significant tailing.

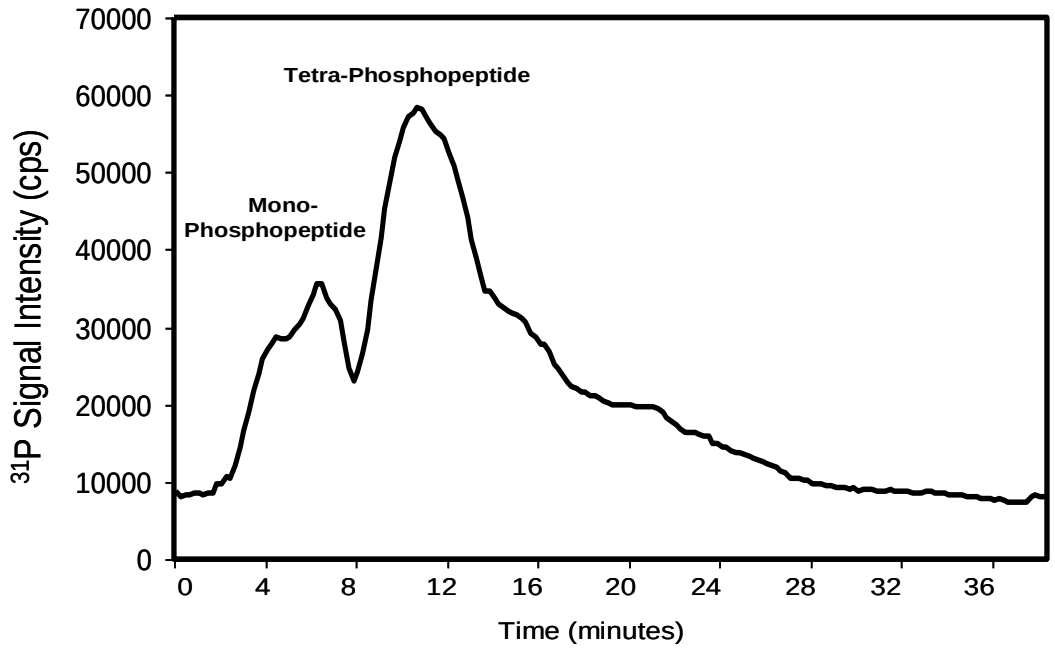


Figure 3.7: GE-ICP-MS on-line separation of the peaks containing mono and tetra-phosphopeptide respectively from the digest mixture of β-casein. 10 µL of the β-casein digest was injected into a 15% tube gel.

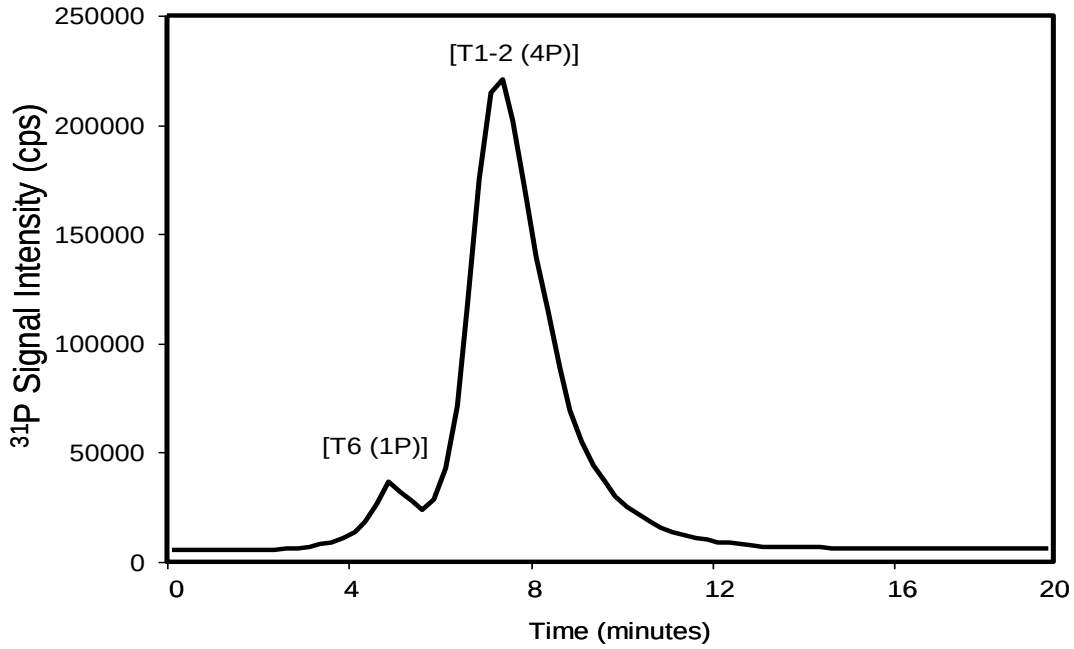


Figure 3.8: GE-ICP-MS on-line separation of the mono and tetra-phosphopeptide standards. 10 µL mixture of 20.2 µM [T6 (1P)] and 16.34 µM [T1-2 (4P)] was injected into 10.5% tube gel.

It is important to note that significant peak tailing was observed from the digest mixture (Fig. 3.6 and 3.7) so that baseline separation was not achieved. The valley between the both peaks in Fig. 3.7 is far behind the baseline that indicates the Rs value of the peaks is much less than the minimum desired value of 1.5. Several contributing factors to peak tailing in the electrophoretic peaks have been discussed in previous studies [127] including overloading of the analytes and weak efficiency of the column. In terms of GE-ICP-MS it means, if PAGE gel is not properly casted or the pores size of the gel are not sufficient for the separation of the proteins then gel will show poor efficiency and broader peaks or tail will be observed in chromatographic peaks

Hence a large number of unresolved ^{31}P peptide impurities were the major likely cause of peak tailing here. The main source for the phosphopeptide impurities could be mis-cleavages during the digestion [8] and α-casein contamination [128], which was also digested with the β-casein. Slow mass transfer to the detector is also an important factor in peak broadening [129]. So by increasing the gel percentage, protein transportation to the ICP-MS slowed down causing broader peaks as shown in Fig. 3.7.

A mass balance study was carried out using [T6 (1P)] and [T1-2 (4P)] and recoveries of 48$\pm$3.1% and 95$\pm$4.0% *(N=3)* respectively were obtained. The cut-off ~1.5 kDa of the dialysis membrane used here may well be the cause of low recovery of [T6 (1P)].

Lower limits of detection (LLD) were achieved as 0.7 and 0.2 µM for [T6 (1P)] and [T1-2 (4P)] respectively using the criteria of three times the standard deviation of the blank. These correspond to 7 pmol and 2 pmol per 10 µL sample of the mono and tetra-phosphopeptide solutions.

3.4 Conclusion

In conclusion, an easy and rapid method for the separation of peptides in low percentage uniform acrylamide gels has been described. This was achieved by modifying the previously described Tricine-SDS-PAGE system. This modified system reduces by 3 to 4 times the cost of the consumables, and using standards, separation with very good resolution was achieved by slab GE. Using low percentage gels and very low reagent concentrations reduces contamination and improves performance for quantitative proteomics and phosphoproteomics. A GE-ICP-MS set-up for the on-

line separation of phosphoproteins/phosphopeptides was also investigated using the modified Tricine-SDS-PAGE system. Whilst several custom made GE-ICP-MS systems have been described previously, here a setup was employed that required only minor modification of the original commercial electroeluter device. Using the modified Tricine-SDS-PAGE enables elemental detection in proteins/peptides such as those typically found in the tryptic digests of phosphorylated proteins. Here, very low ^{31}P background signals were obtained and yielded good LLD values for the phosphopeptides. However, use of the large format tube gels limited peak resolution and this approach may not be useful for the detection of several phosphorylated peptides in digest mixtures in comparison with µLC-ICP-MS systems. Future studies will investigate the use of smaller diameter tube gels, or slab gels, to improve peak resolution.

Chapter 4: A comparison of Tris-glycine and Tris-tricine Buffers for the Electrophoretic Separation of Major Serum Proteins

4.1 Introduction

Despite the advances in separation techniques such as HPLC, micro (μ)-HPLC, SEC and CE, gel electrophoresis (GE) methods are still widely and routinely used for the separation and detection of low and high relative molecular mass (Mr) proteins in biological and biochemical studies. The important merits that determine this widespread application of GE are its ease of use, simpler instrumentation, high resolution and low cost compared to other methods. The recently introduced online coupling of GE with powerful detection techniques such as inductively coupled plasma mass spectrometry (ICP-MS) [22, 24, 27], has further enhanced its potential for the qualitative and quantitative analysis of biomolecules. However, widespread use of the offline coupling of GE with MS based techniques for the determination of phosphorylated and non-phosphorylated protein, such as MALDI-MS, have played a major role in enhancing the scope and applications of the GE based methodologies [130-132]. One dimensional gel electrophoresis involves either SDS-PAGE or native-PAGE methods, however, SDS-PAGE has not been encouraged for the detection and quantitation of metal binding proteins because the metal tends to be lost during the sample preparation [38]. Thus the importance of native-PAGE increases when characterization of the intact metal bound proteins is required.

Mammalian serum is a complex fluid. It is the major transport medium carrying: nutrients to the cells, waste to the kidneys, regulatory molecules to target tissues, antibodies to the invading sites and clotting agents to injured or damaged parts of blood vessels. In human serum 3700 proteins have been discovered and 325 have been identified [133]. Of these the major proteins found in serum are alpha-1 globulin (1-3 mg/mL), alpha-2 globulin (6-10 mg/mL), β-globulin (7-12 mg/mL), γ-globulin (7-16 mg/mL) and albumin (35-50 mg/mL).

The separation of these five major serum proteins by native-PAGE techniques is

difficult to achieve in a single gel. To separate these major proteins in a single gel, a detailed modification of the gel system is required [134].

In SDS-PAGE, Tris-glycine and tricine methods are widely used for the separation of high and low molecular mass proteins respectively. Recently, the tricine-SDS-PAGE method has been modified and its successful online coupling with ICP-MS for the separation and quantitation of low molecular mass proteins has been described in the chapter 3 [27]. Here, the same modified method, but without using SDS (native-PAGE) was used to separate the five major serum proteins in a 7% single PAGE gel.

As described in chapter 1 (section 1.1.1) SPEP is a quick method for the separation of major serum proteins and it is employed for clinical studies. For further information about procedure please see [135]. The main focus for the separation of the major serum proteins by SPEP is to determine their quantities which are good indicators of several diseases in clinical studies. For example, a low level of albumin may be indicative of liver infection and renal loss whilst dehydration may increase albumin level in plasma. For further details the following reference can be consulted [136].

For SPEP Tris-barbital buffer system is used as a running buffer but here for the first time modified Tris-tricine buffer was employed and our results showed enhance resolution of these major serum proteins.

The main objectives of this study were to investigate the potential of a modified Tris-tricine system for the separation of serum proteins by native-PAGE, and SPEP in comparison with the other traditional methods which employ Tris-glycine, Tris-tricine and Tris-barbital buffer systems.

4.2 Experimental

4.2.1 Chemicals

The chemicals required to prepare the gel and buffers have been described in chapter 3 (section 3.2.1). In addition 5, 5-Diethyl- barbituric acid (Barbital), 5, 5-Diethylbarbituric acid sodium salt (barbitone) for Tris-barbital buffer and Ponceau S for membrane staining were purchased from Sigma-Aldrich, UK. Sepraphore® III (cellulose

acetate) membranes (5.7 x 12.7 cm) for SPEP were purchased from DiaSys, Berkshire, UK.

All the solutions were prepared in ultra-pure water (18 MΩ. cm, from a Milli-Q water purification system, Millipore Corporation, Bedford, MA).

Human blood serum samples were obtained from Dr. Imre Lengyel, Institute of Ophthalmology, University College London, Bath Street, London, EC1V 9EL, UK.

4.2.2 PAGE

4.2.2.1 Buffer system for native and SDS-PAGE

To prepare the stacking and resolving gels and running/electrode buffers for native and SDS-PAGE three buffer systems were employed as described below. In stacking and resolving gels pH was adjusted using HCl. For running/electrode buffers, pH adjustment is not required.

4.2.2.2 Tris-glycine

To prepare the stacking and resolving gels for native and SDS-PAGE, 2.5 M Tris-buffer with a pH of 8.8 were prepared. The pH of the gel buffers was adjusted using HCl. Running buffers were prepared with concentrations of 192 mM glycine and 50 mM Tris without SDS for native PAGE and with 0.1% (m/v) SDS for SDS-PAGE.

4.2.2.3 Tris-tricine original

Stacking and resolving gels were prepared using 2.5 M Tris-buffer with a pH of 8.45 for native and SDS-PAGE. For running/electrode buffer 100 mM Tris-tricine without SDS for native-PAGE and with 0.1% SDS for SDS-PAGE were prepared. (Note: original Tris-tricine method requires two different running/electrode buffers i.e., 100 mM Tris-tricine (pH 8.25) on cathode chamber and 100 mM Tris (pH 8.9) on anode chamber. Here, to compare with modified Tris-tricine system, only one buffer (100 mM Tris-tricine with pH 8.25) was employed on both electrodes in original system.

4.2.2.4 Tris-tricine modified

For modified Tris-tricine system both stacking and resolving gels were prepared by employing 2.5 M Tris buffer with a pH of 8.8. The gels were prepared using same buffer for both native and SDS-PAGE. Similarly, 25 mM Tris-tricine buffer was prepared for native-PAGE without SDS and 0.05% SDS was included into the buffer for SDS-PAGE.

4.2.2.5 Sample buffer

Non-reducing sample buffer for native-PAGE and SPEP was prepared by mixing 1 mL of 1 M Tris with 3 g glycerol and 0.02 mg Bromo Phenol Blue (BPB) and making up the solution to 10 mL with deionized water. 2x reducing sample buffer for SDS-PAGE was purchased from Sigma Aldrich, Poole, UK containing 100 mM Tris-HCl (pH 6.8), 1% (m/v) SDS, 4% (v/v) 2-mercaptoethanol, 0.02% (m/v) Coomassie Brilliant Blue (CBB) and 24% (m/v) glycerol.

4.2.2.6 Gel casting

For native-PAGE and SDS-PAGE, 4% stacking gel (%T = 4 m/v, %C = 3.3 m/v) and 7% resolving gel (%T = 7 m/v, %C = 3.3 m/v) were cast as described in Chapter 3 [27]. (*Note*: %T represents the total concentration of acrylamide and bis-acrylamide and %C represents the concentration of cross linker or bis-acrylamide). Urea, SDS and glycerol were not included in casted gels. For the stacking gel 0.66 mL of acrylamide/bis-acrylamide, 0.76 mL of 2.5 M Tris buffer, 100 µL of APS, and 6 µL of TEMED were dissolved in 3.5 mL of deionized water. Similarly, for the 7% resolving gels 2.33 mL of acrylamide/bis-acrylamide, 5.60 mL of 2.5 M Tris buffer, 150 µL of APS, and 6 µL of TEMED were dissolved in 1.91 mL of deionized water. The mini-gel casting apparatus was from Bio-Rad UK (Mini-PROTEAN® 3 Cell) and all the gels were cast at 7.3 cm height x 8 cm width and 0.75 mm thickness. (*Note*: acrylamide is recognised as a neurotoxin [137], so gloves must be worn all the time and the work must be done in a properly ventilated area).

4.2.2.7 Sample preparation and loading onto the gel

Human serum was diluted 10 fold with deionized water and mixed with sample buffer in a 1:1 ratio (*Note*: serum samples were not centrifuged before or after the dilution). 10 µL of this sample was injected onto a 7% gel and run at room temperature using a PowerPac™ HC, high current power supply system (Bio-Rad Laboratories, Hemel Hampstead, UK) at an applied voltage of 150V (0.08-0.12 mA current).

4.2.3 Serum protein electrophoresis (SPEP)

SPEP was performed for the separation of serum proteins by employing the conventional Tris-barbital running buffer (pH 8.6), Tris-glycine and the modified Tris-tricine-native-PAGE buffer system. Tris-barbital buffer was prepared in 800 mL of deionized water by dissolving 5.8 g Tris-base (60 mM), 2.5 g barbital (16 mM) and 9.8 g barbitone (59 mM). Each membrane was soaked in each of the three buffers for 5-10 minutes. Human serum was prepared (as described above) and run by injecting 5 µL on the Sepraphore® III (cellulose acetate) membrane and running for 30 minutes at 300V (DC power supply, Consort E832, Belgium).

4.2.3.1 Staining and de-staining

For PAGE, a 0.025% (m/v) Coomassie Brilliant Blue solution was prepared in 10% (v/v) acetic acid and each gel was stained for 20 minutes after washing with deionized water. To enhance the band visibility/contrast, the staining was repeated by replenishing the staining solution. Gels were washed with deionized water and de-staining was performed using 10% acetic acid for 20-25 minutes. Similarly, for the membrane, staining was performed using 5% (m/v) Ponceau S (prepared in 5% (v/v) dicholoro acetic acid) for 10 minutes and after washing with water the membrane was stained with 0.025% Coomassie staining solution for 5 minutes and de-stained with 10% acetic acid for 5-10 minutes.

4.3 Results and discussion

4.3.1 Electrophoretic separation of serum proteins by native-PAGE

The Tris-tricine buffer system is usually employed as an electrode/elution buffer for the analysis of low molecular mass proteins in tricine-SDS-PAGE [27, 39]. The original tricine-SDS-PAGE method has been simplified for the separation of low molecular mass proteins by changing the pH and concentration of the buffers used and more effective separation was achieved by employing this modified method, as described in Chapter 3 [27]. The simplified procedure works with only one electrode buffer i.e., 25 mM Tris-tricine and 0.05% (m/v) SDS (pH 8.25) with a gel buffer pH of 8.8. By contrast, the original method used two different electrode buffers i.e. 100 mM Tris-tricine and 0.1% (m/v) SDS (pH 8.25) for the cathode and 100 mM Tris (pH 8.9) for the anode, with a pH of 8.45 for the gel buffer. Here, for the first time this buffer system (without SDS) was used for the separation of high molecular mass serum proteins. To evaluate the potential of this Tris-tricine system for the separation of serum proteins, a comparative study was carried out using Tris-glycine and Tris-tricine original and modified systems. Fig. 4.1 shows head-to-head comparative results obtained after electrophoretic separation: (a) Tris-glycine (b) Tris-tricine original and (c) Tris-tricine modified methods. It is clear from Fig. 4.1 that the five most abundant serum proteins were only resolved in the Tris-tricine modified buffer system whilst in Tris-glycine only three bands were observed in a 7% (m/v) gel. Similarly, the Tris-tricine original native-PAGE method was also unable to fully resolve these most abundant serum proteins.

In an SDS-PAGE system, the migration speed of the ions such as glycine or tricine plays a very important role in the separation of the analytes. It has been observed in previous investigations that the high pH of the resolving gel enhanced the migration speed of the glycine and tricine and as a result the bands were further resolved in the SDS-PAGE system [27, 126].

In SDS-PAGE mobility primarily depends on the molecular masses of the proteins whilst in native-PAGE, proteins retain their folded conformation so electrophoretic

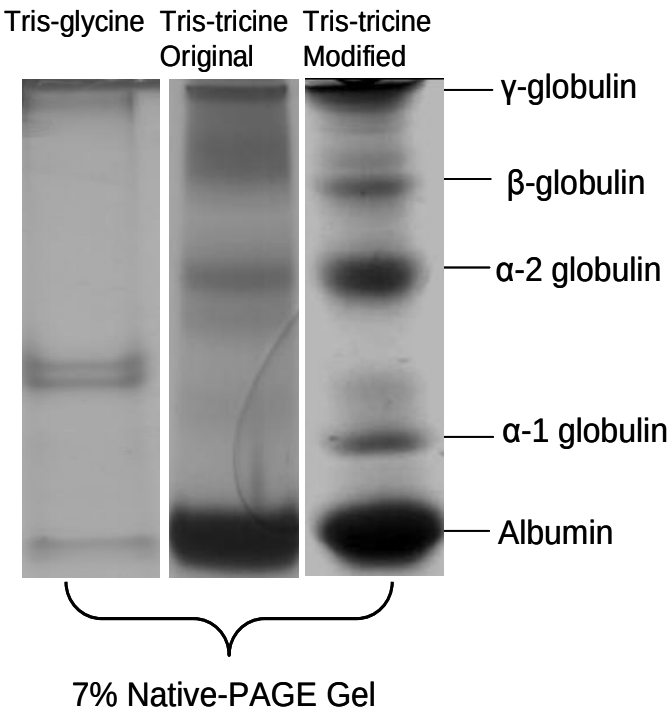


Figure 4.1: Head-to-head comparison of the different buffer systems in native-PAGE. The modified Tris-tricine-native PAGE system efficiently resolved all the five most abundant serum proteins in a 7% gel. 10-µL of the serum sample was injected and separation was performed at the applied voltage of 150V at room temperature with the gel size of 7.3 cm height x 8 cm width and 0.75 mm thickness.

mobility of the proteins depends on several factors such as size, shape and protein's native or intrinsic charges. Thus in alkaline conditions proteins with high negative charge density will migrate faster from cathode to anode than the proteins with low negative charges [43, 138]. Highly abundant proteins in serum have pI values below 7 (Swiss Prot). Hence they are negatively charged, and increasing the pH of the gels from 8.45 to 8.8 provides a more favourable environment for protein movement from cathode to anode and the separation of the five most abundant serum proteins was observed with enhanced resolution. Interestingly, the Tris-glycine system did not show a similar level of separation at pH 8.8. The apparent reason for this poor resolution of the Tris-glycine system is the slow migration speed of the glycine. Glycine is a weaker ion and migrates much more slowly than tricine in a basic environment even at high pH [43], so at pH 8.8, tricine migration speed further enhances the separation of serum proteins in native-PAGE.

4.3.2 SDS-PAGE

Despite the fact that the Tris-tricine buffer system effectively resolves serum proteins in native-PAGE, Tris-tricine-SDS-PAGE system is not very useful in resolving these serum proteins. As shown in Fig. 4.2 very poor separation of the serum proteins was achieved using the Tris-tricine-SDS-PAGE system, in a 7% (m/v) gel. The Tris-glycine system showed comparatively better resolved serum proteins bands. For the Tris-tricine original and modified methods similar results were obtained. As mentioned above, 3700 proteins have been discovered in serum, covering a wide mass range.

The Tris-tricine-SDS-PAGE system has good capability for resolving 3-200 kDa molecular mass proteins in a 7% (m/v) acrylamide gel [27] and due to the large number of proteins, the bands were not properly resolved and poor separation was achieved.

The Tris-glycine SDS-PAGE system resolves only very high molecular mass proteins in a 7% (m/v) PAGE gel, hence better separation was achieved for the major plasma proteins. Note, because of the severe loss of loosely bound metals in the different steps of SDS-PAGE, such as sample preparation [38], staining and de-staining process [139, 37], it has not been recommended for quantitative studies of metal bound proteins.

4.3.3 Serum protein electrophoresis

SPEP is not a widely employed electrophoretic method due to the poorly resolved bands obtained on the cellulose acetate membrane, however it is a quick separation method and frequently used in hospitals for the screening of blood samples because of its speed and ease of coupling with a densitometer. Tris-barbital buffer of pH 8.6 is considered to be the standard running buffer for protein separation in this system.

Here, this method was further investigated and it was found that the Tris-tricine running buffer enhanced protein separation. As shown in Fig. 4.3, the major serum proteins were better resolved using the modified tricine compared with the conventional Tris-barbital buffer system.

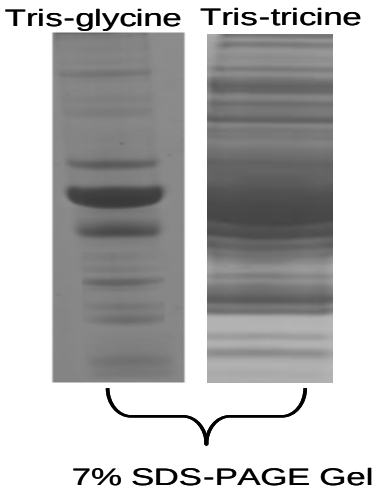


Figure 4.2: Separation of the serum proteins via Tris-glycine and Tris-tricine-SDS-PAGE (10-µL sample injection) showing that Tris-tricine produces a much more complex electropherogram than Tris-glycine. This electrophoretic separation was performed at applied voltage of 150V at room temperature in 7% PAGE gel with the size of 7.3 cm height x 8 cm width and 0.75 mm thickness.

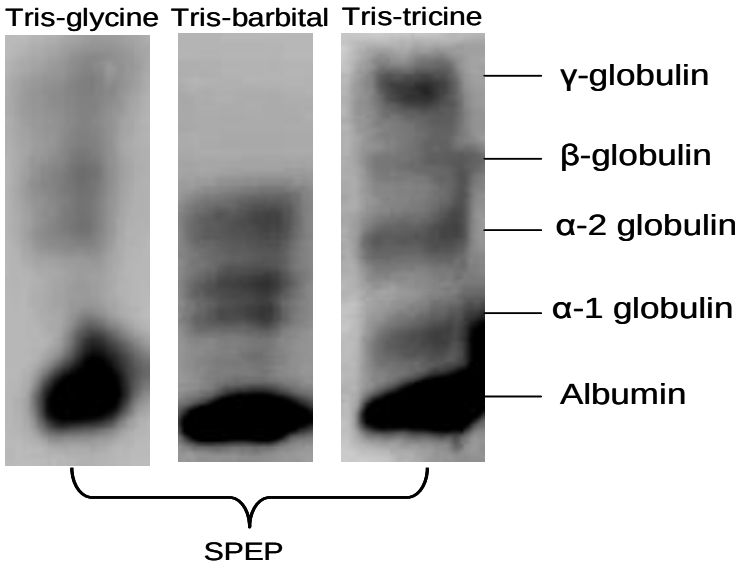


Figure 4.3: A comparison of the separation of serum proteins on a Sepraphore® III (cellulose acetate) membranes (5.7 x 12.7 cm) using conventional Tris-barbital, Tris-glycine and the modified Tris-tricine buffer systems. Modified Tris-tricine showed the best resolution of the five major protein bands. 5 µL of the human serum was injected onto the membrane and run for 30 minutes at 300V.

It is clear from Fig. 4.1 and Fig. 4.3 that, despite improvement in SPEP separation by employing the tricine buffer system, it still cannot compete with the slab PAGE separation methodology in terms of resolution. However, with respect to the total time consumption, SPEP could be a better choice than native-PAGE. As described above, in SPEP the good separation was achieved within 30 minutes by applying the voltage of 300V. Interestingly, native-PAGE gel takes 1 to 2 hours for this separation of serum proteins at applied voltage of 150V. It was observed that in native-PAGE separation, increasing the voltage up to 250V, separation can be achieved within an hour, however this high voltage increases the temperature inside the gel and running buffers which may cause change of the pH of the gel and running buffer [27, 126] whilst the cellulose acetate membrane can tolerate the high voltage current and achieve separation without any proteolysis of the serum proteins. major protein bands. 5 µL of the human serum was injected onto the membrane and run for 30 minutes at 300V.

4.4 Conclusion

An easy and rapid method for the separation of serum proteins in native-PAGE was achieved using the modified tricine SDS-PAGE method described in the previous chapter. Results indicate that the five major proteins were separated with enhanced resolution (using Tris-tricine buffer system), compared to the Tris-glycine and Tris-tricine original methods. The Tris-tricine running buffer was also found to be much more effective in the separation of serum proteins in cellulose acetate electrophoresis (or SPEP) as compared with conventional buffer systems.

Chapter 5: Preventing Protein Fragmentation and Metal Loss in SDS-PAGE

5.1 Introduction

Gel electrophoresis based methodologies play a vital role in the separation, detection and quantification of bio-molecules, as discussed in Chapter 1 (section 1.1), with SDS-PAGE being the most widely used method due to its high reproducibility and resolving power.

Despite the benefits of employing SDS-PAGE, several publications have reported the limitations and problems associated with SDS-PAGE [36-38]. The two most important problems which have been frequently addressed are the fragmentation of the proteins [140] and loss of the metals in metalloproteins [36-38]. The fractional loss of a specific metal from a protein depends on its binding affinity. The binding affinity of the metals with proteins can be classified into two categories [38] (a) high affinity binding, which characterises the so-called metalloproteins and (b) weak affinity binding, usually non-covalent in nature, which characterises the so-called metal-binding proteins.

For the separation of proteins using GE or SDS-PAGE methods, three main steps are involved: (a) sample preparation, (b) running the sample in the gel using the electrode buffers and (c) staining and destaining. Previous studies suggest that step (c) may cause metal loss [37], however, staining and de-staining are not necessary for qualitative or quantitative studies of metal-binding proteins performed by coupling gel separation with ICP-MS detection. Running the sample in high percentage gels could also be an important factor for the protein fragmentation and metal loss [3]. This problem can be resolved using either the tricine-SDS-PAGE or modified tricine-SDS-PAGE system previously described in Chapter 3 [3] as they employ low percentages gels. In SDS-PAGE, the most apparent and important factor in protein fragmentation and loss of the metals is the sample preparation step so the main objective of this study was to develop a strategy to avoid this problem.

5.2 Experimental

5.2.1 Chemicals

All the solutions were prepared in ultra-pure water (18 MΩ. cm, from a Milli-Q water purification system, Millipore Corporation, Bedford, MA).

Chemicals for the preparation of the gels and buffer were as described in Chapter 3 (section 3.2.1) with the addition of human serum albumin (HSA, (molecular mass=66.5 kDa) as a model protein purchased from Sigma-Aldrich.

5.2.2 SDS-PAGE

To investigate protein fragmentation in SDS-PAGE a modified tricine-SDS-PAGE system was employed as described in Chapter 3 [27]. The stacking and resolving gels (4 and 7%) (m/v) were cast as described in chapter 3 (section 3.2.4 and Table 3.2).

Reducing sample buffers were prepared for SDS-PAGE with pH values of 6.8, 7.0 and 8.8 (pH was adjusted using HCl). 1 mL of 1 M Tris was mixed with 0.31 g of DTT, 0.2 g SDS, 2% Coomassie Brilliant Blue and 3 g glycerol and the solution was made up to 10 ml using deionized water (glycerol and tracking dye were not included in the sample buffer for the metal loss studies).

For the study of the protein fragmentation, 10 µL of 1 mg/mL HSA was mixed with 10 µL of sample buffer (pH of 6.8, 7.0 or 8.8) and after heating the mixture at 70ºC, 90ºC and 100ºC,10 µl was injected into the 7% gel. Similarly, for the metal loss studies 1 mL of 1 mg/mL HSA was mixed with 1 mL of one of the above described sample buffers and each mixture was heated at 70ºC, 90ºC and 100ºC.

To investigate any changes in pH during the heating and cooling process, the sample buffer pH was measured before heating, after 10 min heating and after cooling to room temperature.

For SDS-PAGE, a 0.025% (w/v) Coomassie Brilliant Blue solution was prepared in a 10% (v/v) acetic acid and each gel was stained for 20 minutes after washing with deionised water. For sharp bands, the staining was repeated by replenishing the

staining solution. Gels were washed with deionized water and destaining was performed using 10% acetic acid for 15-20 minutes.

5.2.3 Ultra-filtration

Ultra-filtration centrifuge tubes (2 mL), incorporating a polyethersulfone membrane with molecular mass cut-off 3 kDa (Vivaspin 2), were purchased from Sartorius Corporation UK. An ultra-filtration process was performed for the study of zinc and copper loss in the samples. The tubes were filled with 2 mL of each mixture of sample and sample buffer (1:1) and centrifuged at 1000 x g for 30 minutes. The supernatants were collected and 1 mL of each supernatant was mixed with 10 mL of deionized water before analysis by ICP-MS.

5.2.4 Instrumentation

ICP-MS analysis for ^{63}Cu and ^{66}Zn was performed on an Element 2 XR Sector Field ICP-MS instrument (Thermo Scientific, Bremen, Germany) using a PFA micro-flow LC nebulizer (Elemental Scientific, Omaha, USA) with a flow rate of 400 µL/min and a cyclonic glass spray chamber (Glass Expansion, Victoria, Australia). The instrument was operated at medium resolution (R=4000) to avoid interferences on ^{63}Cu and ^{66}Zn. The flows of the gases were adjusted as: Outer = 15.5 L/min, Auxiliary = 0.88 L/min, Nebulizer = 0.997 L/min with forward power of 1250 W. Ni sampler and skimmer cones were used throughout the analysis.

To perform the pH measurements a Jenway pH meter was from Bibby Scientific Limited, Beacon Road, Stone, Staffordshire, ST15 0SA, UK.

For sample preparation in SDS-PAGE, proteins require mixing with a volume of reducing sample buffer, which is usually prepared by dissolving glycerol, tracking dye (Bromo Phenol Blue or Coomassie), anionic detergent SDS and a reducing agent such as DTT or 2-mercaptoethanol in Tris buffer at pH 6.8. In the presence of DTT 70°C is enough to reduce disulfide bonds in proteins. SDS binds with proteins with the constant ratio of 1.4 g/g. Here, the role of sample buffer in protein fragmentation and metal loss was investigated. Cannon-Carison *et al.* previously investigated the role of the pH of the sample buffer in protein fragmentation using three pH values, pH 6.8, pH 7.0 and pH 8.8 at a constant temperature of 100ºC and it was suggested that

phosphate buffer (instead of Tris) at pH 7.0 could be a good buffer to prevent fragmentation of the proteins [140]. Buffering efficiency of phosphate buffer lies in the pH region 6.4-7.4 so it will be a good buffer below pH 7.4 [141]. However, using phosphate buffer in the sample increases the phosphorus background and hampers the use of ICP-MS detection and quantification of phosphoproteins. Here, this work was extended using the same sample buffers with the same 3 pH values but with different temperatures, however only Tris base was used to prepare these sample buffers as most of the SDS-PAGE systems employ Tris buffer. Sample buffers of each pH value were mixed with HSA in a 1:1 ratio. Each mixture was heated to 70 and 100ºC for 10 minutes and run on a 7% modified tricine-SDS-PAGE system. The results obtained from this investigation are discussed below.

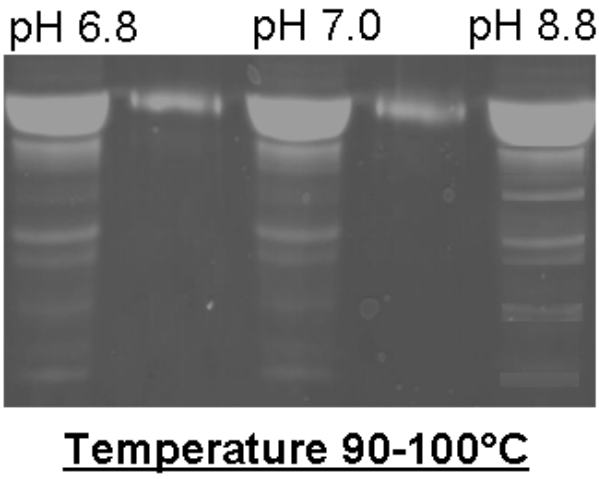
5.3 Results and discussion

5.3.1 Protein fragmentation

It is clear from Fig. 5.1 that at 90-100°C considerable protein fragmentation occurred at all 3 pH values. However, at 70°C there was very little fragmentation at all 3 pH values. This indicates that 70ºC provides a suitable environment for sample preparation at any pH. However, in comparing the temperature and pH values the high temperature was observed to be a dominating factor in protein cleavages during sample preparation, especially at boiling temperature as it is clear from Fig. 5.1a that similar protein fragmentation without any significant difference in proteolysis was observed at all three pH values.

Measurements of the sample buffer pH, presented in Table 5.1, show that heating up to 90-100ºC can also significantly decrease the pH of the sample. This low pH, high temperature environment favours the fragmentation of the peptide bonds. The major factor involved in this reduction of the pH of the sample is the sensitivity of the Tris buffer to temperature and its weak buffering efficiency below pH 7.5 [141]. Interestingly, Tris buffer at pH 8.8 only dropped down to pH 7.2 (Table 5.1) at 90-100ºC but still showed the fragmentations. It has been previously reported that at mild to high alkaline pH a number of chemical reactions take place at elevated temperature (above 85ºC) in different positions of the amino acid chain which can cause hydrolysis of the sample proteins.

(a)



(b)

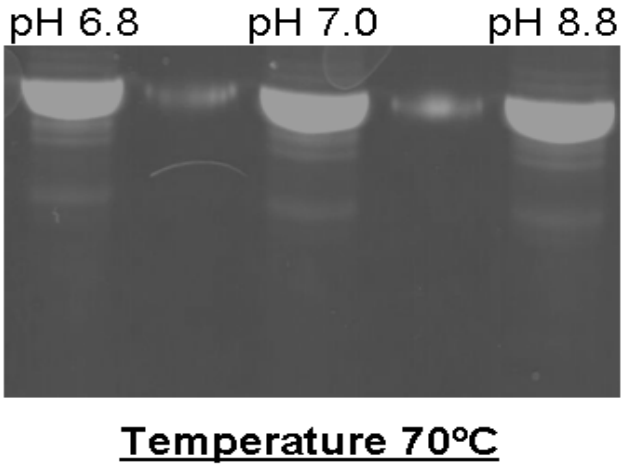


Figure 5.1: Head to head comparison of protein (HSA) fragmentation for sample buffers at pH values of 6.8, 7.0 and 8.8 and temperatures of (a) 100ºC and (b) 70ºC in SDS-PAGE.

Many of these involve reaction with cysteine, for example the base catalyzed beta elimination of sulphur to yield dehydroalanine, which can react with lysine to form lysinoalanine [142-144]. On the other hand, the opposite proteolytic trend was observed at 70ºC as compared to 90-100ºC. As shown in Fig. 5.1b heating the sample at 70ºC did not show protein fragmentation at all three pH values. As listed in Table 5.1, whilst pH was reduced on heating at all temperatures, a relatively small reduction of the pH was observed from all three initial pH values on heating at 70ºC. These

findings confirm that high acidic or alkaline medium at 90-100ºC produces an environment to cleave the amino acid chains. Thus selecting the pH of the sample buffer from 6.8, 7.0 and 8.8 is not important to prevent protein fragmentation, but selecting the temperate is crucial and plays a major role. Thus choices of the DTT is crucial as compared to 2-mercaptoethanol as the latter requires 100ºC for efficient use as a reducing agent.

Table 5.1: pH of the sample buffer before, after 10 minutes heating and cooling at room temperature (n=3)

pH of the sample buffer before heating	**pH of the sample buffer after 10 minute heating at 70, 90 and 100°C and cooling at room temperature (°C)**		
Room Temp	**70**	**90**	**100**
6.8	6.4±0.1	6.2±0.1	6.1±0.1
7.0	6.7±0.1	6.4±0.2	6.3±0.2
8.8	8.4±0.2	8.1±0.1	8.1±0.1

5.3.2 Metal loss study

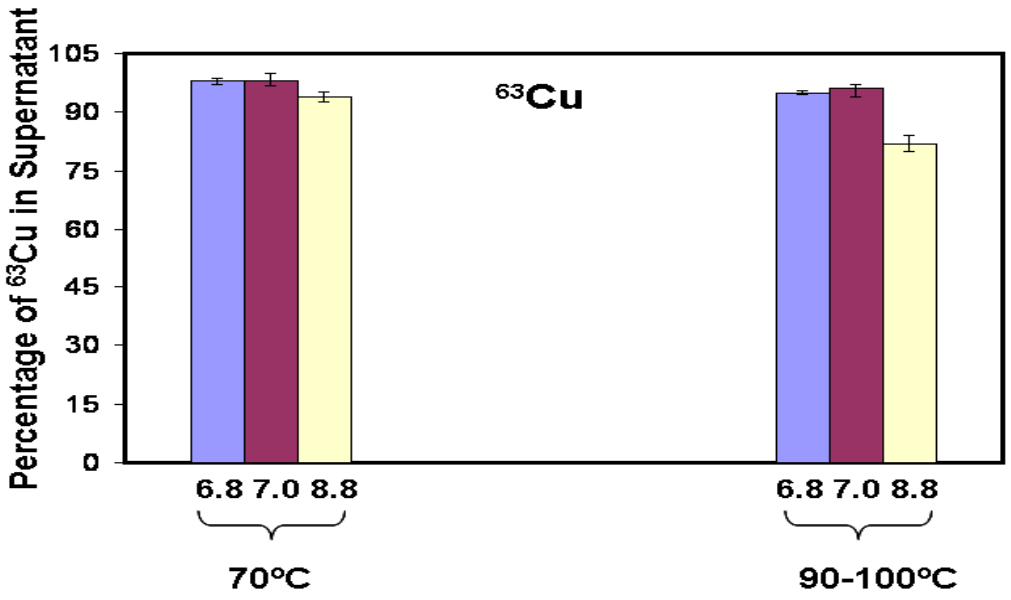
Previous studies have reported the loss of metals from proteins in the sample preparation step [38]. The major reason described was unfolding of the 3 dimensional or tertiary structures of proteins [38]. Thus if the metal is not tightly bound (bound with weak affinity) with the amino acid, it will be lost when protein unfolds its tertiary structure. However, if the metal is bound with high affinity it can be retained (depends on the binding constant, log *K*, values). Here the factors affecting the metal loss on the high binding affinity sites were investigated using HSA. In this study HSA was selected as a model protein because it contains zinc and copper with high Zn(II)-His and Cu(II)-His affinity respectively [145]. The former's binding affinity with denatured HSA can be affected with the change of the pH [145] as described below.

Albumin is the most abundant protein (40 mg mL^{-1}) in blood plasma and 98% of the exchangeable zinc in blood plasma (9-14-µM) is bound with albumin [146-148]. Swiss Prot data indicates that each molecule of HSA binds with one copper and four zinc ions. Half of the total zinc (two zinc ions) in albumin binds with Histidine (His) residues at position 91 and 271 whilst the other two bind with Asparagine (Asn) and Aspartic acid (Asp) at 123 and 273 positions of the amino acid chain. Similarly copper binds with His at position 127. Masuoka *et al.* [145] have reported that binding affinities of the Zn(II) and Cu(II) are higher at His sites of the amino acid chains, however these binding affinities in Zn(II)-His as compared to Cu(II)-His are affected by change in pH of the buffer or chelates. The affinity of the metal-protein binding depends on pH. The binding constant (log *K*) of Zn(II) and Cu(II) with the highest affinity sites in HSA [Zn(II) – His], [Cu(II)-His] were reported to be 7-7.5 and 11.1 respectively [145, 149]. Masuoka *et al.* have further reported no binding of Zn(II)-His at pH 5.0, however, Cu(II)-His binding is not significantly affected at acid or alkaline pH [145]. The Zn (II)-His binding constant value was determined at pH 7.4 [145, 150]. Thus zinc binding affinities can be decreased by increasing the acidic environment.

Fig. 5.2 shows the percentages of ^{63}Cu and ^{66}Zn remaining in the supernatant after centrifuging the mixture of HSA and sample buffer at pH 6.8, 7.0 and 8.8 with temperatures of 70 and 90-100ºC. These percentages were calculated against the percentages of the metal remaining in the supernatant without heating. Fig. 5.2 shows the percentages of ^{63}Cu and ^{66}Zn remaining in the supernatant after centrifuging the mixture of HSA and sample buffer at pH 6.8, 7.0 and 8.8 with temperatures of 70 and 90-100ºC. These percentages were calculated against the percentages of the metal remaining in the supernatant without heating. For copper studies, very low level of the metal loss was observed (shown in Fig. 5.2a). In contrast, variations with respect to the temperature and pH were observed in zinc.

Table 5.1 shows heating the sample for 10 minutes, decreases its pH, however, cooling to room temperature recovers it with only a slight decrease compared to the pH before heating. This indicates that zinc detached from the protein by heating will rebind after cooling down to room temperature, however, binding will depend on the recovered pH of the medium. This pH recovery at room temperature was much closer to the actual pH values at 70°C than 90-100°C.

a)



b)

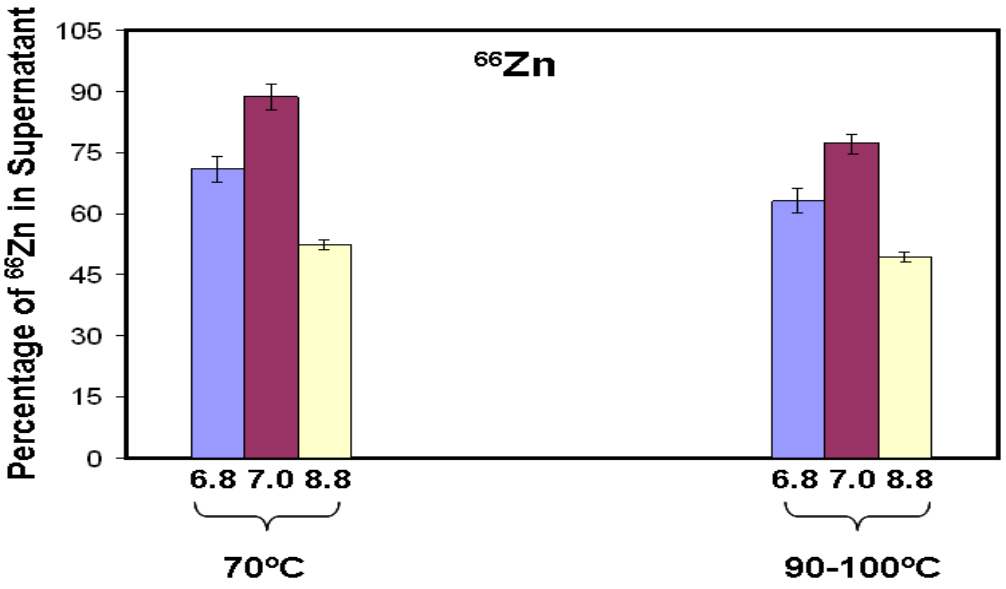


Figure 5.2: Shows Percentages of (a) ^{63}Cu and (b) ^{66}Zn in HSA remaining in the ultra-filtrate supernatant after heating at 70, 90-100ºC with the sample buffer pH 6.8, 7.0 and 8.8 (n=3) as compared with the original ^{63}Cu and ^{66}Zn concentration before heating.

Thus zinc showed maximum retained percentages at 70 ºC (Fig. 5.2b) i.e., 77, 90 and 50% for pH 6.8, 7.0 and 8.8 respectively. These results clearly show that the metal loss in HSA can be minimized in SDS-PAGE by preparing the protein sample at 70ºC. Here, at 70ºC, minimum zinc loss was observed which suggests 70ºC is an optimal temperature. This indicates that all those metals whose binding affinities with the proteins are affected by a change in pH, can be retained by selecting pH values close to the optimum pH as indicated by their log *K* values and by preparing the sample at 70ºC. The similarities between the proteolysis and metal loss are important to note and indicate that similar factors were involved in the proteolysis and metal loss.

5.4 Conclusion

In conclusion, the factors involved in fragmentation of a protein sample and metal loss in the sample preparation stage of SDS-PAGE have been investigated. It was found that application of high temperature i.e. 90-100ºC at sample buffer pH values of 7.0, 6.8 and 8.8 led to unwanted protein fragmentation, which clearly suggests that high temperature plays a dominating role in fragmentation. A reducing sample buffer (containing Tris, SDS and DTT) at a temperature of 70ºC was found to be optimal in order to avoid fragmentation. Measuring ^{63}Cu and ^{66}Zn in HSA showed that prior information about the binding constant values of the metal-amino acid is very important in order to avoid metal loss. At 70ºC, the pH of the Tris-sample buffer does not significantly change so this could be the best temperature for sample preparation with the sample buffer prepared at the optimal pH for the binding constant value of the metal-amino acid bond. It is important to note that this work focused only on the Tris sample buffer because popular SDS-PAGE methods like Laemmli and Tricine SDS-PAGE employ Tris in the sample buffer. However, in future work, other buffers like Bis-Tris, HEPES etc can be employed which may produce much better results.

CHAPTER 6: Laser Ablation (LA)-ICP-MS Determination of Metal Bound Proteins

6.1 Introduction

Laser ablation coupling with ICP-MS (LA-ICP-MS) is a powerful detection method for the characterization of specific elements present in a given sample. It has frequently been used for the characterization of metal and non-metal bound proteins in the last few years [16, 19, 36-38]. LA-ICP-MS can easily be combined with GE techniques via off-line separation of the proteins followed by GE-LA-ICP-MS analysis of the gel, however, this strategy suffers from several problems, especially in PAGE-LA-ICP-MS such as: (a) high background signal intensity due to contamination in the gel which results in poor detection limits and may hamper quantitative studies, (b) curling of the gel during PAGE-LA-ICP-MS analysis occurs due to gel drying. GE-LA-ICP-MS is described in detail in chapter 1 (section 1.4). It was observed in this work that the use of a cellulose acetate membrane in place of a polyacrylamide gel offers the advantages of much reduced contamination from P, S, Fe, Cu and Zn together with greater physical robustness.

The main objectives of this study were to investigate a novel SPEP-LA-ICP-MS strategy for the detection of Fe, Cu and Zn to confirm the separation of the serum proteins.

6.2 Experimental

6.2.1 Chemicals

Chemicals and materials used in this work were as listed in Chapter 4 (section 4.2.1) In addition, ICP-MS elemental standards for the quantification of P, S, Fe, Cu and Zn were purchased from Merck Chemical UK.

6.2.2 Preparation for sample and standards

Sample was prepared as described in chapter 4 (section 4.2.2.7). The 6 standards for each of P, S, Fe, Cu and Zn for external calibration were prepared with the concentration of 20, 40, 60, 80, 100 and 200 µg/L

6.2.3 Serum Protein electrophoresis

SPEP procedure was followed as described in chapter 4 (section 4.2.3)

6.2.4 Electroelution

Electroelution was performed to quantify the concentrations of P, S, Fe, Cu and Zn in the blank polyacrylamide gel and blank Sepraphore III membrane i.e. 7% gel and a membrane of the same size were cut using a clean, sharp blade and loaded into the electroeluter glass tubes separately after chopping into small pieces and electroeluted using the Bio-Rad 422 electroeluter. Modified tricine running buffer was used as an elution buffer and 0.01-0.02 A current applied to each tube.

6.2.5 Instrumentation

6.2.5.1 ICP-MS and LA

ICP-MS analysis for ^{31}P, ^{32}S, ^{56}Fe, ^{63}Cu and ^{66}Zn was performed on an Element 2 XR Sector Field ICP-MS instrument (Thermo Scientific Corporation, Bremen, Germany). The instrument was operated at medium resolution (R=4000) to avoid interferences. A Nd:YAG (UP-213) LA system from ESI, New Wave Research, Fremont, CA, USA was coupled with ICP-MS for the detection of ^{56}Fe, ^{63}Cu and ^{66}Zn. Helium was used as a carrier gas to transport the ablated material to the ICP-MS instrument. The operating parameters of the ICP-MS and LA instrument are described in Table 6.1 and Table 6.2 respectively.

6.3 Results and discussion

6.3.1 SPEP-LA-ICP-MS coupling

To confirm the separation of the serum proteins on the cellulose acetate membrane, SPEP was coupled with LA-ICP-MS for the detection of ^{63}Cu, ^{66}Zn and ^{56}Fe. This strategy has not been described previously. Initial optimization was performed with the ablation of the blank membrane and no peak was observed. Table 6.3 shows the metals bound to the proteins and their functions corresponding to the most abundant proteins found in human serum.

Table: 6.1 ICP-MS Parameters

Instrument (ICP-MS)	Thermo Scientific Element 2 XR, Bremen, Germany
Cones	Ni sampler and Ni skimmer
Nebulizer	PFA micro flow LC (Elemental Scientific, Omaha, USA)
Flow rate	400 µL min^{-1}
Spray Chamber	Cyclonic (Glass Expansion, Victoria, Australia)
Elements Analyzed	^{31}P, ^{32}S, ^{56}Fe, ^{63}Cu, ^{66}Zn
Resolution	Medium (4000)
Forward power	1250 W
Gas flows	Cool = 15.5 L min^{-1}, Auxiliary = 0.88 Lmin^{-1}, Nebulizer = 0.996L min^{-1}

Table: 6.2 LA Parameters

Instrument (LA)	Nd:YAG, UP 213 Laser System, New Wave Research, CA, USA Fremont,
Elements Analyzed	^{56}Fe, ^{63}Cu, ^{66}Zn
Light Source	Ring
Laser Energy	1.35 mJ
Repetition rate	20 Hz
Spot size	200-µm
Laser firing mode	Continuous
He carrier gas flow rate	0.7 L min^{-1}

Table 6.3: Major classes of the serum proteins and their associated metal bound proteins (Swiss-Prot)

Major Protein	Metal Bound Sub proteins	Bound Metals	Function
Albumin	Albumin(65.5 kDa)	Cu & Zn	1- Regulate colloidal osmotic pressure of the blood (the pressure exerted in the cardio vascular system by plasma proteins) 2- Major transporter of Zn in plasma, binds about 80% of all plasma Zn
Alpha-1 Globulin	1-Antitrypsin(46.7kDa)	-	Inhibitor of serine proteases
	2-Transcortin (45 kDa)	-	Major transport protein for glucocorticoids and progestins in the blood
	3-Thyroxine binding globulin (46 kDa)	-	Major thyroid hormone transport protein in serum
Alpha-2 Globulin	1-Macroglobulin(163.2 kDa)	Zn	Inhibit proteinase activities of several enzymes
	2-Ceruloplasmin(122.2 kDa)	Cu	Involve in Iron transfer across the cell membrane
	3-Haptoglobin(45.2 kDa)	-	Combine wit free haemoglobin to protect kidneys from damaging by hemoglobin
	4-Hemoglobin (16.0 kDa)	Fe	
Beta globulin	Transferrin(77 kDa)	Fe	Iron transport from site of absorption
Gamma globulin	-	-	-

As described in Table 6.3 albumin is a Cu and Zn binding protein. Swiss Prot data indicates that each molecule of albumin binds with one Cu and four Zn ions, as discussed in Chapter 5 (section 5.3.2). Fig. 6.1a and Fig. 6.1b show detection of ^{63}Cu and ^{66}Zn respectively in the first band which is consistent with the presence of albumin. Very good signals for ^{63}Cu were observed.

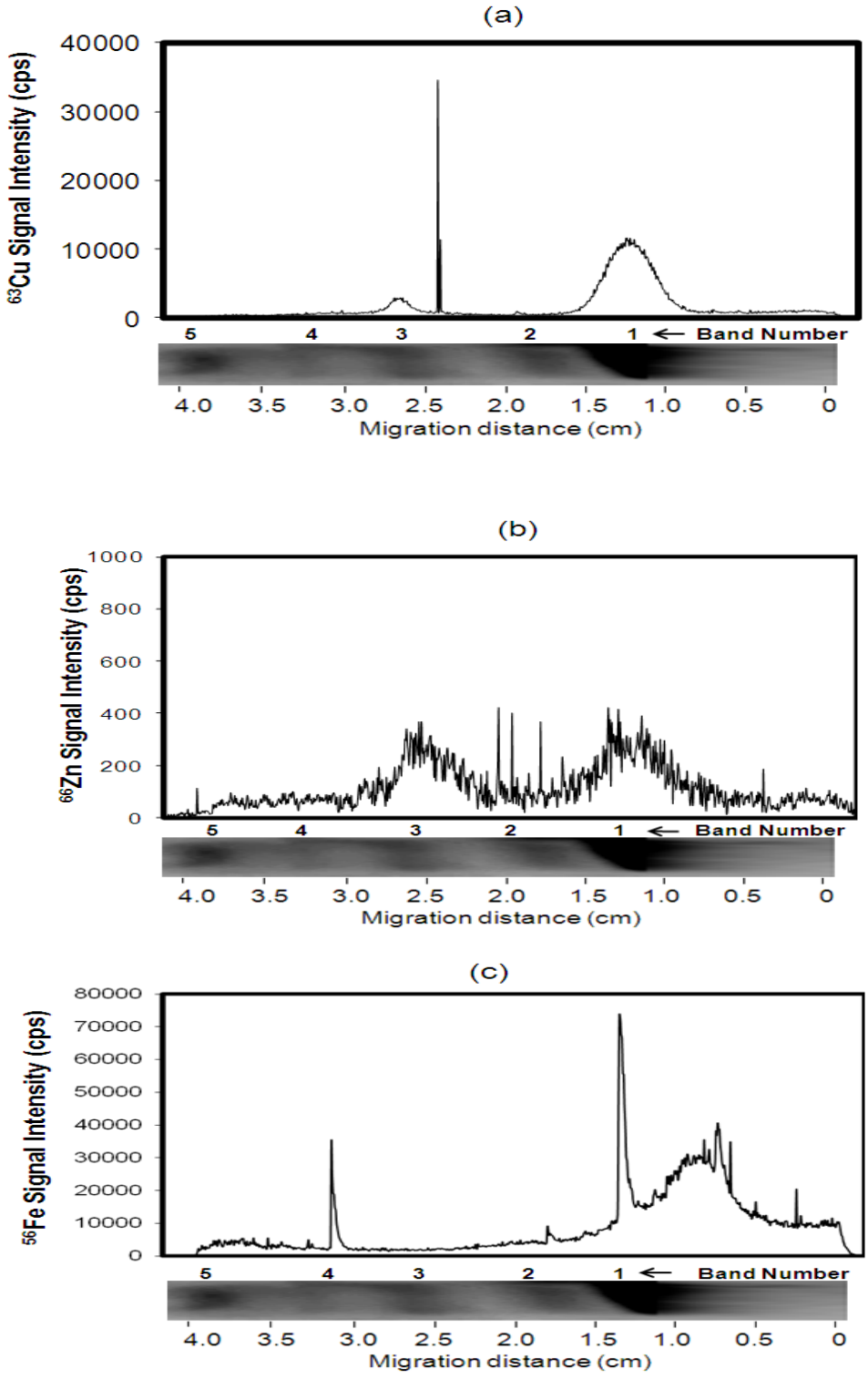


Figure 6.1: SPEP-LA-ICP-MS coupling for the detection of: (a) ^{63}Cu (b) ^{66}Zn and (c) ^{56}Fe in human serum (un-stained gel). Gel images are shown below each graph for peak reference. Some spikes were observed which may be artefacts and need further investigation.

Good peak shape and base line separation was achieved without peak tailing, which suggests that the system has very good compatibility for the detection of ^{63}Cu, however ^{66}Zn showed very poor signal intensity. The exact reasons for this are not known, however, the following may be Good peak shape and base line separation was achieved without peak tailing, which suggests that the system has very good contributing factors: (a) unlike Cu, Zn in albumin is not firmly bound [151] and may have been lost during the electrophoretic run [152], and (b) the first ionization potential (IP) value of Zn is higher (906.4 kJ mol^{-1}) than the first IP value of Cu (745.5 kJ $mole^{-1}$) and Fe (762.5 kJ mol^{-1}), hence it is not so well ionized in ICP-MS.

Similarly, the detection of the ^{63}Cu in band 3 indicates that band 3 is alpha-2 globulin. Here again ^{63}Cu has shown very good peak shape and no tailing. Each ceruloplasmin monomer (the most likely source of Cu) firmly binds with six Cu atoms, however low signal intensity of the ^{63}Cu was observed as compared to the ^{63}Cu in albumin. This was due to very high concentration of albumin in serum. Macroglobulin in alpha-2 globulin has also been investigated as a Zn binding protein [151, 153], however most of the Zn in serum/plasma is associated with albumin. Fig. 6.1b indicates that ^{66}Zn again has not shown good sensitivity or not enough and the factors described above for albumin can also be involved here.

Table 6.3 shows that betaglobin (band 4) contains transferrin which is an iron binding protein. Each transferrin binds with eight Fe atoms. An iron (^{56}Fe) peak at band 4 was observed which confirms the presence of transferrin in human serum. However, peak is sharp and more work is required to verify that this is not an experimental artefact. Similarly Fe was detected just before first band which could be experimental artefact, sample contamination or free iron and require further investigation.

The very low background signals of ^{56}Fe, ^{63}Cu and ^{66}Zn show the high compatibility of the SPEP membrane for LA-ICP-MS detection of these elements. In this preliminary study, a few spikes were observed which may well be artefacts, and the experiments need to be repeated to confirm the findings. Further investigations regarding the blank background of these biologically important elements is discussed in the next section.

6.3.2 Quantification of P, S, Fe, Cu and Zn in blank membrane and PAGE gel

To investigate the potential of the membrane system for quantitative study of metal binding and non-binding proteins, electroelution of the blank membrane and blank polyacrylamide gel was performed for 3 hours (*N=3*). Using these electroeluates, ICP-MS determination of ^{31}P, ^{32}S, ^{56}Fe, ^{63}Cu and ^{66}Zn was carried out using external calibration ($R^2 = 0.999$ for all elements).

Table 6.3 shows the very low concentrations of ^{31}P, ^{32}S, ^{56}Fe, ^{63}Cu and ^{66}Zn in the membrane as compared to the polyacrylamide gel. These low backgrounds make the membrane potentially more suitable for quantitative studies than the polyacrylamide gel.

Table 6.3: Concentrations of ^{31}P, ^{32}S, ^{56}Fe, ^{63}Cu and ^{66}Zn in 7% blank gel and cellulose acetate membrane eluents after 3 hours of electroelution (N=3)

Element	7% Blank gel Concentration in solution	Blank membrane Concentration in solution
	ppb	ppb
^{31}P	3±0.03	1±0.04
^{32}S	40±0.6	10±0.4
^{56}Fe	30±0.4	20±0.2
^{63}Cu	10±0.2	10±0.5
^{66}Zn	20±0.9	10±0.5

The major factor involved in the high background signals obtained from the PAGE methods is the polyacrylamide gel materials which are not free from impurities (despite the use of very high purity grade chemicals). For example APS contains S, P, and metal impurities and is the likely reason for the very high background signals of ^{32}S, as well as raised levels of the other elements.

6.4 Conclusion

To confirm that the desired separation of the five major serum proteins, with metal retention, was achieved in the cellulose acetate membrane, an LA-ICP-MS analysis of the membrane was carried out. The results showed very low background signals for ^{56}Fe, ^{63}Cu, and ^{66}Zn and very good signal intensities for ^{56}Fe, and ^{63}Cu, however, the detection of ^{66}Zn was relatively poor. The exact factors of this poor detection are not cleared.

To investigate the potential efficiency of the cellulose acetate membrane for the quantitation of biologically important compounds, quantitative analyses of blank polyacrylamide gel and blank membranes were performed and the results indicated very low concentrations of P, S, Fe, Cu and Zn in the blank membrane compared with the polyacrylamide gel. This suggests that the cellulose acetate membrane could be more useful for ICP-MS quantitative studies than polyacrylamide gel, provided adequate resolution can be obtained.

Chapter 7: Role of Zn Metal in Age-Related Macular Degeneration (AMD)

7.1 Introduction

Age-related macular degeneration (AMD) is the most common cause (approximately 50%) of legal blindness in people over the age of 55 years in the developed countries [154]. It begins with the degeneration of the macula in the central part of the retina and in advanced stages results in total disability of reading, writing, driving, moving etc. One estimate indicates that 8 million Americans are affected with early stage AMD and one million develop advanced AMD [155]. In the UK, AMD is the cause of 42% of total blindness in the age range 65-74 years [156]. AMD in the early stages develops slowly and can be arrested, but later stages of this disease results in visual loss that cannot be repaired. The major factor involved in the progression of AMD is the formation of the whitish yellow lesion clinically called drusen [157] which lies in the Bruch's membrane (BM) between the RPE and choroid and becomes thicker by this deposition [158]. The etiology of AMD is still not very well understood but previous studies suggest that several risk factors including age [159-160], mutations in compliment factor H [161-162], smoking [163-164], genetic factors [165], cardiovascular risk factors [166], race [167] etc may be involved in developing this disease.

Opposing roles for Zn have recently been reported in both the development and potentially the cure (or slowed progression) of AMD. Thus there has been increased interest in therapeutic interventions with Zn. Lengyel *et al.* reported the presence of free or loosely bound Zn in the protein in drusen [158]. The presence of Zn in dietary antioxidant supplementation containing vitamins E, C and beta- carotene has been suggested to be beneficial in delaying and preventing AMD [168]. The literature however is contradictory with Evans *et al.* [169] reporting no evidence to support prevention or delay of the AMD process, whereas the Age-Related Eye Disease Study Research Group (AREDS Report No. 8) indicated benefits in taking a combination of antioxidants and Zn supplementations in early and advanced AMD [170]. Recently epidemiological research has indicated that the supplement combination of adequate dietary antioxidants and Zn may reduce by 35% the risk of AMD [168]. Sub-optimal Zn levels may be indicated by Zn concentrations in serum/plasma and the circulating Zn

metallome hence accurate measurement of Zn may play a significant role in the discovery of the biomarkers for this disease.

As discussed earlier in this thesis, gel electrophoresis plays a vital role and is recognised as the gold standard method for the separation of large molecules such as proteins and DNA. Separation of the highly abundant serum/plasma proteins such as albumin and globulin is mostly performed using the native-PAGE method for the following reasons: (a) serum/plasma contains hundreds of proteins and use of SDS-PAGE in a single gel leads to a complex and un-resolved electropherogram, (b) albumin and globulin proteins are good indicators of disease states and so their separation in intact form provides vital information and (c) the chance of protein fragmentation and metal loss is very high in SDS-PAGE, as discussed in detail in Chapter 5 [19, 37].

The emerging role of ICP-MS has been recognised widely for protein analysis [116-118], and ICP-MS detection and quantification of metals (and sometimes non-metals) bound with protein provides a great deal of useful information about potential disease states. For example: Wilson's disease, which is associated with a low level of Cu [171], and hemochromatosis, which is associated with a high level of Fe in the plasma [172] can be readily diagnosed with the aid of ICP-MS.

The main objective of this study was to investigate whether the accumulation of the free (defined here as Zn in the < 2 kDa fraction) or protein bound Zn in sera correlated with AMD status.

7.2 Experimental

7.2.1 Chemicals

To prepare the mobile phase sodium dihydrogen phosphate dihydrate (NaH_2PO_4. $2H_2O$), sodium chloride (NaCl) and sodium hydroxide (NaOH) were purchased from Sigma-Aldrich, UK. Standard reference material Seronorm^{TM} trace elements serum, level 2 (6 x 3mL) was purchased from Alere, UK.

Cu and Zn standards were purchased from Merck Chemical, UK.

All the solutions were prepared in ultra-pure water (18 MΩ. cm, from a Milli-Q water purification system, Millipore Corporation, Bedford, MA).

Human blood serum samples were obtained from Moorfields Eye Hospital as mentioned in chapter 4 (section 4.2.1).

All the necessary ethical approval and safety precautions were taken before handling the blood samples.

A lyophilized powder, ≥97% (agarose gel electrophoresis) HSA was purchased from Sigma-Aldrich and used as a standard in SEC-HPLC analysis for the quantification of serum albumin in AMD patients.

A small scale (n=16) study of the levels of Zn in the sera of AMD patients was performed. Samples were obtained from male and female participants affected by AMD within various age groups. The investigation was based on 8 serum samples each from the male and female AMD patients within the age range of 64-90 years. All the samples were analysed by ICP-MS and quantified using external calibration. The concentrations of Zn and Cu were determined in Seronorm^{TM} trace elements level 2 reference standard alongside the samples to verify the accuracy of the assay.

7.2.2 Sample preparation

Human serum was diluted 5000 fold with deionized water. For ultra-filtration they were diluted initially 10 fold with deionized water and 100 folded after ultra-filtration.

7.2.3 Preparation for standards

7.2.3.1 SEC

Stock concentration of HSA was prepared by dissolving 2 mg in 1 mL of deionized water. Total 6 standards with the concentration of 2, 1, 0.7, 0.5, 0.3 and 0.2 ppm were prepared from this stock concentration.

7.2.3.2 Quantification of Zn and Cu.

Stock concentration of 100 ppb Zn solution and 10 ppb Cu solution were prepared. Both stock solutions were further diluted to prepare the 6 standard of each. Final concentration of Zn standards was, 10.04, 8.02, 6.02, 4.0, 2.02 and 0 ppb. Final concentration of Cu standards was, 0.64, 0.32, 0.16, 0.08, 0.04 and 0.02 ppb.

7.2.3.3 Standard reference material (Seronorm^{TM})

To prepare the stock solution of the reference material lyophilized powder was dissolved in 3 mL of deionized water (recommended by manufacturer). This stock concentration was further diluted 5000 folded for ICP-MS analysis.

7.2.4 SEC-UV

A Superdex^{TM} 200 10/300, gel filtration column (average particle size = 13 µm, bed dimension = 10x300-310mm, bed volume 24 mL, length=30 cm, molecular mass range = 10-600 kDa) was purchased from GE Healthcare, 71 Great North Road, Hertfordshire, UK. This was employed to separate the sera proteins. Separations were performed using an Agilent Hewlett Packard (HP) HPLC system with UV detection at 280 nm, using isocratic elution with sodium phosphate buffer, prepared as detailed below. The injection volume for each sample and standard was 100 µL.

7.2.4.1 Mobile phase

Sodium phosphate buffer was used as a mobile phase in SEC-HPLC separation of the serum proteins with the flow rate of 500 µL/min. It was prepared by dissolving 8.5 g sodium dihydrogen phosphate and 1.64 g sodium chloride in 1 litre of deionized water (pH was adjusted to 7 using NaOH).

7.2.5 Ultra-filtration

Ultra-filtration centrifuge tubes (2 mL), incorporating a Hydrosart® membrane with molecular mass cut-off 2 kDa (Vivaspin 2), were purchased from Sartorius Corporation UK. An ultra-filtration process was performed for the study of Zn loss in the samples. Tubes were filled with 1 mL of each of 16 AMD and 1 mL of each of 3 control

samples and centrifuged at 1000 g for 30 minutes. The supernatants were collected, diluted 500 times and nebulised to the ICP-MS for Zn analysis using auto-sampler self aspiration method.

7.2.6 Instrumentation

ICP-MS analysis for ^{66}Zn was performed on an Element 2 XR Sector Field ICP-MS instrument (Thermo Scientific, Bremen, Germany) using a PFA micro-flow LC nebulizer (Elemental Scientific, Omaha, USA) with a flow rate of 400 µL/min and a cyclonic glass spray chamber (Glass Expansion, Victoria, Australia). The instrument was operated at medium resolution (R=4000) to avoid interferences on ^{66}Zn. The flows of the gases were adjusted as: Outer = 15.5 L/min, Auxiliary = 0.88 L/min, Nebulizer = 0.997 litres/min with forward power of 1250 W. Ni sampler and Ni skimmer cones were used throughout the analysis.

7.3 Results and discussion

7.3.1 Quantitation of Zn and Cu in sera from AMD patients

Good external calibration lines were obtained for Zn and Cu i.e., R^2 = 0.9993 and R^2 = 0.9988 respectively and good agreements were shown between the found and certified values for the SeronormTM standard. The values obtained were 0.95 ± 0.08 mg/L for Zn and 2.7 ± 0.05 mg/L for Cu compared with the certified values of 0.92 ± 0.07 mg/L and 2.6 ± 0.2 mg/L respectively. As shown in Fig. 7.1 there was variation in circulating Zn level in AMD patients (as for healthy individuals). Importantly, no correlation with age was observed in either the male or female participants as some of the older people (85 years or older) were found with higher Zn concentrations. Comparing males and females, Zn was higher in the males than females, which supports previous work [160].

The major portion of the total Zn in serum is associated with albumin [151] and it is likely that the low Zn level in AMD is actually associated with low Zn concentration bound to albumin. Albumin is not only bound with Zn but also with Cu. Thus ICP-MS measurement of Cu was also performed. As shown in Fig. 7.1 there was no significant variation observed in Cu concentration, however slightly higher concentrations of Cu were observed in females than in males [Fig. 7.1]. [The T values between males and females for Zn and Cu were calculated as 0.17 (P = 0.89) and 0.16 (P=0.89)

respectively. In statistics the P value less than 0.05 is considered to be significant thus the above P values (0.89) of Zn and Cu are statistically not significant]. This is expected as a higher concentration of Cu in females has been reported elsewhere [173]. The high level of Zn in drusen [158] suggests that Zn may have a causative role in protein oligomerization. However, it is not known whether the lowered Zn levels in the circulation are a cause or effect of AMD, but given that the major Zn pool in the circulation is albumin, some initial work on fractionation of Zn according to its molecular weight was performed.

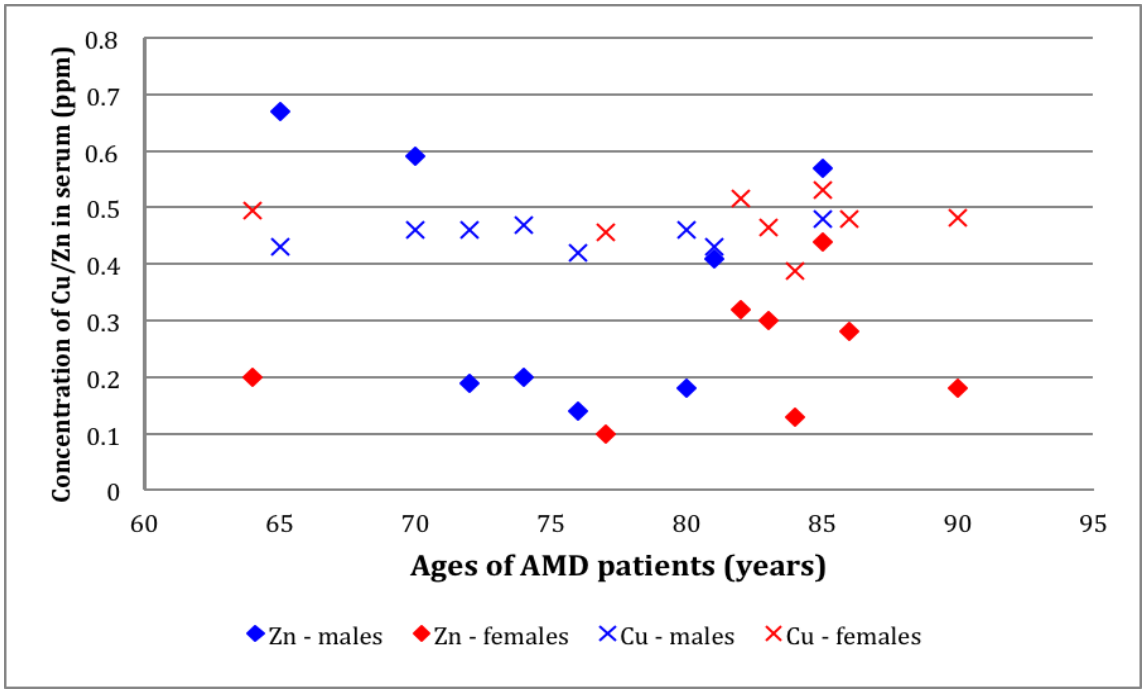


Figure 7.1: A comparison of Zn and Cu concentrations in serum samples from AMD males and females within the age range 64-90 years.

7.3.2 Determination of Zn in the high molecular weight fraction of serum

As mentioned in Chapter 5 (section 5.1), the binding of metals with proteins can be classified into two categories [38] (a) high affinity binding, which characterises the so called metalloproteins, and (b) weak affinity binding, usually non-covalent in nature, which characterises the so called metal-binding proteins. In the serum proteins,

albumin and alpha-2 macroglobulin are the Zn binding proteins. As detailed in section 5.3.2, Swiss Prot data indicates that each molecule of albumin binds with one Cu and four Zn ions whilst only one Zn ion binds with alpha-2 macroglobulin, hence albumin is highly abundant with Zn ions.

Previous studies reported that Zn is loosely bound in albumin [173] and can quickly detach in SDS-PAGE separation methodologies [19, 37]. To estimate the amount of Zn bound to albumin and alpha-2 macroglobulin, ultra-filtration at 1000 g using 2 ml centrifuge tubes containing 2.0 kDa molecular mass cut-off membranes was carried out and the supernatant of each sample was collected separately. These supernatants were then analysed by ICP-MS, by monitoring the ^{66}Zn ion. It was found that there was no significant difference between AMD and normal serum. The retained Zn in the supernatants, as percentages, were found to be 88 $\pm$ 4% and 91 ± 2% (probability two tail P value was calculated as 0.83, which is statistically not significant) respectively, hence it was concluded that the lower concentration of Zn in AMD was not because of differences in binding of Zn ions to large proteins in normal and AMD sera.

7.3.3 Quantification of HSA by SEC method

As described above, there was no significant difference observed in the unbound fraction of Zn in AMD and normal serum samples. To check that the data were directly comparable, it was necessary to confirm that the albumin level did not vary. For this purpose, a size exclusion chromatography (SEC) separation of serum proteins was performed. Fig. 7.2 shows the separation of serum proteins performed by SEC. Quantification of the concentration of the albumin was performed by external calibration (R^2 = 0.9989). As shown in Fig. 7.3 there was no significant difference in the concentration of the albumin between the AMD patients and controls.

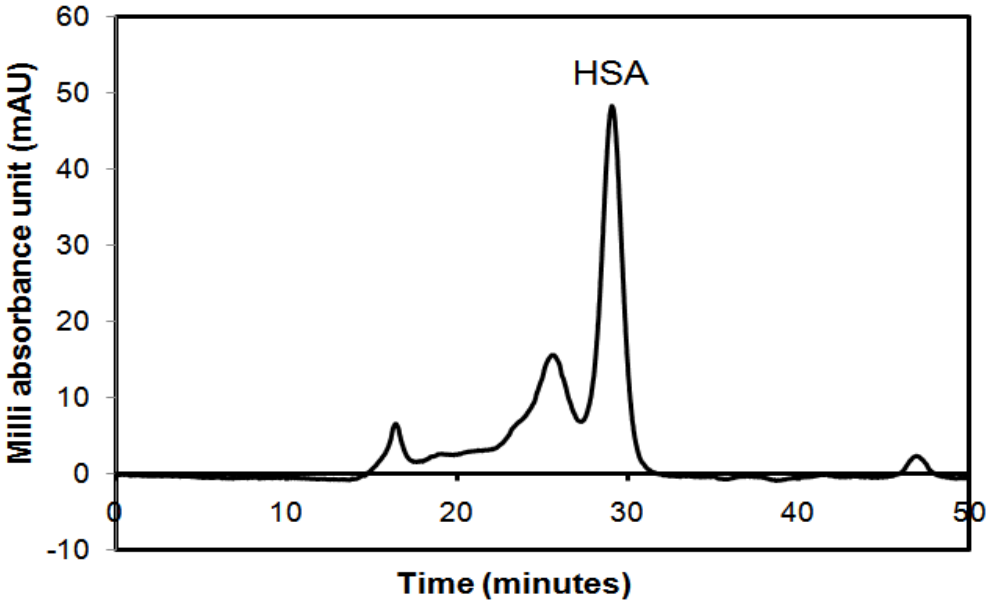


Figure 7.2: Separation of serum proteins by size exclusion chromatography (SEC)

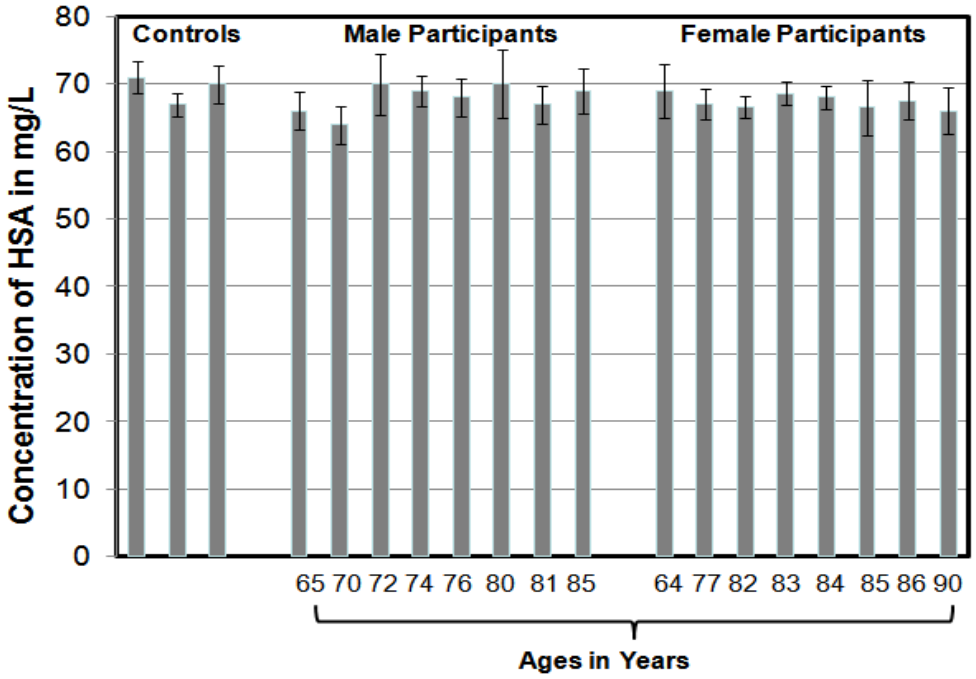


Figure 7.3: Quantification of HSA using SEC method. There was no significant difference observed in HSA concentration between male and female AMD patients and controls.

This was the same for both males and females and is evidence that the low level of Zn in AMD was not associated with lower amounts of albumin. It is interesting to note that the range of the concentration of HSA which is normally found in human serum is

35-60 mg/mL but here a slightly higher concentration was observed. This was probably due to contamination of HSA with other proteins unresolved by the poor separation efficiency of the SEC method.

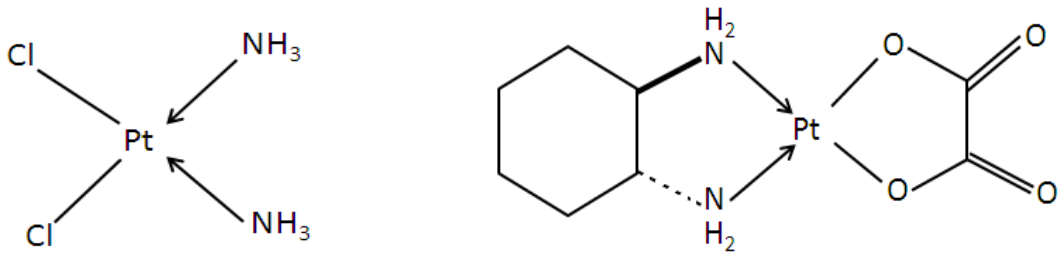
7.4 Conclusion

In conclusion an early stage work performed for the purpose of developing a metallomics approach to studying the role of Zn in Age Related Macular Degeneration (AMD). This work carried out on serum samples collected from 16 AMD patients of different age groups and at different stages of disease development. Three controls samples of the serum were also obtained. These investigations confirmed some of the previous findings about AMD but also revealed new information about the disease. Zn and Cu concentrations were measured by inductively-coupled plasma mass spectrometry (ICP-MS) and the method was validated by the analysis of Seronorm standard reference material. It was not possible to obtain a satisfactory set of control samples (e.g. from siblings or matched age relatives) and therefore the data obtained is not sufficiently powered for publication. However, the results obtained are significant and have been useful in directing our current research activity. In this work significant variation in serum Zn concentration was observed for the AMD patients whereas Cu concentrations remained approximately constant. Male participants showed higher concentrations of Zn and lower concentrations of Cu than female participants which confirm previous findings. In an ultra-filtration assay, a similar percentage of Zn remained in the supernatant in control and AMD sera, suggesting the level of the unbound (Zn-protein fraction < 2kDa) Zn in AMD is the same as in normal sera. Further, SEC results showed no significant variation in albumin concentration within AMD patients (nor between AMD patients and control serum) thus suggesting the variations found in serum Zn were not due to variation in albumin levels. It was not possible to obtain a satisfactory set of control samples (e.g. from siblings or matched age relatives) and therefore the data obtained is not sufficiently powered for publication. However, the results obtained are significant and have been useful in directing our current research activity.

Chapter: 8 A comparison of human serum albumin and human serum transferrin binding efficiencies with cisplatin and oxaliplatin using coupled SEC-ICP-MS technique

8.1 Introduction

The anti-cancer properties of cisplatin [cis-diamminedichloro platinum (II)] were discovered in 1960s and the first patient was treated in 1971 [174]. Since 1978 cisplatin has been widely used, with or without other drugs, for the treatment of the solid tumours as a chemotherapeutic agent [174]. Cisplatin has successfully treated several cancers e.g., ovarian, and testicular tumours [175] but still some tumours acquire resistance during the course of therapy, perhaps because of reduced platinum transport [176-177]. This stimulated a great interest to develop a novel platinum drug. In 1972 the first oxaliplatin [1R, 2R-diaminocyclohexane oxalate platinum (II)] drug was synthesized by substituting the amine radicals of cisplatin with a carrier ligand called 1, 2-diaminocyclohexane (DACH) which had capability of circumventing the cisplatin-mediated resistance [174-175, 178]. Oxaliplatin was first given to a patient in 1986 [175]. The oxaliplatinum synthesized in early stages was not water soluble, however, in the late 70s a modified form of the water soluble oxaliplatinum [176] was introduced which was effectively used for the treatment of advanced colorectal cancer in combination with 5-fluorouracil [176]. Structures of cisplatin and oxaliplatin are shown in Fig. 8.1



(a) Cisplatin (b) Oxaliplatin

Fig. 8.1 Structures of a) cisplatin and b) oxaliplatin

Those proteins which contain sulphur containing amino acids such as cysteine (Cys) and Methionine (Met) play very important role in platinum based anticancer therapy because of the tendency of sulphur to bind with platinum drugs [179-181]. The binding of the Pt dugs with sulphur containing compounds is not well understood (at least in terms of its clinical effect) and has potentially both negative and positive outcomes. For example, the irreversible binding of the platinum drugs with Cys and Met residues is considered to be a major step to inactivate these drugs [179, 182-183]. On the other side, Pt-S interaction can be used to decrease the side effects of the platinum drugs in clinical applications [179]. Also the discovery of the new drugs can be benefited by deep understanding of this interaction.

The role of the platinum bound protein complexes in the mechanism of the action of the Pt-drug is still not well understood [184]. HSA contains 585 amino acids residues with 17 disulphide bond and a molecular mass of 66.5 kDa. HSA is a zinc and copper bound protein and its main function involves zinc transportation in the plasma (Swiss-Prot). Human serum transferrin is one of the major proteins of beta-globulin and a low concentration shows malnutrition. It contains 679 amino acids residues and its molecular mass is 80 kDa. It is an iron bound protein and it is involved in iron transportation from the sites of absorption (Swiss-Prot).

The interactions between HSA and Pt-drugs and transferrin and Pt-drugs have been studied extensively using different techniques [185-189], however, only few publications show ICP-MS based strategies to study HSA and transferrin interaction with Pt drugs as compared to DNA-Pt drugs interactions. So, the main objectives of this study were to determine the effect of the binding of the cisplatin and oxaliplatin with HSA and transferrin after 4 and 24 hours incubation at physiological temperature (37ºC).

8.2 Experimental

8.2.1 Chemicals

Chemicals for the preparation of gels and buffer are described in chapter 3 (section 3.2.1). Some additional chemicals for this work were also purchased from Sigma-Aldrich as follows: HSA and human serum transferrin (molecular mass, 80 kDa), as model proteins; cisplatin [cis-diamminedichloro platinum (II)] and oxaliplatin [1R,

2R-diaminocyclohexane oxalate platinum (II)] as Pt-drugs; boric acid and ethylene diammine tetra acetate (EDTA) for mobile phase.

For cisplatin and oxaliplatin study by SEC-ICP-MS, Pt standards were purchased from Merck Chemical UK.

All the solutions were prepared in ultra-pure water (18 MΩ. cm, from a Milli-Q water purification system, Millipore Corporation, Bedford, MA).

8.2.2 Sample preparation

A sample solution of 0.5 mg/mL HSA and transferrin was prepared by dissolving 5 mg of each of HSA and transferrin in 10 mL of deionized water. Solutions of 10 µM cisplatin and 10 µM oxaliplatin were prepared and 100 µL of each of these solutions were mixed with the mixture of 900 µL of HSA and transferrin and incubated for 4 and 24 hours at 37°C. The control samples were also prepared by adding 100 µL of deionized water in 900 µL of the 0.5 mg/mL HSA and transferrin solution and incubated at 37°C for 4 and 24 hours.

8.2.3 Modified tricine-SDS-PAGE

To confirm whether cisplatin and oxaliplatin causing protein fragmentation the serum proteins, 10 µL of each of the above sample (section 8.2.2) was mixed with 10 uL of tricine-SDS-PAGE sample buffer. 10% tricine SDS-PAGE and buffers were prepared as described in chapter 3 (section 3.2.4, Table 3.2)

8.2.4 SEC-UV

For the investigation of a suitable buffer system SEC-HPLC was performed (please see chapter 7 section (7.2.4) for information about SEC column and parameters used in HPLC).

8.2.4.1 Buffer system (mobile phase)

Three different buffers were prepared for SEC-HPLC and SEC-ICP-MS investigation. (a) sodium phosphate buffer by dissolving 8.5 g sodium phosphate dihydrogen and 1.64 g sodium chloride in 1 Litre of deionized water (pH was adjusted to 7 using NaOH), (b) 20 mM Tris borate EDTA (TBE) by dissolving 5.86 g Tris, 1.23 g boric acid and 5.84 g EDTA in Litre of deionized water (pH was adjusted to 8.2 using NaOH) and (c) 25 mM Tris-tricine buffer by dissolving 3 g Tris and 4.5 g tricine in 1 Litre of deionized water (pH was not adjusted),

8.2.4.2 Instrumentation

An Agilent Hewlett Packard (HP) HPLC system with UV detection at 280 nm, was coupled with Element 2 XR Sector Field ICP-MS instrument (Thermo Scientific, Bremen, Germany). For the detection of ^{195}Pt, the ICP-MS instrument was operated at low resolution. The following parameters were used for ICP-MS analysis, Forward Power, 1300 W, Cool gas, 15.5 $Lmin^{-1}$, Auxiliary gas, 0.88 $Lmin^{-1}$, Sample gas, 0.997 $Lmin^{-1}$, Cones, Ni sampler and Ni skimmer, Nebulizer, PFA micro-flow LC nebulizer (Elemental Scientific, Omaha, USA) with a flow rate of 500 $\mu Lmin^{-1}$, Spray Chamber, Cyclonic (Glass Expansion, Victoria, Australia). For separation, 50 µL of each sample was injected into the gel filtration column and analyzed through ICP-MS detection. The column was equilibrated for 1 hour with mobile phase prior to the injection.

8.3 Results and discussion

8.3.1 Choice of the buffers

In SEC, phosphate buffer is mostly employed as a mobile phase for the separation of serum proteins. Although, the phosphate buffer provides good separation of the serum proteins, it also severely affects the sensitivity of the ICP-MS in SEC-ICP-MS based separation by depositing a layer on the injector of the torch and cones. So, initial investigation was carried out by optimising a suitable buffer system for the separation of the platinum adducts. For this purpose SEC-UV separation of the mixture of HSA and transferrin was performed using three different buffers system i.e., 50 mM phosphate buffer, 20 mM TBE and 25 mM Tris-tricine. As it is clearly shown in Fig.

8.2, the three buffers do not show any significant difference for the separation of HSA and transferrin. This indicates that a modified Tris-tricine buffers system can also be employed for the separation of these proteins instead of using phosphate or other buffer systems. Thus modified Tris-tricine buffer system can be employed SEC-ICP-MS based investigation of the serum proteins.

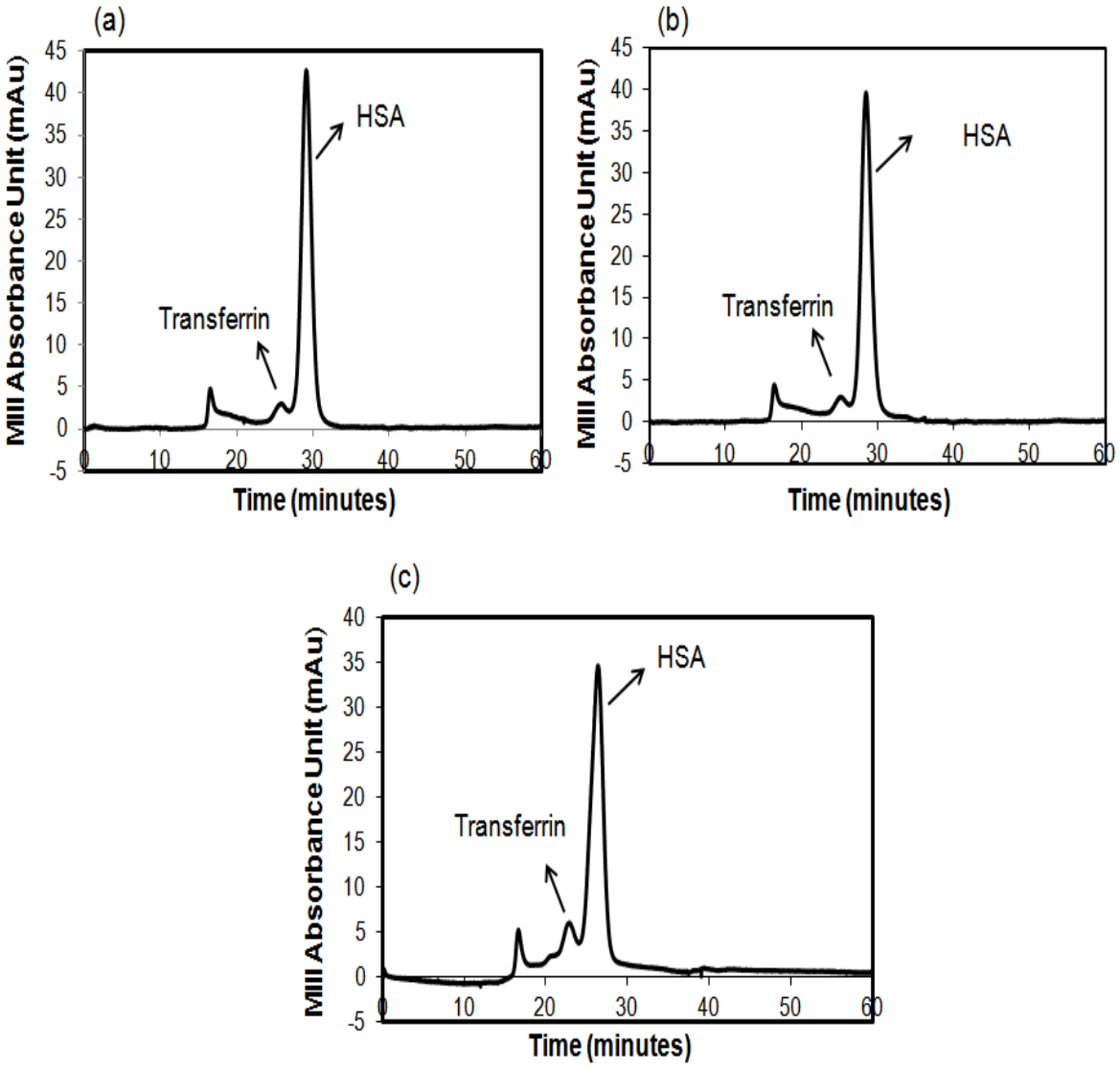


Figure 8.2: (a) HSA and Transferrin using phosphate buffer (b) HSA and Transferrin using 20 mM TBE (c) HSA and transferrin using 25 mM Tris-tricine

8.3.2 Effect of cisplatin and oxaliplatin binding on serum proteins

After investigating a suitable buffer for the separation of HSA and transferrin, the SEC-HPLC system was coupled with ICP-MS for the investigation of the binding tendencies of the cisplatin and oxaliplatin with HSA and transferrin. For this purpose 10-µM concentration of each drug was incubated with the mixture of HSA and transferrin at 37ºC for 4 and 24 hours. Finally, the analytes were separated using the SEC column described above and eluted to ICP-MS for the detection of ^{195}Pt signals using 25 mM Tris-tricine buffer with the flow rate of 500 µL min^{-1}. Fig. 8.3 shows the binding of cisplatin and oxaliplatin with HSA and transferrin after 4 and 24 hours incubation respectively at 37ºC. A volume of 50-µL of each of 7.5-µM HSA and 6.5-µM transferrin was injected into the SEC column via an HPLC auto-sampler.

HSA shows a higher signal intensity for ^{195}Pt in both drugs than transferrin which indicates HSA has more interaction with Pt-drugs than transferrin. Pt- drugs bind with specific sites of the proteins and in most cases they are Met and Cys residues. However, some of the Met and Cys are more favourable for binding than others for example in HSA Met 298 is more favourable because it is most surface accessible residue [179]. Beside Met and Cys several other binding sites have also been reported in HSA and transferrin [179, 184] including the O donor sites of tyrosine and aspartate (Y150 or Y148) and (D375 or E376) for HSA and O donor sites E265, Y314, E385 and T457 for transferrin [184]. However, Szpunar et al. have reported the poor stability of Pt-transferrin adduct for complex formation [185]. Thus signal intensity of ^{195}Pt is observed lower in transferrin than HSA.

After 4 hours incubation the rate of the cisplatin binding is almost twice that of oxaliplatin for HSA. The rate of reactivity of the cisplatin and oxaliplatin with Met residue is different. Oxaliplatin reactivity is lower and it forms unstable Pt-Met adducts compared with cisplatin. The apparent reason for this behaviour of oxaliplatin is the presence of the highly stable DACH-Pt moiety [179].

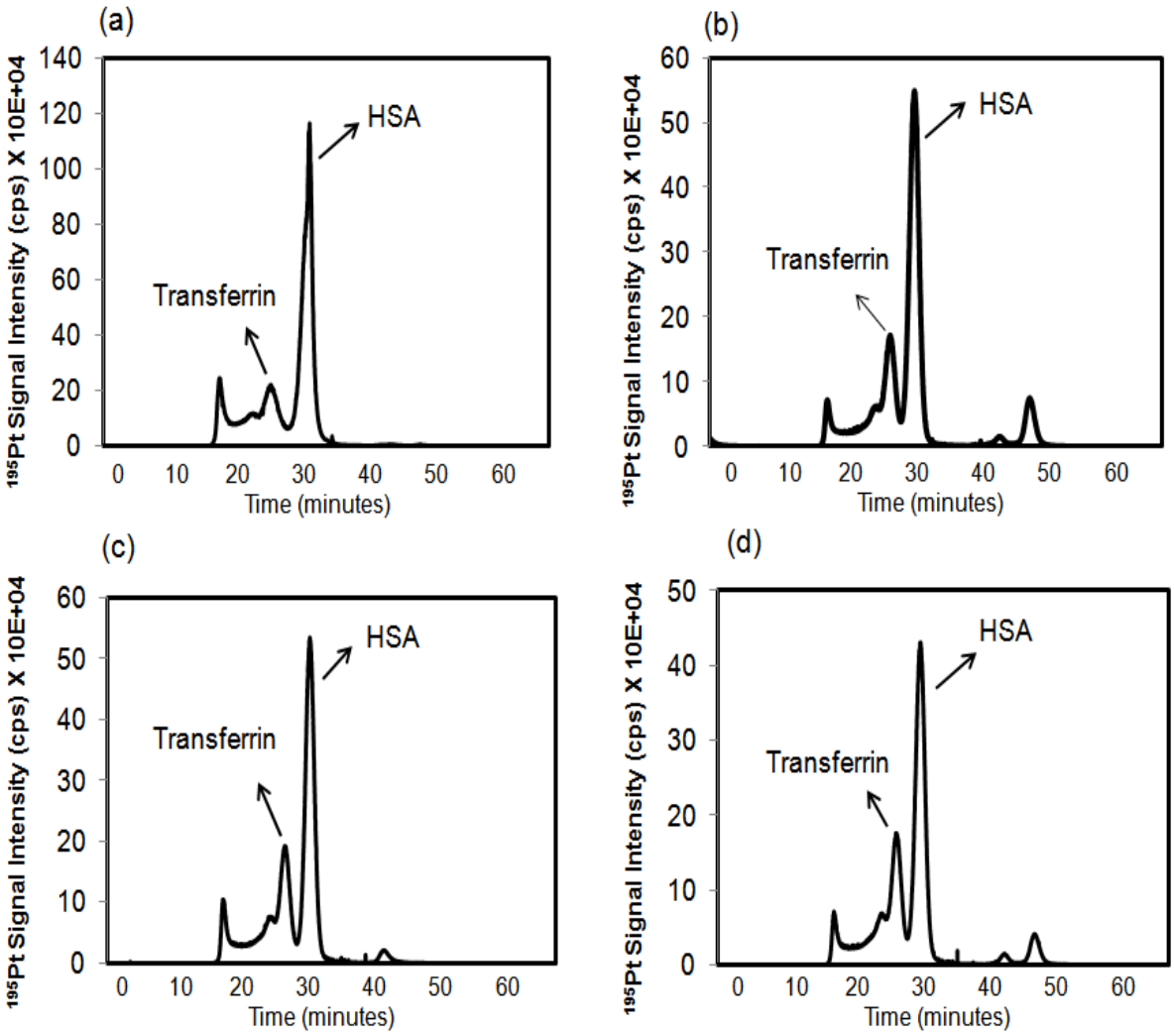


Figure 8.3: Comparison of cisplatin and oxaliplatin for the binding efficiencies with HSA and transferrin: (a) cisplatin, HSA and transferrin after 4 hours (b) oxaliplatin, HSA and transferrin after 4 hours (c) cisplatin, HSA and transferrin after 24 hours (d) oxaliplatin, HSA and transferrin after 24 hours.

After 24 hours incubation of Pt-drugs with the mixture of proteins, significant decrease in the binding of cisplatin was observed for HSA which indicates the interaction of the HSA binding sides with cisplatin decreases by increasing the time.

It is important to note that there were total three peaks observed in cisplatin binding with serum proteins after 4 hours incubation (Fig. 8.3a) but after 24 hours incubation one additional peak observed as shown in Fig. 8.3c. There could be several hypothesises for this behaviour of the cis-Pt-drugs. One possibility could be the

proteolytic effect of these drugs on the serum proteins. To confirm the proteolytic activities of cisplatin modified tricine-SDS-PAGE was employed. For this purpose cisplatin and oxaliplatin bound serum proteins (incubated for 4 and 24 hours) were separated on a 10% gel (Fig. 8.4) It is clear from Fig. 8.4 that there were not any additional protein band found on a 10% gel which suggests no proteolytic activities of these Pt-drugs. These findings suggest the additional peaks observed were due to unbound cisplatin and oxaliplatin. Szpunar et al. also reported the unbound Pt peak in their investigation of metallodrug-protein interactions using SEC [185]. After 4 hours incubation the binding of the cisplatin with the serum proteins was much higher thus there was no additional peaks observed but after 24 hours incubation a small peak of unbound cisplatin was observed which further confirmed that by increasing the incubation time cisplatin binding affinities with the serum proteins decrease. In contrast, the oxaliplatin did not show any significant decrease in the binding affinities after 24 hours incubation, thus the two similar peaks were observed both after 4 and 24 hours of incubation at 37ºC.

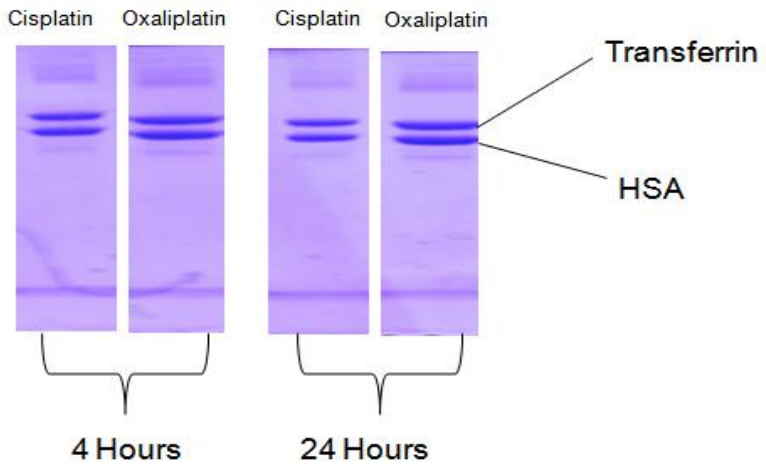


Figure 8.4: Tricine-SDS-PAGE (10% gel) for the detection of additional proteins band. No additional protein band was observed after 24 hours incubation.

8.4 Conclusion

A preliminary investigation of the interaction of cisplatin and oxaliplatin with two of the serum proteins is described using coupled online SEC and ICP-MS. For the first time, modified tris-tricine buffer was employed instead of the traditional phosphate buffer in SEC-ICP-MS analysis which is an important development. To confirm whether

cisplatin and oxaliplatin damaged the serum proteins or not, modified tricine-SDS-PAGE was successfully employed. It is clear from the results that cisplatin binds more strongly than oxaliplatin with both proteins. After 24 hours incubation cisplatin showed decreased signal intensity which indicates cisplatin binding with proteins decreased with increasing time. Cisplatin binding with transferrin, as compared to has, was not significant which could be the result of an unstable Pt-transferrin complex. Oxaliplatin did not show a high binding trend with either protein, perhaps due to the presence of the bulky, non polar DACH ligand. The above are not novel findings, however here the focus was to develop a new method that may be more useful for detailed investigation in future research.

Chapter 9: Future Work

Methods developed in this study can be further improved and applied for several applications. Some recommendations for their further enhancement and applications are described below:

9.1 Modified tricine SDS-PAGE Method

- This method has shown very good separation of small molecular mass proteins and peptides (≥ 1 kDa) in a low percentage gel. Thus an enhanced detection of the elements in GE-LA-ICP-MS can be achieved using this method.

- The distances between the separated bands are not large which may create smearing in case of several smaller peptides being present. Thus further improvement in this method is needed. Perhaps this improvement could be achieved either by further adjusting the pH of the gel and running buffers, replacing Tris-base with any other suitable base or increasing the cross-linking of acrylamide/bis-acrylamide.

- For the separation of phosphopeptides SDS-PAGE methods have not been commonly used because of their poor separation for smaller peptides. Now, the modified tricine-SDS-PAGE can be successfully applied for the detection, identification and separation of phosphorylated peptides.

- GE is frequently used in combination with ESI-MS, MALDI-MS for protein sequencing, protein mass finger printing and identification of smaller peptides. The modified method has shown successful separation of phosphopeptides after tryptic digest. The method can be coupled with ESI-MS, or MALDI-MS in future work.

- This method is coupled on-line with ICP-MS. Further work is needed to couple this method on-line with ESI-MS. This perhaps could be achieved by further decreasing the buffer concentration or replacing Tris with a volatile buffer such as ammonium acetate.

- This modified method can also be applied in 2D-GE in second dimension instead of Tris-glycine-SDS-PAGE system.

9.2 Native-PAGE

Native-PAGE methods have special importance because they can be employed for the detection and quantification of intact metalloproteins. However, the main problem associated with this methodology is poor resolving efficiency as compared to SDS-PAGE. An enhanced native-PAGE method is described in this study which improved its resolving efficiency by employing the modified Tris-tricine buffer system. Using the traditional methods the five major proteins cannot be separated in a single gel, but employing this method all the major serum proteins can be resolved successfully in a 7% gel and there is still more scope to separate complex mixtures. The method was applied here only for the separation of the major serum proteins, but it could also efficiently resolve mixtures of proteins in other biological samples such as saliva, urine, sweat etc., and in cancer studies where metal-protein binding plays the key role in investigation of the efficacy of the drug and all the drug binding proteins require to be separated in a single gel. The above method can be applied to separate proteins with intact metals on gel plates which can be further investigated through LA or electroelution. The developed method used a 29:1 percent acrylamide/bis-acrylamide solution for the preparation of the gel. Increasing the cross linking may further improve the separation.

9.3 SPEP and SPEP-LA-ICP-MS

SPEP methods are frequently applied in clinical studies for the separation of serum proteins. This study described an improved method of SPEP for the separation of major serum proteins. This is a very simple and quick approach for the investigation of disease and separated proteins can be compared with the standards and quantified using a densitometer. The enhanced method is simple and increases the resolution between the separated proteins which will be helpful in quantification studies. However, it is not clear if non-covalently bound metals remain intact with the proteins or not, which opens the doors for future investigation. The SPEP strategy was coupled for the first time with LA for the detection of the metals in serum proteins. However, the method was not fully investigated as LA-ICP-MS method development was not the focus of this study. SPEP-LA-ICP-MS is an interesting strategy and much better

than GE-LA-ICP-MS because of the several advantages described in Chapter 6 (section 6.1). The future work should focus on detection of metalloproteins and phosphoproteins from a wide variety of the biological samples. In comparison with native-PAGE separation, this method still does not show well resolved proteins bands. So, there is still room to enhance further this outstanding methodology in future research.

9.4 GE-ICP-MS

GE-ICP-MS online coupling is in its early stages. We enhanced this method by developing a single buffer reservoir system. As discussed in Chapter 1 (section 1.3), this methodology has a great potential for a wide range of the applications. The following issues can be resolved in the future studies.

- Commercially available GE electroeluter devices are usually coupled with ICP-MS. These devices contain separated cathode and anode buffer chambers which are required to be connected with each other through dialysis membranes or plug gels. Sometimes as a result of bubble generation on the dialysis membrane, both electrodes are disconnected from each other and as a result the migration of the analytes from cathode to anode is stopped. Also, the available dialysis membranes have very poor efficiency to connect the electrodes thus a a high voltage is required to enable fast migration of analytes which may break the gels and increase the temperature of the system. The future work needs to develop well connected cathode and anode buffer reservoirs for this system.

- For metalloproteins analysis GE-ICP-MS is required to use native-PAGE. This strategy does not use anionic detergent such as SDS. The absence of anionic detergent slows down the migration speed of the analytes and several hours may be required to analyse one sample. This problem can be resolved by employing a suitable buffer system or adjusting the pH of the gels and tank buffer.

- GE is still not coupled online with ESI-MS. Future work may include the

development of GE-ESI-MS online coupling setup which will be very useful in protein sequencing and protein/peptide identification and quantification.

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Chapter: 11 Appendices

Appendix 1: Professional Development Training Record

Event	Date Attended	Organization/Department	Duration
Postgraduate research student Induction	15th January 2008	Professional Development	1 day
Poster Competition for PGR's-Rules Clinic	1st April 2008	Professional Development	½ day
Introduction to the Job of Lecturers for Postgraduates and RAs	2nd April 2008	Professional Development	½ day
Designing and Producing Conference Posters	15th April 2008	Professional Development	½ day
Power Points for Presentations	16th April 2008	Professional Development	½ day
Conference Presentation Skills	29th April 2008	Professional Development	½ day
Getting Articles Published for Researchers	6th May 2008	Professional Development	½ day
Teaching Skills-Preparing. To Teach and Promoting Learning	14th May 2008	Professional Development	½ day
Report Writing	15th May 2008	Professional Development	½ day
Tools for Creative Thinking	16th May 2008	Professional Development	½ day
Poster Competition for PGRs	21st May 2008	Professional Development	1 day

Teaching Skills for PGRs and RAs with Supervising Practical Activities	4th June 2008	Professional Development	½ day
Ref Works	10th June 2008	Professional Development	½ day
What is the Literature Review	18th June 2008	Professional Development	½ day
Networking Skills Attending Conferences	20th June 2008	Professional Development	½ day
Managing your PhD as a Project	25th June 2008	Professional Development	½ day
Mass Frontier Training	15th June 2008	Chemistry	½ day
Viva What Happens?	26th June 2008	Professional Development	½ day
Reading for Research	1st July 2008	Professional Development	½ day
Marking for Postgraduates and RAs	2nd July 2008	Professional Development	½ day
Ethical Thinking in Research	3rd July 2008	Professional Development	½ day
Rapid Trace Instrumental Training	3rd October 2008	Chemistry	½ day
Health and Safety Training	03rd December 2008	Chemistry	½ day
Gel Electrophoresis (Oral Demonstration to MSc Students)	6th December 2008	Chemistry	½ day
Gel Electrophoresis Oral Demonstration to MChem Students	12th December 2008	Chemistry	½ day

Winter Plasma Conference	15th – 20 February 2009	Chemistry	6
Poster Competition for PGRs	19th April 2009	Professional Development	1 day
Qualitative Analysis-an Introduction to Collecting and Analysing	12th October 2009	Professional Development	½ day
RSC Postgraduate Symposium	13th November 2009	Chemistry	½ day
BNASS Conference	7th July 2010	Chemistry	1 day
RSC Analytical Research Forum (ARF)	26-28th July 2010	Chemistry	3 days
Reflective Activities for Research	29th September 2010	Professional Development	½ day
Career Planning Career Management for Research	1st October 2010	Professional Development	½ day
Writing Up Your PhD Thesis	5th October 2010	Professional Development	½ day
Second Zn UK meeting	15th October 2010	Chemistry	1 day
Risk Assessment	24th November 2010	Professional Development	½ day
Departmental Based Group Meetings and Seminars	December 2007-November 2010	Chemistry	15 days
Demonstrations in Teaching Lab (F3.02)	October 2009-November 2010	Chemistry	1 day
Control of Substances Hazardous to Health	8th February 2011	Professional Development	½ day
Third Zn UK Meeting	28th February 2011	Chemistry	1 day
Gary Hieftje Lecture	29th March 2011	Chemistry	½ day

Appendix 2: Copies of Published Papers