RELATIVE QUANTITATION OF PEPTIDES BY INDUCTIVELY COUPLED PLASMA MASS SPECTROMETRY

by

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ABSTRACT

Relative Quantitation of Peptides by Inductively Coupled Plasma Mass Spectrometry

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Currently, the detection/quantitation of biomolecules using Inductively Coupled Plasma Mass Spectrometry (ICP-MS) is focused on heteroatom containing (e.g. S, P, Se, Cu) compounds. This limits the scope of ICP-MS as a routine analytical tool in the bioanalytical sciences. To increase this scope novel strategies to incorporate elemental labels have been developed. Chemical derivatisation is one such strategy. Cyclic diethylenetriaminepentaacetic acid anhydride (cDTPA), a bifunctional chelating agent (BFCA), is reacted with N-terminal amines and is subsequently chelated with trivalent metals. This allows the detection of peptides that contained no heteroatom using liquid chromatography (LC)-ICP-MS. The derivatisation efficiency, optimised using LC-UV/vis detection, is approximately 99%. The derivatisation and chelation reactions products are characterised using LC-electrospray ionisation tandem mass spectrometry (ESI-MS-MS).

The labelling procedure allowed the relative quantitation of peptides, by differential isotopic labelling. Two individually derivatised peptide samples are chelated with natural isotopic abundance and isotopically enriched ^{151}Eu respectively. The Eu labelled peptides were combined and analysed by LC-ICP-MS. The resulting ^{153}Eu:^{151}Eu ratio, measured using the pseudo steady state approach for transient signals; is then used to calculate the original peptide proportions using a modified isotope dilution equation. The ICP-MS measured peptide ratio was within 2.8% of the theoretical peptide ratio. The absolute detection limit for the relative quantitation of Eu labelled bradykinin was 5 pmol, which is comparable to current ESI-MS methods. When cDTPA derivatisation was applied to a complex sample multiple by-products were observed in the LC-UV/vis chromatogram. However, the corresponding LC-ICP-MS chromatogram suggested that only singly derivatised peptides were chelated with the metal.

To overcome by-product formation monoreactive BFCAs, namely isothiocyanate-benzyl-EDTA (SCN-Bz-EDTA) and isothiocyanate-benzyl-DTPA (SCN-Bz-DTPA), were evaluated as derivatisation reagents. Solid phase analytical derivatisation (SPAD) and solid phase extraction (SPE) were also evaluated for speed and ease of use. The monoreactive nature of SCN-Bz-EDTA and SCN-Bz-DTPA gave singly derivatised peptides when applied to complex samples. However, the resulting derivatisation efficiency was low and no significant improvement in efficiency was noted when SPAD was used. SCN-Bz-EDTA derivatised peptides could not be chelated to trivalent metals, whilst chelation occurred with SCN-Bz-DTPA derivatised peptides. Eu labelled peptides were isolated and selectively extracted by SPE for relative quantitation by LC-ICP-MS. However, due to the instability of the isothiocyanate reagent and instrumental effects the relative error on the measured peptide ratio was greater than 45% when compared to the theoretical ratio. Although the peptide ratio obtained using the SPE method agreed with the ratio from the non extracted sample.

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A quote that has kept me inspired.

"Live as if you were to die tomorrow. Learn as if you were to live forever."

Mohandas Gandhi

xx

GLOSSARY OF TERMS

1-DE	One dimensional gel electrophoresis
2-DE	Two dimensional gel electrophoresis
ACN	Acetonitrile
ALICE	Acid labile isotope coded extractant
BFCA	Bifunctional chelating agent
cDTPA	cyclic diethylenetriaminepentaacetic acid anhydride
CE	Capillary electrophoresis
CID	Collision induced dissociation
Da	Dalton
DDW	Deionised water
DIGE	Differential gel electrophoresis
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DTPA	Diethylenetriaminepentaacetic acid
DTTA	Diethylenetriaminetetracetic acid
EDTA	Ethylenediaminetetraacetic acid
EDF	Epidermal growth factor
ESI-MS	Electrospray ionisation mass spectrometry
ESI-MS-MS	Electrospray ionisation mass spectrometry mass spectrometry
Eu	Europium
FI	Flow injection
FTICR	Fourier transform ion cyclotron resonance
GC	Gas chromatography
HPLC	High performance liquid chromatography
HR	High resolution

IC	Ion chromatography
ICAT	Isotope coded affinity tag
ICP-MS	Inductively coupled plasma mass spectrometry
IDA	Isotope dilution analysis
IDMS	Isotope dilution mass spectrometry
In	Indium
LC	Liquid chromatography
MALDI	Matrix assisted laser desorption ionisation
MC	Multi-collector
MCAT	Mass coded affinity tags
mDTPA	Monoreactive DTPA
MDTPA	Malamide DTPA
MRI	Magnetic resonance imaging
MRM	Multiple reaction monitoring
mRNA	Messenger ribonucleic acids
MT	Metallothionein
MudPIT	Multi dimensional protein identification technology
m/z	Mass to charge
NMR	Nuclear magnetic resonance
NTA	Nitrilotriacetic acid
N-terminal	Amino terminal
ODS	Octodecyl silica
PEEK	Polyetheretherketone
pH	Potential hydrogen ions
pI	Isoeletric point
PTM	Post-translational modifications

RAM	Relative atomic mass
rf	Radio frequency
RNA	Ribonucleic acids
RPLC	Reverse phase liquid chromatography
SCN-Bz-DTPA	Isothiocyanate-benzyl-DTPA
SCN-Bz-EDTA	Isothiocyanate-benzyl-EDTA
SCX	Strong cation exchange
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEC	Size exclusion chromatography
SELDI	Surface enhanced laser desorption ionisation
SILAC	Stable isotope labelling by amino acid in cell culture
SPAD	Solid phase analytical derivatisation
SPE	Solid phase extraction
Sm	Samarium
TFA	Trifluoroacetic acid
TIC	Total ion count
TMPP	Tris(2,4,6-trimethoxyphenyl)phosphonium propylamine
TOF	Time of flight
UV/vis	Ultra violet/visible

AUTHORS DECLARATION

At no time during the registration for the degree of Doctor of Philosophy has the author been registered for any other university award. This study was jointly supported by the Analytical Trust Fund of Royal Society of Chemistry and Engineering and Physical Sciences Research Council.

Relevant scientific seminars and conferences were regularly attended at which work was usually presented and several papers were prepared for publication. The research undertaken was regularly disseminated to fellow research students at the University of Plymouth through oral presentations and research seminars.

Publications

Patel, P.J.; Jones, P.; Handy R.; Harrington, C.; Marshall, P.; Evans, E. Hywel. Isotopic labelling of peptides and isotope ratio analysis using LC–ICP–MS: a preliminary study.

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Oral Presentations

"Comparison of Derivatisation Methodologies for the Relative Quantitation of Peptides by LC-ICP-MS" P.J. Patel, E. H. Evans, R. Handy and P. Jones. Paper presented at the 13th Biennial National Atomic Spectroscopy Symposium, 12th – 14th July 2006, Glasgow, UK.

Poster Presentations

"Comparison of Derivatisation Methodologies for the Relative Quantitation of Peptides by ICP-MS" P.J. Patel, E. H. Evans, and P. Jones. Paper presented at the Analytical Research Forum, 17th – 19st July 2006, Cork, Ireland.

"Isotopic Tagging for Relative Peptide Quantitation by LC-ICP-MS" P.J. Patel, E. H. Evans, R. Handy, and P. Jones. Paper presented at the Analytical Research Forum, 19th – 21st July 2005, Plymouth, UK.

"Relative Quantitation of Peptides by Differential Isotopic Tagging Using Isotope Dilution Mass Spectrometry." P.J. Patel, E. H. Evans, R. Handy, P. Jones and P. Marshall. Paper presented at the European Winter conference on Plasma Spectrochemistry, 29th Jan – 2nd Feb 2005, Budapest, Hungary.

"Amine Chelate Coupling and Isotopic Tagging for Peptide Determination using LC-ICP-MS" P.J. Patel, E. H. Evans, R. Handy, P. Jones, P. Marshall, and Bill

Leavens. Paper presented at the Analytical Research Forum, 19th – 21st July 2004, Preston, UK.

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"Amine Chelate Coupling and Isotopic Tagging for Peptide Determination using LC-ICP-MS" P.J. Patel, E. H. Evans, R. Handy, P. Jones, P. Marshall, and Bill Leavens. Paper presented at the 12th Biennial National Atomic Spectroscopy Symposium, 12th – 14th July 2004, Plymouth, UK.

Academic Conferences and Meetings attended

6th Analytical Research Forum, 17th – 19th July 2006, Cork, Ireland.

13th Biennial National Atomic Spectroscopy Symposium, 10th – 12th July 2006, Glasgow, UK.

Atomic Spectroscopic Group in Conjunction with the BMSS meeting, 16th March 2006, Leicester, UK.

5th Analytical Research Forum, 18th – 20th July 2005, Plymouth, UK.

European Winter conference on Plasma Spectrochemistry, 29th Jan – 2nd Feb 2005, Budapest, Hungary.

4th Analytical Research Forum, 19th – 21st July 2004, Preston, UK.

Western Region of the Analytical Divisions 50th Anniversary, 14th July 2004, Plymouth, UK.

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CHAPTER 1 INTRODUCTION

1. PROTEOMICS

The term proteomics[1] was coined in the mid 1990's to describe the process of structural identification and cataloguing of the entire protein complement expressed by a specific genome at any given time. This was analogous to the term 'genomics', which was used to describe mapping of the human genome that was being undertaken at the time. Since then, analysis of the proteome has rapidly grown in importance, becoming the leading discipline in life sciences, and which is also considered to be one of the greatest challenges in the post genomic era. Figure 1-1 illustrates the evolution of bio-molecular research over the past twenty years.

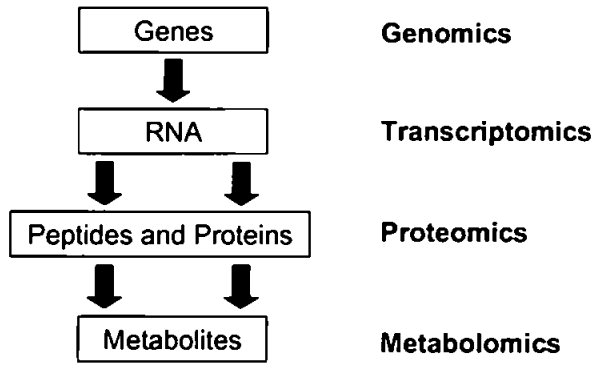

Figure 1-1 Evolution of bio-molecular research from genomics to metabolomics through proteomics.

Early proteomic research concentrated on the cataloguing of proteins and development of protein databases, with currently over 6000 scientific papers published in this field alone[2]. To date, proteomes of numerous prokaryotic and eukaryotic species have been characterised, resulting in extensive information on the proteomes of these organisms. Modern proteomic research has now focused

on the characterisation of the proteome of human cells, organelles, specific tissues and organs.

Research into proteomics has highlighted the complexity of the proteome. For example, while there only 25,000 genes in the human genome, it is estimated that there can be up to 100,000 functionally unique proteins expressed across the assorted human cell types. Given that multiple modified forms of each protein exists, including variations in amino acid sequence at specific sites (polymorphism) and post-transitional modifications (e.g. phosphorylation), the number of proteins could approach one million[3].

Proteomics has been defined as the large scale analysis of the protein properties (expression levels, post-translational modifications, interactions etc.) of a cell or organism[3]. The aim is to obtain a global and integrated view of disease processes, environmental effects, cellular processes and networks at the protein level by studying all the proteins of a cell rather than each one individually.

Several different sub-disciplines can be grouped under the term proteomics to describe specific areas of proteomic research such as, protein expression proteomics, functional proteomics, protein modification, protein interaction, structural proteomics and metallomics. Since the advent of the term proteomics, many other "omic" terms have been coined to describe various different areas of research in proteomics. Sub-disciplines such as interactomics (the study of interactions between proteins, RNA and DNA); ligandomics (the study of biological molecules and metals); functomics; separomics and many more. However, none

of these "omics" refer to new areas of research that did not exist prior to coining of these new words[4].

1.1 Proteomics Sub-Disciplines

Figure 1-2 shows some of the important sub-disciplines associated with proteomics and their potential applications to biology.

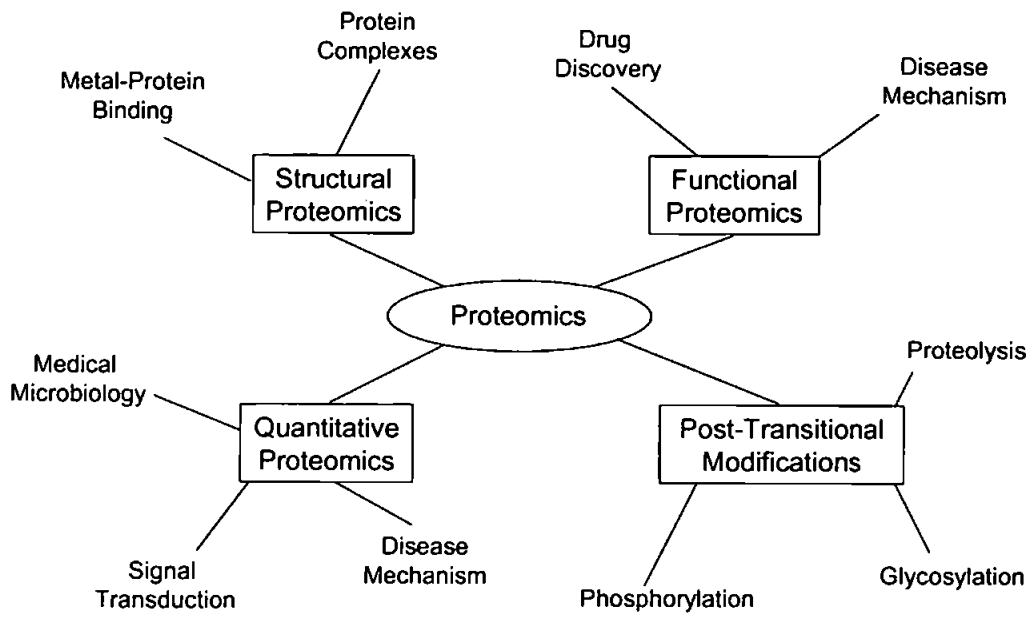

Figure 1-2 Sub-disciplines of proteomics and applications to biology. Modified from Graves *et al.*[5]

Functional proteomics is the study of specific proteins in order to gain information about protein signalling, disease mechanisms, or protein-drug interactions. Typically, selected proteins are isolated by either affinity chromatography or by using protein ligands.

Structural proteomics attempts to identify all the proteins within a protein complex or organelle, determine their location and to characterise all protein-protein interactions, resulting in a cell map[6]. The information can help to elucidate the overall architecture of a cell and explain how certain proteins give a cell its unique characteristics[7].

Post translational modifications (PTMs) are of extreme biological importance. The presence or absence of a particular protein often determines if its designated biological function is carried out, however, this may not always be the case. It has been shown that PTM's can influence a number of cellular processes and an enormous number of proteins have been shown to be post-translationally regulated by a variety of different modifications. More than a hundred protein modifications are known and more will likely be discovered[8]. Proteins are known to be modified post-translationally in response to a variety of intracellular and extracellular signals. The effects of PTMs include change in enzymatic activity, interactions with other proteins, subcellular localisation, targeted degradation etc.

Phosphorylation is probably the most widespread, biologically important of the post-translational modifications[8], and has been found in nearly all cellular processes[9]. Reversible phosphorylation occurs at the Ser, Thr, Tyr residues on proteins and peptides where a phosphoryl group ($-PO_3$) is attached or removed through protein kinases or phosphatase. The majority of methods published on the study of PTMs are based on profiling phosphorylation, whether to identify phosphorylated peptides or to determine the degree of phosphorylaytion[10]. The analysis of phosphorylated peptides can be performed using many analytical

techniques, hence the popularity of such methods as two-dimensional gel electrophoresis (2-DE), Edman degradation, molecular mass spectrometry and Inductively Coupled Plasma Mass Spectrometry (ICP-MS).

1.1.1 Quantitative Methods

A major goal of proteomic research is the quantitative analysis of all proteins expressed in a cell, tissue, or an organism at a specific moment and under certain conditions. Traditionally, quantitative proteome analysis was performed by the differential staining of 2-DE gels, however, the inherent disadvantages of using 2-DE gels has prompted new approaches to quantitation.

It was previously thought that the analysis of messenger ribonucleic acid (mRNA) expression would provide quantitative data on the proteome. However, experimental observation in studies comparing the amount of mRNA to protein levels showed there was no correlation[11]. In hindsight this was unsurprising because proteins catalyse all biological functions and the levels are determined by key effector molecules. In addition, proteins undergo activation and deactivation through post-translational mechanisms which cannot be predicted by studying mRNA levels.

Quantitative analysis using mass spectrometry has historically employed stable isotopes such as ^{2}H, ^{13}C, ^{15}N, for the absolute quantitation of peptides. The method requires the addition of an internal standard which is chemically identical to the analyte of interest, apart from the isotopic enrichment. The absolute amount of the analyte is determined by comparison with a standard curve[12]. The addition

of an internal standard is a necessity for quantitation using techniques such as electrospray ionisation (ESI) mass spectrometry because of the variable ionisation efficiencies for analytes of different composition and from different elution times during chromatographic analysis.

1.2 Expression Proteomics

When exposed to different environmental, pathological, pharmacological or genetic perturbations, cells respond by altering protein abundances via transformations, or changes in protein structure by post-translational modification[13]. Therefore, it is an essential part of proteomics to quantify these changes in protein abundance. Expression proteomics (relative quantitation) is the method of choice for comparing the differences in protein expression between samples that differ by some variable, such as between diseased and healthy cells. The approach compares the expression of either the entire proteome or sub-proteome, and which can be used to identify novel proteins in signal transduction or disease specific proteins. For example, Mann and co-workers[14] relatively quantified the differences between epidermal growth factor (EGF)-stimulated and non-stimulated (EGF) cells, and identified 28 specifically enriched proteins which provided information on related biochemical pathway.

In practice, relative quantitation of proteomic samples is based on the isotope dilution theory and is achieved when a protein mixture (reference sample) labelled with natural or light isotopes is compared to a second sample containing the same proteins at different abundances, but labelled with enriched or heavy isotopes. In theory, all the sample components will exist in peptide pairs after tryptic digestion

with identical sequences, but differ in mass due to the isotope incorporation. The peptide pairs have the same physico-chemical properties so they are expected to behave in the same manner during sample isolation, separation and ionisation. Hence, the ratio of the isotopic intensities of the spiked and unspiked samples is related to the relative abundances of proteins in the original sample.

The past few years have seen the development of several labelling strategies for the purposes of expression proteomics illustrated in Figure 1-3. These approaches have been used with ESI-MS to allow simultaneous quantitation and identification.

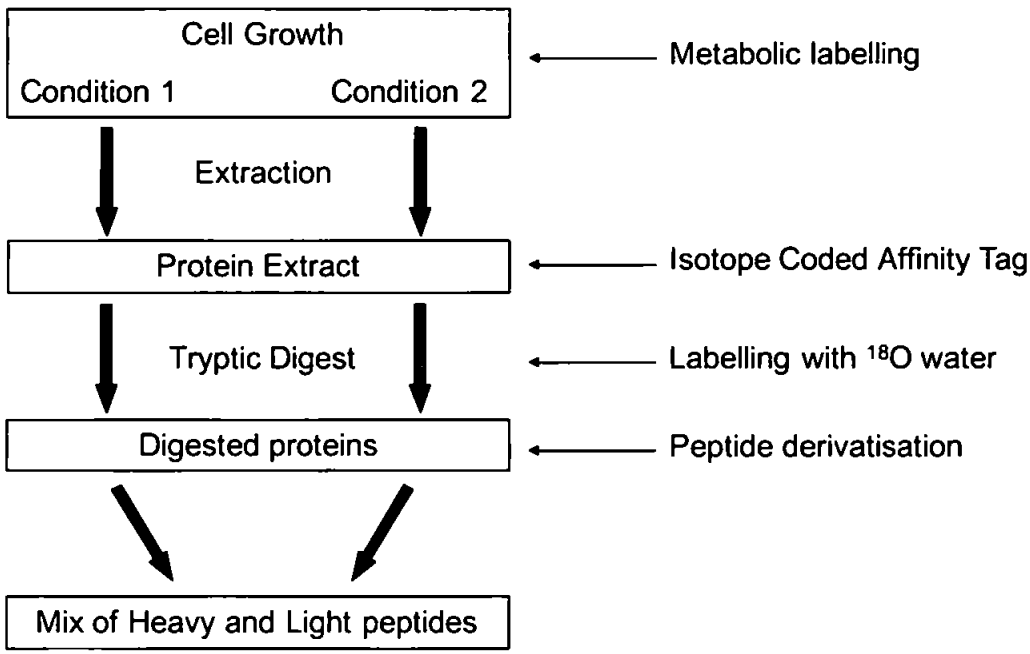

Figure 1-3 Strategies for labelling proteomic samples with stable isotopes.

Stable isotopes labels can be introduced into proteins at various stages of sample preparation before mass spectrometric analysis. However, the ideal stratagem is that labelled (heavy and light) samples should be combined directly after the

labelling step. Since any variations caused by subsequent sample preparation would be equal and relative for the combined samples.

1.2.1 Metabolic Isotope labelling

A critical part of the isotope labelling process is the assurance of complete derivatisation (labelling) of all proteins within a sample. Metabolic labelling is the method of choice when it is required to cover the entire proteome, because the stable isotopes are introduced at the earliest possible time, during the processes of cellular metabolism (Figure 1-3). Hence, the two samples can be combined prior to protein extraction, thus any variations from subsequent sample preparation would be relative for the combined samples. The strategy of metabolic isotope labelling differs to that of the chemical isotope labelling method by targeting the entire protein/peptide sample rather than a specific amino acid residue (cysteine in the case of isotope coded affinity tags, and lysine in the case of mass coded affinity tags). Labelling through metabolic incorporation is one of the most accurate procedures for quantitation of proteins from a cell line because every peptide produced by the cell and cleaved by enzymatic digestion will have an isotopically identical internal standard. Several groups[14-17] have developed metabolic labelling strategies that enable the stable isotope coding of all proteins within a sample. These strategies used various stable isotopes to label the internal standards such as ^{2}H, ^{13}C, ^{15}N and ^{18}O.

1.2.1.1 ^{15}N Labelling

Oda *et al.*[15] described the first method of metabolic labelling, using yeast cells and growing them on a natural N and uniformly labelled ^{15}N enriched media. At the end

of the growing period the cell cultures were combined and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) or reverse phase high performance liquid chromatography (LC) and were relatively quantified and identified using ESI-MS. The authors reported the relative quantitation of 42 high abundant proteins and were able to quantitate and identify *in vivo* phosphorylation sites[15]. There was no fixed mass increase between the resulting labelled and unlabelled peptides, because the number of nitrogen atoms in amino acids ranges from one (as in glycine) to four (as in arginine).

The incorporation of ^{15}N into proteins and peptides is advantageous because it can also be used for structural proteomics[18] by following the phase shifts using nuclear magnetic resonance (NMR). This has provided insight into biochemical functions that were not detectable from sequence analysis alone. The ^{15}N metabolic labelling technique has been limited to microorganisms, because the isotopically substituted media is difficult and expensive to produce for the mammalian system[19]. Though, Conrads *et al.*[20] successfully labelled bacteria and mammalian cells in culture, the resulting peptide pairs were analysed using liquid chromatography - fourier transform ion cyclotron resonance (LC-FTICR) to obtain relative quantitation. The approach of ^{15}N metabolic labelling has been demonstrated to be an accurate quantitation methodology and which has been applied to various modes of separation[21].

1.2.1.2 ^{13}C Enrichment and Depletion

Metabolic labelling using ^{13}C isotopically modified media has been reported[22], though this method has not been extensively employed for protein and peptide

quantitation. The technique involved positive and negative labelling for the quantitation of peptides by isotope dilution, in which one sample was grown in ^{12}C at 99.98% enrichment (^{13}C-depleted, negative labelling) and the other grown in ^{13}C-enriched (^{12}C-depleted, positive labelling) media prior to analysis by matrix assisted laser desorption ionisation - time of flight - mass spectrometry (MALDI-TOF-MS). Carbon is an ideal stable isotope to use rather than nitrogen, because the two most abundant elements found in living organisms are carbon and hydrogen. ^{13}C carbon can be supplied in two forms for stable isotope labelling; either as pure carbon sources to synthetically defined media for microbes, or as carbon dioxide for photosynthetic organisms. Recently, ^{13}C metabolic labelling using carbon has been applied to the field of quantitative metabolomics[23, 24].

An advantage of using either ^{15}N or ^{13}C for isotopic labelling is that the methods do not rely upon one single amino acid residue for labelling, as is the case with other stable isotope labelling methods such as ICAT. In addition, ^{15}N or ^{13}C labelled peptides do not display significant isotope dependent shifts compared with their naturally labelled counterparts during chromatography hence, improving relative quantitation. The popularity of these metabolic labelling methods has resulted in the development of software tools such as TurboSequest and Xcalibur (Thermo Finnigan, San Jose, USA) to allow quicker identification and quantitation.

1.2.1.3 Stable Isotope Labelling by Amino Acid in Cell Culture

The technique of metabolic labelling was further improved by Ong *et al.*[19] for the relative quantitation of mammalian cells. Mammalian cells cannot synthesise a number of amino acids, which must be supplied in the cell culture medium as free

amino acids to support cell growth. The stable isotope labelling by amino acid in cell culture (SILAC) technique involves incorporation of deuterium enriched leucine (Leu-$^{2}H_3$) and normal leucine in the medium respectively. The samples are combined and the resulting analysis performed by 2-DE gel separation with LC-ESI-MS detection.

The advantage of using amino acid incorporation of stable isotopes is that proteins in mammalian cells can be quantitated. Leucine was selected because it is the most abundant amino acid at 9.4% of total amino acid abundance. This allows 68% of the tryptic peptides to be differentially labelled, compared to 20% of tryptic peptides using the ICAT method (calculated on the basis of the relative abundance of cysteine and leucine residues, and an average length of 14 amino acids for tryptic peptides that can be sequenced by mass spectrometry[19]). Compared to the original method of metabolic labelling there is a fixed increase in mass between labelled and unlabelled tryptic peptides that is proportional to the number of leucine residues incorporated. Ong *et al.*[16] reported the complete incorporation of the stable isotope labelled amino acid after five doublings of the cell lines. Furthermore, it was found that the enriched growth media did not alter cell growth, cell morphology, or doubling time. However it was noted that incorporation times varied from cell line to cell line, so longer incubation times may be necessary[16].

The SILAC approach allows specific amino acids to be incorporated for relative quantitation, so the proteome coverage can be reduced by incorporating cysteine or tryptophan residues. In addition, amino acids can be labelled with either ^{2}H, ^{13}C, ^{15}N, or ^{18}O. Deuterium labelling is the obvious choice, because it is a cheaper

label, however, its physico-chemical properties are different from hydrogen, which results in an isotope effect during reverse phase liquid chromatography[25] which leads to variations in isotope ratios. This hydrogen isotope effect is caused by early elution of deuterated peptides compared to non-deuterated counterparts[26]. When peptides elute at different times the ionisation conditions may differ, particularly during gradient elution, which can cause bias in the relative quantitation.

A second generation SILAC approach has been developed to overcome the isotope effects of using deuterated leucine. The method uses $^{13}C_6$-Arginine as a label, the advantage being that trypsin cleaves after the arginine and lysine residues, so generates peptides with these residues at the carboxyl terminus in equal abundance[17].

A limitation of the metabolic labelling technique is that the procedure has only been applied to organisms that can be grown in defined media, such as unicellular organisms and cells in culture as described previously. However, unique strategies have been recently employed for the relative quantitation of multicellular organisms by the complete labelling of potato plants[27], the nematode *(Caenorhabditis elegans)* and the fruitfly *(Drosophila melanogaster)*[28]. Plants were labelled using $K^{15}NO_3$ added to the growth media as the only source of nitrogen[27]. Krijgsveld *et al.*[28] used a two step labelling procedure to incorporate stable isotopes into proteins and peptides, by growing bacteria and yeast on ^{15}N-enriched media which were then used to feed the nematode and fruitfly respectively.

The incorporation of stable isotopes to a selected amino acid is an ideal method for quantitating proteins or natural cell lines because the method is inexpensive, analytically accurate, and simple to carry out. The stable isotopes are incorporated at the earliest possible stage, so experimental errors are minimised and mass spectrometric analysis does not discriminate between unknown(s) and internal standards because they both have the same ionisation efficiencies. The advantage of using amino acid stable isotope incorporation over the ^{15}N metabolic labelling technique is that the isotope shift mass can be predefined (determined by which amino acid is incorporated) which facilitates peptide sequencing and identification.

Metabolic labelling is a limited technique because it relies on proteomic samples derived from cell cultures, and cannot be applied to other samples of interest such as tissue samples or body fluids[29]. When metabolic labelling is not suitable for analysis, alternatives such as proteolytic labelling or chemical labelling techniques have to be explored.

1.2.2 Proteolytic Labelling

Several procedures have been described for the incorporation of isotopic labels after proteolysis. Proteolysis incorporates oxygen atom(s) from the solvent into the C-terminus of the digested peptide using either ^{18}O or ^{16}O respectively. Mirgorodskaya *et al.*[30] and Yao *et al.* [31], describes the cleavage of protein in heavy ($H_2{}^{18}O$) water with the other sample being cleaved in light ($H_2{}^{16}O$) water. This approach was used to label all proteolytic peptides and compare protein extracts from serotypes of adenovirus[30].

The proteolytic labelling procedure is simple, because the stable isotope is not incorporated during protein synthesis, and all peptides are labelled. In addition, oxygen atoms of the C-terminus carboxylates do not exchange with the solvent in the absence of enzyme (trypsin). A drawback to this type of quantitation is that isotopic labelling occurs late in the sample preparation process, which can lead to incomplete labelling and unequal losses due to sample handling; also, many peptides cannot be enzymatically cleaved. In addition, the tryptic lysate results in a very complex sample which can cause problems during analysis. For example, a total *Escherichia coli* lysate would produce 132,768 peptides; a total yeast lysate produces 333,247 peptide fragments, and a total human digest would produce a nominal 2,641,532 peptides[31].

1.2.3 Chemical Labelling Strategies

Chemical labelling or derivatisation of specific amino acid residues can be used to incorporate both stable isotopes for relative quantitation and to attach affinity tags to specifically enrich and isolate peptides. Considering that there are twenty natural amino acids, the selection of functional groups to be tagged is rather limited. This section will describe stable isotope labelling techniques on whole proteins and peptides.

Recent reviews by Leitner *et al.*[32, 33] discusses isotopic tagging strategies of various amino acids, primarily for proteomic analysis. Cysteine is frequently labelled because the thiol group can be specifically modified by reagents containing iodoacetyl or vinyl functionalities. These reagents have been in use for a long time to alkylate free cysteine residues after the reduction of the disulphide

bonds. In addition, cysteine is a rare amino acid with an average relative abundance of 1.1% across several species[34]. Tagging of other amino acids has also been extensively described, such as lysine residues after tryptic digestion, or N-terminal amines[35].

1.2.3.1 Isotope Coded Affinity Tag

Stable isotope quantitation strategies have been demonstrated, with a wide range of residue labelling specificities and with several different reagents, to provide relative quantitation using a variety of technologies. Isotope coded affinity tag[36] (ICAT) developed by Aebersold and co-workers has made the biggest impact in proteomics since the advent of soft ionisation mass spectrometry.

Figure 1-4 shows the structure of the first generation ICAT reagent split into three distinctive functional groups. The iodoacetamide group is used to incorporate stable isotopes into proteins or peptides via a selective alkylation of the thiol groups of cysteine residues at a pH of about 7-8. The isotope coded region of the tag is used to distinguish between the two forms, the light form containing eight hydrogens and the heavy form containing eight deuteriums. The biotin moiety is used for specific isolation of labelled peptides by means of biotin-avidin affinity chromatography.

The ICAT strategy for quantifying differential protein expression is based on the detection of the relative amounts of heavy and light reagents by mass spectrometry (MS). The ICAT reagent is added to two different cell states, one state is derivatised with the isotopically light reagent, and the second state is

derivatised with the isotopically heavy reagent. The binding of the ICAT to the cystienyl residues on the proteins is performed via an alkylation reaction.

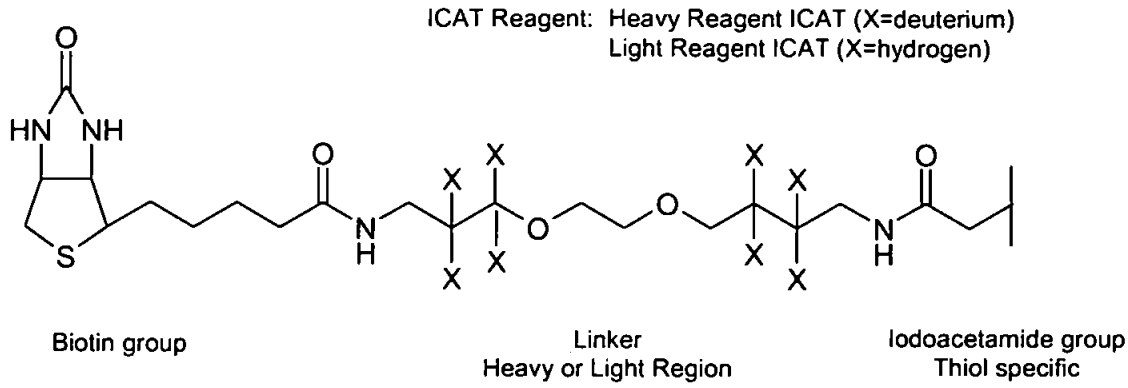

Figure 1-4 Structure of the first generation isotope coded affinity tag (ICAT) reagent. The X functional groups represents whether the compound is deuterated or not.

The alkylation reaction is highly specific, and is limited to the thiol group of each cysteine residue. The two samples are then combined together and digested using protease to generate peptide fragments, of which some are tagged with the ICAT reagent. The next step is to separate and isolate the tagged peptides (peptides containing cysteine) from the untagged peptides by using avidin affinity chromatography. The biotinylated peptides selectively bind to avidin because of the high affinity for binding between biotin and avidin. This separation step allows biotinylated peptides (tagged) to be isolated from compounds that were not biotinylated (untagged). The isolated peptides from the affinity chromatography are then separated via reverse phase μLC, and analysed by mass spectrometry.

The mass spectrometer can be used in dual mode for relative quantitation and sequencing of the peptides. The mass difference between the heavy (deuterated)

and light (hydrogenated) is 8 Da, therefore, pairs of peptides with identical peptide sequences only differ physically and not chemically so chromatographic separation can be used. Relative quantitation is achieved by comparing the signal intensities for the pairs of peptide ions of identical sequence that are tagged with either the heavy or light form of the ICAT reagent.

The ICAT strategy has the ability to provide relative quantitation to a degree that has not been surpassed, and, due to the success of the ICAT reagent many other similar reagents have been produced and are currently being used for protein relative quantitation. These second-generation compounds have been produced in light of some shortfalls experienced with the first generation ICAT reagent..

A distinct advantage of the ICAT strategy is the selective isolation of the biontinylated cysteine residues on peptides using biotin/avidin affinity chromatography. The strong binding between the biotin tag and avidin provides an enrichment of peptides of interest and the selective nature of the ICAT reagent reduces the complexity of the peptide mixture. For example, the tryptic digest of the entire yeast proteome (6,113 proteins) produces 344,855 tryptic peptides, but only 30,619 of these peptides contain a cysteine residue[36]. However, the dependence on the biotin/avidin binding system for the isolation step may lead to additional difficulties, such as non-specific binding of peptides and incomplete elution of undesirable peptides.

The cysteine specificity of the ICAT reagent can be a notable disadvantage because one out of seven proteins in the human proteome does not contain the

cysteine residue[37]. The work carried out by Gygi *et al.* used a yeast cell (*Saccharomyces cerevisae*) that contained 30,619 cysteine containing peptides which provided ~92% coverage of the proteome[29].

Another significant problem with the first generation ICAT reagent is that separation through a reverse phase column can lead to a well-known isotope effect, in which the deuterated peptide elutes earlier than the non-deuterated counterpart[25]. This effect can lead to large variations in measured isotope ratios because of variations in ionisation during gradient elution. The hydrogen isotope effect can lead to peptide expression errors, hence, protein expression errors of greater than 25%, which have been reported, though the isotope effect was addressed by Smolka *et al.*[38] by utilising two dimensional gel electrophoresis (2-DE) separation for ICAT tagged peptides followed by MS analysis. The research showed that there was co-migration of the heavy and light forms of the ICAT reagent on the 2-DE gel. Another critical issue was that the relatively large biotin tag would also fragment under collision induced dissociation (CID) MS, complicating the mass spectra and hindering computer aided interpretation.

1.2.3.2 Second Generation Isotope Coded Affinity Tags

Second generation ICAT reagents were synthesised to address the issues with the original ICAT reagent (Figure 1-5a) described in 1.2.3.1. Zhou *et al.*[39] described the use of a solid phase capture and release method to introduce stable isotopes, in addition to isolating the tagged peptides (Figure 1-5c). The tag was attached to a solid phase resin containing pore glass beads consisting of a photo-cleavable linker, an isotope tag (either heavy or light), and a specific reactive group. The

advantage of using this method was that the peptides were isolated and tagged in one step, which meant minimal sample handling. The photo-cleavable linker allowed a smaller tag to be used (170 Da compared to 442 Da for the original ICAT reagent), hence improving identification during CID MS. A side-by-side comparison of the solid phase and solution phase methods indicated that more proteins were identified from *Saccharomyces cerevisae* using the solid phase capture-release ICAT method[40].

However, the significant isotope effect remains when chromatography is used because of the hydrogen/deuterium isotope effect. This was addressed by Qui *et al.*[40] who synthesised an acid labile isotope coded extractant (ALICE) shown in Figure 1-5d, wherein the reagent incorporates nine ^{13}C atoms as the heavy reagent, this permits the ^{13}C heavy/light-modified peptides to co-elute by reverse phase chromatography, making relative quantitation much more reliable. In addition, they used a solid phase capture and release method to enrich Cys-containing peptides and a acid labile linker was used to reduce the mass of the tag for MS. A similar tag was developed by Applied Biosystems Inc. (Foster City, CA) which contained ^{13}C atoms as the isotopic label and with an acid cleavable biotin linker (Figure 1-5b). The method was identical to the original ICAT method except that, as an additional step, the biotin group was cleaved off prior to MS. A, schematic of the different types of tagging reagents described are shown in Figure 1-5.

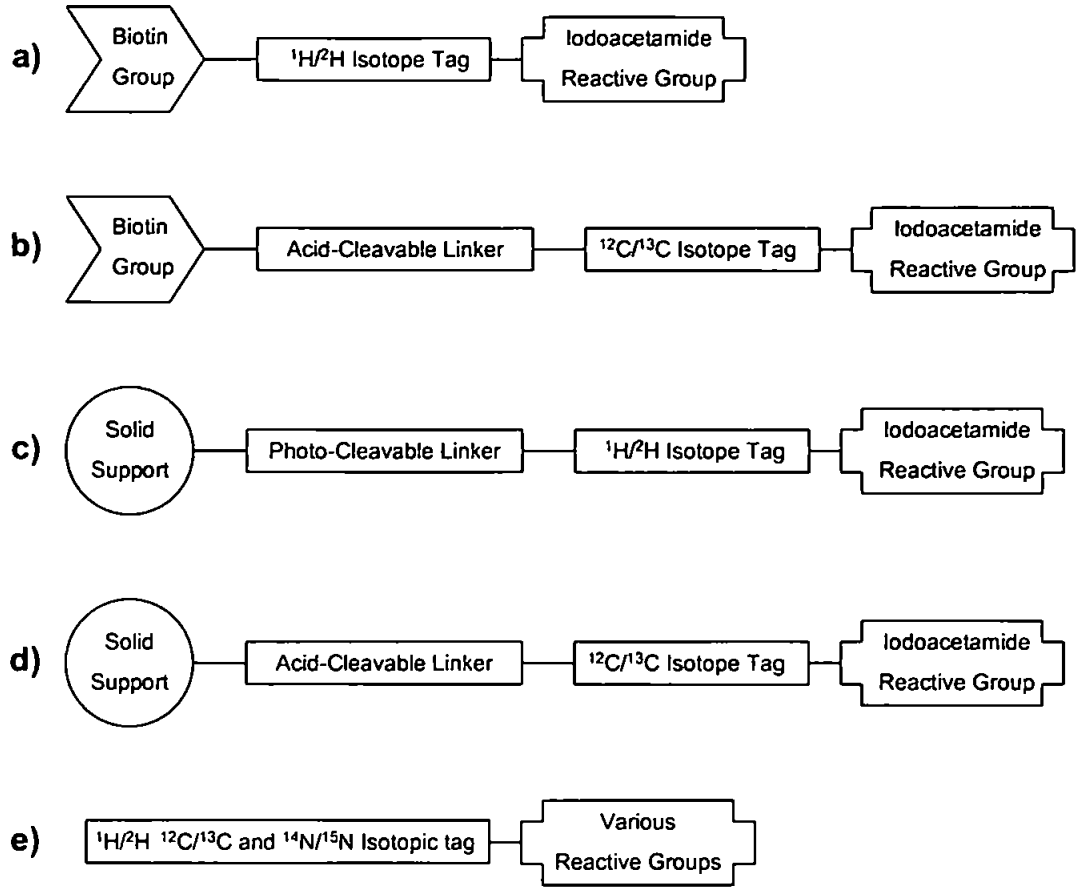

Figure 1-5 Summary of the available ICAT based tagging reagents (a-d) and comparison to (e) a standard stable isotope tag for peptides: a) First generation ICAT, with biotin affinity group, hydrogen or deuterium based tag and a iodoacetamide reactive group introduced by Gygi *et al.*[36]; b) Second generation ICAT with an acid cleavable biotin tag, carbon based tag and a iodoacetamide reactive group introduced by Applied Biosystems; c) Second generation ICAT with an photo-cleavable solid phase group, hydrogen or deuterium based tag and a iodoacetamide reactive group introduced by Zhou *et al.*[39]; d) Second generation ICAT with an photo-cleavable solid phase group, hydrogen or deuterium based tag and a iodoacetamide reactive group (ALICE) introduced by Qui *et al.*[40]. Modified from Tao *et al.*[41].

1.2.3.3 Mass-Coded Abundance Tagging

In 1999 it was reported that MALDI-TOF ionisation is biased towards peptides that contain arginine residues compared to peptides containing lysine residues. The detection intensity was found to be 4-18 times more intense for arginine than lysine[42]. Different research groups[43-45] used this phenomenon to derivatise the lysine group of terminated tryptic peptides through the guanidination of the ε-amino group of the C-terminal. The derivatisation converted the lysine into a homoarginine which exhibits enhanced intensities using MALDI-TOF for protein identification.

Cagney and Emili[46], introduced the mass-coded abundance tagging (MCAT) abbreviation, applied the guanidination derivatisation procedure and demonstrated its use with LC/MS-MS-MS for the relative quantitation of proteins in mixtures. The strategy for MCAT involves digesting two samples using trypsin, and the resulting tryptic peptides of one of the samples is modified with O-methylisourea for the guanidination of the lysine to homoarginine, while the other sample remains unmodified. The two samples are combined together in a 1:1 ratio, and seperated by reversed phase capillary LC before being introduced into an ESI source for MS and MS/MS analysis. The resulting spectra are used to compare ion intensities for relative quantitation.

Two qualitative differences are apparent between the MCAT and ICAT methods. The MCAT derivatisation adds a 42 Da difference between two identical peptides while ICAT adds a 8 Da difference between the light and heavy forms of the

peptides. Additionally, ICAT is performed on intact proteins prior to digestion whereas MCAT guanidination is conducted on tryptic digested peptides.

The main advantage of using the MCAT method is that the reaction of unhindered peptides proceeds to completion in 1hr at 37°C, which is ideal for high through-put analysis. The disadvantage is that relative quantitation does not take into account differences in ionisation efficiency between lysine (untreated) and homoarginine (treated sample), which can bias the peptide ratio.

1.2.3.4 N-Terminal Labelling

In addition to specific amino acid residues such as cysteine and lysine, the primary amine group of the N-terminal can also be targeted. The method developed by Regnier and co-workers[47, 48] is the acylation of primary amino groups of peptides, after tryptic digestion, using either *N*-acetoxysuccinimide or *N*-acetoxy-[$^2D_3$]succinimide. The method employed a global internal standard strategy (GIST), due to the fact that all peptides, regardless of composition, were labelled. However, this can result in a very complex sample so, in order to reduce sample complexity, the group introduced a capture/enrichment strategy whereby peptides that contain Cys and His were isolated[49]. This allowed a greater proportion of the proteome to be analysed compared to the ICAT method, though the authors failed to mention the percentage of coverage over the proteome.

1.2.3.5 Summary of Relative Quantitation Techniques

Relative quantitation has been well documented in several reviews[19, 50-53]. The "pros" and "cons" of current methods are summarised in Table 1-1. The most

fundamental factor to be considered when selecting a method or reagent, is the accuracy of the relative quantitation. It is important to note the observation by Gygi *et al.*[54], when discussing the error associated with metabolic labelling method using ^{15}N, "The percentage error of the experimental technique was found to be excellent (±10%)."

The large errors observed in these experiments were not caused by the tagging method but the resulting method(s) of detection and isotope ratio analysis. Molecular mass spectrometry, when used for relative quantitation, has proved to exhibit poor linearity, precision and accuracy. Due to the poor accuracy and precision it is difficult to determine small differences in protein expression between control and disease states.

Table 1-1 Summary of proteome relative quantitation methods.

	Labelling method	Overview of methodology	Change in peptide mass	Isotope coding	Advantages	Disadvantages
Biological incorporation	N^{15} Labelling[15]	Growth on general or isotopic media	Depends on composition of peptides	$^{14}N/^{15}N$	Earliest isotope incorporation Samples combined at earliest stage Complete proteome coverage	Expensive Limited to cells that can be grown in cell cultures Complex samples require fractionation prior to MS
	SILAC[17, 19, 27, 28]	Incorporation of isotope coded AA from growth media		$^{1}H/^{2}H$ $^{12}C/^{13}C$ $^{14}N/^{15}N$	Choose specificity of isotope incorporation Can cover entire proteome Earliest incorporation and sample combination	Expensive Limited to cells that can be grown in cell cultures Complex samples require fractionation prior to MS
Chemical incorporation	First generation ICAT reagent[36]	Cys residue modification followed by selective capture	8 Da for labelled Cys residue	$^{1}H/^{2}H$	Reduced sample complexity by enrichment of Cys-peptides Universally applicable	Limited to proteins containing Cys Isotope effect affects chromatography Non-Cys containing peptides enriched by avidin chromatography Biotin tag complicates MS analysis
	Acid cleavable ICAT reagent[40]		9 Da for labelled Cys residue	$^{12}C/^{13}C$	Reduced sample complexity by enrichment of Cys-peptides Universally applicable Cleavable biotin tag	Limited to proteins containing Cys Non-Cys containing peptides enriched by avidin chromatography
	MCAT[46]	Guanidination of C-terminal lysine	42 Da for labelled peptides		No stable isotopes required Inexpensive Universally applicable	Complex samples require fractionation prior to MS Require multiple derivatisation steps
	Enzymatic Digestion $^{16}O/^{18}O$[30, 31]	C-terminal modification during proteolytic cleavage	2-4Da dependant on peptides	$^{16}O/^{18}O$	Complete proteome coverage Universally applicable Simple derivatisation	The 2-4Da mass difference limits the analysis of larger peptides Complex samples require fractionation prior to MS
	Derivatisation of N- and C- terminus[50-53]	Tagging of stable isotopes are proteolysis	Dependant on tag	$^{1}H/^{2}H$ $^{12}C/^{13}C$ $^{14}N/^{15}N$	Universally applicable Complete proteome coverage	Complex samples require fractionation prior to MS No selectivity – large dynamic range

1.3 Proteomic Technology

Traditionally, proteomic technology has focused on protein identification using Edman sequencing to obtain N-terminal amino acids sequences[55]. However, molecular mass spectrometry is now the method of choice to obtain structural information, though Edman sequencing still finds a role in proteomics because of the well established nature of the technique.

The basic instrumental analytical requirements in proteomic analysis are high sensitivity, high resolution, high throughput and high-confidence in protein identification. In addition, proteins should be quantified and post-translational modifications identified.

The following general considerations should be taken into account during proteome analysis (Figure 1-6):

- Sample preparation – whether samples require treatment such as enrichment, extraction from matrix, reduction of sample complexity, tagging of stable isotopes.
- Sample separation – baseline resolution of analytes, time for analysis, fractionation to reduce complexity.
- Sample detection – identification, qualitative or quantitative, protein expression, mass resolution.

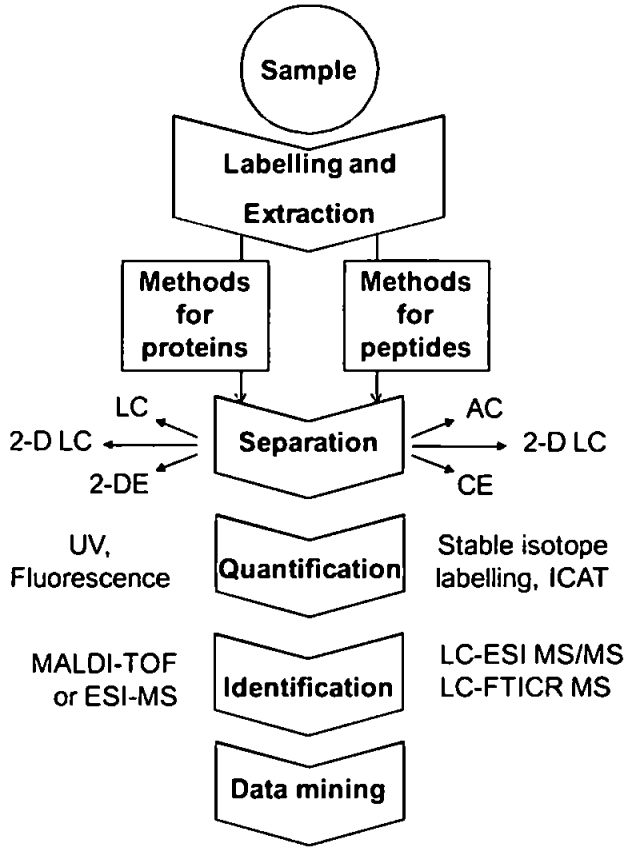

Figure 1-6 Summary of technologies used at each step of proteomic analysis of intact proteins or complex mixtures of peptides.

1.3.1 Separation Technology in Proteomics

In order to reduce sample complexity prior to mass spectrometry one of two approaches can be used: (1) proteins are separated, then digested – top down proteomics[56]; (2) or bottom up proteomics where a complex protein mixture is digested then the resulting peptides are chromatographically resolved, also known as shotgun proteomics[57]. Whichever method of analysis is selected the separation plays an essential role in the analysis.

Several separation approaches have been employed for the analysis of complex mixtures prior to mass spectrometric analysis. These separation methods range from high through-put techniques such as chromatography to high peak capacity methods such as electrophoresis.

1.3.1.1 One Dimensional Gel Electrophoresis

The most common application of one dimensional gel electrophoresis (1-DE) is the characterisation of proteins. A purification step to reduce the complexity of the sample is necessary because of the limited resolving power of 1-DE. The limited resolution is due to the way proteins are separated, they are separated in one dimension by mass, with a mass range of 10 to 300k Da being separated. 1-DE has the advantages of being simple and a very reproducible method of separation.

1.3.1.2 Two Dimensional Gel Electrophoresis

For the last 25 years, two-dimensional gel electrophoresis (2-DE) has been the method of choice for the analysis of complex protein mixtures (e.g. crude cell lysate), and can be used to separate as many as 5000 different proteins in a single

experiment[58]. The technique separates proteins in two dimensions: the first dimension resolves proteins depending on their net charge, separating proteins within an isoelectric point (pI) range of 3.5-10; and the second is based on molecular weight. Once separated, samples can be visualised by staining the proteins with silver stains, fluorescent stains or coomassie blue. Once located proteins can be eluted from the gel for analysis.

The primary application of 2-DE is in protein expression profiling, where protein samples can be compared qualitatively and quantitatively. The appearance or disappearance of a spot can indicate the difference between normal and diseased cell. The intensity of the spots can also provide quantitative information though it is not reliable as other techniques such as stable isotope labelling.

An improvement in protein expression profiling has been achieved using differential gel electrophoresis (DIGE). The technique uses fluorescent tagging of two protein samples using two different amine reactive dyes. The dyes are designed to ensure that the proteins have the same mobility regardless of which dye is attached. The samples are then run on the same gel plate and compared by digital imagining using the optimum fluorescence of the respective dyes. The technique circumvents the need to compare several 2-DE gels.

A major advantage of 2-DE is the ability to resolve proteins that have undergone certain post-transitional modifications, because such proteins undergo a change in net charge as well as a change in mass. An example of this is protein phosphorylation, where the phosphorylated protein can be resolved from its non-

phosphorylated form as several spots on the 2-D gel rather than a single spot for the non-phosphorylated protein[59].

Developments of 2-DE over a period of years has resulted in an increase in reproducibility through the introduction of immobilised pH gradients, which improves resolution. However, large and hydrophobic proteins still cannot be resolved because they cannot enter the gel in the first dimension, in addition, to highly acidic and basic proteins[60].

1.3.1.3 Liquid Chromatography

Liquid chromatography (ion exchange, size exclusion, affinity and reverse phase) and electrophoretic separations in the liquid-phase (capillary zone and free flow electrophoresis) are well established techniques that have been used to purify or separate organic molecules, DNA or peptides and proteins.

1.3.1.4 Reversed Phase LC

Reversed phase chromatography is used to describe the state in which the stationary phase is less polar than the mobile phase[61]. A chemically bonded octadecylsilane (ODS, C_{18}), an *n*-alkane chain, is the most frequently used stationary phase. Shorter alkyl groups including C_4, C_8 (which is used for protein separation) and also cyclohexyl and phenyl groups provide other alternatives to aid chromatographic separation of compounds. The mobile phase (eluent) normally contains a mixture of water or aqueous buffer solution with water miscible solvents, such as methanol and acetonitrile. Typically, acid (formic, acetic or trifluoroacetic) is added to the mobile phase to render all of the component

proteins and peptides positively charged and to reduce unwanted ionic interactions with the stationary phase. In addition, the type of mobile phase used for separations can have a large effect on the retention and it can also promote or suppress ionisation of the analyte molecules.

In reversed phase chromatography partition occurs between the bonded organic phase and mobile liquid phase. Consider the following separation of a two component mixture, component A has the same interaction with the adsorbent surface as the eluent, and component B has strong interaction with the stationary phase. An injection of the sample into the mobile phase, generally a narrow band (5 - 20 μL injection volume), results in the sample flowing with the mobile phase onto the column. Molecules of component A will interact with the adsorbent surface and be retained to the same extent as eluent molecules. Thus, as an average result, component A will move through the column with the same speed as the eluent. Molecules of component B, being more strongly adsorbed on the stationary phase will interact with it much longer. Thus, they will move through the column slower than the eluent flow. This results in two distinct separated peaks. The first peak to elute will be due to component A, followed by a second peak to elute, component B.

Retention of the analyte depends on its affinity for the mobile phase and stationary phase, so extensive method development is often necessary to enable adequate resolution between analytes. As a general rule for reversed phase chromatography, polar substances interact better with the mobile phase and elute first, and as the hydrophobic character of the analyte increases, retention

increases[61]. Separation efficiency of the stationary phase is determined on the adsorbent particle sizes, porosity, pore size, column size, shape, and packing performance. Typically particle size of 3 and 5μm are used for separation involving proteins, peptides and other small molecules, however, reducing the particle size from 5 to 3μm for the same column length can increase separation efficiency by 30-40%[62]. In general, the longer the component is retained on the column, the broader its zone (peak on the chromatogram).

1.3.1.5 Multi-dimensional Liquid Chromatography

One dimensional-liquid chromatography (1D-LC) techniques are routinely used for protein and peptide identification, though applications in proteomics are restricted by the complexity of the sample. A typical proteolytic digestion can yield hundreds of thousands of peptides, all of which must be separated. This exceeds the analytical range of most 1D-LC methods because of insufficient peak capacity, so this has led to development of high resolution separation techniques such as multi-dimensional chromatography.

In order to improve separation and resolution of proteomic samples, multi-modular combinations of high performance liquid chromatography (LC), ion-exchange chromatography (IC), size exclusion chromatography and capillary electrophoresis (CE) have been developed. Most multidimensional systems are coupled with mass spectrometry so the choice of the second dimension is limited to reversed phase chromatography. The choice of the first dimension separation must be complementary to the reversed phase separation, e.g. CE-RPLC, IC-RPLC.

A limitation to using multidimensional chromatography systems is the requirement for two or more valves and pumps, and more complex 2D-LC systems cannot be automated due to the requirement for multiple sample loops, splits and switching valves to transfer samples between columns. This problem was solved with the creation of biphasic columns[63], capillary columns packed with strong cation exchange (SCX) resin followed by RP resin, thereby allowing the automated analysis of proteomic separations. Biphasic columns are the key component in the development of multidimensional protein identification technology (MudPIT), which has been the most successful for the global analysis of complex protein mixtures. The elution profile contains typical eluents used for reverse phase chromatography (water, acetonitrile and an acidic modifier), in addition to SCX eluents such as moderate amounts of methanol, organic solvents or ionic species in the form of a buffer. This allows the separation of compound in both reverse and ion exchange phases respectively

Washburn *et al.*[64] described the use of MudPIT and assigned over 5,000 separated peptides to 1484 proteins in *Saccharomyces cereviseae* using SCX chromatography in the first dimension followed by RPLC in the second dimension. Using the same criteria described by Washburn the separation of more than 12,000 unique peptides and more than 1600 unique proteins was achieved from a whole yeast lysate[65].

1.3.2 Mass Spectrometry Technology in Proteomics

Mass spectrometry is capable of providing information about: (1) the elemental composition of analyte; (2) structures of inorganic, organic, and biological

molecules; (3) the qualitative and quantitative data; and (4) isotopic ratios of atoms in samples.

1.3.2.1 Electrospray Ionisation Mass Spectrometry

Electrospray ionisation mass spectrometry (ESI-MS) which was first described in 1984 by Fenn and co-workers[66, 67], who successfully demonstrated the basic experimental principles and methodologies of the ESI technique, including soft ionisation of involatile and thermally labile compounds, multiple charging of proteins and intact ionisation of complexes. Since then, LC-ESI-MS has made rapid progress with extensive literature, many reviews, special journal issues and books, and has now become one of the most important techniques for analysing biomolecules such as peptides, proteins and oligonucleotides.

In ESI-MS, the sample is typically dissolved in a volatile solvent and is delivered to the ionisation source through a narrow metal microcapillary tube into the orifice of the mass spectrometer, where a potential difference between the capillary and the inlet to the mass spectrometer results in the generation of a fine aerosol of analyte containing droplets. As the solvent evaporates the droplets decrease in size resulting in the formation of desolvated ions; this evaporation leaves positive ions originating from the addition of one (MH^+) or more [$(M + nH)^{n+}$] protons to the analyte[68]. These ions are then extracted into the high vacuum of the analyser system of the mass spectrometer where the ions are separated according to their m/z ratio.

Acid modifiers such as formic acid have been used in LC eluents when combined with ESI-MS to provide additional benefits to mass spectrometry for example in peptide analysis acid modifiers can protonate or form ion pairs with the peptides and elute them in sharper chromatographic bands. Also due to the excess protons available when using acidic modifiers, peptides will be readily protonated forming MH^+ ions or even $[(M + nH)^{n+}]$ ions, thus promotes efficient ionisation in the ESI source. However care must be taken when selecting acid modifiers, trifluoroacetic (TFA) acid can readily form ion pairs and produce sharper chromatographic bands, however TFA can also reduce ionisation has it can "soak up" a lot of protons during analysis.

1.3.2.2 Matrix Assisted Laser Desorption/Ionisation Mass Spectrometry

Matrix-assisted laser desorption/ionisation (MALDI) was developed by Karas and Hillenkamp[69]. In 1987, it was shown that if a low concentration of analyte was mixed with a small organic molecule (matrix) onto a probe or metal plate and introduced into a pulsed laser beam. A substantial burst of ions was produced with each laser pulse. This formed the foundation of modern MALDI based techniques.

In MALDI-MS the sample is incorporated into an energy absorbing matrix such as 2,5-dihydroxybenzoic acid or α-cyano-4-hydroxycinnamic acid. The mixture is then applied to metal plates, such as the 96-well format, dried and then subjected to a pulse of UV laser light. The specific target of the laser is directed into the vacuum of the instrument. The ionisation of the sample predominately results in the formation of protonated molecular ions (MH^+).

A time of flight (TOF) mass spectrometer is traditionally used in conjunction with MALDI. The time taken for an ion to traverse the flight tube is determined by its m/z ratio, the lighter ions travelling faster than the heavier ions. Improvements in mass accuracy have been achieved by developments such as the reflectron lens and delayed extraction, which result in an increase in mass resolution. A major advantage of MALDI is that samples can be directly analysed without any purification, after digestion from 2DE gels. Typically MALDI analysis has been used for qualititative analysis such as the identification of proteins and peptides.

A different approach using MALDI-MS for peptide fractionation and identification is the use of ProteinChip technology (Ciphergen) known as Surface Enhanced Laser Desorption Ionisation (SELDI)-MS. This employs a surface capture method using either antibodies or chemically modified surfaces to bind specific classes of proteins.

1.3.2.3 Inductively Coupled Plasma Mass Spectrometry

Atmospheric-pressure inductively coupled plasmas (ICPs) are flame-like electrical discharges that have transformed the practice of elemental and isotopic ratio analysis[70]. The argon ICP has now become the most widely used atomic spectroscopy source in routine analytical chemistry. In 1983 Date and Gray[71, 72] and Houk and Fassel[73] developed the use of ICPs as an ion source for mass spectrometry.

Liquid sample introduction is the most common way of introducing a sample into the ICP (Figure 1.7). Typical introduction systems consist of a nebuliser, where the

liquid sample is converted to an aerosol, and a spray chamber which transports the aerosol into the plasma, where the desolvation - vaporisation - atomisation - excitation - ionisation processes occur. The hot plasma gas is transmitted into a low pressure interface containing both sampler and skimmer cones. The ions formed in the plasma are focused through a lens system and transmitted to a mass analyser prior to detection by either an ion counting or an analogue detector.

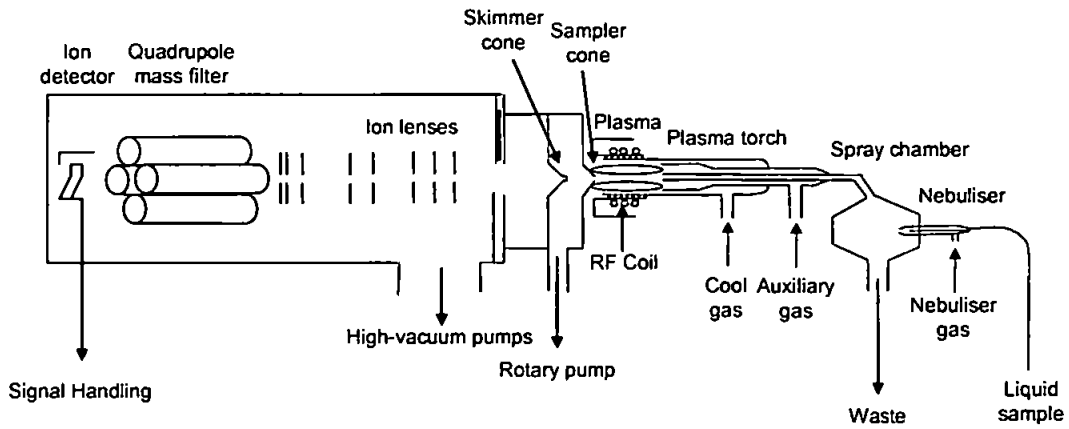

Figure 1-7 Schematic of a quadrupole ICP-MS instrument

1.4 Inductively Coupled Plasma Mass Spectrometry in Proteomics

Since its introduction in the early 1980's, ICP-MS has been widely used for trace element detection and speciation of inorganic species. However, recently ICP-MS has been increasingly used in the field of proteomics because it can provide complementary information on metal protein complexes or metal dependent biochemical pathways. Molecular mass spectrometry alone cannot provide information on these processes, and ESI-MS detection limits suffer when salt rich real-world samples are analysed. Also, the ionisation efficiency of ESI-MS is analyte specific so an internal standard is required. Likewise, information on metal-protein interactions is often lost in the gas phase during MALDI-MS[74]

The advantages of using elemental mass spectrometry ICP-MS are: large linear dynamic range; low detection limits; selective for most elements in the periodic table; detection independent on structure and matrix; ease of coupling with all forms of chromatography; high throughput; mass balance; capable of accurate and precise isotope ratios. However, ICP-MS detection has still not been utilised to its full potential in the analysis of protein and peptide based samples compared to organic mass spectrometry. Most likely because it cannot be used to detect the major constituents of proteins or peptides namely C, H, N and O. These elements have extremely high background signals because they are either present in the sample matrix, solvents used during sample introduction, or atmospheric air entrained in the plasma. Hence, ICP-MS is generally limited to the element specific detection of bio-organic compounds containing a hetero-atom such as P, S, Se, Br, Cl, I, a metal or metalloids.

Past and current research into life sciences involving ICP-MS has primarily focused on the speciation and quantitation of organic molecules where a hetero-atom is incorporated into the molecule or metal complex, such as metalloproteins[75]. Improvements in instrumentation have recently allowed the routine analysis of phosphorus and sulphur[76, 77] in biological samples by employing a quadrupole ICP-MS with a collision/reaction cell or a sector field (SF) instrument.

Current strategies on offer using ICP-MS detection of metalloproteins, proteins and peptides from various biological samples using several approaches have been comprehensively reviewed[74, 78, 79]. However, the analysis of metalloproteins, phosphorous and sulphur covers only a small aspect of the proteomic challenge. For ICP-MS to be used more extensively in life sciences applications there is a requirement for methods that can incorporate elemental or isotopic species into the organic molecules.

1.4.1 Speciation of Proteomic Samples

Various separation techniques have been coupled with ICP-MS for both multi-element and elemental speciation analyses, including liquid chromatography, gas chromatography, supercritical fluid chromatography and capillary electrophoresis[80]. Size exclusion chromatography (SEC) has been most utilised for proteomic speciation and ICP-MS analysis, primarily as a first step in studies involving metal containing proteins such as seleno-proteins, metallothioneins, and metalloproteins.

1.4.2 Natural Heteroatom Peptides and Proteins

With improvements in instrumentation over the last decade, the analysis of naturally occurring heteroatom analysis has been achieved. Elements such as S,P, or Se, have been readily quantitated using a variety of different methods involving either quadrupole ICP-MS or high resolution ICP-MS.

1.4.2.1 Sulphur

The majority of sulphur containing cysteine amino acid residues are found in a specific group of proteins known as Metallothioneins. The difficulty in measuring sulphur (m/z 31.972) for quantitative analysis is mainly due to the high background counts from the $^{16}O^{16}O$ interference at m/z 31.989. In order to spectrally resolve the interference from the main sulphur isotope a resolution of 2500 or greater is required. The quantitative analysis of proteins using LC-HR-ICP-MS was reported by Wind *et al.*[81] for the sulphur-specific detection of insulin.

1.4.2.2 Phosphorous

Up to a third of all proteins and peptides in the human body are phosphorylated at any given moment. Given their importance in a variety of bodily functions a great deal of attention has been focused on the detection, and quantitation of this heteroatom. Several papers on the ICP-MS detection of phosphorous have been published[10, 76, 77]. Wind's group have determined the degree of phosphorylation using the stoichiometric phosphorus to sulphur ratio ($^{31}P/^{32}S$), determined in phosphopeptides containing cysteine and/or methionine residues from various proteins[77]. This was then used to calculate the degree of phosphorylation using

protein/peptide sequence information. The major drawback of this approach is that the number of cysteine and/or methionine residues must be known.

1.4.2.3 Selenium

Selenium can be in found two amino acids selenomethionine and selenocysteine, analogues of methionine and cysteine. Selenocysteine is genetically encoded and considered to be the 21st amino acid. Typically, ICP-MS detection of Se (m/z 74-82) is plagued with interference from polyatomic ions. It was not until the advent of high resolution and/or reaction/collision cell technology for ICP-MS that selenium could be determined at the proteomic scale. The primary method applied by researchers for the determination and characterisation of selenium, is to use SEC-ICP-MS to screen the sample for Se-containing fractions, then to characterise the Se-containing bio-molecule by LC-ESI-MS/MS[82]. These methods have been reported in many reviews concerning ICP-MS and proteomics[74, 79].

1.4.2.4 Metallothioneins

Metallothioneins (MT's) are a class of proteins that transport essential metals such as Cu and Zn as well as to remove heavy metals such as Cd. These MT's are rich in cysteine residues and typically contain 20 or so of these residues (up to 30% cysteine) which are primarily used for the binding of metals, thus making the protein an ideal candidate for ICP-MS detection. As with most ICP-MS methods involving proteins and peptides, the primary separation and purification is undertaken using SEC-ICP-MS, followed by chromatography of higher resolution, followed by ICP-MS and ESI-MS detection for characterisation.

1.4.3 Elemental Tags for Proteomics

Some work involving the elemental tagging of organic molecules has begun to emerge but there has not been a concerted effort to design and evaluate isotopically labelled tags for element specific detection. Few methods exist for incorporating metal tags into proteins and one favoured method is via an immunoassay-based reaction to incorporate various metal tags such as gold-tagged antibodies[83, 84] or europium labelled antibodies[85]. The recent development of metal-tagged antibodies has made it possible to use element specific detection in bio-techniques. However, antibodies containing metal tags have not yet been produced specifically for elemental analysis, so many of the tags used in the ICP-MS detection have been produced for other purposes, such as the gold-tagged antibodies that are used in the localisation of cellular protein in electron microscopy[86]. These tags seem ideal for the quantitation of antigens with ICP-MS detection because of the uniform size and number of the gold particles per antibody (conjugate).

Lanthanide (Eu, Tb, Dy and Sm) tags have also been used in an automated fluoroimmunometric system (Wallac AutoDELFIA), e.g. isothiocyanate-benzyl-DTPA-Eu, which can used to tag the free amino group on peptides. Such tagging procedures provide a simple efficient method for quantifying the specific proteins or peptides of interest.

In recent years several methods have been developed involving chemical derivatisation to incorporate elemental tags into organic molecules for ICP-MS analysis. For example, in our laboratory the reagent

tris(trimethoxyphenyl)phosphonium (TMPP) has been used to tag various organic carboxylic acids with phosphorus to make them amenable to detection using ICP-MS.[87, 88]. Another chemical derivatisation method is the use of cyclic diethylenetriaminepentaacetic acid anhydride (cDTPA) which has been used for the chemical derivatisation of primary amines to allow the tagging of copper[89]. The use of chemical derivatisation for the analysis of compounds in biological systems has been used in many other aspects of analytical chemistry with the prime example being molecular mass spectrometry and, in particular, the advent of isotopic tagging techniques for relative quantitation. Hence, it will only be a matter of time before elemental tags will be in used routine analysis for proteomic samples using ICP-MS.

1.5 Aims and Objectives

The aim of this work is to investigate whether ICP-MS can be used to relatively quantitate peptides through heteroatom labelling and assess through isotope dilution mass spectrometry the methods accuracy and precision compared to current methods described in section 1.2.

The objectives of this programme of study were, therefore to, develop techniques for:

- The derivatisation of peptides through bifunctional chelating agents;
- Labelling derivatised peptides with trivalent metals such as indium and europium;
- Relative quantitation using isotope dilution mass spectrometry on labelled peptides;

Primarily, the investigation will establish whether peptides can be derivatised and labelled with cDTPA and europium respectively. These labelled peptides then can be used to accurately determination the isotope amount ratio hence peptide ratio determinations performed using LC-ICP-MS. Different analytical techniques will be investigated to improve derivatisation and sample handling to improve the overall methodology for relative quantitation for complex samples. Electrospray Ionisation Mass Spectrometry will be employed to characterise the various species formed during derivatisation and chelation in order to verify the complexes formed.

CHAPTER 2 DERIVATISATION OF PEPTIDES WITH CYCLIC DIETHYLENETRIAMINEPENTAACETIC ACID ANHYDRIDE

2. INTRODUCTION TO LABELLING

Chemical derivatisation (labelling) is an established analytical process that is used to modify the properties of an analyte molecule to facilitate detection using a given technique[90]. Derivatisation has been used in many analytical disciplines to either enhance detection, such as in mass spectrometry[88], ultraviolet/visible (UV/Vis) and fluorescence spectroscopy, or to improve chromatographic properties in techniques such as gas chromatography (GC) and liquid chromatography. In this chapter, chemical derivatisation is used to incorporate elemental labels into peptides to facilitate element specific detection using LC-ICP-MS.

Naturally occurring peptides, as opposed to peptides generated from protein digestion, have been largely overlooked in proteomics, even though they are important in a myriad of biological functions. For example, substance P a small 11-amino acid peptide with a sequence of Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met, located in the central nervous system, acts as a neurotransmitter, and has been associated with several physiologic activities including the vomiting reflex, stress, mood disorders, smooth muscle contraction, vasodilation and pain[91]. Hence, it is essential to study naturally occurring peptides in a similar manner to that of proteins.

2.1 Elemental Labelling

The analysis of biomolecules by ICP-MS has principally focused on compounds that contain a heteroatom such as sulphur, phosphorous or selenium, and this has limited the potential of element specific detectors in proteomic research. In addition, when these elements are determined the use of either high resolution or expensive upgrades to ICP-MS such as a collision/reaction cell is required. Hence, alternative strategies for analysing biomolecules by ICP-MS must be considered, especially for those compounds that do not contain a heteroatom.

Two approaches, namely chemical and biological labelling, have been developed for the incorporation of elements and isotopes into biomolecules. As quoted by Quinn *et al.*[85] "As tags have not yet been developed specifically for elemental analysis, we have made use of immunoreagents that already contain element tags." Some research has just begun to emerge, however, there has not been a concerted effort to design and evaluate isotopically labelled tags for element specific detection.

2.1.1 Biological Labelling

One of the first methods for element labelling of proteomic samples was reported by Baranov *et al.*[83] who developed an immunoassay coupled to ICP-MS detection to label specific antigens using gold tagged antibodies. The same research group introduced the use of lanthanide tags to be used in conjunction with immunoassays[85]. These tags were not originally designed for ICP-MS detection but for the localisation of cellular proteins using electron microscopy or fluoroimmunometric analysis. The main advantage of using immunoassays for

elemental incorporation is that specific antigens can be isolated from their biological matrix, resulting in simplified detection and quantitation.

Harrington *et al.*[75] successfully labelled metalloproteins by growing cultures on media containing natural copper or enriched ^{65}Cu and suggested that there is potential for analysis by species specific isotope dilution analysis on proteins labelled in this manner.

2.1.2 Chemical Labelling

In recent years several methods have been developed involving chemical derivatisation of organic molecules to incorporate elemental tags for ICP-MS analysis. In our laboratory the reagent tris(trimethoxyphenyl)phosphonium (TMPP) has been used to tag various organic carboxylic acids to improve their ionisation and detection using ESI-MS[88]. In addition, because the tag contained a phosphorus group this allowed the subsequent elemental specific detection of carboxylic acids using ICP-MS[87]. Another chemical derivatisation method employed cyclic diethylenetriaminepentaacetic acid anhydride (cDTPA) for the chemical derivatisation of primary amines to allow the tagging of copper[89] and subsequent detection using LC-ICP-MS.

2.1.3 Bifunctional Chelating Agents

Bifunctional chelating agents (BFCAs) are a class of organic compounds that exhibit two different functions; reaction with a specific organic moiety and chelation with a metal. Traditionally BFCAs have been extensively used as metal chelators in the development of radiopharmaceuticals and contrast agents for magnetic

resonance imaging (MRI). In the former application tumour targeting compounds, such as monoclonal antibodies and receptor directed peptides, are conjugated using BFCAs to deliver radioactive metals such as ^{111}In and ^{67}Cu. Many bifunctional compounds have been synthesised to react with different organic moieties, though they have mostly been based on known chelators such as, ethylenediaminetetraacetic acid (EDTA), diethylenetriaminepentaacetic acid (DTPA), and 1,4,7,10-tetraazacyclododecane-*N,N',N'',N'''*-tetraacetic acid (DOTA).

Two strategies commonly employed to label organic molecules are; 1) The metal is chelated to the BFCA prior to the reaction with the organic molecule e.g. Eu-Isothiocyanate-benzyl-DTPA used in the AutoDELFIA labelling strategy[92]. 2) The BFCA is allowed to react with the organic molecule, with the metal chelation following to produce a metal-BFCA-organic complex[89]

2.1.3.1 Derivatisation of Amino groups with cDTPA

Acid anhydrides such as cDTPA react primarily with nucleophiles such as alcohols or amines to form esters or amides respectively. The reaction between a primary amine group on a peptide and cDTPA is an acylation reaction, where a hydrogen atom in the primary amine (peptide) is replaced by an acyl group (RCO, where R = an organic group) to form an amide bond shown in Figure 2-1 and the detailed mechanism for the reaction in Figure 2-2.

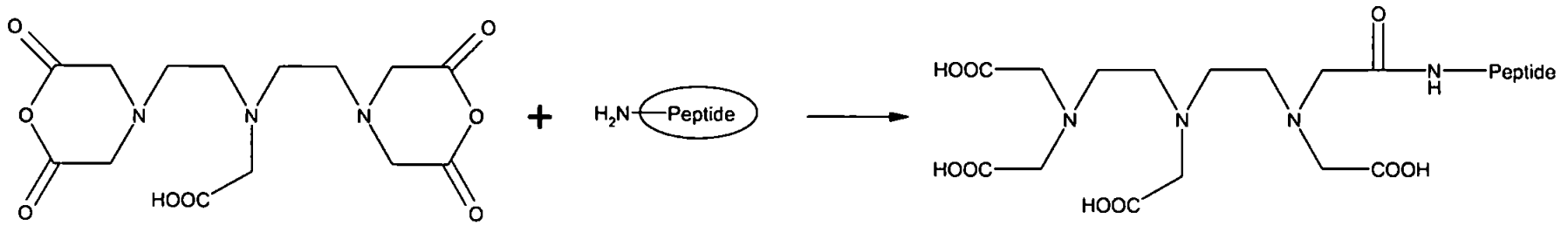

Figure 2-1 Reaction scheme for the derivatisation of a peptide using cDTPA.

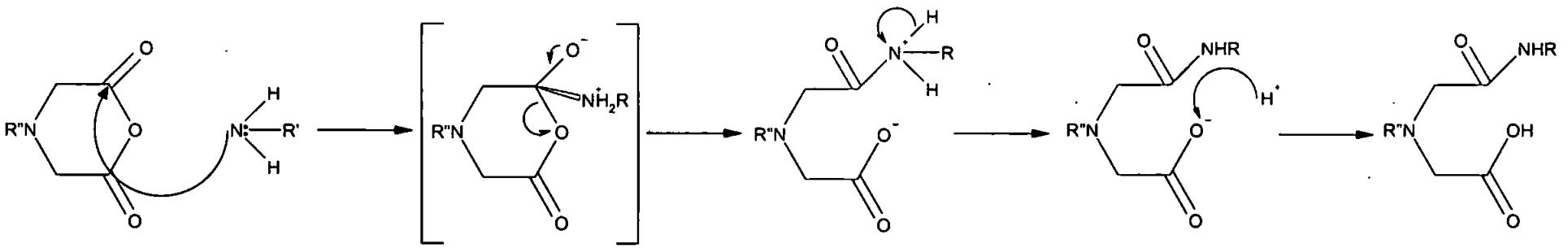

Figure 2-2 Reaction mechanism for the derivatisation of a peptide using cDTPA.

2.2 Experimental

The methods in the following section describe peptide derivatisation using cDTPA and the subsequent optimisation of the reaction. In addition, the characterisation of the derivatisation reaction using ESI-MS and LC-ESI-MS/MS is described, as well as, the method for peptide labelling with indium.

2.2.1 Reagents and Standards

Naphthylamine, bradykinin acetate, cyclic diethylenetriaminepentaacetic anhydride (cDTPA), sodium bicarbonate, and sodium acetate were purchased from Sigma Aldrich, (Gillingham, Dorset, UK). Dimethyl sulfoxide (DMSO), ammonium acetate, nitric acid (trace analysis grade), LC grade acetonitrile (ACN) and methanol were obtained from were purchased from Fisher Scientific UK Ltd, (Loughborough, Leicestershire UK). Ammonium bicarbonate was purchased from Fluka, Sigma Aldrich, (Gillingham, Dorset, UK). Distilled deionised water (DDW) 18.2 MΩ, was obtained using an Elga Maxima water purifying system. Aristar grade Indium atomic spectroscopy standard was purchased from VWR International (Poole, Dorset, UK).

2.2.2 Membrane Desolvation

A major limitation of HPLC-ICP-MS is the low tolerance of the plasma for organic solvents or dissolved salts used in HPLC mobile phases. The ICP is intolerant to large amounts of either of these, so mobile phase selection must be carefully considered. The most critical consideration when coupling ICP-MS to HPLC is to maximise analyte transport but minimise solvent transport to the plasma.

The underlying problem is that organic solvents have a higher vapour pressure than water which leads to an increased solvent loading on the plasma[93]. This reduces the stability of the plasma and also causes a change in the capacitance of the plasma resulting in a higher reflected power[94], which results in the shut down of the power supply. Further problems are encountered with gradient elution[95]. As the eluent composition changes (to an increased organic content in reversed phase chromatography) the nature of the plasma changes, meaning that the ion lens settings and nebuliser gas flows that gave an optimal signal at the start of a gradient may not be optimal at the end of an elution gradient. This results in varying sensitivity over a chromatographic run leading to sloping baselines, calibration and quantification problems.

From previous studies in our laboratory by Cartwright *et al.*[98] the most promising approach to remove the solvent vapour was to use a membrane desolvation device. A membrane desolvator used at the interface between the spray chamber and torch may be used to remove close to 100% of organic solvent39. Within a membrane desolvator, the sample vapour enters a heated membrane and a counter-current flow of sweep gas (usually argon) is used to remove solvent vapours that diffuse across the membrane. The theory is that the solvent molecules are so small that they diffuse through the membrane, whereas the larger analyte molecules are too large and are not removed by the membrane. The removal of the solvent vapour, and thus the sensitivity of the instrument, is dependent on optimising the sweep gas flow.

2.2.3 *Instrumentation*

All LC analyses were performed using the Luna C18 (2) column (Phenomenex, Macclesfield, UK) for reversed phase LC-UV/vis, LC-ICP-MS and LC-ESI-MS-MS analysis. The mobile phase comprised of a binary system of: eluent A, water containing 0.05% formic acid (v/v); and eluent B, acetonitrile containing 0.05% (v/v) formic acid. The linear gradient employed started at 100% A, changing to 67% A and 33% B over 20 minutes. The flow rate was 1 ml min^{-1} with an injection volume of 25 µL.

The LC system used for the UV/vis and UV/vis-ICP-MS analysis was a HP 1090 liquid chromatograph (Agilent Technologies, Stockport, UK). Element specific detection was performed using quadrupole ICP-MS (VG PlasmaQuad 3, Thermo Elemental, Winsford, UK). The ICP-MS sample introduction system consisted of a 1.0 ml min^{-1} microMist nebuliser and a cyclonic spray chamber both purchased from Glass Expansion, AUS. The LC outlet was connected to the ICP-MS nebuliser via 2 m of Teflon tubing (1/16" x ID 0.18mm). A membrane desolvator (Vestec Corporation, Texas, USA) was connected between the spray chamber (Nebuliser) and torch to remove organic solvents. The dry aerosol exiting the desolvator was then transferred to the ICP torch via 1 m of 0.25 in. i.d. Tygon tubing. Operating and data acquisition conditions are shown in Table 2-1.

LC-UV/vis-ESI-MS analysis was performed by using a Waters 2695 Separation Module and Waters 2487 dual wavelength absorbance detector. ESI-MS was achieved in positive ion mode using a Micromass Quattro Ultima Pt. The LC outlet was connected to the ESI-MS and UV/vis detector by a 30 cm length of PEEK

tubing (1/16" x ID 0.25mm) connected in line with a flow splitter (Upchurch Scientific, Washington, USA), the flow of 1 ml min^{-1} from the LC column was split at a ratio of 1:9, where 0.1 ml min^{-1} was sent to the electrospray ionisation source and 0.9 ml min^{-1} to the UV/vis detector. Typical ESI-MS and ESI-MS-MS conditions are given in Table 2-2.

Table 2-1 Operating and data acquisition conditions for Quadrupole ICP-MS.

ICP	
Nebuliser gas flow / L min^{-1}	1.2
Auxiliary gas flow / L min^{-1}	0.8
Coolant gas flow / L min^{-1}	14.0
RF forward power / W	1350
Nebuliser	1.0 ml min^{-1} Micromist
Spray chamber	Jacketed quartz cyclonic (room temperature)
Torch	Quartz fassel-type
Interface	
Sampling cone	Nickel, 1mm i.d.
Skimmer cone	Nickel, 0.7mm i.d.
Time Resolved Analysis	
Masses monitored	^{115}In
Dwell time / ms	10
Channels per mass	5
Membrane Desolvation	
Sweep gas	Argon
Desolvation gas flow / L min^{-1}	1.0
Desolvation temperature / °C	80.0

Table 2-2 Operating conditions for ESI-MS and ESI-MS/MS.

ESI-MS Potentials	
Capillary voltage / kV	3.5
Cone voltage / V	0.8
RF lens 1 / V	100
Aperture / eV	0,5
RF lens 2 / V	1.0
Multiplier / V	650
MS-MS Potentials	
Low mass resolution 1	19.0
High mass resolution 1	17.0
Ion energy / V	1.0
Collision cell entrance / V	80.0
Collision cell	Argon 1bar
Collision gradient	0.0
Collision cell exit / V	50.0
Low mass resolution 2	15.0
High mass resolution 2	15.0
Ion energy / V	1.0
Gas	
Desolvation gas flow / Lhr^{-1}	452
Cone gas flow / Lhr^{-1}	40.0
Temperatures	
Source temperature / °C	80.0
Desolvation temperature / °C	150.0

2.2.4 Preparation of Solutions

All buffer solutions were prepared gravimetrically, stored at room temperature and discarded a month after preparation. 0.1 M solutions of sodium bicarbonate and ammonium bicarbonate were adjusted to pH 8 using hydrochloric acid and 0.1 M solutions of sodium acetate and ammonium acetate were adjusted to pH 6 using acetic acid. pH was measured using a calibrated Jenway 3010 pH meter (Jenway, UK). Molecular sieves (Sigma Aldrich, Gillingham, Dorset) were used to prepare the anhydrous DMSO. All peptide stock solutions were prepared in sodium bicarbonate pH 8. The cDTPA was prepared in anhydrous DMSO so no inadvertent hydrolysis of cDTPA would occur.

2.2.5 Derivatisation and Chelation with In^{3+}

Initial attempts to derivatise amines using the method of Marshall *et al.*[89] were unsuccessful, the method required the addition of 800 µL of sodium bicarbonate (100 mM) to 100 µL of naphthylamine (1 mM) and 100 µL of cDTPA (1 mM). The solution was then left to react at room temperature for 60 minutes after mixing. A possible explanation for the unsuccessful derivatisation could be due to the reaction proceeding in a highly aqueous environment, which would favour the hydrolysis of the anhydride over the acylation reaction of the anhydride.

Subsequently, the original method for the reaction of cDTPA with proteins by Hnatowich *et al.*[93] was modified and applied to peptide derivatisation. The modified method required the addition of 0.2 g of cDTPA (4.6 mM) in anhydrous DMSO to 0.2 g of peptide (2.3 mM), finally 0.1 g of sodium bicarbonate solution (100 mM) adjusted to pH 8 was added, the solution was left to stand after mixing for 60

minutes at room temperature. Derivatised peptides were chelated with indium; the indium stock solution was prepared in 0.1 M sodium acetate at pH 6. For ICP-MS analysis, 0.1 g of the derivatised peptide sample was added to 0.1 g of indium (3.6 mM) then a further 0.8 g of 0.1 M sodium acetate pH 6 was added and the solution was agitated and left at room temperature for 30 minutes. The sample was further diluted 100 fold prior to analysis.

2.2.6 Optimisation of Derivatisation Reaction

Factors that were considered when optimising the derivatisation reaction were: reaction time; molar ratio of cDTPA to peptide; and stability of the cDTPA reagent, while factors such as room temperature were not considered as the acylation and hydrolysis of anhydrides proceeds from 4°C to 37°C to completion[110]. In addition, buffer and pH conditions were selected according to Paik *et al.*[97], who concluded that the acylation reaction of the anhydrides is buffer and pH dependent, and suggested that derivatisation was more efficient at pH 8.2 using a 0.1 M bicarbonate buffer. The chromatographic separation time was reduced, by increasing the gradient elution to 100% eluent B over 10 minutes to allow for high throughput. UV/vis detection of 220nm was selected as this seemed to offer much more sensitive detection and small changes in derivatisation can be quantified.

Reaction times of 0 minutes to 90 minutes were selected as this was considered the time required for the reaction to progress to completion and adequate time for the hydrolysis of unreacted anhydride. Molar ratio of reagents is considered one of the most important factors in analytical studies as excess reagents have to removed or inactivated as not to interfere with analysis, conversely excess

reagents are typically required to improve derivatisation efficiencies. The stability of the cDTPA reagent was also observed due to the highly reactive nature of anhydrides in atmospheric and aqueous solutions.

2.2.7 Mass Spectrometry Studies

2.2.7.1 ESI-MS

Soft ionisation methods such as ESI-MS have been at the forefront of proteomic research for the analysis of intact chemical species and for structural information. ESI-MS can be employed in several modes to gather various different information from a single sample, which can either be inserted directly into the ionisation source or can undergo some type of chromatography prior to the ionisation source.

Scanning ESI-MS was used to confirm the masses of reaction products. The mass scan spectrum was obtained by ramping the amplitude of the direct current (dc) and radio frequency (rf) voltages while keeping rf/dc ratio constant over a required mass range. The sensitivity of scanning ESI-MS is therefore determined by the mass range, scan speed and resolution.

Multiple reaction monitoring (MRM) ESI-MS or tandem MS, was used to generate structural information. MRM-ESI-MS is a highly specific method with very high sensitivity due to fragmentation being known prior to analysis. The mass analyser of a triple quadrupole instrument consists of two quadrupoles separated by a collision cell. The first quadrupole is used to select a first ion (precursor) which is fragmented in the collision cell. This collision induced dissociation (CID) is

achieved by accelerating the ions in the presence of a collision gas such as argon or helium. The collision energy of the gas can be varied to allow different degrees of fragmentation. The resulting fragments (daughter ions) are then analysed by the second quadrupole. Typically ESI-MS-MS both quadrupoles are operated in static mode to increase sensitivity. Molecular masses to be monitored during MRM were determined by positive ion mode infusion ESI-MS-MS, where the derivatised sample was infused at 0.1 ml min^{-1}.

LC hyphenated with ESI-MS and ESI-MS/MS was used to characterise the products of the cDTPA derivatisation reaction. Derivatisation for ICP-MS was performed using sodium bicarbonate buffer, however, this was changed to ammonium bicarbonate for ESI-MS to prevent sodium adducts forming during the ionisation process and to improve ionisation of the sample in the electrospray source.

2.2.7.2 LC-ICP-MS

Gradient LC-ICP-MS was used to determine whether the chelation of cDTPA with In^{3+} was successful. Research by Cartwright *et al.*[98] showed that 100% acetonitrile gradient elution can be run in conjunction with ICP-MS detection without having a detrimental effect on the plasma and signal by using a membrane desolvator. In addition, membrane desolvation has the advantage of removing many polyatomic ion interferences thereby enabling selective detection of elements such as phosphorus using quadrupole ICP-MS.

2.3 Results and Discussion

2.3.1 Derivatisation of Peptides with cDTPA

Bradykinin, a nine amino acid peptide chain was used has a principle test peptide, because the peptide contains no readily detectable heteroatom by ICP-MS, its physiological important role in vasodilation especially with contraction of non-vascular smooth muscle, increase vascular permeability and associated effects in pain mechanisms. The amino acid sequence is Arg - Pro - Pro - Gly - Phe - Ser - Pro - Phe - Arg and molecular weight of 1060.21 with an empirical formula $C_{50}H_{73}N_{15}O_{11}$.

In order to test the efficacy of the derivatisation of bradykinin with cDTPA, preliminary studies were performed using LC-UV/Vis detection at 254 nm. Chromatograms of underivatised and derivatised peptide are shown in Figure 2-3. As can be seen, the underivatised bradykinin eluted at 9.8 minutes whereas two peaks, corresponding to the derivatised peptide, eluted at 14.6 and 14.8 minutes respectively. The presence of two separate peaks for the derivatised bradykinin may possibly be explained by the fact that the cDTPA reagent contains two functional groups; hence it is possible that the derivatisation reaction yielded more than one product, though confirmation using ESI-MS would be required to confirm the hypothesis.

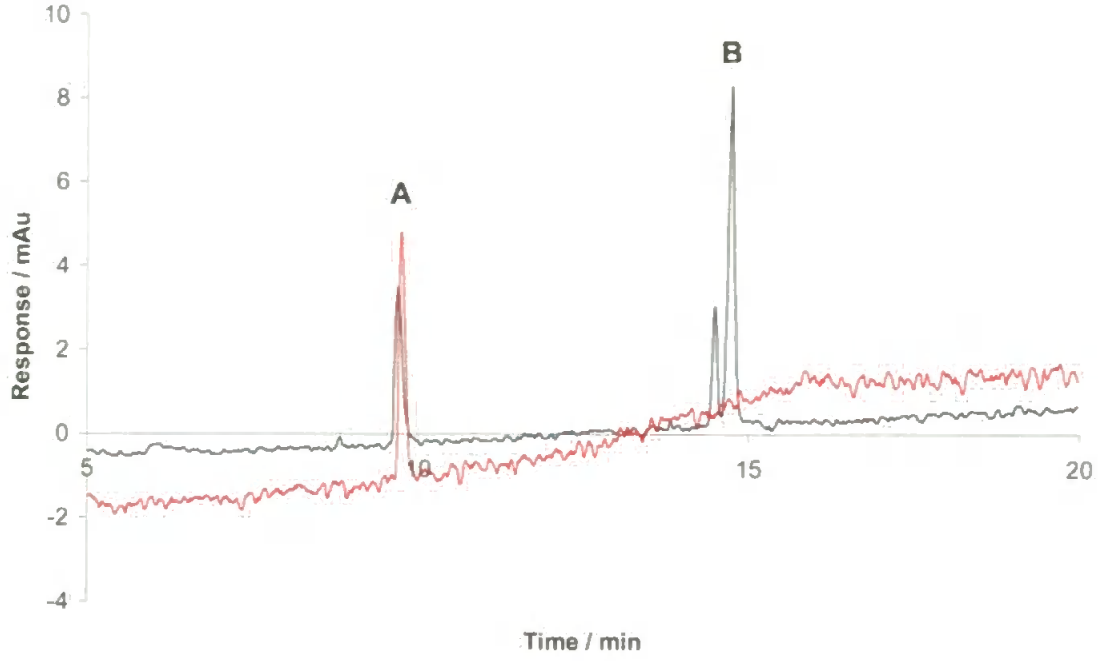

Figure 2-3 UV/vis Chromatograms at 254nm of bradykinin (red) and cDTPA derivatised bradykinin (black) using gradient elution: A, bradykinin; B, cDTPA-bradykinin.

2.3.2 Optimisation of Derivatisation Reaction

The effect of reaction time on the efficiency of derivatisation is shown in Figure 2-4a. It is apparent that there was no significant difference in reaction efficiency regardless of the length of time after the initial derivatisation that the samples were analysed. This data is summarised in Table 2-3, which shows that the reaction efficiency was > 94% over a period of 16 hours for the derivatisation reaction when the cDTPA reagent was dissolved. However, stability of the reagent did have a significant effect on the reaction efficiency (Figure 2-4b, Figure 2-4c, Table 2-3), with ~ 87% of bradykinin being derivatised when the cDTPA reagent was 2 hrs. old and falling to between 29 - 49% when using 4 hr. old cDTPA reagent. This is probably because the cDTPA, which is susceptible to hydrolysis, was hydrolysed by residual water in the 'dry' DMSO. It is apparent from Table 2-3 that provided derivatisation was performed immediately after preparation of the reagent, it was not necessary to increase the reaction time past 30 minutes

Hence, the reaction time was reduced to 15 min (to allow for the hydrolysis of the unreacted cDTPA) and additional molecular sieves were used to 'dry' the DMSO. These improvements were implemented prior to optimisation of the molar ratio of cDTPA to bradykinin. The effect of the ratio of cDTPA to bradykinin is shown in Figure 2-5. The efficiency of the derivatisation reaction was observed to increase from 70% to >99% when the cDTPA:bradykinin molar ratios was increased from 1:1, 2:1 and 5:1 respectively. The efficiency of derivatisation was calculated from integrated peak areas of bradykinin remaining after derivatisation compared to a bradykinin standard using HP chemstation software. Bradykinin and cDTPA-bradykinin was identified as peak A and B respectively based on retention times. The change in retention time of the bradykinin peak in the 2:1 (red trace) sample

could be either due to the reproducibility of the manual injection or due to the amount of underivatised bradykinin associated with the sample respectively.

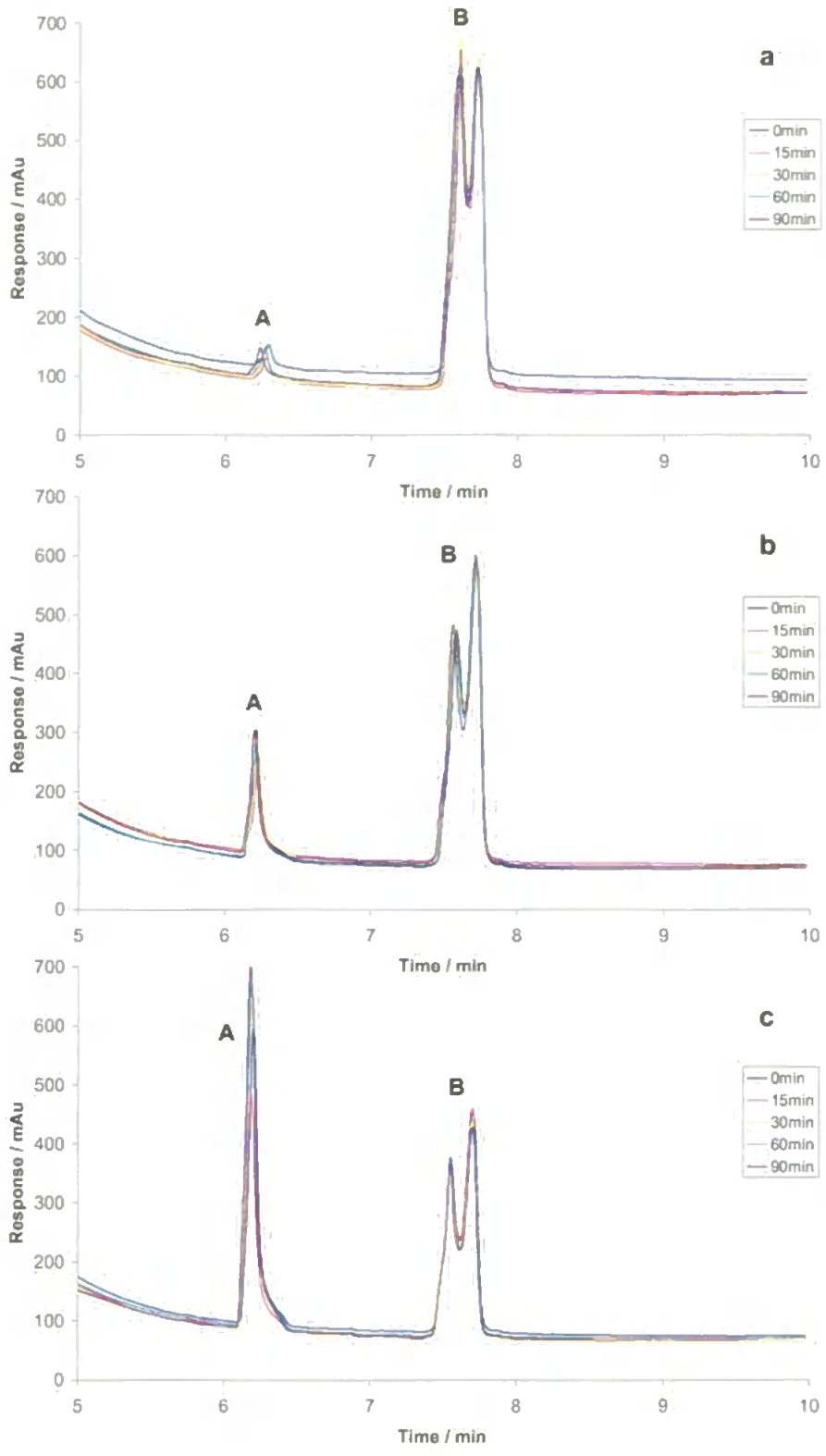

Figure 2-4 Chromatograms (UV/Vis at 220nm) of bradykinin derivatised with cDTPA obtained at intervals after the initial derivatisation and reagents prepared: a, 0 hrs; b, 2 hrs; c, 4 hrs prior to the derivatisation. Peaks identified as: A, bradykinin; and B, cDTPA-bradykinin

Table 2-3 Percentage peak area data of derivatised bradykinin remaining after optimisation of reaction time and stability of cDTPA reagent.

Injection time after derivatisation / min	Percentage of bradykinin derivatised after sample preparation		
	0 hrs	2 hrs	4 hrs
0	96.4	86.6	39.1
15	96.5	89.3	29.6
30	96.3	86.7	45.9
60	96.4	87.6	48.7
90	96.3	87.3	48.3
16 hrs	94.8	87.5	44.0

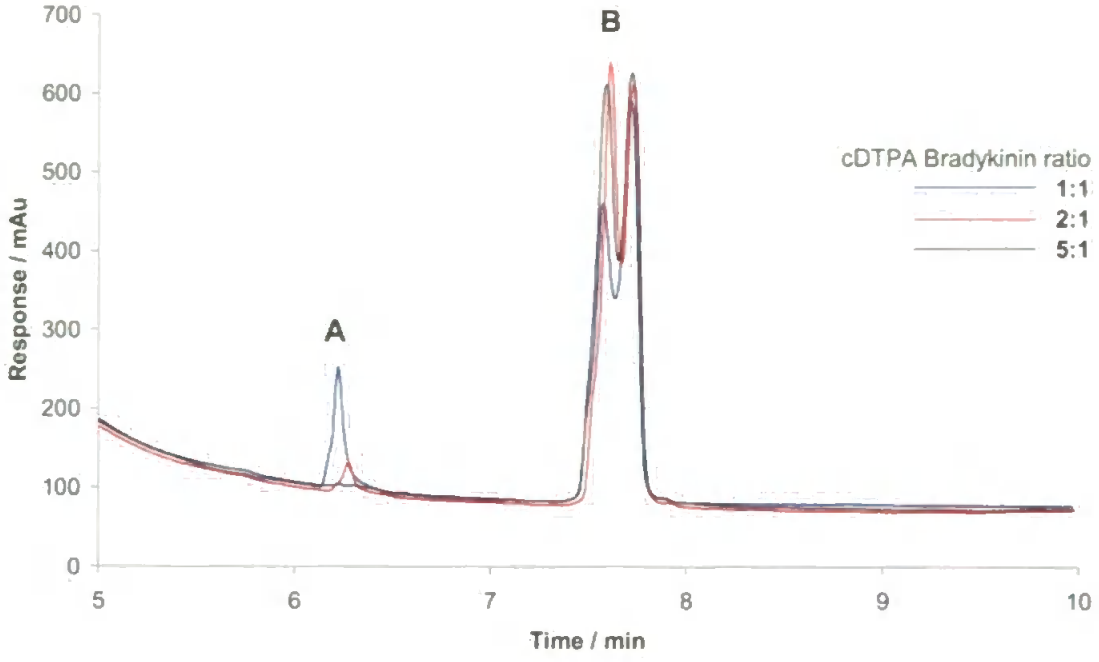

Figure 2-5 UV/vis chromatogram at 220nm of cDTPA derivatised bradykinin at molar ratios of 1:1, 2:1 and 5:1 of increasing cDTPA concentration respectively. Peaks identified as; A, bradykinin; and B, cDTPA-bradykinin.

2.3.3 Mass Spectrometry Studies

The infusion mass spectrum of the cDTPA derivatised bradykinin solution is shown in Figure 2-6, the sample was prepared using ammonium bicarbonate as the buffer in order to increase ionisation efficiency of the electrospray source. The ion lenses were tuned to m/z 1436 because this was the m/z of the cDTPA derivatised bradykinin, and the mass range was limited to 100-1500 m/z to increase the sensitivity because only products of derivatisation within this range were expected.

2.3.3.1 Peptide Fragmentation

A notation has been developed for indicating peptide fragments that arise from a tandem mass spectrum (Figure 2-6). Peptide fragment ions are indicated by a, b, or c if the charge is retained on the N-terminus and by x, y or z if the charge is maintained on the C-terminus. The subscript indicates the number of amino acid residues in the fragment.

Fragmentation of bradykinin typically involves the loss of either the N-terminal or C-terminal arginine amino acid depending on the energy of collisions in the second quadrupole respectively. The collision energies were typical set to remove the C-terminal arginine residue and this was additionally confirmed by the fragmentation of the arginine residue attached to the C-terminal during MRM studies as the N-terminal attached cDTPA was not fragmented (Figure 2-8d).

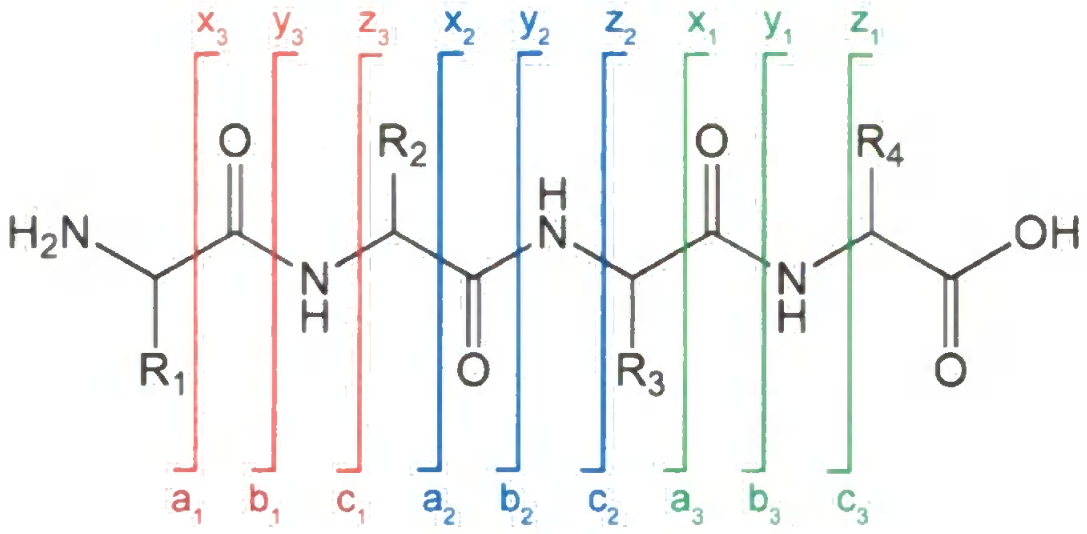

Figure 2-6 Peptide fragmentation notation using the scheme of Roepstorff and Fohlman[99].

The mass spectrum shows that bradykinin-cDTPA and bradykinin were present along with fragments, formed during the ionisation process, of both the bradykinin-cDTPA and/or bradykinin. To confirm the origin of the fragments it would be necessary to isolate the bradykinin-cDTPA complex from the unreacted components of the derivatisation process. However, it is probable that m/z 1280 originated from $[bradykinin\text{-}cDTPA]^+$ minus an arginine residue and m/z 905 formed from either $[bradykinin\text{-}cDTPA]^+$ minus a cDTPA-arginine or $[bradykinin]^+$ minus an arginine residue. There was no evidence in the mass spectrum that cDTPA formed a conjugate with two bradykinin moieties, as what seemed to be the case from Figure 2-3. Further investigations were performed using LC-ESI-MS-MS to determine whether two bradykinins are derivatised by a single cDTPA reagent, with results shown in Figure 2-7.

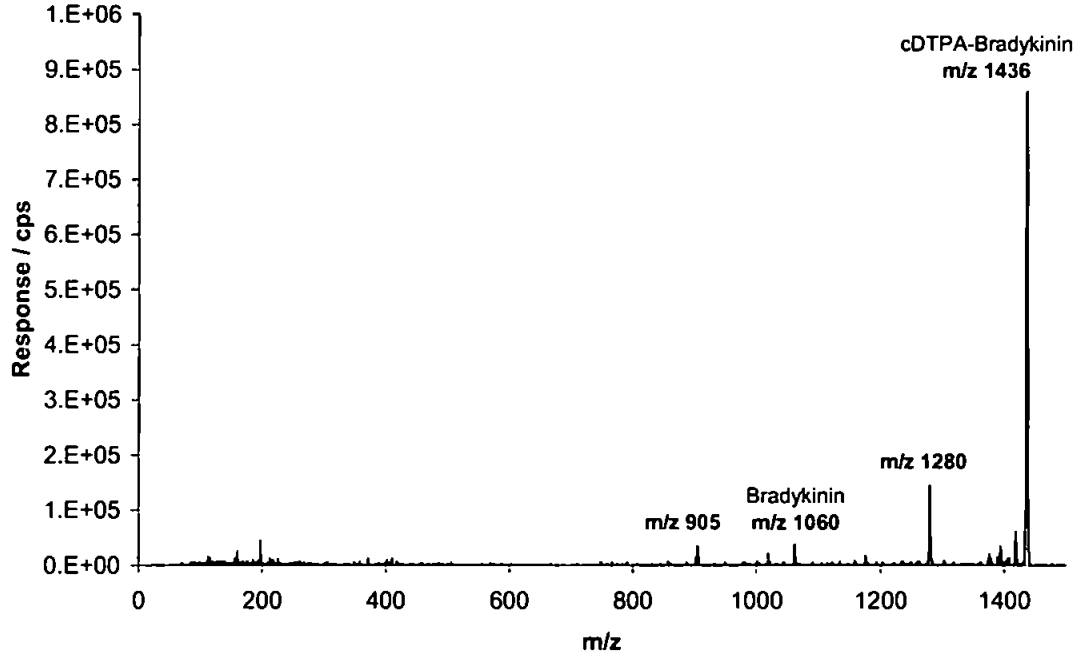

Figure 2-7 Infusion molecular mass spectrum of cDTPA derivatised bradykinin in ammonium based buffers using positive ion mode ESI-MS (scan range m/z 100-1500).

Figure 2-8a shows the UV/vis chromatogram for derivatised bradykinin. The bradykinin-cDTPA complex eluted in two peaks at 13.5 and 14.8 minutes, peaks B and C respectively, while the underivatised bradykinin eluted at 10.3 minutes peaks A. These peaks were confirmed by analysis using ESI-MS (Figure 2-8b) as well as retention times of the respective bradykinin standard. In order to identify the peaks eluting at 13.5 and 14.8 minutes ESI-MS was used in MRM mode to monitor the transitions 1061>905 (Figure 2-8c) and 1436>1280 (Figure 2-8d). These MRM transitions were monitored because they were fragmented during infusion ESI-MS and are associated with the loss of arginine residue from bradykinin and bradykinin-cDTPA respectively. This confirmed the presence of a single peak due to bradykinin at 10.3 minutes and two peaks due to bradykinin-cDTPA at 13.5 and 14.8 minutes.

The formation of the extra peak during derivatisation in ammonium bicarbonate compared to the sodium bicarbonate buffer could be explained by the buffering environment containing extra NH_4^+ ions which could react/bind to the cDTPA molecule. In addition the peak splitting seen in Figure 2-4a, may have been caused by column degradation due to high concentrations of cDTPA and trivalent metal ions over a period of time.

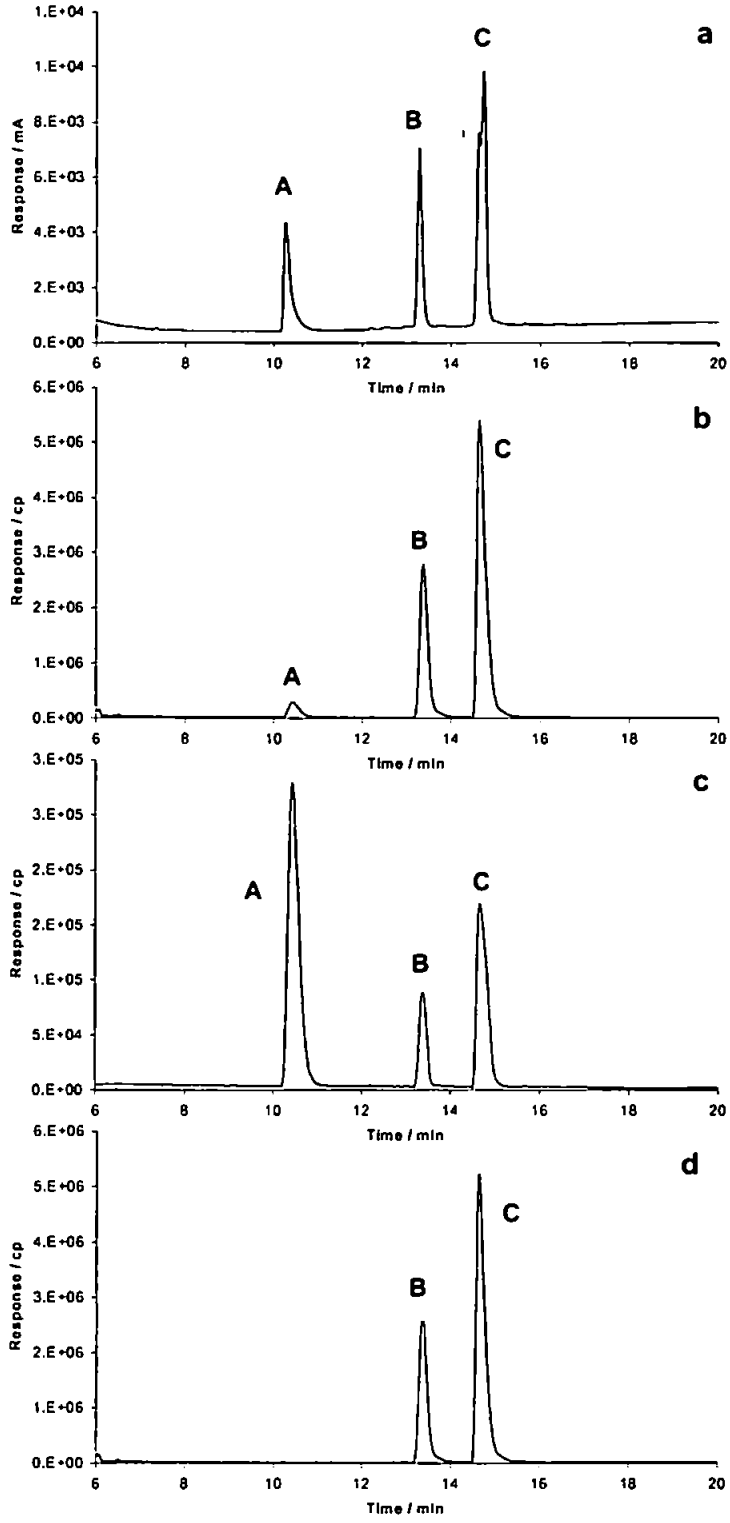

Figure 2-8 Chromatograms of derivatised bradykinin in ammonium bicarbonate buffer: A, UV-vis chromatogram at 254nm; B, Total ion count using positive ion mode ESI-MS; C, MRM transition 1061>905 using positive ion mode ESI-MS-MS; D, MRM transition 1436>1280 using positive ion mode ESI-MS-MS. Peaks identified as: A, bradykinin; B and C, cDTPA-bradykinin.

2.3.4 *Elemental Mass Spectrometry Studies*

Figure 2-9 shows a chromatogram of derivatised bradykinin with detection using ICP-MS at m/z 115. The chromatogram shows three resolved peaks which were A, excess In^{3+} from the chelation step; B, excess cDTPA from derivatisation step chelated with In^{3+}; and C, bradykinin tagged with In^{3+}. The bradykinin-cDTPA-In complex was detectable at a concentration of 2.3 μM of bradykinin, corresponding to an indium concentration of 260 ng ml^{-1}. The chromatogram showed the derivatisation and chelation at a 1:2:4 molar ratio of bradykinin: cDTPA: indium respectively. Increasing the amount of excess cDTPA improved the derivatisation efficiency, however, chromatographic resolution between cDTPA-indium and indium tagged bradykinin peaks was reduced.

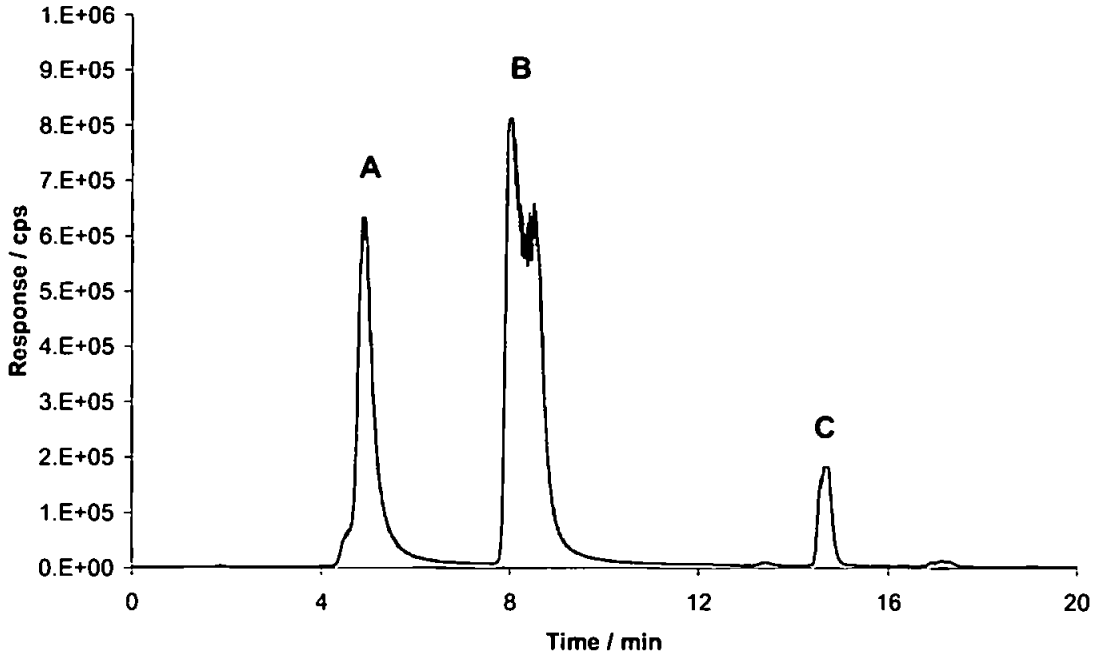

Figure 2-9 Chromatogram of derivatised bradykinin chelated with indium using ICP-MS detection at m/z 115. A, uncomplexed In^{3+};B, cDTPA-In; C, bradykinin-cDTPA-In.

Conclusions

LC-ICP-MS has been used for the detection of peptides after derivatisation with a bifunctional chelating agent and subsequent chelation with In^{3+} metal. This allowed element specific detection to be performed on peptides, which are typically not amenable for detection by ICP-MS. The derivatisation reaction was optimised to less than 30 min, with a derivatisation efficiency of ~95% at a molar ratio of 2:1 cDTPA to bradykinin. However, there is scope for improvement such as the removal of unreacted reagents prior to analysis, the use in complex samples, which are both considered in chapter 4, and improvement in sensitivity of the technique. LC-ESI-MS/MS was used for the characterisation of the derivatisation reaction using multiple reaction monitoring, which suggested that only single derivatised cDTPA had been formed and not a multiple derivatised cDTPA. However, switching buffers from sodium to ammonium bicarbonate resulted in an additional bradykinin-cDTPA conformer which complicated identification. Current work is progressing to show that the relative quantitation of peptides is achievable by LC-ICP-MS using a modified form of the isotope dilution equation.

CHAPTER 3 RELATIVE QUANTITATION OF PEPTIDES BY ISOTOPIC LABELLING

3. INTRODUCTION

The relative quantitation of peptides by molecular mass spectrometry has been at the forefront of proteomic research, however, one problem is the accuracy and precision of the measured peptide ratio. Gygi *et al.*[54] quoted "The percentage error of the experimental technique was found to be excellent (±10%)." when commenting on the accuracy of the relative quantitation obtained by the metabolic labelling technique[15]. The error arises not from the labelling strategy but from the method of detection, and it is well known that labelling strategies which use molecular mass spectrometry struggle to differentiate between small differences in peptide expression.

In this chapter, we describe the relative quantitation of differentially isotopically labelled peptides using ICP-MS detection. Gravimetrically prepared peptide samples were derivatised with cyclic diethylenetriaminepentaacetic acid anhydride and chelated with either natural or enriched europium. The $^{153}Eu/^{151}Eu$ isotope ratio was measured using LC-ICP-MS and this was used to calculate relative peptide ratios. LC-ESI-MS-MS was used to confirm europium chelation and to compare relative quantitation between organic and inorganic MS.

3.1 Relative Quantitation by ICP-MS

The approach used for relative quantitation using europium labelling is shown schematically in Figure 3-1. The strategy is based on relative quantitation techniques described in Chapter 1. Two peptide samples, of a standard sample (n_x) and an unknown sample (n_y) were derivatised with cDTPA and chelated with either natural Eu or enriched Eu respectively. Equal amounts of the labelled peptides were then combined and the resulting europium ratio (R_b) can be determined from the LC-ICP-MS chromatogram.

3.1.1 Theory

Consider the situation where two separate solutions, one containing a known amount of peptide labelled with natural Eu (n_x) is mixed with another containing a known amount of peptide labelled with enriched ^{151}Eu (n_y). Equation 3-1 shows how the isotope amount ratio (R) of $^{153}Eu/^{151}Eu$ in the resultant solution can be expressed.

Equation 3-1 $$R = \frac{\text{Moles of } ^{153}\text{Eu}}{\text{Moles of } ^{151}\text{Eu}}$$

This is the basis for the isotope dilution equation that can be further expanded to the generic form shown in Equation 3-2.

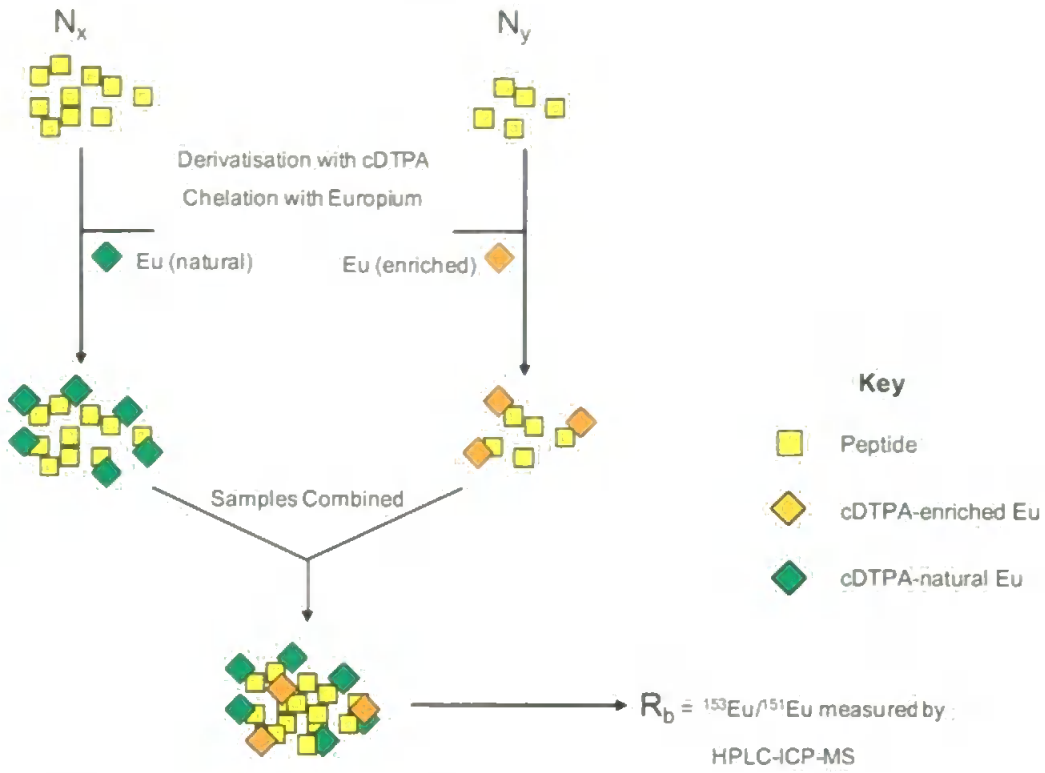

Figure 3-1 A schematic representation of the tagging of peptides for the relative quantitation by LC-ICP-MS.

Equation 3-2 $$R = \frac{(n_x A_x) + (n_y A_y)}{(n_x B_x) + (n_y B_y)}$$

Where: n_x is the amount of peptide labelled with natural Europium; n_y is the amount of peptide labelled with enriched ^{151}Eu; A_x is the atom fraction of natural ^{153}Eu; B_x is the atom fraction of natural ^{151}Eu; A_y is the atom fraction of enriched ^{153}Eu; B_y is the atom fraction of enriched ^{151}Eu. The equation can be rearranged to Equation 3-3 and the relative amount of peptide in the two samples can be determined.

Equation 3-3 $$\frac{n_x}{n_y} = \frac{(A_y - RB_y)}{(RB_x - A_x)}$$

3.2 Isotope Dilution Mass Spectrometry for Speciation

The measurement of stable isotope ratios has been used for a wide variety of applications, including geochronology, food authenticity, isotope tracer studies, climate analysis and many others. Isotope dilution mass spectrometry (IDMS) is a quantitative technique used to determine the amount fractions of an element in a sample and can be applied to all elements with two or more isotopes. This is accomplished by the addition of an isotopically enriched spike containing an accurately known amount, with known atom fractions, of the element to be determined in the sample. The technique has been extensively reviewed with advantages and disadvantages discussed by Heumann *et al.*[100].

IDMS has a number of advantages over traditional external calibration techniques, provided that the following pre-requisites are met[101]:

- more than one interference-free, stable isotope must be available for isotope ratio measurement;
- an isotopically enriched analogue, or spike, of the analyte must be available;
- complete equilibration between the spike and sample must be achieved;
- the mass fraction and isotopic abundances of the natural and enriched elements must be well characterised;
- the spike and sample must be chemically stable.

3.2.1 Calculation of Isotope Amount Ratio

A recent review by Clough *et al.*[102] discusses how IDMS has been applied to speciation studies by LC-ICP-MS, focussing on measurement parameters, data extraction and minimising uncertainty. Two approaches can be used to determine the isotope amount ratios resulting from two transient signals from an LC-ICP-MS chromatogram, namely peak integration and pseudo steady state.

3.2.1.1 Peak Integration

Both isotope specific peaks for the species of interest in the LC-ICP-MS chromatogram are integrated; from these integrals the isotope amount ratio for that species can be determined. An advantage of using this approach is that the effect of spectral skew is minimised, (Figure 3-2) however, precision is lost because multiple injections are required to obtain a standard deviation on the measured ratio.

3.2.1.2 Pseudo Steady-State

The pseudo steady state approach for isotope amount ratio measurements assumes that the chromatogram is an undulating signal and that at the apex of the peak a pseudo steady state is achieved (Figure 3-3). In order to achieve the best precision and accuracy, only data-points during the pseudo steady state of each species (peak) for each isotope of a particular species in the chromatogram, are used and baseline signal subtracted. The isotope amount ratio is calculated using each corresponding data-point from the two peaks and subsequently corrected for mass bias effects. The advantage of this approach is that the precision inherent in making isotope ratio measurements using a sequential or simultaneous mass spectrometer is maintained, and it is possible to obtain an estimate of precision from a single peak. However, spectral skew must be minimised in order to obtain an accurate isotope amount ratios.

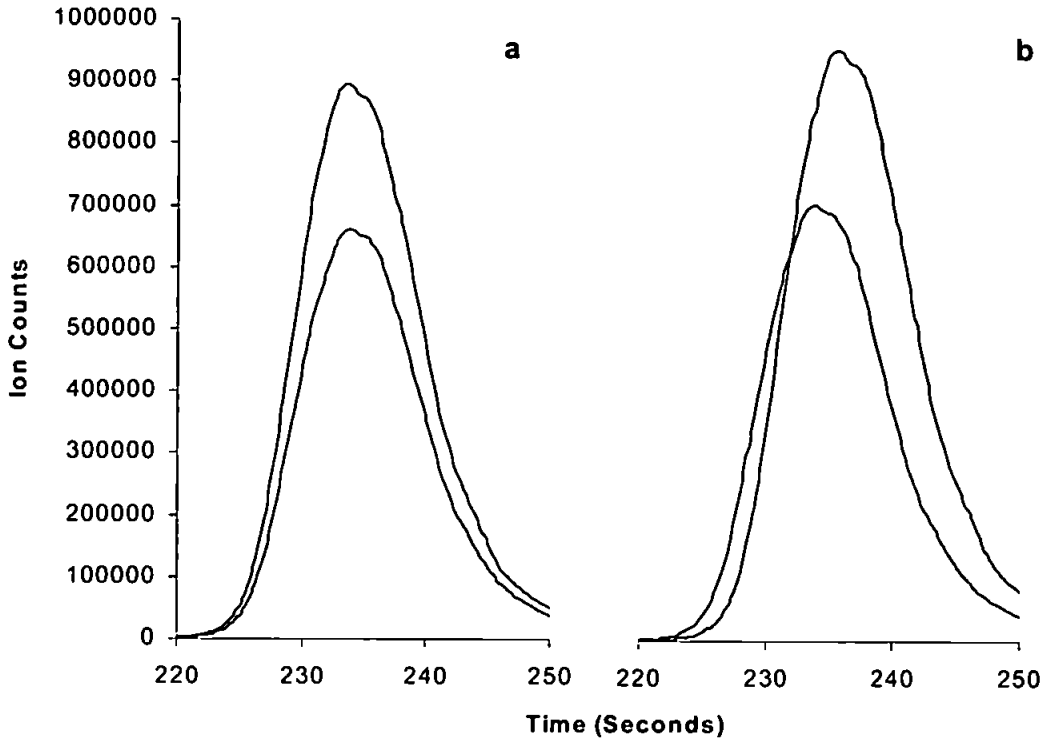

Figure 3-2 Schematic illustrating spectral skew for ^{153}Eu and ^{151}Eu isotopes signals obtained by FI-ICP-MS: A, no spectral skew; B, minor isotopes signal shifted by 2 seconds.

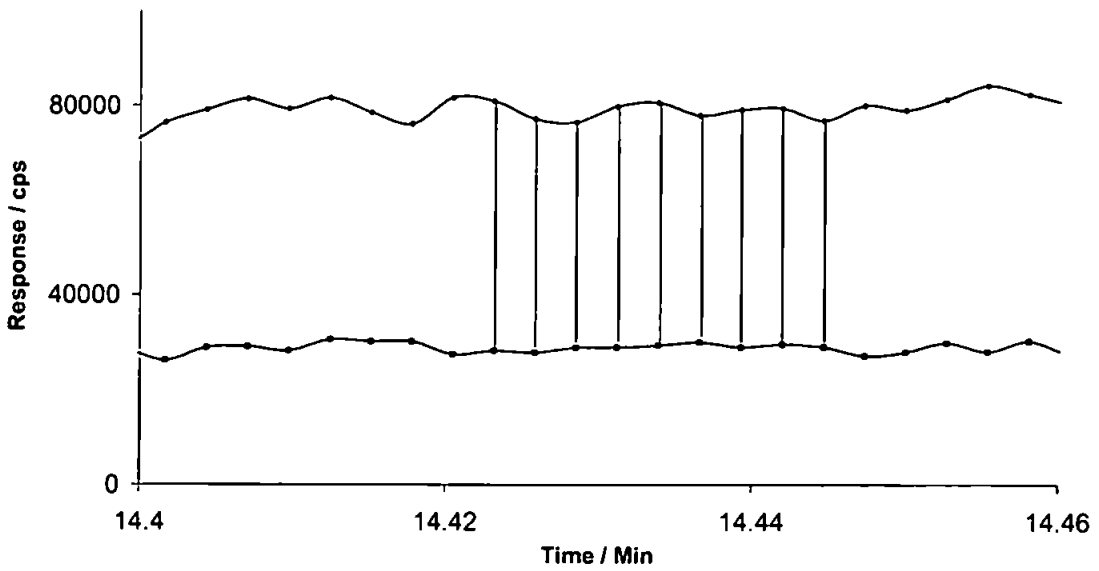

Figure 3-3 Isotope amount ratios measured using the pseudo steady state approach. The isotope amount ratio is calculated by ratioing the ion signals for coincident pairs of data points over the peak maximum.

3.2.2 *Mass Bias Correction*

In order to obtain accurate isotope (and hence peptide) ratios it is necessary to compensate for mass bias in the instrumentation. Quadrupole and sector field ICP-MS instruments transmit ions of different mass with different efficiencies predominantly due to space charge effects in the ion source and interface region[103] resulting in a non uniform response and inaccurate isotope ratio measurements. In other words, if an elemental solution composed of two isotopes with an exactly 1:1 molar ratio is analysed using ICP-MS, an isotope amount ratio of 1:1 will not necessarily be observed. Mass bias depends upon mass and the type of mass spectrometer used, but generally tends to be greatest at low mass and decreases with increasing mass. Mass bias may have deleterious effects on the accuracy of isotope amount ratio determinations, so a correction must always be made, either by bracketing the sample with a certified reference material, or by interpolation from the mass bias observed for an isotope pair of similar mass to the isotope pair under study[102].

The most convenient method for mass bias correction in speciation analysis using LC-ICP-MS is to spike the mobile phase with an internal standard or to add the internal standard post column and to monitor it continuously. Mass bias of the ^{153}Eu:^{151}Eu isotope amount ratio was corrected by interpolation of the mass bias observed for the ^{154}Sm:^{152}Sm isotope amount ratio using the Russell correction expression[104] shown in Equation 3-4, which corrects for mass bias on the basis of the absolute mass of the isotope pairs. The addition of the samarium internal standard was achieved by adding the solution post column via a low dead volume t-piece. The advantage of this approach is that the mass bias correction can be

performed by spiking the sample on-line with the correction standard and measuring this isotope amount ratio at the same time as the sample. Europium is an ideal element for isotope ratio measurements because it is only has two isotopes which are free from polyatomic and isobaric interferences, and mass bias corrections can easily be made using a samarium standard.

$$\left(\frac{^{153}Eu}{^{151}Eu}\right)_{cor} = \frac{\left(\frac{^{153}Eu}{^{151}Eu}\right)_{mes}}{\left(\left(\frac{^{154}Sm}{^{152}Sm}\right)_{mes} \Big/ \left(\frac{^{154}Sm}{^{152}Sm}\right)_{cer}\right)^{\left(\ln\left(\frac{RAM^{153}Eu}{RAM^{151}Eu}\right) \Big/ \ln\left(\frac{RAM^{154}Sm}{RAM^{152}Sm}\right)\right)}}$$

Equation 3-4: The Russell correction expression used for mass bias correction of the europium isotope ratio.

Where: *cor* is the corrected isotope amount ratio, *mes* is the measured isotope amount ratio; *cer* is the certified isotope amount ratio and RAM is the relative atomic mass.

3.3 Experimental

The methods in the following section describe europium labelling of cDTPA derivatised peptides for relative quantitation. In addition, the methodology was optimised for isotope ratio measurements, as well as, the characterisation of the europium chelated peptides using ESI-MS and ESI-MS/MS.

3.3.1 Reagents and Standards

Bradykinin acetate, substance P, cyclic diethylenetriaminepentaacetic anhydride (cDTPA), sodium bicarbonate, trifluoroacetic acid (TFA), and sodium acetate were purchased from Sigma Aldrich, (Poole, Dorset, UK). Dimethyl sulfoxide (DMSO), ammonium acetate, nitric acid (trace analysis grade), LC grade acetonitrile and methanol were obtained from Fisher Scientific UK Ltd, (Loughborough, Leicestershire UK). Ammonium bicarbonate was purchased from Fluka, Sigma Aldrich, (Poole, Dorset, UK). Distilled deionised water (DDW) 18.2 MΩ, was obtained using an Elga Maxima water purifying system. ^{151}Eu-enriched europium (Eu_2O_3 99.24% enriched) was purchased from CK Gas Products, (Hampshire, UK). A standard ICP-MS solution of natural europium was purchased from Alfa Aesar (Heysham, UK).

3.3.2 Instrumentation

All LC analyses were performed using the Luna C18 (2) column (Phenomenex, Macclesfield, UK) for reversed phase LC-UV/vis, LC-ICP-MS and LC-ESI-MS analysis. The mobile phase was a binary system of: eluent A, water containing 0.05% formic acid (v/v); and eluent B, acetonitrile containing 0.05% (v/v) formic acid. Two gradient elution profiles were employed: (1) a linear gradient elution,

performed by increasing mobile phase B from 0% to 33% over 20 minutes; (2) a nonlinear gradient elution profile where mobile phase B was increased from 0% to 15% over 5 min and to 18.75% over the next 15 minutes The flow rate was 1 mL min^{-1} with an injection volume of 25 μL. The LC system used for the UV/vis and UV/vis-ICP-MS analysis was a HP 1090 liquid chromatograph (Agilent Technologies, Stockport, UK). Element specific detection was performed using quadrupole ICP-MS (VG PlasmaQuad 3, Thermo Elemental, Winsford, UK). The ICP-MS sample introduction system consisted of a 1.0 mL min^{-1} microMist nebuliser and a cyclonic spray chamber both purchased from Glass Expansion (AUS). A samarium internal standard was added post column through an applied biosystems LC pump (Foster city, USA) via a low dead volume t-piece (Fisher Scientific, Loughborough, UK), which combined the two flows to the nebuliser via a 30 cm PEEK tubing (1/16" x ID 0.18mm). A membrane desolvator (Vestec Corporation, Texas, USA) was connected between the spray chamber (Nebuliser) and torch to remove organic solvents. The dry aerosol exiting the desolvator was then transferred to the ICP torch via 1 m of 0.25 in. i.d. Tygon tubing. Operating and data acquisition conditions are shown in Table 3-1.

Clough *et al.*[105] previously determined the optimised quadrupole ICP-MS data acquisition settings for isotope amount ratio analysis using the pseudo steady state approach. The data acquisition settings were further corroborated by measuring isotope ratios of natural europium samples spiked with known amounts of enriched europium. The resulting isotope ratios, measured by flow injection ICP-MS, were compared to theoretical isotope ratios. Spectral skew was minimised by setting the dwell time to 10 ms, which has the advantage of increasing the number of data points at the apex of the chromatographic peak, for pseudo steady state.

LC-UV/vis-ESI-MS analysis was performed using a Waters 2695 separation module and Waters 2487 dual wavelength absorbance detector. ESI-MS was achieved in positive ion mode using a Micromass Quattro Ultima Pt. The LC outlet was connected to the ESI-MS and UV/vis detector by a 30 cm length of PEEK tubing (1/16" x ID 0.25mm) connected in line with a flow splitter (Upchurch Scientific, Washington, USA), the flow of 1 mL min^{-1} from the LC column was split at a ratio of 1:9, where 0.1 mL min^{-1} was sent to the electrospray ionisation source and 0.9 mL min^{-1} to the UV/vis detector. Typical ESI-MS and ESI-MS-MS conditions are given in Table 3-2.

Table 3-1 Typical ICP-MS operating conditions for isotope ratio measurements.

ICP	
Nebuliser gas flow / L min^{-1}	1.3
Auxiliary gas flow / L min^{-1}	0.8
Coolant gas flow / L min^{-1}	14.0
RF forward power / W	1350
Nebuliser	1.0 ml min^{-1} Micromist
Spray chamber	Jacketed quartz cyclonic (room temperature)
Torch	Quartz fassel-type
Interface	
Sampling cone	Nickel, 1mm i.d.
Skimmer cone	Nickel, 0.7mm i.d.
Time Resolved Analysis	
Masses monitored	^{151}Eu, ^{152}Sm, ^{153}Eu, ^{154}Sm
Data Acquisition	Peak Jump
Dwell time / ms	10
Channels per mass	5
Membrane Desolvation	
Sweep gas	Argon
Desolvation gas flow / L min^{-1}	0.9
Desolvation temperature / °C	80.0

Table 3-2 Typical ESI-MS operating conditions.

ESI-MS Potential	
Capillary voltage / kV	4.0
Cone voltage / V	1.0
RF lens 1 / V	150
Aperture / eV	1.0
RF lens 2 / V	1.0
Multiplier / V	650
MS-MS Potential	
Low mass resolution 1	10.0
High mass resolution 1	10.0
Ion energy / V	2.0
Collision cell entrance / V	80.0
Collision cell	Argon 1bar
Collison gradient	0.0
Collision cell exit / V	70.0
Low mass resolution 2	12.0
High mass resolution 2	12.0
Ion energy / V	1.0
Gas	
Desolvation gas flow / Lhr^{-1}	450
Cone gas flow / Lhr^{-1}	40.0
Temperature	
Source temperature / °C	100
Desolvation temperature / °C	150

3.3.3 Derivatisation and Eu^{3+} Chelation

3.3.3.1 Bradykinin Derivatisation and Eu^{3+} Chelation

Using the optimised method for peptide derivatisation described in section 2.2.4, two bradykinin samples (23 µM, 0.2 g) were derivatised with cDTPA (46 µM, 0.2 g), sodium bicarbonate pH 8 (0.1 M, 0.1 g) was added to give a final mass of 0.5 g. The samples were the left to react for 15 min. either natural or enriched europium (36 µM, 0.1 g) was added to separate 0.1 g aliquots of the two derivatised peptide samples. Sodium acetate pH 6 (0.1 M, 0.8 g) was added to adjust the pH of the resulting solution for ICP-MS analysis. The samples were then left for 30 minutes to ensure complete chelation before analysis. Subsequently the two derivatised, chelated samples were mixed in the proportion of 1:1 (0.25 g). Varying bradykinin concentrations were derivatised with cDTPA (46 µM) and chelated with europium enriched (36 µM) for relative quantitation. Samples for ESI-MS analysis were prepared in ammonium bicarbonate pH 8 and ammonium acetate pH 6 buffers. However, concentrations of reagents were increased ten-fold for ESI-MS detection.

3.3.3.2 Substance P and Peptide Mixture Derivatisation and Eu^{3+} Chelation

Using the method described in section 3.3.3.1, substance P (23 µM) ,and a peptide mixture containing bradykinin (23 µM 0.1g) and substance P (23 µM 0.1 g), were derivatised with cDTPA (46 µM, 0.2 g). A 0.1 g aliquot of the derivatised sample was chelated with either natural and enriched europium (36 µM, 0.1 g). Sodium acetate pH 6 (0.1 M, 0.8 g) was added to adjust the pH of the resulting solution for relative quantitation ICP-MS analysis.

3.3.4 Isotope Ratio Measurement by LC-ICP-MS

Isotope ratio measurement conditions were optimised using several standards of natural europium spiked with known amounts of enriched europium and measured using flow injection ICP-MS, with a samarium internal standard (66.5 nM) in the carrier stream. Various dwell times and channels per mass were used to measure the $^{153}Eu/^{151}Eu$ isotope ratio with the previously described data extraction methods. Analysis of the data suggested that the pseudo-steady state approach, with a dwell time of 10-20 ms and 5 channels per mass, resulted in mass bias corrected isotope amount ratios which were closest to the theoretical values of the gravimetrically prepared samples. In addition, a greater degree of precision was obtained using the pseudo steady approach compared to that obtained by peak integration. This was in agreement with previous findings[106].

3.4 Results and Discussion

3.4.1 Investigation of Derivatisation and Chelation using ESI-MS

Figure 3-4 shows the infusion mass spectrum of natural europium chelated with derivatised bradykinin, (^{151}Eu-cDTPA-bradykinin^{+} m/z 1584 and ^{153}Eu-cDTPA-bradykinin^{+} m/z 1586), confirming the formation of these species. However, due to insufficient resolution the isotopic pattern for europium was not observed. The europium labelled bradykinin (m/z 1584) was further fragmented by collision induced dissociation to determine the molecular masses of possible daughter ions (m/z 905 and m/z 540) that could be subsequently monitored by MRM (multiple reaction monitoring).

Further complementary LC-ESI-MS-MS yielded the chromatograms shown in Figure 3-5. Figure 3-5a shows the LC-ICP-MS chromatogram of bradykinin derivatised in sodium bicarbonate buffer. The derivatisation of bradykinin in ammonium bicarbonate and chelation with europium in ammonium acetate buffer yielded two peaks at 12.3 and 14.8 min respectively (Figure 3-5b, c and d) observed using gradient elution profile 1. Confirmation that these peaks were due to bradykinin-cDTPA-Eu was obtained provided by monitoring the MRM transition corresponding to 1584>905 (Figure 3-5c) and 1584>540 (Figure 3-5d). These transitions resulted from the loss of a cDTPA-Eu-arginine group and bradykinin from the bradykinin-cDTPA-Eu complex respectively. Additional monitoring of 1061>905 (Figure 3-5d) confirmed that there was a separate peak associated with bradykinin. The presence of two eluted Eu-cDTPA-peptide complexes was the result of the initial derivatisation of the peptide with cDTPA; no additional Eu-complexes were formed that were detected by ESI-MS analysis. In addition, Figure 3-5e shows the MRM transition corresponding to bradykinin fragmentation.

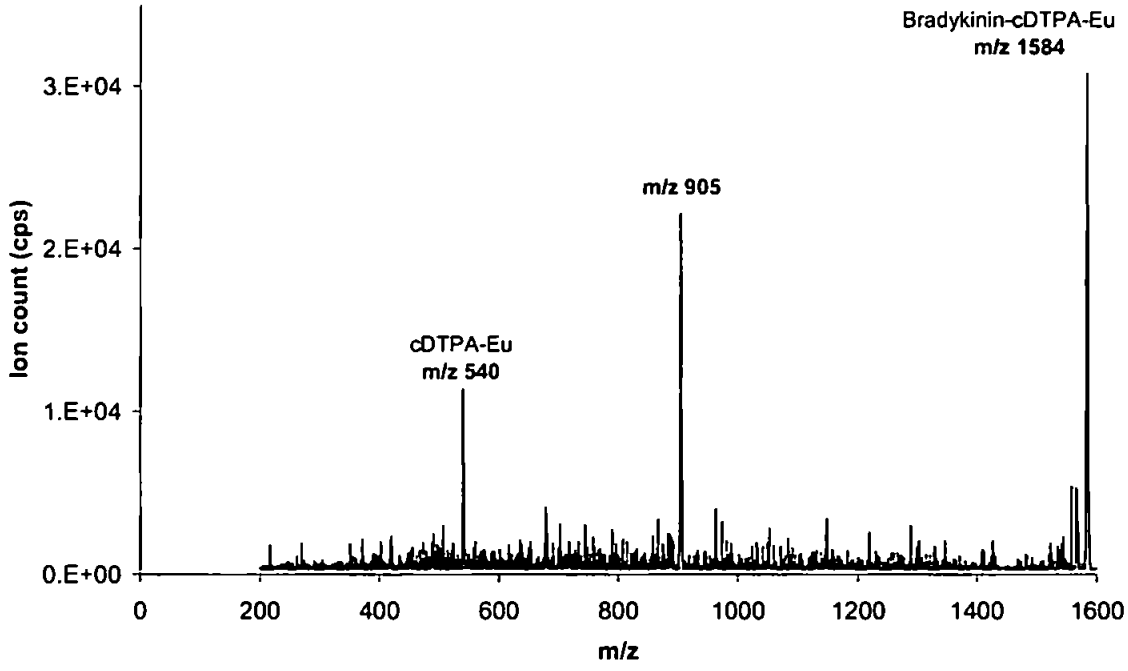

Figure 3-4 Infusion ESI-MS of the daughter ions of bradykinin chelated with enriched europium.

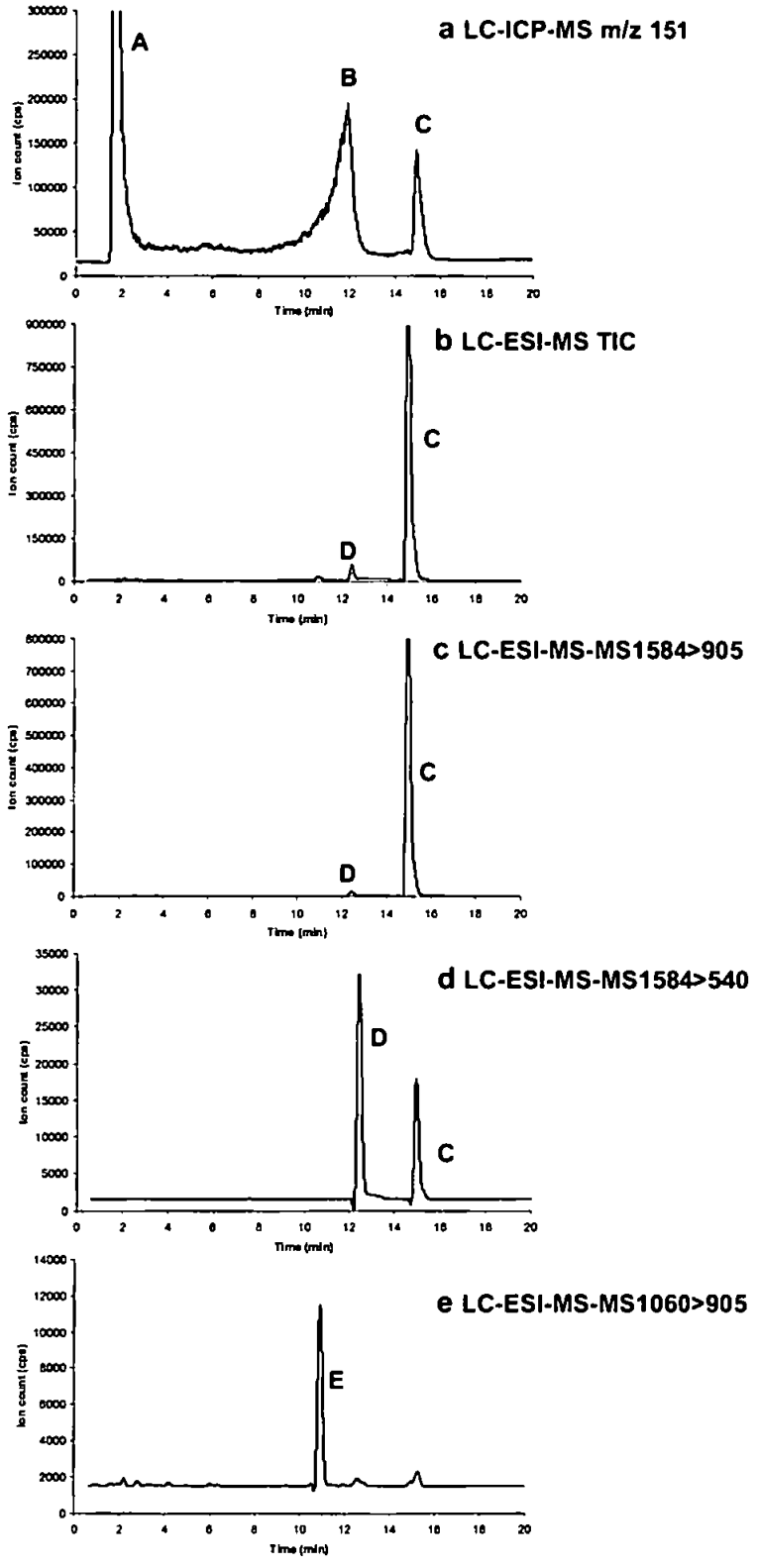

Figure 3-5 Chromatograms of bradykinin derivatised with cDTPA and chelated with natural Eu^{3+}: a, ICP-MS detection at m/z 151; b, ESI-MS total ion count; c, ESI-MS-MS MRM transition 1584>904; d, ESI-MS-MS MRM transition 1584>540; e, ESI-MS-MS MRM transition 1061>905. Peaks identified as: A, free Eu^{3+}; B, cDTPA-Eu; C and D, Eu-cDTPA-bradykinin; and E, bradykinin.

3.4.2 *Interference of Buffers*

The effect that using ammonium based buffers derivatisation with cDTPA can yield multiple by-products has been discussed in chapter 2. Figure 3-6 compares the LC-ICP-MS and LC-ESI-MS chromatograms of bradykinin derivatised in ammonium bicarbonate and chelated with europium in ammonium acetate. The LC-ESI-MS trace results in the elution of bradykinin (D) and two bradykinin-cDTPA-Eu (E and G) peaks at 9.2 min and 13.5 minutes respectively. However, the corresponding LC-ICP-MS yielded several peaks, these were identified as (A) free Eu^{3+}, (F) cDTPA-Eu, (E and G) bradykinin-cDTPA-Eu, and (B and C) unknown Eu species. The origins of these unknown Eu species could possibly be due to the ammonium acetate buffer forming Eu-acetate complexes such as $Eu(Ac)_2^+$ and $Eu(Ac)_3$.

3.4.3 *Relative Quantitation by ESI-MS*

Relative quantitation of europium chelated peptides was attempted by LC-ESI-MS. However, mass resolution was not sufficient to distinguish between ^{151}Eu and ^{153}Eu labelled bradykinin. To resolve the 2Da mass unit difference, a loss in sensitivity was observed, resulting in an ion signal which was not sufficient to accurately quantitate the derivatised chelated peptides.

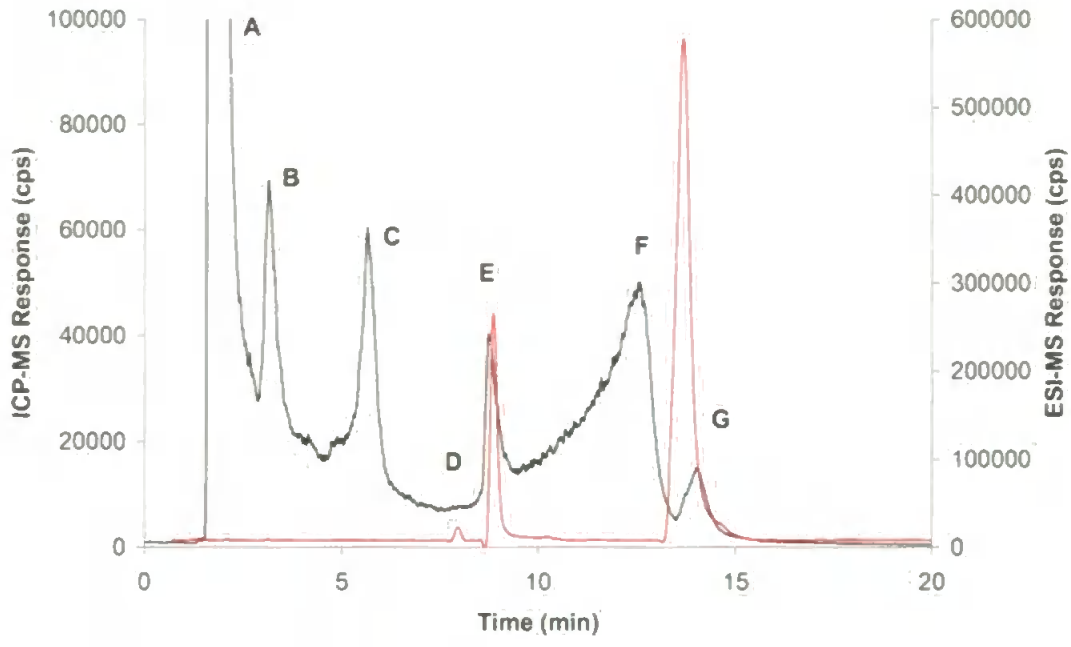

Figure 3-6 ICP-MS m/z 151 (Black) and ESI-MS TIC (Red) chromatogram of derivatised bradykinin chelated with enriched europium in ammonium bicarbonate and ammonium acetate buffers using gradient elution profile 2: A, Free Eu^{3+}; B and C, unknown Eu species; D, bradykinin; E, bradykinin-cDTPA-Eu; F, cDTPA-Eu; G, bradykinin-cDTPA-Eu.

3.4.4 Relative Quantitation using LC-ICP-MS

Figure 3-7, Figure 3-8, and Figure 3-9 show chromatogram obtained for derivatised bradykinin, substance P and a mixture of both the peptides respectively. The chromatogram in Figure 3-7 shows three resolved peaks: free Eu^{3+} at 2 min, a broad peak due to cDTPA-Eu^{3+} at 11 min and bradykinin-cDTPA-Eu^{3+} at 14.8 minutes. The chromatogram in Figure 3-8 shows peaks due to free Eu^{3+} at 2 min, cDTPA-Eu^{3+} at 11 min and substance P-cDTPA-Eu^{3+} at 18 minutes. Derivatised and Eu chelated substance P eluted in several unresolved peaks, possibly due to the methionine residues in the peptide forming disulphide bridges. No ESI-MS analysis was performed to identify whether a disulphide bridge was formed. The chromatogram in Figure 3-9 shows a mixture of derivatised bradykinin and substance P with the peaks due to free Eu^{3+} at 2 min, cDTPA-Eu^{3+} at 11 min, bradykinin-cDTPA-Eu^{3+} at 14.8 min and substance P-cDTPA-Eu^{3+} at 18 minutes.

Comparing the traces of derivatised substance P and the mixture of peptides an additional peak (E) was resolved in Figure 3-8. Though no ESI-MS data was obtained for this complex, it can be assumed that this was caused by the formation of bradykinin-cDTPA-substance P-Eu complex. This complex could be formed because the cDTPA molecule contains two anhydride reactive moieties.

The increase in background counts for ^{151}Eu and ^{153}Eu in the chromatograms is probably due to the incomplete elution of the free europium, the background subsides to normal levels once complete elution of europium species has occurred. Hydrolysed cDTPA was injected post analysis to remove any non-eluted of column.

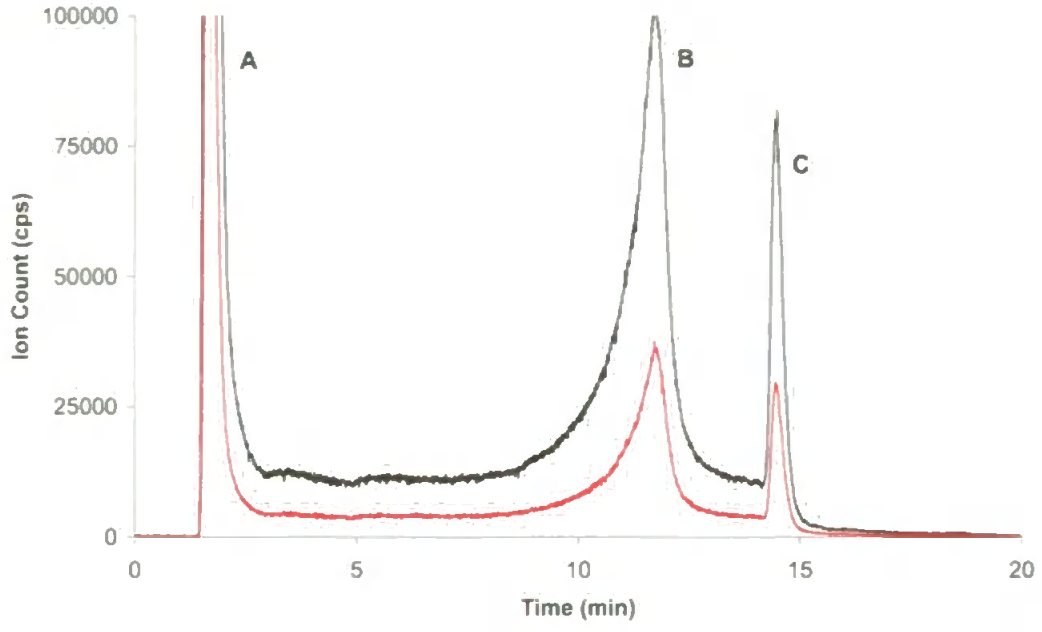

Figure 3-7 Multi-isotope ICP-MS chromatogram of derivatised bradykinin chelated with Eu using gradient elution profile 1. ^{151}Eu black and ^{153}Eu red. Resolved peaks; A, free Eu^{3+}; B, cDTPA-Eu^{3+}; and C, Bradykinin-cDTPA-Eu^{3+}.

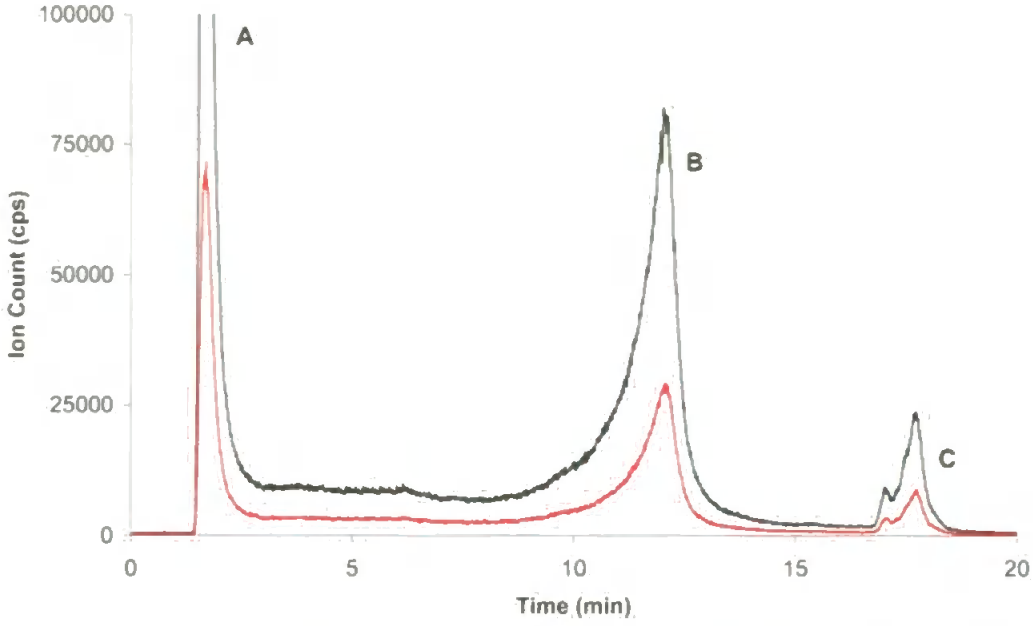

Figure 3-8 Multi-isotope ICP-MS chromatogram of derivatised substance P chelated with Eu using gradient elution profile 1. ^{151}Eu black and ^{153}Eu red. Resolved peaks; A, free Eu^{3+}; B, cDTPA-Eu^{3+}; and C, Substance P-cDTPA-Eu^{3+}.

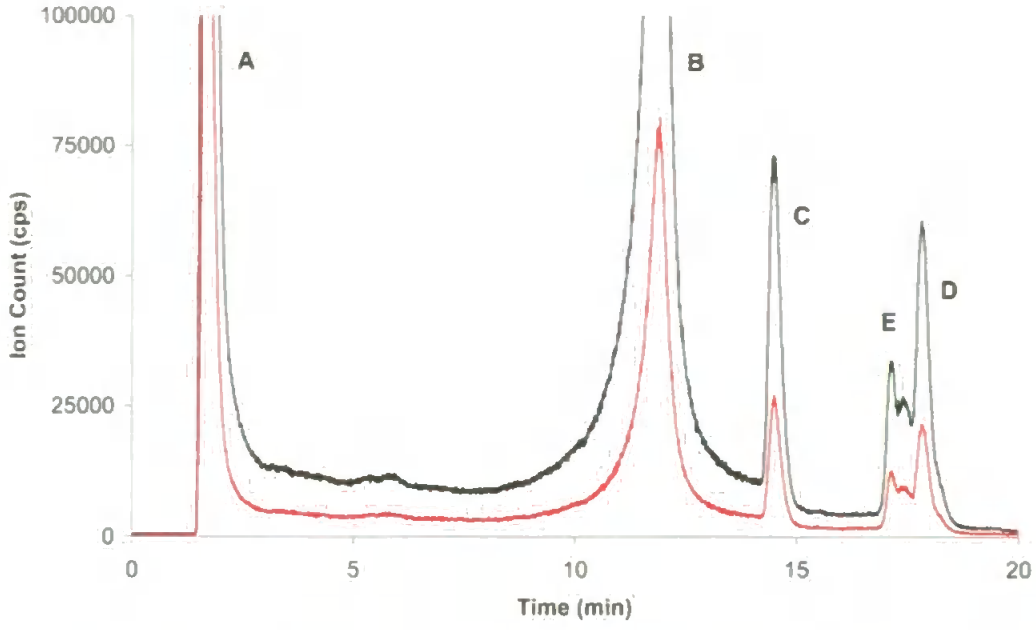

Figure 3-9 Multi-isotope ICP-MS chromatogram of a derivatised peptide mixture containing bradykinin and substance P chelated with Eu using gradient elution profile 1. ^{151}Eu black and ^{153}Eu red. Resolved peaks; A, free Eu^{3+}; B, cDTPA-Eu^{3+}; C, Bradykinin-cDTPA-Eu^{3+}; D, Substance P-cDTPA-Eu^{3+} and E, unknown resolved peak.

A pseudo steady-state approach was used to determine the isotope amount ratios from the multi-isotope chromatograms obtained by LC-ICP-MS. To obtain the best accuracy all co-incident data points were corrected by baseline subtraction. The obtained Eu ratio was mass bias corrected using the isotope amount ratio from the samarium internal standard. The ^{153}Eu:^{151}Eu corrected ratio was then used to calculate the peptide ratio (n_x/n_y) using Equation 3-3.

A comparison of the theoretical ratio, obtained from the gravimetric amount of the peptide, and the measured peptide ratios for bradykinin, substance P and a mixture of peptides is shown in Table 3-3. As can be seen, the measured peptide ratios agreed extremely well with the theoretical ratios, with relative errors of between 0.1 and 2.8% so, in principle, small differences in peptide expression should be detectable. Additionally, the relative quantitation of substance P was hindered by the fact that this peptide forms disulphide bridges relatively easily so elutes as several peaks at approximately 17 min. The largest relative error in the peptide ratio occurred with the mixture of peptides (Figure 3-8). is most likely due to the formation of di-peptide Eu complexes. The relative errors were significantly larger on samples containing substance P; this was probably due to the inefficient chromatography and the possible disulphide bridge formation. The two peptide mixture also had large errors associated with the relative peptide amounts this is probably due to intermolecular binding associated with cDTPA.

Table 3-3 Relative quantitation of peptides comparing theoretical and measured peptide ratios.

Sample	Peptide	Concentration (μM)	Theoretical	Measured		
			Peptide ratio nx/ny	Peptide ratio* nx/ny	± Relative error (%)	± Relative Expanded Uncertainty (%)
1	Bradykinin	0.6	1.0016	1.0002	0.134	5.420
2	Bradykinin	0.6	1.0016	1.0023	0.078	6.152
3	Bradykinin	0.6	1.875	1.864	0.562	3.694
4	Bradykinin	0.6	1.875	1.9015	1.411	5.746
5	Substance P	0.7	1.021	1.037	1.925	3.438
6	Substance P	0.7	1.021	1.032	1.492	9.800
7	Bradykinin	0.6	1.029	1.010	1.897	13.034
	Substance P	0.7	1.027	1.002	2.470	15.432
8	Bradykinin	0.6	1.029	1.057	2.790	7.392
	Substance P	0.7	1.027	1.003	2.322	8.586

The overall relative expanded uncertainty on the measured isotope amount ratio was determined by taking into account all the individual uncertainty components and combining them, after conversion to a standard uncertainty, using the spreadsheet method first described by Kragten *et al.*[107]. Each spreadsheet constructed during this work was separately validated by manual calculation. In this particular case the relative expanded uncertainties were quite high up to 15%. This large uncertainty was associated with precision of the measured isotope ratios namely $^{153}Eu:^{151}Eu$ and $^{154}Sm:^{152}Sm$. This could possibly be due to plasma instability. Previously, our experience of using isotope dilution analysis for the speciation of merthylmercury[106] it should be possible to reduce the standard uncertainty to less than 6% relative.

To improve the precision on the measured isotope ratios multi-collector (MC)-ICP-MS can be employed. Quadrupole ICP-MS measure isotopes sequentially therefore, any temporal fluctuations of the ion beam affects the accuracy and precision of the measured isotope amount ratio. Improvement in precision of the measured isotope amount ratio using MC-ICP-MS is because each isotope ion is measured simultaneously on appropriately spaced collectors. Thus, any fluctuations on one isotope ion will be equal on other isotope ions.

The typical concentration of peptide that could be easily detected was approximately 0.6 μM, which equated to 23 μM of original peptide solution that was derivatised and chelated. The lowest concentration of peptide for which relative quantitation could be accurately performed, was 5 μM which equated to an absolute detection limit of 5 pmol injected on to the LC column. The lowest

detectable concentration of Eu-peptide complex was 100 nM which equated to an absolute amount of 0.1 pmol of peptide detected. While ICP-MS is capable of much lower detection limits, in this method the efficiency of the derivatisation reaction was the limiting factor. This was because, at low peptide concentrations, hydrolysis of cDTPA was favoured over the acylation reaction with cDTPA, resulting in lower derivatisation efficiency.

3.5 Conclusions

The relative quantitation of peptides *via* the use of differential isotopic tagging has been achieved using LC-ICP-MS detection. Peptides were derivatised using cyclic diethylenetriaminepentaacetic anhydride and labelled with natural and enriched europium to differentiate between samples. This allowed ^{151}Eu and ^{153}Eu detection of bradykinin and substance P, which are not amenable to detection by ICP-MS. The technique of derivatisation involving differential isotopic tagging, has expanded the scope of ICP-MS allowing the detection of proteins and peptides, rather than being restricted to the speciation of metallo-proteins or heteroatom containing proteins/peptides. This method has the potential for accurate relative and absolute quantitation of peptides, especially if a multicollector instrument is used, which should result in a much better precision for the isotope ratio measurement. A number of challenges still need to be overcome to make this a viable approach, namely to improve detection limits, remove excess reagents and the relative quantitation of complex samples.

CHAPTER 4 INVESTIGATION OF NEW DERIVATISATION AGENTS AND TECHNIQUES

4. INTRODUCTION

It has been shown in chapter 3 that ICP-MS can be used to relatively quantitate peptides with high accuracy and precision. However, the current technique of peptide derivatisation is limited to single peptide samples and cannot be directly compared to current methods, which are used to relatively quantitate hundreds to thousands of peptides. Hence, for the methodology developed to be comparable complex samples must be analysed with comparable accuracy and precision.

In this chapter the derivatisation of complex peptide samples with various bifunctional chelating agents is described. The concept of solid phase analytical derivatisation[108] (SPAD) to improve relative quantitation is also introduced. The SPAD technique utilises a solid phase support for derivatisation and removal of excess reagents, and derivatised peptides can be selectively extracts for LC-ICP-MS.

4.1 Derivatisation of Complex Samples

In previous studies using cDTPA for derivatisation it was determined that the reagent was unsuitable for reaction with complex samples because the resulting derivatisation reaction could yield multiple unwanted by-products[109]. These by-products were easily formed because cDTPA contains two reactive anhydride moieties. This is a significant problem because the majority of proteomic samples will contain a large number of peptides. The formation of unwanted side products with cDTPA has been demonstrated with insulin by Maisano *et al.*[109]. Figure 4-1 shows the potential by-products that can be formed from the derivatisation reaction of peptides with cDTPA. An intermediate (A) is formed when the primary amine of the peptide is derivatised by the first anhydride group, and, depending on how the second anhydride group reacts, three different derivatised peptide complexes can occur: complex B, a single derivatised peptide with the second anhydride group hydrolysed; complex C, intermolecular derivatisation, a cDTPA complex that has been derivatised with another peptide; and complex D, intramolecular derivatisation, a cDTPA complex that has derivatised an amine group within the original peptide.

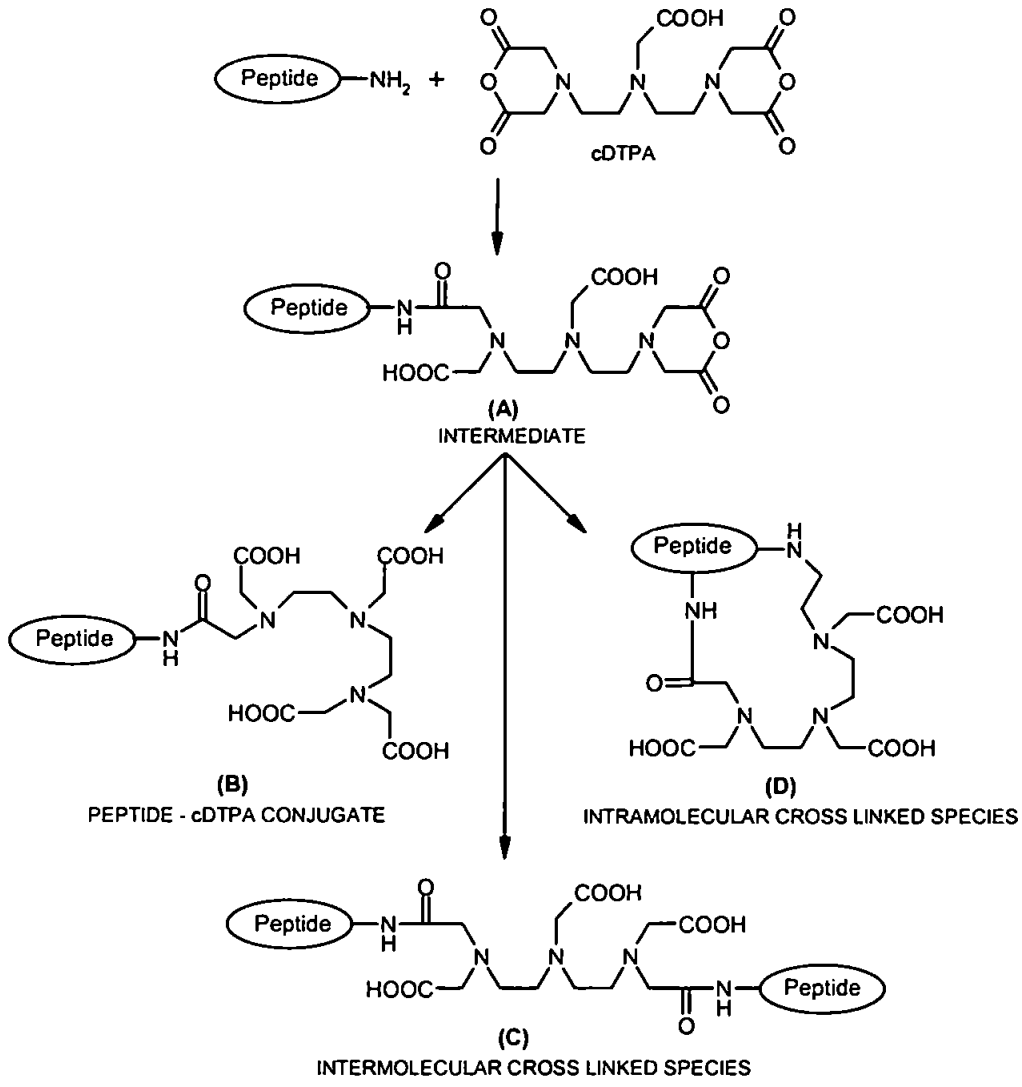

Figure 4-1 A schematic representation of the potential by-products that can be formed from the derivatisation reaction between cDTPA and peptide. Figure modified from Bennett *et al.*[110]

4.1.1 Monoreactive Bifunctional Chelating Agents

In order to prevent the formation of multiple by-products, monoreactive bifunctional chelating agents have been synthesised. This class of compounds contain only one reactive moiety, thereby eliminating the possibility of multiple by-product formation. Figure 4-2 shows structures of the bifunctional chelating agents that have been considered for peptide derivatisation. These BFCAs are based on various chelating agents such as ethylenediaminetetraacetic acid (EDTA), nitrilotriacetic acid (NTA), diethylenetriaminetetraacetic acid (DTTA), and diethylenetriaminepentaacetic acid (DTPA), with the most popular being DTTA and DTPA. These modern BCFAs have also been synthesised with various reactive moieties such as those specifically designed to react with amines i.e. isothiocyanate, carbonates, anhydrides or designed to react with specific functional groups such as, thiols ε-terminal lysines and amines, these reaction mechanisms and conjugations have been extensively discussed[111].

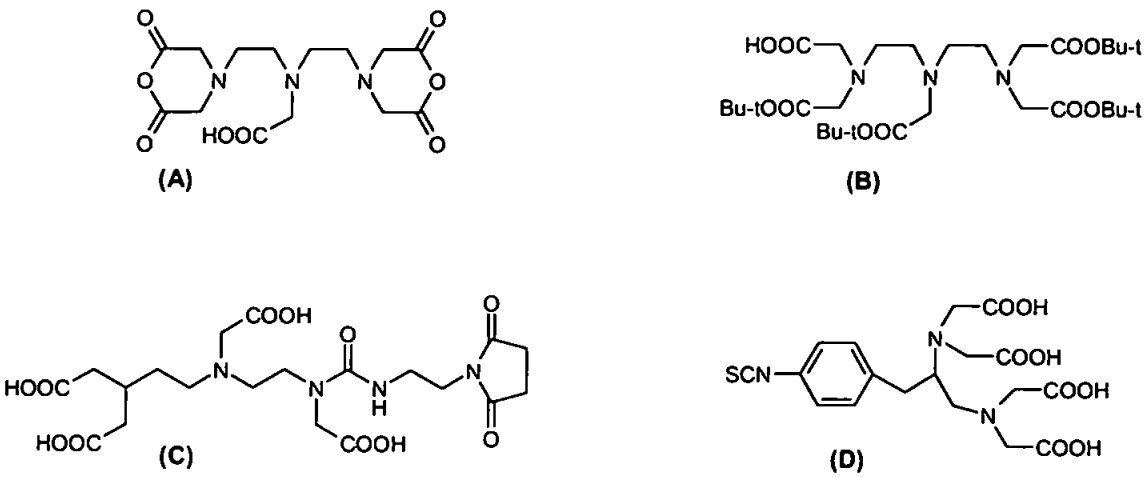

Figure 4-2 Chemical structures of (A) cDTPA, (B) Monoreactive DTPA, (C) Monoreactive maleimide DTPA (D) Isothiocynanate-benzyl-EDTA.

4.1.1.1 Monoreactive Diethylenetriaminepentaacetic Acid

Monoreactive Diethylenetriaminepentaacetic Acid (mDTPA) structure B in Figure 4-2 has been synthesised by Arano *et al.*[112] for use in the synthesis of radioisotopic labelled antibodies by solid phase. A major advantage of mDTPA is that it is soluble in a variety of solvents, making it useful in either solution or solid phase derivatisation. However, the major disadvantage is that mDTPA is not commercially available and, according to Arano *(personal communication)*, the synthesis is difficult and could take months to complete.

4.1.1.2 Maleimide Diethylenetriaminepentaacetic Acid

Maleimide diethylenetriaminepentaacetic acid (MDTPA) was synthesised by Arano *et al.*[113] to overcome the problems caused by inter- and intramolecular cross linking with cDTPA derivatisation. MDTPA (Figure 4-2C) contains a maleimide group which reacts readily with thiol containing molecules (e.g. proteins and peptides). The original MDTPA synthesis took several steps, though recently Lattuada *et al.*[114] published a simple synthetic route yielding gram quantities.

4.1.1.3 Isothiocyanate-Benzyl-ethylenediaminetetraacetic Acid

Isothiocyanate-benzyl-ethylenediaminetetraacetic acid (SCN-Bz-EDTA) (Figure 4-2D) was synthesised by Meares *et al.*[115] for the purpose of attaching various metal ions to monoclonal antibodies. The isothiocyanate group reacts with nucleophiles such as amines, sulfhydryls and the phenolate ion of tyrosine side chains. However, the only stable product of the reaction is with primary amine groups[111]. Therefore, isothiocyanate compounds are almost entirely selective for modifying ε-

terminal and N-terminal amines in proteins, or primary amines in other molecules[116].

4.1.2 Complex Samples

When considering the analysis of 'real' samples the analytical method will only be as good as the sample preparation employed. For example, the analysis of sea water by ICP-MS often requires some form of sample preparation such as the removal of matrix components and interfering ions, or detrimental effects on chromatography, detection, accuracy and precision can be observed. Most proteomic methods isolate the analytes of interest to reduce sample complexity. For example, the ICAT[36] method uses biotin avidin chromatography to isolate and preconcentrate derivatised peptides and remove excess reagent and underivatised peptides.

4.1.2.1 Solid Phase Analytical Derivatisation

A major requirement of derivatisation reactions is that the reaction must work at a low analyte concentration. However, at low levels, reaction kinetics are reduced resulting in a lower derivatisation efficiency. Other disadvantages of solution phase derivatisation are:

- Many reagents are unstable.
- Yields multiple by-products.
- The analyte is typically diluted by the addition of reagents.
- Reaction rates in general are poor.
- Excess reagent must always be removed or deactivated.

One of the advantages of solid phase analytical derivatisation (SPAD) is that it improves efficiency at low concentrations so it seems an attractive alternative derivatisation method[117]. The SPAD technique typically uses solid phase extraction (SPE) tips for sample preparation, derivatisation and elution. The basic strategy for SPAD, is that the sample is loaded onto the solid phase sorbent, and derivatised, followed by elution in gradient fractions.

Several reviews and publications[108, 118, 119] have been presented on solid phase analytical derivatisation. Keough *et al.*[118] reported the solid phase sulfonation of tryptic peptides absorbed onto C_{18} μZiptip™. The technique involved the sample clean up of the tryptic peptides, which was sorbed on the C_{18} zip tip and then sulphonated prior to MALDI-MS analysis.

Sample Isolation

Solid phase sorbents have previously been used to isolate and preconcentrate analytes of interest. Once the sample is loaded onto the sorbent, matrix components can be removed from the SPE cartridge using mild eluents. The advantage of this approach is that the analyte is isolated in a narrow band on the solid phase tip. Preconcentration of the analyte can also result in a two-fold improvement in derivatisation efficiency. First, the analyte is isolated in a defined volume by the chromatofocusing effect, resulting in the 'local' concentration being higher than in solution. Another effect of preconcentration is that the load to elution volume ratio can be varied, thus determining the level of preconcentration[117].

Reagent Loading

Reagents used for solid phase derivatisation should be chosen to yield a rapid reaction, produce no by-products, not interfere with subsequent analysis and should be soluble in a weak loading solvent. The latter is the most important aspect of SPAD; if a strong loading solvent is used the reagent is not likely to be retained on the solid phase along with the analytes.

Derivatisation

As with solution phase derivatisation, reaction time, temperature and pH can all be optimised to improve reaction efficiency.

Elution

Derivatised analytes can be selectively eluted from the solid phase by increasing the eluent strength in a stepwise gradient. In addition, excess reagent can be used for derivatisation because unreacted reagent can be removed or isolated from analytes prior to analysis. Elution of the sample in this manner can be advantageous to the subsequent HPLC separation, as the complex sample is fractionated prior to LC-UV/Vis and LC-ICP-MS.

4.2 Experimental

The methods in the following section describe the derivatisation of cDTPA and SCN-Bz-EDTA to peptide(s) for qualitative and relative quantitation analysis. In addition, the derivatisation methodology was further developed to improve derivatisation efficiency and complex sample handling with the use of SPAD.

4.2.1 Reagents and Standards

Bradykinin acetate, HPLC peptide mix, cyclic diethylenetriaminepentaacetic anhydride (cDTPA), sodium bicarbonate, sodium acetate, sodium carbonate, ammonium carbonate and trifluoroacetic acid (TFA) (reagent grade) were purchased from Sigma Aldrich, (Poole, Dorset, UK). Isothiocyanate-benzyl-EDTA was purchased from Dojindo Laboratories (Kumamoto, Japan). Dimethyl sulfoxide (DMSO), ammonium acetate, nitric acid (trace analysis grade), LC grade acetonitrile and methanol were obtained from Fisher Scientific UK Ltd, (Loughborough, Leicestershire, UK). Ammonium bicarbonate was purchased from Fluka, Sigma Aldrich, (Poole, Dorset, UK). Distilled deionised water (DDW) 18.2 MΩ, was obtained using an Elga Maxima water purifying system. ^{151}Eu-enriched europium (Eu_2O_3 99.24% enriched) was purchased from CK Gas Products, (Hampshire, UK). A standard ICP-MS solution of natural europium was purchased from Alfa Aesar (Heysham, UK). Aristar grade indium atomic spectroscopy standard was purchased from VWR International (Poole, Dorset, UK).

4.2.2 Instrumentation

All LC analyses were performed using the Luna C18 (2) column (Phenomenex, Macclesfield, UK) for reversed phase LC-UV/vis and LC-ICP-MS analysis. The

mobile phase was a binary system of: eluent A, water containing 0.05% formic acid (v/v); and eluent B, acetonitrile containing 0.05% (v/v) formic acid. Various gradients were used in this chapter of research therefore the LC gradient profiles will be mentioned with in the appropriate sections. The flow rate was 1 ml min^{-1} with an injection volume of 25 µL for all analyses.

The LC system used for the UV/vis and UV/vis-ICP-MS analysis was a HP 1090 liquid chromatograph (Agilent Technologies, Stockport, UK). Element specific detection was performed using quadrupole ICP-MS (VG PlasmaQuad 3, Thermo Elemental, Winsford, UK). The ICP-MS sample introduction system consisted of a 1.0 ml min^{-1} microMist nebuliser and a cyclonic spray chamber both purchased from Glass Expansion (AUS). The LC outlet was connected to the ICP-MS nebuliser via 2 m of Teflon tubing (1/16" x ID 0.18mm). A membrane desolvator (Vestec Corporation, Texas, USA) was connected between the spray chamber (Nebuliser) and torch to remove organic solvents. The dry aerosol exiting the desolvator was then transferred to the ICP torch via 1 m of 0.25 in. i.d. tygon tubing. Operating and data acquisition conditions are shown in Table 4-1.

Table 4-1 Operating and data acquisition conditions for Quadrupole ICP-MS.

ICP	
Nebuliser gas flow / L min^{-1}	1.3
Auxiliary gas flow / L min^{-1}	0.8
Coolant gas flow / L min^{-1}	14.0
RF forward power / W	1350
Nebuliser	1.0 ml min^{-1} Micromist
Spray chamber	Jacketed quartz cyclonic (room temperature)
Torch	Quartz fassel-type
Interface	
Sampling cone	Nickel, 1mm i.d.
Skimmer cone	Nickel, 0.7mm i.d.
Time Resolved Analysis	
Masses monitored	^{115}In or ^{151}Eu, ^{152}Sm, ^{153}Eu, ^{154}Sm
Data Acquisition	Peak Jump
Dwell time / ms	10
Channels per mass	5
Membrane Desolvation	
Sweep gas	Argon
Desolvation gas flow / L min^{-1}	0.9
Desolvation temperature / °C	80.0

4.2.3 cDTPA Derivatisation

The HPLC peptide mixture contained five peptides Gly-Tyr, Val-Tyr-Val, Leu-enkephalin, Met-enkephalin and angiotensin II at approximately 0.5mg per peptide. The peptide mixture was derivatised according to the modified method in section 2.2.4, 0.2 g of the peptide mixture sample (0.2 mM) was derivatised with 0.2 g of cDTPA (0.4 mM) in anhydrous DMSO, finally 0.1 g of sodium bicarbonate solution (100 mM) adjusted to pH 8 was added, the solution was left to stand, after mixing, for 30 min. at room temperature. An aliquot of the derivatised peptide was analysed by LC-UV/vis prior to LC-ICP-MS. The derivatised peptide mix was chelated with Eu^{3+} for ICP-MS analysis by adding 0.1 g of a 60 µM Eu^{3+} solution in 0.1 M sodium acetate (pH 6) to 0.1 g of the derivatised peptide solution, then a further 9.8 g of 0.1 M sodium acetate pH 6 was added and the solution was agitated and left at room temperature for 30 minutes prior to analysis. The gradient chromatography for these samples was a step gradient, starting with 0% eluent B from 0-5 minutes with a gradient to 50% eluent B over 20 minutes, and then up to 100% the over next 5 minutes.

4.2.4 Isothiocyanate-Benzyl-EDTA Derivatisation

SCN-Bz-EDTA peptide reaction shown in Figure 4-3 involves nucleophilic attack on the central, electrophilic carbon of the isothiocyanate group. The resulting electron shift and proton loss creates a thiourea linkage between the isothiocyanate-containing compound and the amine, with no leaving group involved[107]. The SCN-Bz-EDTA compound is monoreactive and reacts with the target amine between pH 8-9 where the amine groups are mainly unprotonated. Reaction times vary from 4 to 24 hrs at 4°C. Various buffers can be selected for

this reaction such as sodium carbonate, sodium phosphate and borate buffered saline.

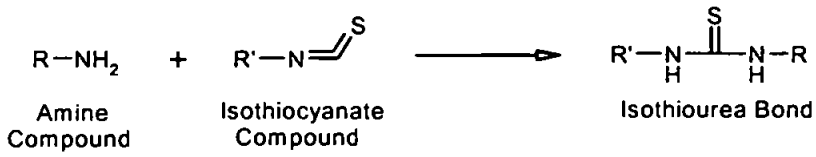

Figure 4-3 Reaction scheme for the derivatisation of peptides using SCN-Bz-EDTA

The derivatisation reaction was performed in a similar manner to the cDTPA derivatisation method (section 2.2.3) in terms of concentrations and molar excess of reagents. All reagents were prepared in 0.1 M sodium carbonate pH adjusted to 8 using hydrochloric acid. 0.2 g SCN-Bz-EDTA (0.4 mM) was added to 0.2 g peptide sample (0.2 mM), and a further 0.1 g of 0.1 M sodium carbonate was added to give a final mass of 0.5 g.

The derivatisation was optimised for three different reaction factors: time; pH and temperature. The chromatographic separation for the optimisation experiments was a step gradient by increasing eluent B to 100% over 20 minutes and then held isocratically at 100% B for a further 10 minutes.

The chelation of metals, europium and indium, was performed in 0.1 M ammonium citrate[115] adjusted to pH 6 using citric acid. A 0.1 g aliquot of the SCN-Bz-EDTA derivatised peptide sample was added to 0.1 g of trivalent metal (320 μM), the pH of the resulting solution was adjusted to pH 6 using 9.8 g of 0.1M ammonium citrate.

4.2.5 Solid Phase Extraction Peptide Recovery

Disposable solid phase extraction (SPE) cartridges C_{18} (octadecyl, 18% carbon) columns containing 500 mg of sorbent (40µm, 60Å), with a column volume of 2.8 ml were obtained from Varian (Harbour City, CA, USA) and used for SPE and SPAD experiments. The peptide mix was dissolved in 0.1 M sodium carbonate buffer to give a total peptide concentration of 0.05 mg/ml. The peptide concentration was sufficient to be detected by the LC-UV/vis analysis but not overload the SPE column.

To determine if the individual peptides in the HPLC peptide could be separated by SPE the following procedure was employed. The SPE procedure was modified from a method by Herraiz *et al.*[120]. The C_{18} sorbent was conditioned prior to peptide loading by passing successively, 1mL of methanol, 1 mL of Milli-Q water and 1 mL 0.1% TFA. Once the sorbent was conditioned, peptide loading and SPE were performed by passing a 1 mL portion of the peptide mix through the SPE column at a flow rate of 1 mL/min maintained using a peristaltic pump. The peptides retained on the SPE column were washed with 1 mL of 0.1% TFA. Subsequently, peptides were eluted in fractions *via* a stepwise ACN gradient. ACN fractions were 25% ACN, 50% ACN, 75% ACN, and 100% ACN eluted in this order through the SPE column. Each collected fraction was individually analysed using LC-UV/vis. The HPLC gradient elution profile for these analysis was a linear gradient over 10 minutes from 0% - 100% eluent B. The peak area of each peak arising from each SPE elution fraction was compared to peak areas of a non SPE extracted reference sample.

4.2.6 *Solid Phase Extraction of SCN-Bz-EDTA Derivatised Peptides*

Subsequently, SCN-Bz-EDTA derivatised peptide(s) were solid phase extracted using the method described in section 4.2.5. The peptide(s) sample were derivatised according to the method in section 4.2.4 but without metal chelation. This procedure was investigated to determine whether SPE can isolate metal labelled peptides from excess reagent used in the derivatisation and chelation steps. In addition, three more eluent fractions were included these included 0.1% TFA in 5%, 10% and 15% ACN to remove any excess metal from the chelation reaction. For purposes of high through-put LC analysis time was reduced to 15 min per sample by increasing the gradient to 100% ACN over 10 min and holding isocratically for a further 5 min.

4.2.7 *Solid Phase Analytical Derivatisation*

To achieve SPAD, additional steps were added to the SPE methodology (section 4.2.4). 1 mL of bifunctional chelating agent was passed through the column after the 0.1% TFA peptide wash step, followed by 1 ml of trivalent metal addition. Figure 4-4 shows the apparatus required for SPAD.

Table 4-2 Comparison of SPE and SPAD methods.

	Solid Phase Extraction	Solid Phase Analytical Derivatisation
Column conditioning	1ml MeOH, 2ml 0.1% of TFA	
Sample loading	1ml Peptide(s) in buffer	
Sample wash	0.1% of TFA	
Derivatisation	N/A	1ml BFCA
Chelation	N/A	1ml trivalent metal
Elution	1ml of 0.1% TFA in 0-100% ACN	

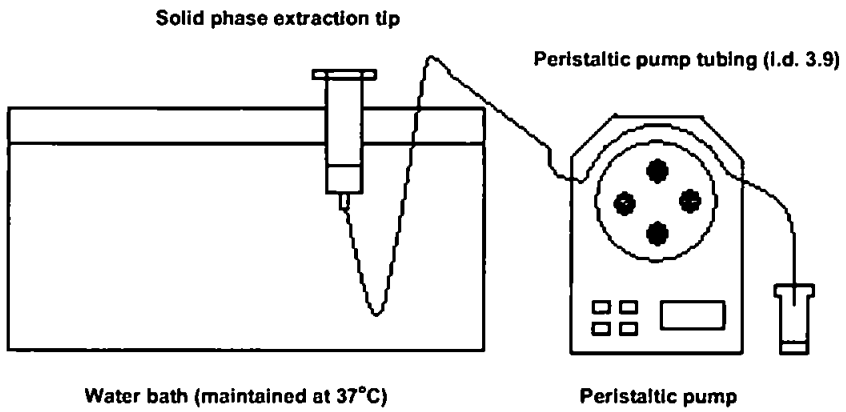

Figure 4-4 A schematic representation to show the apparatus required for solid phase analytical derivatisation

4.3 Results and Discussion

4.3.1 cDTPA Derivatisation of Complex Samples

The UV/vis chromatogram in Figure 4-5 shows both an underivatised and cDTPA derivatised peptide mix. The peptide mix contained five peptides, labelled A-E (Figure 4-5 red trace). When the peptide mix was derivatised, more than the expected number of derivatised products resulted (Figure 4-5 black trace). Typically, derivatisation of five peptides should result in five derivatised products; however, because the cDTPA reagent contains two reactive groups, complexes described in Figure 4-1 can be formed, resulting in extra peaks. The formation of the extra by-products would result in inaccurate relative quantitation because it is impossible to predict how much of each peptide would be derivatised in these additional by-products.

The LC-ICP-MS chromatogram in Figure 4-6 shows the result of chelation of natural europium to the cDTPA derivatised peptide mix. The chromatogram shows five resolved Eu peaks possibly representing the singly derivatised peptides (Figure 4-6 A-E). A comparison of the respective UV/vis and ICP-MS chromatograms, suggests that intramolecular and intermolecular cDTPA species cannot chelate with trivalent metals, possibly due to the reduced number of co-ordination sites available for chelation. It was concluded that new reagents such as monoreactive BFCA would have to be evaluated if the relative quantitation of complex samples was to be achieved.

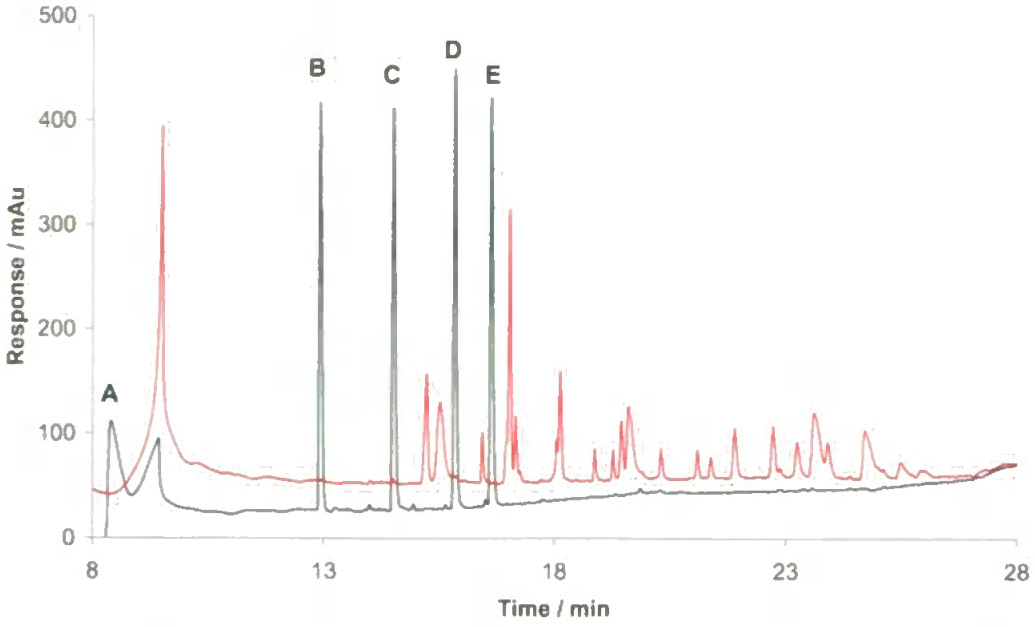

Figure 4-5 LC-UV-vis chromatograms, with detection at 220nm, of underivatised (black) and derivatised (red) peptide mix using cDTPA. Original peptides are labelled A-E.

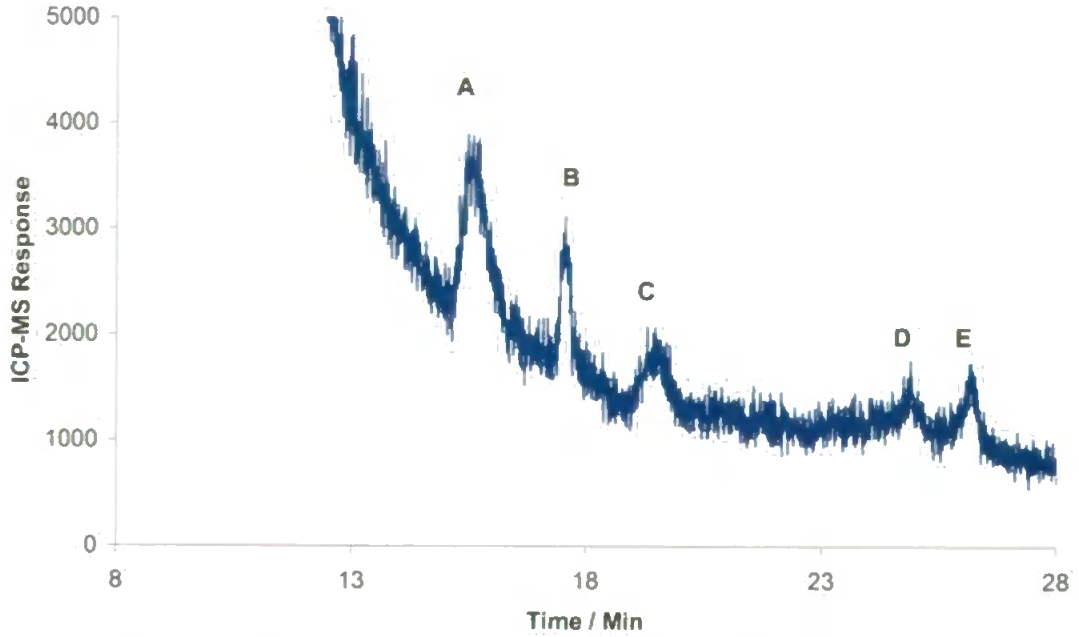

Figure 4-6 LC-ICP-MS chromatograms at m/z 153 of cDTPA derivatised peptide mix with Eu natural chelation. Peaks A-E represent Eu labelled peptides from peptide mixture.

4.3.2 *SCN-Bz-EDTA Derivatisation*

The derivatisation reaction between peptides and SCN-benzyl-EDTA was optimised for reaction time, temperature and pH. Figure 4-7 to 4-10 show the UV/vis chromatograms of optimised derivatised samples of bradykinin and the HPLC peptide mixture respectively. The factors and levels used for the optimisation were: time, 2 and 24 hours; temperature, 4°C and 37°C; and sodium carbonate buffer, pH 8 and pH 9 respectively. Derivatisation efficiency was calculated by comparing the peak area of the underivatised peptide(s) to the standard HPLC peptide mixture and a bradykinin sample respectively. Figure 4-7 and Figure 4-8 shows the derivatisation of bradykinin with SCN-Bz-EDTA after a reaction time of 2 and 24 hours respectively. The eluted peaks were identified as A, bradykinin, B, SCN-Bz-EDTA-Bradykinin, C, SCN-Bz-EDTA and D, SCN-Bz-EDTA by-products, from the respective retention times. From chromatograms Figure 4-7a and 4-7b it is apparent that the derivatisation efficiency was affected by reaction temperature, increasing temperature from 4°C to 37°C improved derivatisation efficiency from 4% to 55%. Increasing derivatisation reaction time from 2 to 24 hours (Figure 4-7a and Figure 4-8a), further increased derivatisation efficiency by 15%. Changing buffer pH from 8 to 9 resulted in no significant increase or decrease in derivatisation efficiency.

It was noted that the SCN-Bz-EDTA reagent was unstable in solution over a longer reaction time and at higher reaction temperatures. This instability is well documented for reagents containing isothiocyanates and isocyanates; this instability results in the breakdown of the isothiocyanate reagent into several species (labelled D in Figure 4-8b and 4-8d). However, molecular mass spectrometry would be required to identify whether these peaks are products of

the SCN-Bz-EDTA or bradykinin-SCN-Bz-EDTA. Figure 4-9 and Figure 4-10 shows the derivatisation of a peptide mixture. Peaks A-E was identified as underivatised peptides, based on the respective retention times from standards; F, SCN-Bz-EDTA; and G, SCN-Bz-EDTA by-products, and peaks 1-5 were assumed to be derivatised peptides. Derivatised and underivatised peptides were determined on retention times and based on the disappearance of peptides and SCN-Bz-EDTA derivatisation reagent (Figure 4-10b and Figure 4-10d). The chromatograms showed that the optimal derivatisation condition was 24hrs, in pH 9 buffer at a reaction temperature of 37°C. However, when using these derivatisation conditions a greater amount of SCN-Bz-EDTA by-products were formed. To distinguish between peptide-SCN-Bz-EDTA and SCN-Bz-EDTA by-products would require LC-ESI-MS confirmation. Further complimentary LC-ICP-MS data should also confirm the number of derivatised peaks and whether derivatisation was successful.

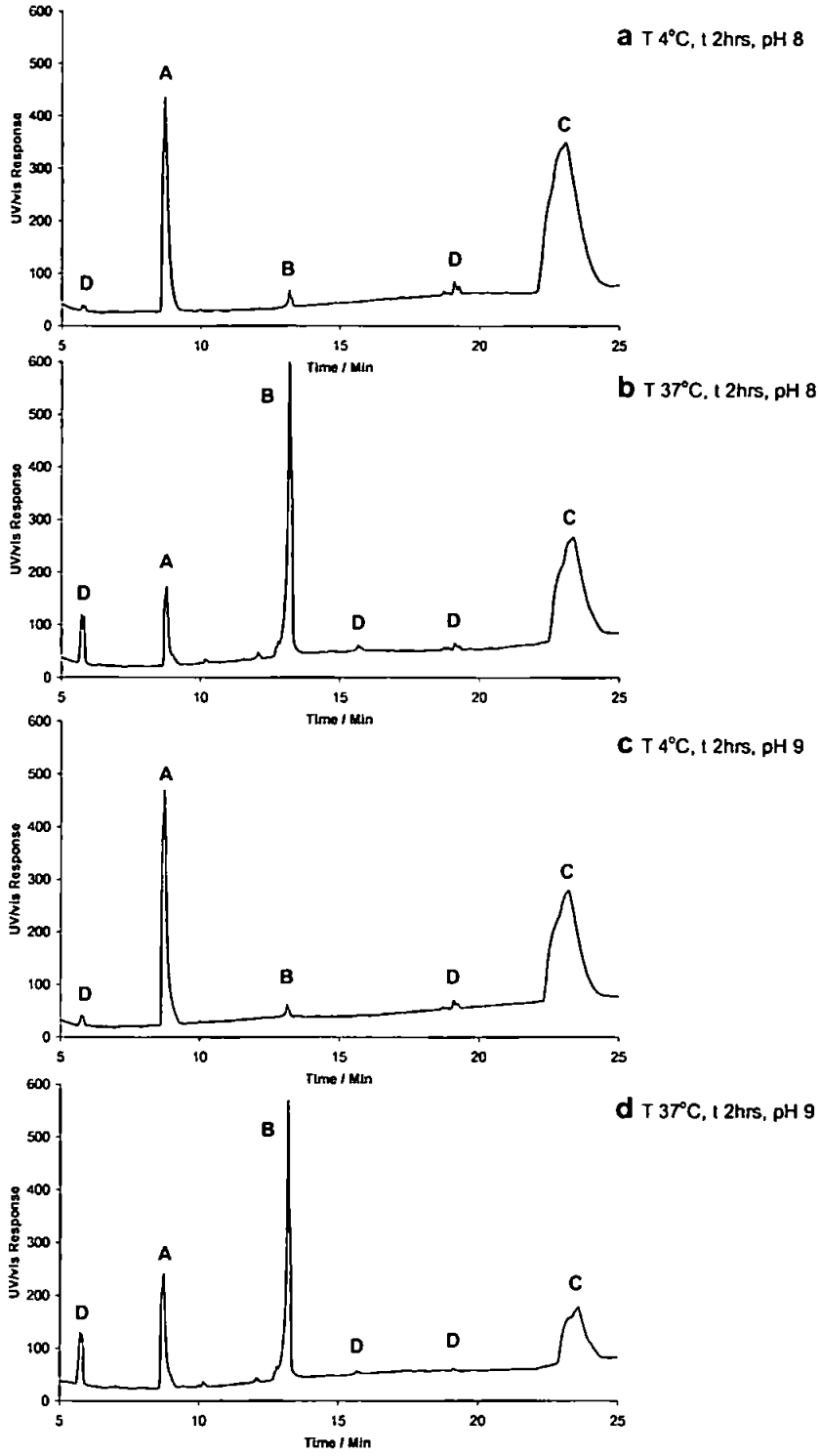

Figure 4-7 HPLC chromatograms, with UV/vis detection at 220nm, of bradykinin derivatised (2hrs) with SCN-Bz-EDTA: A, Bradykinin; B, SCN-Bz-EDTA-Bradykinin; C, underivatised SCN-Bz-EDTA; D, SCN-Bz-EDTA by-products.

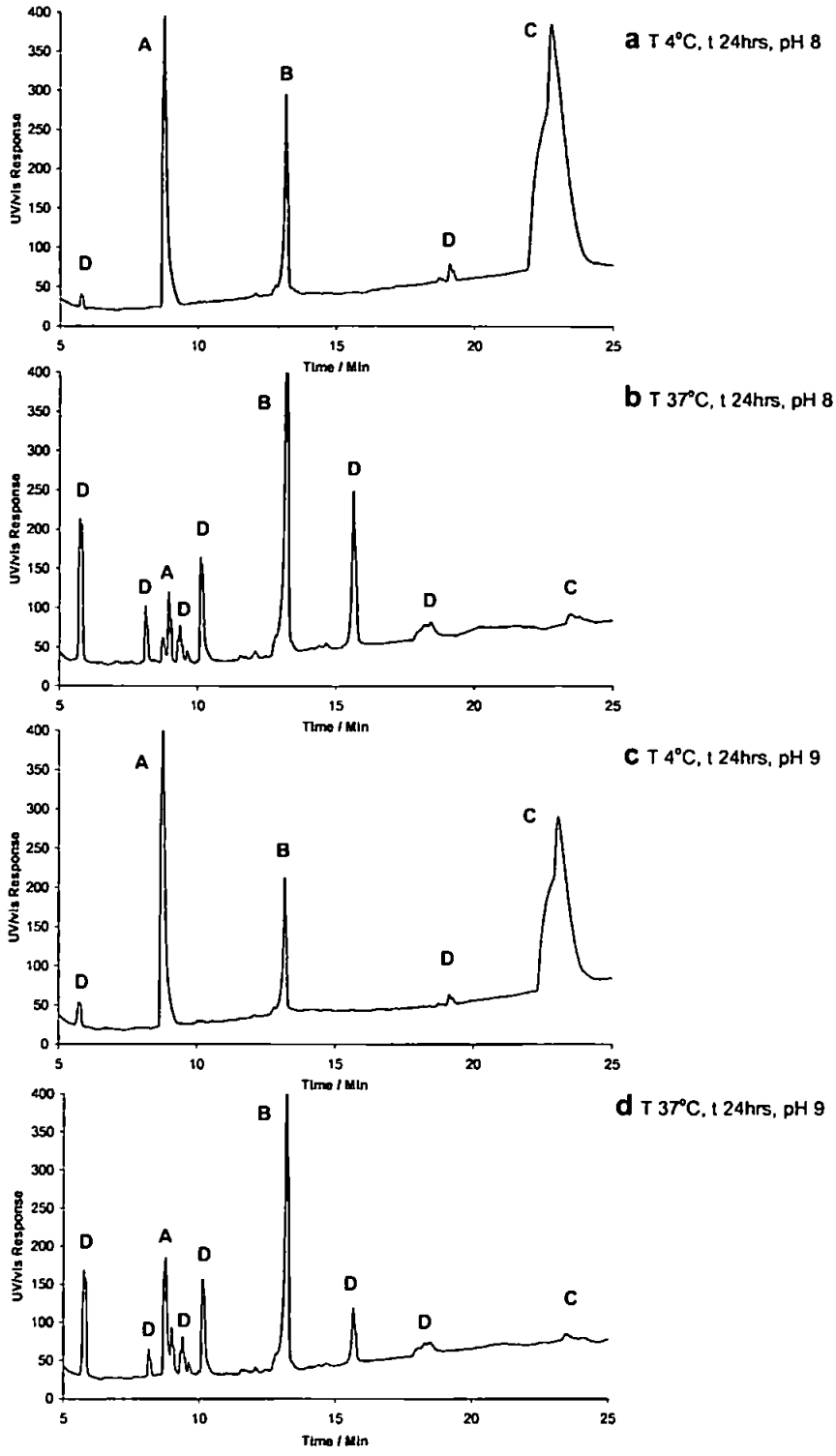

Figure 4-8 HPLC chromatograms, with UV/vis detection at 220nm of bradykinin derivatised (24hrs) with SCN-Bz-EDTA: A, Bradykinin; B, SCN-Bz-EDTA-Bradykinin; C, underivatised SCN-Bz-EDTA; D, SCN-Bz-EDTA by-products.

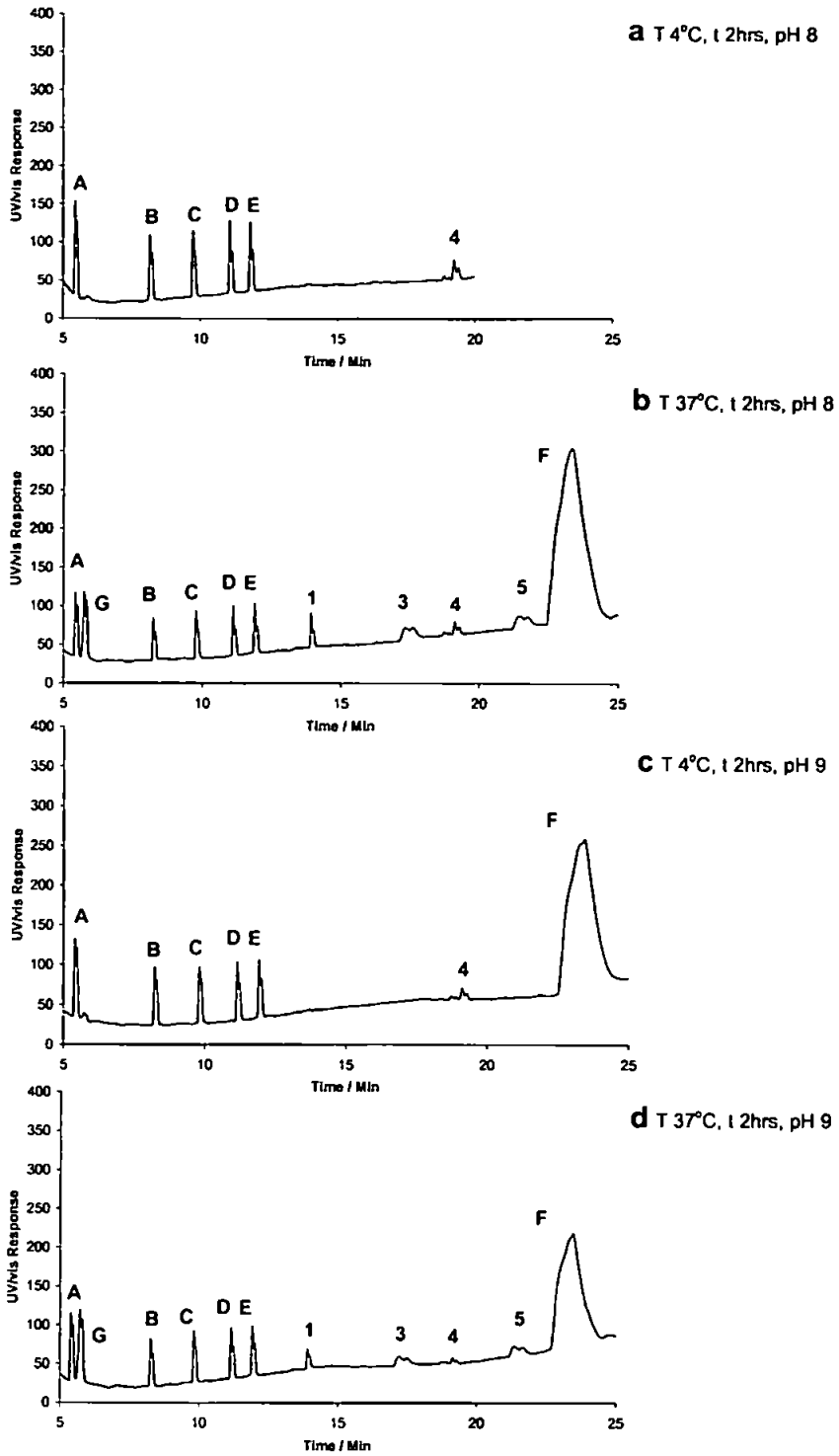

Figure 4-9 HPLC chromatograms, with UV/vis detection at 220nm, of the HPLC peptide mix derivatised (2hrs) with SCN-Bz-EDTA. Underivatised peptides: A; B; C; D; E. Derivatised peptides: 1; 2; 3; 4; 5 in the same order. F, SCN-Bz-EDTA; G, SCN-Bz-EDTA by-products

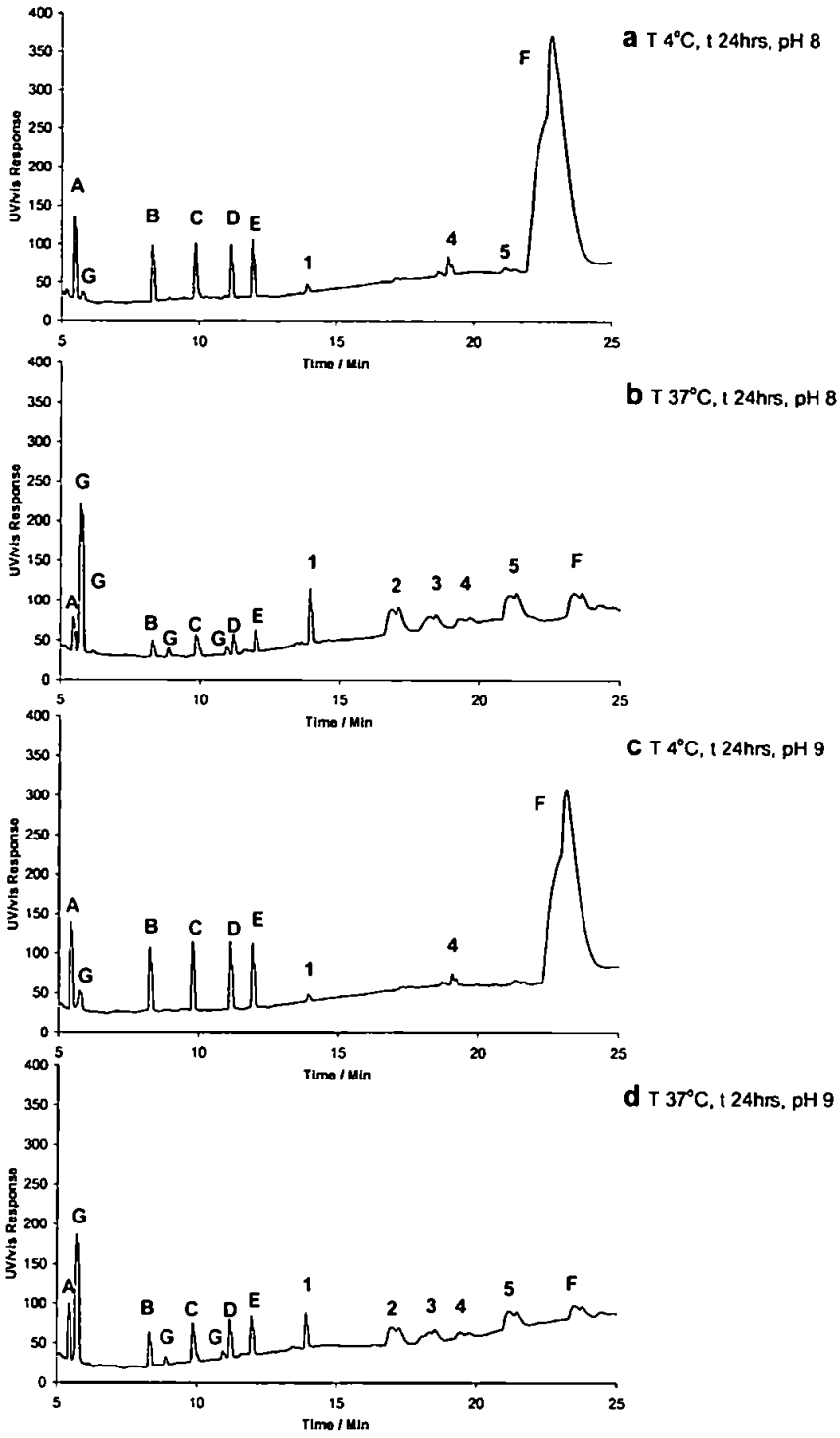

Figure 4-10 HPLC chromatograms, with UV/vis detection at 220nm, of the peptide mix derivatised (24hrs) with SCN-Bz-EDTA. Underivatised peptides: A; B; C; D; E. Derivatised peptides: 1; 2; 3; 4; 5 in the same order. F, SCN-Bz-EDTA; G, SCN-Bz-EDTA by-products

4.3.2.1 LC-ICP-MS of SCN-Bz-EDTA Derivatised Peptides

The procedure to chelate indium with derivatised SCN-Bz-EDTA complexes, developed by Meares *et al.*[115] recommended using 0.1 M ammonium citrate buffer adjusted to pH 6 using citric acid. Figure 4-11 shows the chelation of indium to SCN-Bz-EDTA in ammonium citrate and sodium acetate pH 6 (Figure 4-11a and Figure 4-11c) respectively. Previously, when chelating hydrolysed cDTPA, only two chromatographic peaks would elute; free metal and cDTPA-metal complex. However, when SCN-Bz-EDTA was chelated with indium several chromatographic peaks were resolved. The only peak that could be identified with confidence was the unreacted SCN-Bz-EDTA-In, eluting at 22 min (peak A). This was suggested because of the reduction in peak area that occurred when the SCN-Bz-EDTA sample contained bradykinin. Molecular mass spectrometry would be required to identify the In-complexes formed during chelation. Figure 4-11b and Figure 4-11d show the LC-ICP-MS chromatograms of bradykinin derivatised with SCN-Bz-EDTA and chelated with indium in ammonium citrate and sodium acetate respectively. The LC-UV/vis traces prior to indium chelation suggested that derivatisation of bradykinin was successful. However, the similarity of the two corresponding LC-ICP-MS traces suggested that no derivatised bradykinin had been chelated with indium. A possible explanation for this is that, once derivatised, the peptide(s) blocked the chelation sites.

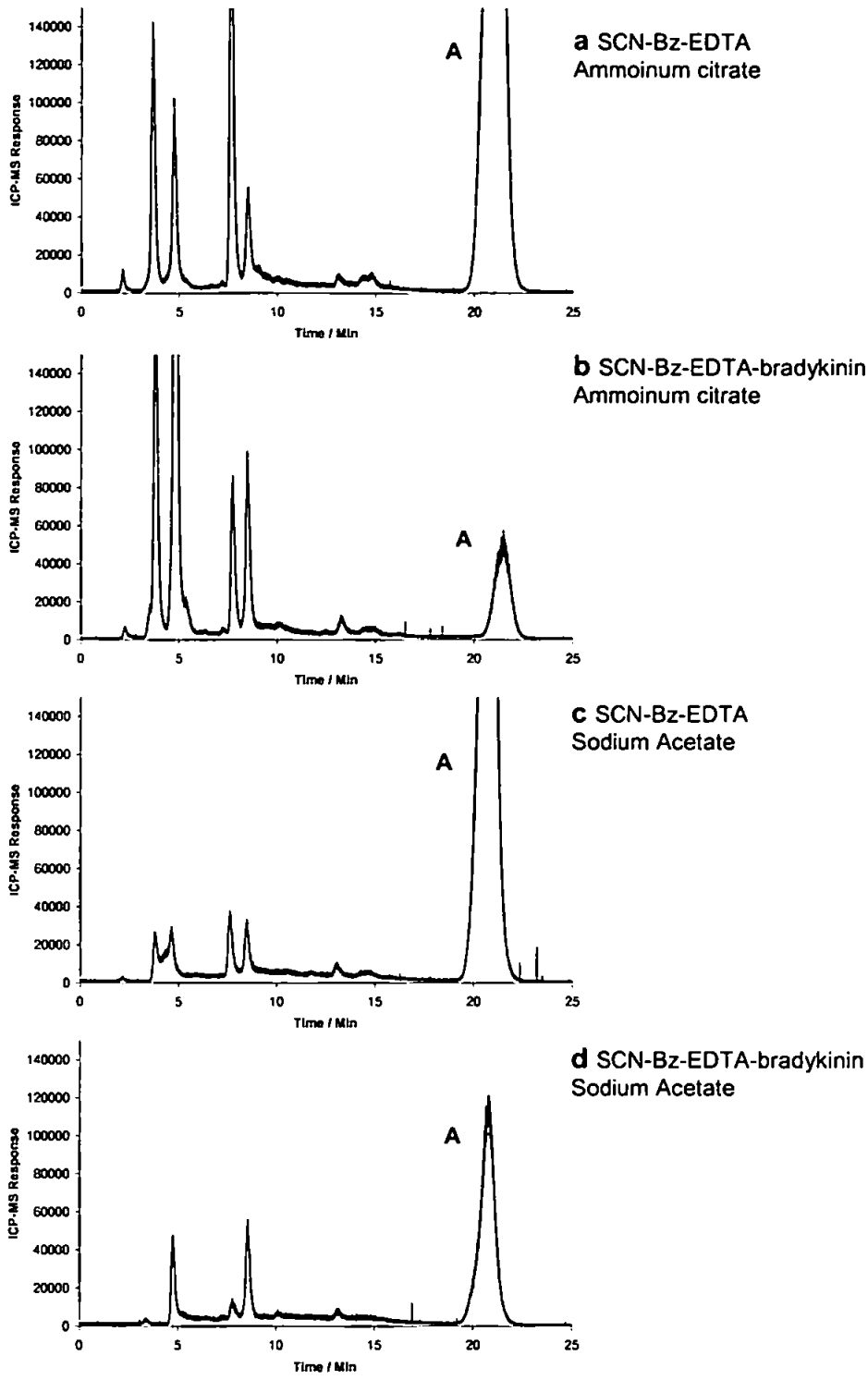

Figure 4-11 LC-ICP-MS chromatograms, with detection at m/z 115 of SCN-Bz-EDTA chelated to indium and SCN-Bz-EDTA derivatised bradykinin chelated to indium in ph 6 ammonium citrate or ph 6 sodium acetate buffer respectively. Peak labelled A, SCN-Bz-EDTA-In. Chromatograms: a, SCN-Bz-EDTA-In in ammonium citrate pH 6; b, SCN-Bz-EDTA-bradykinin-In in ammonium citrate pH 6; c, SCN-Bz-EDTA-In in sodium acetate pH 6; d, SCN-Bz-EDTA-bradykinin-In in sodium acetate ph 6.

4.3.3 Solid Phase Extraction

The LC/UV-Vis chromatogram in Figure 4-12 shows a separation of the peptide mixture used as a reference for solid phase extraction. Peptides loaded onto the SPE tip were recovered into six fractions: unretained fraction (sample loading); 0.1% TFA wash fraction; and the four ACN fractions (representing a step gradient elution). Table 4-3 shows the recoveries of the peptides from the C18 SPE column, the recoveries were calculated as a percentage by comparing the peak area of the fractionated peptides to the total peak area obtained from a reference sample. The peptides were identified as the following: peptide 1, Gly-Tyr (Mw 238.2, hydrophilicity -1.1); peptide 2, Val-Tyr-Val (Mw 379.5, hydrophilicity -1.8); peptide 3, Leu-enkephalin (Mw 555.6, hydrophilicity -1.3); peptide 4, Met-enkephalin (Mw 573.7, hydrophilicity -1.2); and peptide 5, angiotensin II (Mw 1046.2, hydrophilicity -0.3); based on molecular weights and hyrdophilicity of each peptide respectively using a peptide property calculator[121].

The SPE of the peptide mixture confirmed that peptides can be retained on the SPE column and extracted into fractions. The first fraction was the peptide loading itself, however, it was calculated that up to 55% of the first peptide was not retained. The second fraction was a peptide wash, used to remove any unwanted sample matrix, two of the peptides were eluted during this step suggesting that relatively small and polar peptides were not retained on the SPE column. The next fraction was a 25% ACN which removed the remaining peptides in the fraction. The next three fractions increased the ACN elution strength; however, these fractions did not contain any retained peptides. The SPE investigation suggested that SPAD can be utilised to derivatise peptides on solid phase sorbents.

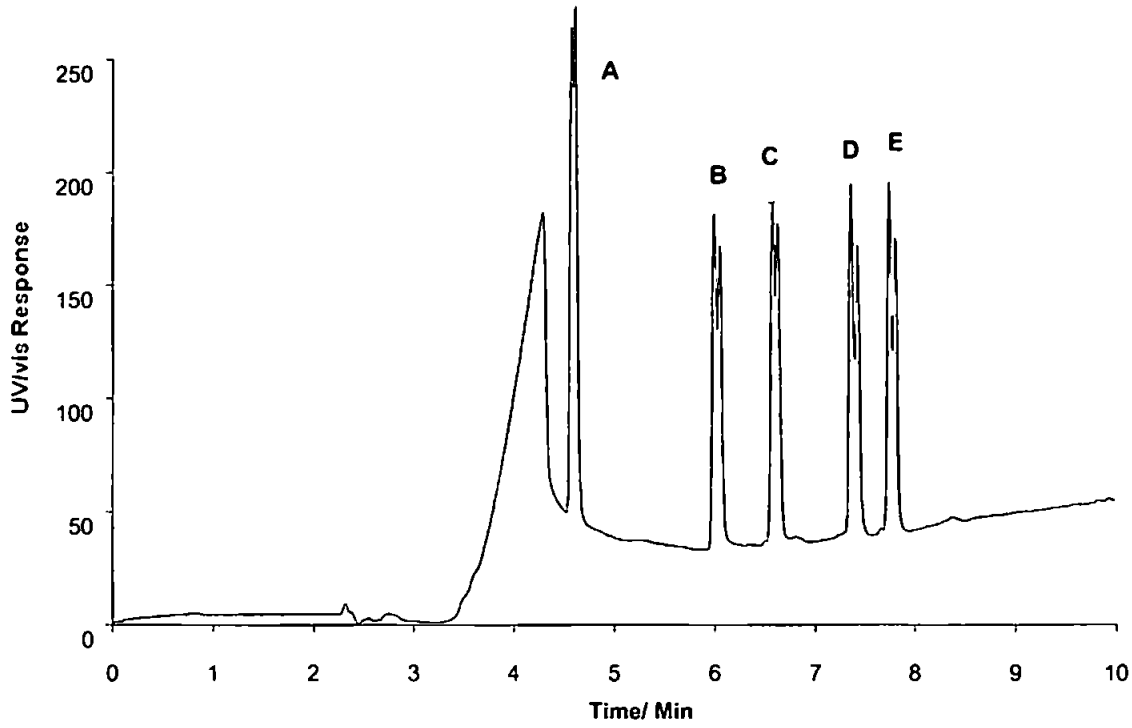

Figure 4-12 HPLC chromatogram with UV/vis detection at 220nm of a reference peptide mixture sample.

Table 4-3 Fractionation and recovery of LC peptide mix using C18 SPE column.

Peptide	Retention Time (Min)	Recovery in the fraction [a] (%)		
		1	2	3
1	4.55	55	28	15
2	6.0	0	17	79
3	6.55	0	0	99
4	7.40	0	3	97
5	7.8	0	0	100

[a] **Fractionation of peptides carried out as described in experimental section (5.2.3). After loading three fractions were collected (1) unretained, (2) peptide wash (0.1% TFA) and (3) peptide elute (0.1% TFA in 25% ACN).**

In addition to SPAD, SPE can be used to isolate analytes of interest from other interfering analytes as shown in Figure 4-13, the solid phase extraction of bradykinin derivatised with SCN-Bz-EDTA. The sample was extracted into several fractions, Figures 4-13b to Figure 4-13j. Figure 4-13a shows the original sample prior to SPE with peaks labelled: A, bradykinin; B, SCN-Bz-EDTA derivatised bradykinin; C, SCN-Bz-EDTA; and D, SCN-Bz-EDTA by-products. Figure 4-13b shows the sample loading step, in that fraction some of the unreacted SCN-Bz-EDTA was eluted from the SPE tip, however no other sample analytes were eluted, this would suggest that either the SPE tip was overloaded with SCN-Bz-EDTA or the SCN-Bz-EDTA formed into by-product complex and was eluted in this fraction. Subsequent fractions increased in eluent (ACN) strength starting from 0.1% TFA to 0.1% TFA in ACN. No analytes of interest where eluted in the three fractions. Bradykinin was eluted in the 15% and 25% ACN fractions (Figures 4-13f and 4-13g), though majority of the bradykinin was eluted in the latter. In addition, the 25% ACN fraction eluted the majority of the derivatised bradykinin as well as a SCN-Bz-EDTA by-product. The remaining unreacted SCN-Bz-EDTA was eluted in the remaining ACN fractions. No LC-ICP-MS data was obtained from the SPE of derivatised bradykinin because of the inefficient chelation method. However, it has been shown that derivatised peptide can be isolated from excess reagents; therefore the SPE method can be applied to isolate chelated peptides.

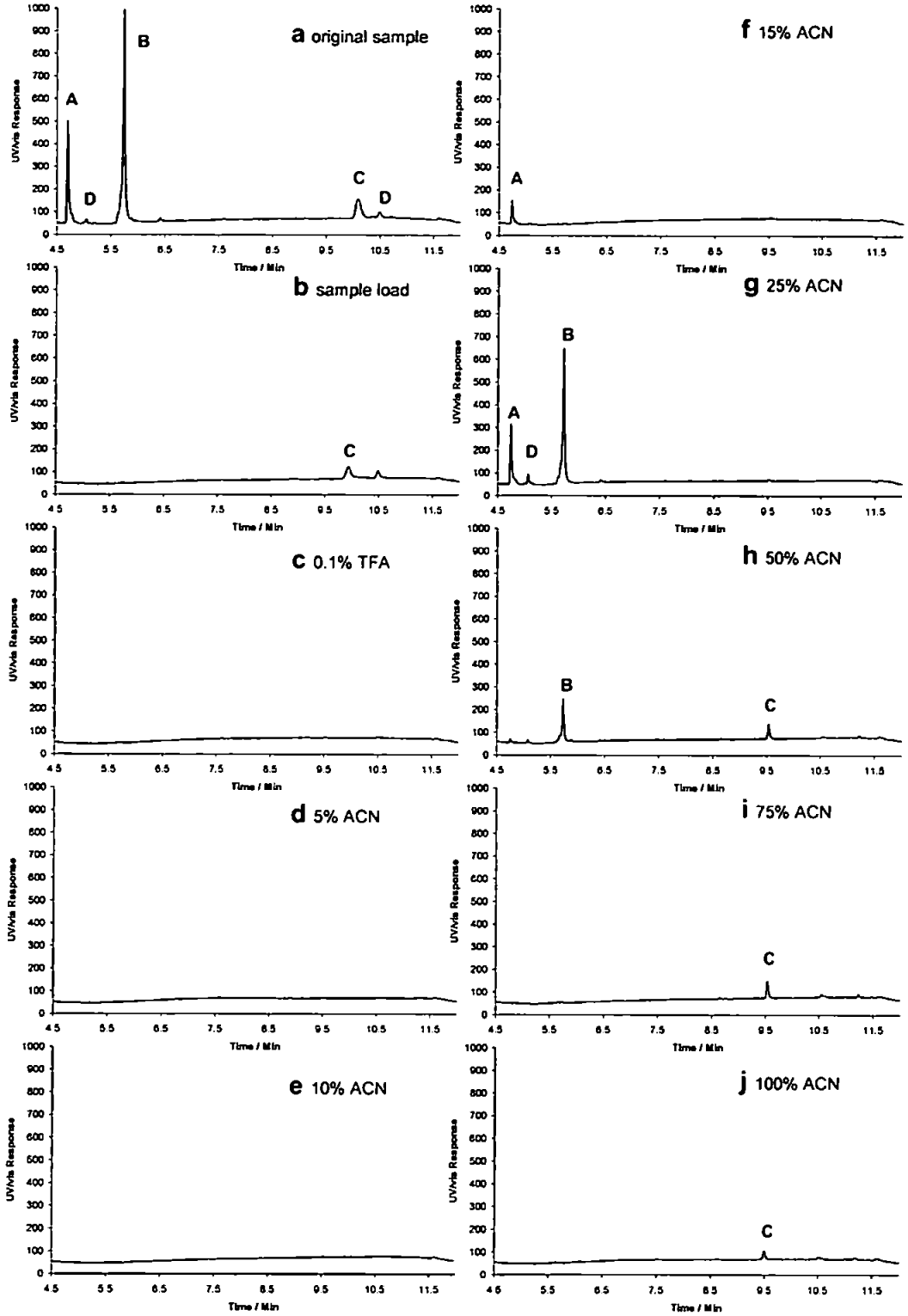

Figure 4-13 HPLC chromatograms with UV/vis detection at 220nm of the solid phase extracted fractions from the derivatisation reaction of bradykinin with SCN-Bz-EDTA. Peaks identified from retention times as: A, bradykinin; B, bradykinin-SCN-Bz-EDTA; C, SCN-Bz-EDTA; and D SCN-Bz-EDTA by-products

4.3.4 *Solid Phase Analytical Derivatisation*

Initial experimental data showed that the derivatisation of peptides on the solid phase using cDTPA was not successful, as all peptides and reagent were eluted from the solid phase sorbent by the DMSO loading solvent in the derivatisation fraction. The peptide eluted from the solid phase sorbent due to the polarity of DMSO. Unfortunately, cDTPA was not soluble in any of these solvents, and hydrolysed when dissolved in water, so DMSO was the only effective solvent for derivatisation. Table 4-4 shows some of the other solvents and their respective polarities compared to water that were used for solid phase derivatisation.

Table 4-4 Relative polarity and the solubility of cDTPA in the organic solvents.

Organic Solvent	Relative Polarity [a]	cDTPA solubility
DMSO	0.444	Yes
Acetonitrile	0.460	No
Tetrahydrofuran	0.207	No
Ether	0.117	No
Acetone	0.335	No
Water	1.000	Yes

[a] **Relative polarity compared to water**

Hence, SCN-Bz-EDTA (Figure 4-2) was selected as the reagent of choice for SPAD, because the reagent is soluble in water, a major factor for SPAD. The derivatisation conditions used for SPAD were similar to the optimised conditions used for the solution phase derivatisation of bradykinin. The trivalent metal addition step was excluded, due to lack of chelation with derivatised peptides. The reaction temperature was 37°C, maintained using a water bath, with a reaction pH of 8. The flow rate through the solid phase column was maintained at 0.1 ml min^{-1}.

The eluent from each step in the SPAD method was collected and analysed by separately by HPCL-UV/vis. All peaks were identified by retention times.

Figure 4-14a shows the peptide load and, as expected, no peptide was eluted from the column. The next step was the on-column derivatisation, this resulted in Figure 4-14b, which shows elution contained a small amount of SCN-Bz-EDTA. Next, an incremental elution of the retained compounds was performed, starting with 25% ACN (Figure 4-14c) which eluted underivatised bradykinin. Next, 50% ACN (Figure 4-14d) eluted bradykinin and SCN-Bz-EDTA derivatised bradykinin. All the remaining bradykinin was eluted in the 75% ACN fraction, along with more derivatised bradykinin and unreacted SCN-EDTA (Figure 4-13e). The final fraction was the 100% ACN fraction which eluted the remaining derivatised bradykinin and unreacted SCN-Bz-EDTA (Figure 4-13f). The derivatisation efficiency was calculated based on peak area of the underivatised bradykinin, and was determined to be 5%. This suggested that derivatisation of peptides using SCN-Bz-EDTA on a solid phase sorbent was not feasible. The low derivatisation efficiency was probably due to the actual derivatisation being slow and solid phase derivatisation reactions are required to be rapid due to the short interaction time for the derivatisation reaction to occur. The preconcentration effect was not observed during these experiments as the elution volume was greater than the sample loading volume. However, the majority of the SCN-Bz-EDTA derivatised bradykinin was separated from the derivatisation reagent which suggests that solution phase derivatisation can be utilised prior to SPE.

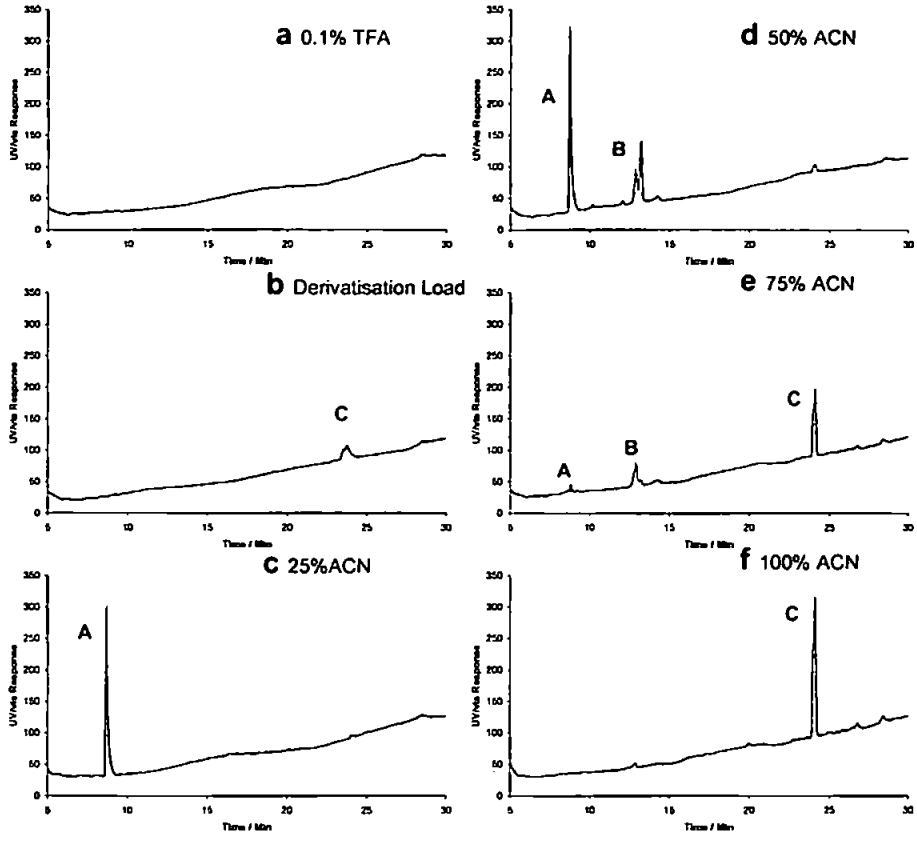

Figure 4-14 HPLC chromatograms, with UV/vis detection at 220nm of the fractions collected fromsolid phase analytical derivatisation of bradykinin with isothiocyanato-benzyl-EDTA. Peaks were identified: A, bradykinin; B, SCN-Bz-EDTA derivatised bradykinin; C, SCN-Bz-EDTA.

4.4 Conclusions

Different techniques have been investigated for the derivatisation of complex samples such as the evaluation of a monoreactive bifunctional chelating agent and solid phase analytical derivatisation and solid phase extraction. These investigations have stemmed from the inherent disadvantage of using cDTPA as a derivatisation agent. cDTPA derivatisation of a peptide mixture resulted in the formation of additional by-products from intermolecular and intramolecular species due to the reagent containing two reactive groups.

The use of a monoreactive isothiocyanate-benzyl-EDTA for the derivatisation of a peptide mixture resulted in no additional by-products being formed. However, it was noted that the instability of the reagent over a period of time complicated analysis. In addition, the reagent was found not to chelate to either indium or europium once derivatised which rendered ICP-MS detection and quantitation ineffective. To improve the derivatisation efficiency between peptide(s) and SCN-Bz-EDTA, the use of solid phase analytical derivatisation was investigated; subsequent analysis suggested that efficiency could not be improved compared to the original solution phase derivatisation. However, a benefit was that the selective elution of the derivatised products and isolation from reagents suggests that SPE can be utilised prior to LC-UV/vis analysis. Further investigations in to improving the current labelling strategy is ongoing, reagents such as SCN-Bz-DTPA. The reagent was selected due to the success of chelation of indium and europium to the DTPA chelating agent. As well as, utilising solid phase extraction to isolate derivatised and chelated peptides.

5. INTRODUCTION

The analysis of complex samples requires the use of various sample handling techniques, such as the solid phase extraction and pre-fractionation to remove matrix interferences and reduce sample complexity respectively. We have previously shown that monoreactive BFCAs such as, isothiocyanate-benzyl-EDTA can be used to derivatise relatively complex samples, without the formation of unwanted peptide by-products. In addition solid phase extraction allowed the isolation of the analyte from excess reagent prior to analysis.

In this chapter, the relatively quantitation of peptide samples is described by combining the methods and techniques discussed in the previous chapters using isothiocyanate-Bz-DTPA as the BFCA.

5.1 Relative Quantitation of Complex Samples

Current relative quantitation methods employ a variety of techniques to either remove excess reagent, underivatised peptides or enrich specific peptides. For example, the first generation ICAT[36] reagent contains a biotin group attached to afford the isolation of tagged peptides by biotin/avidin chromatography or by using a selective capture/enrichment step to isolate peptides containing cysteine and histamine[49].

In the previous study it is shown that derivatised peptides can be isolated from the derivatisation reagents using solid phase extraction (section 4.3.3). This isolation and selective extraction allows derivatisation and chelation reagents to be used excess reagents. Hence, increasing derivatisation efficiency and allowing fractionation of complex samples.

5.1.1 Isothiocyanate-benzyl-DTPA

Based on previous research isothiocyanate-benzyl-DTPA (SCN-Bz-DTPA) was selected as an alternative BFCA to SCN-Bz-EDTA, because of the relative optimised conditions for isothiocyanate reactions with peptides and successful chelation of trivalent metals to DTPA.

5.2 Experimental

The methods described in this section utilise the procedures described in the previous chapters such as peptide derivatisation with isothiocyanate, differential isotopic labelling using europium for relative quantitation and solid phase extraction.

5.2.1 Reagents and Standards

Bradykinin acetate, HPLC peptide mix, sodium acetate, sodium carbonate, ammonium carbonate and trifluoroacetic acid (reagent grade) were purchased from Sigma Aldrich, (Poole, Dorset, UK). HPLC grade acetonitrile and methanol were obtained from Fisher Scientific UK Ltd, (Loughborough, Leicestershire UK). Ammonium bicarbonate was purchased from Fluka, Sigma Aldrich, (Poole, Dorset, UK). Isothiocyanate-benzyl-DTPA was purchased from Macrocyclics (Dallas, Texas, USA). Distilled deionised water (DDW) 18.2 MΩ, was obtained using an Elga Maxima water purifying system. ^{151}Eu-enriched europium (Eu2O3 99.24% enriched) was purchased from CK Gas Products, (Hampshire, UK). A standard ICP-MS solution of natural europium was purchased from Alfa Aesar (Heysham, UK). Indium atomic spectroscopy standard was purchased from VWR International (Poole, Dorset, UK).

5.2.2 Instrumentation

All LC analyses were performed using the Luna C18 (2) column (Phenomenex, Macclesfield, UK) for reversed phase LC-UV/vis and LC-ICP-MS analysis. The mobile phase was a binary system of: eluent A, water containing 0.05% formic acid (v/v); and eluent B, acetonitrile containing 0.05% (v/v) formic acid. The linear

gradient employed started at 100% A, changing to 100% B over 20 minutes. The flow rate was 1 ml min^{-1} with an injection volume of 25 µL for all analyses.

The LC system used for the UV/vis and UV/vis-ICP-MS analysis was a HP 1090 liquid chromatograph (Agilent Technologies, Stockport, UK). Element specific detection was performed using quadrupole ICP-MS (VG PlasmaQuad 3, Thermo Elemental, Winsford, UK). The ICP-MS sample introduction system consisted of a 1.0 ml min^{-1} microMist nebuliser and a cyclonic spray chamber both purchased from Glass Expansion (AUS). The LC outlet was connected to the ICP-MS nebuliser via 2 m of Teflon tubing (1/16" x ID 0.18mm). A membrane desolvator (Vestec Corporation, Texas, USA) was connected between the spray chamber (Nebuliser) and torch to remove organic solvents. The dry aerosol exiting the desolvator was then transferred to the ICP torch via 1 m of 0.25 in. i.d. Tygon tubing. Operating and data acquisition conditions are shown in Table 5-1.

Table 5-1 Operating and data acquisition conditions for Quadrupole ICP-MS.

ICP	
Nebuliser gas flow / L min^{-1}	1.2
Auxiliary gas flow / L min^{-1}	0.8
Coolant gas flow / L min^{-1}	14.0
RF forward power / W	1350
Nebuliser	1.0 ml min^{-1} Micromist
Spray chamber	Jacketed quartz cyclonic (room temperature)
Torch	Quartz fassel-type
Interface	
Sampling cone	Nickel, 1mm i.d.
Skimmer cone	Nickel, 0.7mm i.d.
Time Resolved Analysis	
Masses monitored	^{115}In or ^{151}Eu, ^{152}Sm, ^{153}Eu, ^{154}Sm
Data Acquisition	Peak Jump
Dwell time / ms	10
Channels per mass	5
Membrane Desolvation	
Sweep gas	Argon
Desolvation gas flow / L min^{-1}	0.9
Desolvation temperature / °C	80.0

5.2.3 Peptide Derivatisation and chelation

Bradykinin (0.2 g 0.2 mM) was derivatised according to the optimised method in section 4.2.3 with 0.2 g of SCN-Bz-DTPA (0.4mM) with a futher 0.1 g of 0.1 M sodium carbonate buffer (pH 8) was added to give a final mass 0.5 g. The sample were left to react over 2 hrs in a 37°C water bath. The chelation of trivalent metal to the SCN-Bz-DTPA derivatised peptides was performed according to the method in section 2.2.4. After two hours of derivatisaiton, a 0.1 g aliqout of the derivatised bradykinin sample was added separately to 0.1 g indium (0.32 mM). The resulting was then further diluted to 10 g using 0.1 M sodium acetate buffer adjusted to pH 6 using acetic acid and left to stand for 30 minutes. The remaining derivatised bradykinin sample was analysed by LC-UV/vis to determine whether derivatisation was successful.

5.2.3.1 Peptide Mix Derivatisaiton and Chelation

A HPLC peptide mixture (0.2 g) with a total peptide concentration of 0.2 mM was derivatised with SCN-Bz-DTPA (0.2 g 0.4 mM) with a futher 0.1 g of 0.1 M sodium carbonate buffer (pH 8) was added to give a final mass 0.5 g in a similar manner to the method described in section 5.2.3. The sample was derivatised for 24 hours at 37°C in a water bath. The chelation of indium to the derivatised peptides was performed by adding 0.1 g of indium (0.32 mM) to 0.1 g of derivatised peptides with further dilution using 10 g of 0.1 M sodium acetate buffer pH 6 and left to stand for 30 minutes.

5.2.4 Solid Phase Extraction and Relative Quantitation

Relative quantitation was performed according to the method described in section 3.3.3. Bradykinin (0.2 g 0.5 mM) was derivatised with SCN-Bz-DTPA (0.2 g 2 mM) the sample was futher diluted to 0.5 g with 0.1 M sodium bicarbonate pH 8. A 0.1 g aliquot of the derivatised sample was chelated with natural europium (0.1 g 1 mM). Using the chelation method described in section 2.2.4, the sample was then further diluted using 9.8g of sodium acetate to adjust the pH to 6. Additionally samples were prepared containing 0.5 mM, 0.25 mM and 0.125 mM of bradykinin using the same method derivatisation and chelation as above, however chelated with enriched europium (1mM). The chelated samples were left to stand for 30 minutes to ensure chelation.

Once chelated equal gravimetric amounts of the enriched ^{151}Eu labelled peptides (0.5 g) were combined with natural Eu labelled peptides (0.5 g) and left to stand for 10 minutes for equilibration prior to SPE or LC-ICP-MS analysis. Solid phase extraction followed the method described in section 4.2.6, where labelled samples were extracted into several fractions, using incremental eluent strenght. The solid phase extracted sample was analysed by LC-ICP-MS to validate the Eu isotope ratio obtained from the SPE method.

5.3 Results and Discussion

5.3.1 Peptide Derivatisation and Chelation

The LC-UV/vis chromatogram in Figure 5-1 and 5-2 shows the derivatisation of bradykinin and a HPLC peptide mixture using SCN-benzyl-DTPA respectively. The method for derivatisation was previously described in section 4.3.4 for the reaction between isothiocyanate and peptides. The derivatisation efficiency of the reaction between bradykinin and isothiocyanate-benzyl-DTPA was determined to be 55% based on peak areas of underivatised bradykinin in Figure 5-1B. In addition, the derivatised bradykinin chromatogram (Figure 5-1B) suggested that more than the expected number of peaks were eluted. The masses of the derivatised products could not be determined by infusion molecular mass spectrometry due to the high sodium content in the derivatised products.

Figure 5-2 shows the derivatisation of a HPLC peptide mixture, from the peak areas of the underivatised peptides, the derivatisation efficiency of reaction between SCN-Bz-DTPA and peptide mixture was determined to be 46% based on the disappearance of the peptides. However, it was observed that though the underivatised peptides reduced in peak area by 46% between the reference and derivatised samples there was no increase in SCN-Bz-DTPA derivatised peptide peaks. This could either suggest that the derivatisation efficiency was affected at low analyte concentration or that the derivatised peptide complexes are not stable in solution and broken down during the derivatisation reaction.

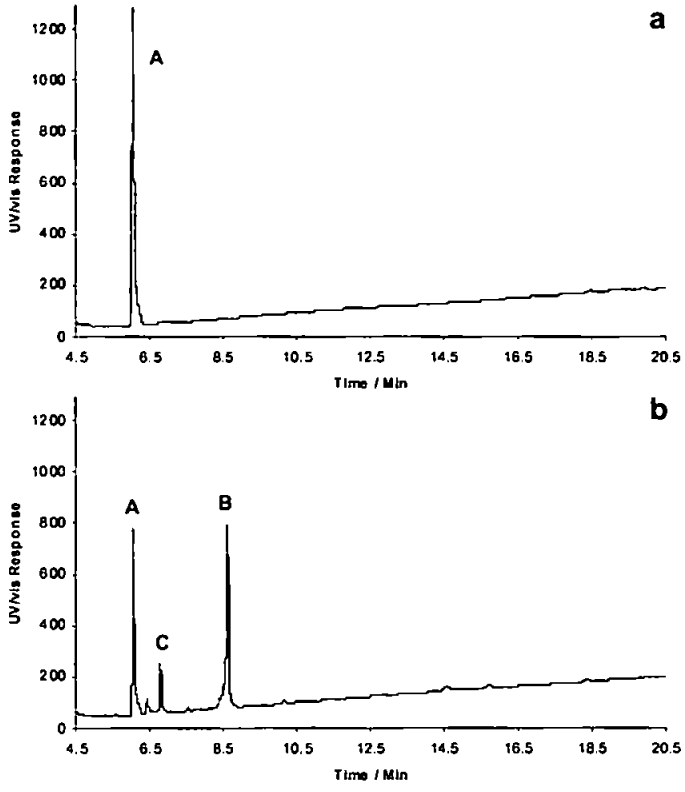

Figure 5-1 HPLC chromatograms with UV/vis detection at 220nm of bradykinin derivatised with SCN-Bzl-DTPA: a, bradykinin; b, SCN-Bz-DTPA derivatised bradykinin; Peaks identified as: A, bradykinin; B, SCN-Bz-DTPA-bradykinin and C, SCN-Bz-DTPA by-products.

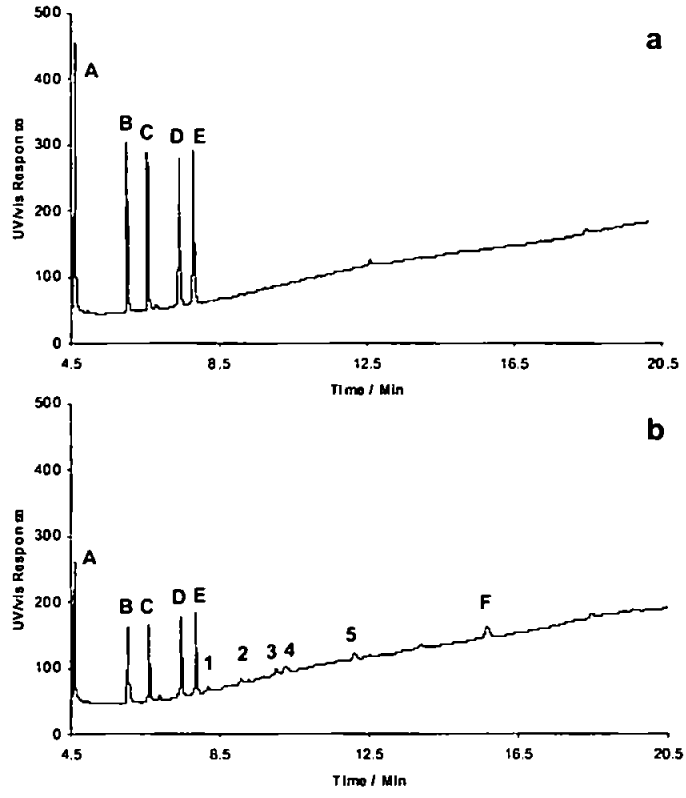

Figure 5-2 HPLC chromatograms with UV/vis detection at 220nm of a HPLC peptide mixture derivatised with SCN-Bzl-DTPA: a, bradykinin; b, SCN-Bz-DTPA derivatised bradykinin; Peaks identified as: A-E, underivatised peptides; (1-5), SCN-Bz-DTPA derivatised peptides, and F, SCN-Bz-DTPA by-products.

The corresponding LC-ICP-MS chromatograms for the derivatised bradykinin and peptide mixture are shown in Figure 5-3 and 5-4 respectively. The derivatised peptides were chelated with indium in sodium acetate buffer at pH 6.1 and 6.5. The effect of pH of the sodium acetate buffer was critical to chelation, it was seen that more indium was chelated to DTPA at a sodium acetate buffer of pH 6.1 than at buffer pH of 6.5. The chromatogram in Figure 5-3 contains three resolved peaks that correspond to: A, free In^{3+}; B, SCN-benzyl-DTPA-In; and C, bradykinin-SCN-Bz-DTPA-In based on the respective retention times. Figure 5-4 shows the chromatogram of derivatised and indium chelated peptide mix. Peaks that are can be determined are: A, free In^{3+} and B, SCN-benzyl-DTPA-In. In addition, two smaller peaks were visible at 9.8 min (C) and 10.2 min (D). These could be derivatised peptides, however mass spectrometry confirmation would be required.

A possible explanation to the reduced derivatisation efficiency for the HPLC peptide mixture is that the relative concentration of the individual peptides was approximately 5 times less than the bradykinin. This would suggest that the reagent is not suitable for derivatisation at low concentrations; this has been previously seen with cDTPA that below certain concentration efficiency is greatly reduced. According to literature on SCN-benzyl-DTPA, the typical molar ratio of reagent to analyte is 100:1 in excess of SCN-benzyl-DTPA. However, using this amount of excess reagent for derivatisation would not be feasible for the current method of analysis by LC-ICP-MS as it would result in inefficient chromatography, column and ICP-MS detector deterioration. A possible solution would be to remove excess reagents, prior to analysis such as using solid phase extraction.

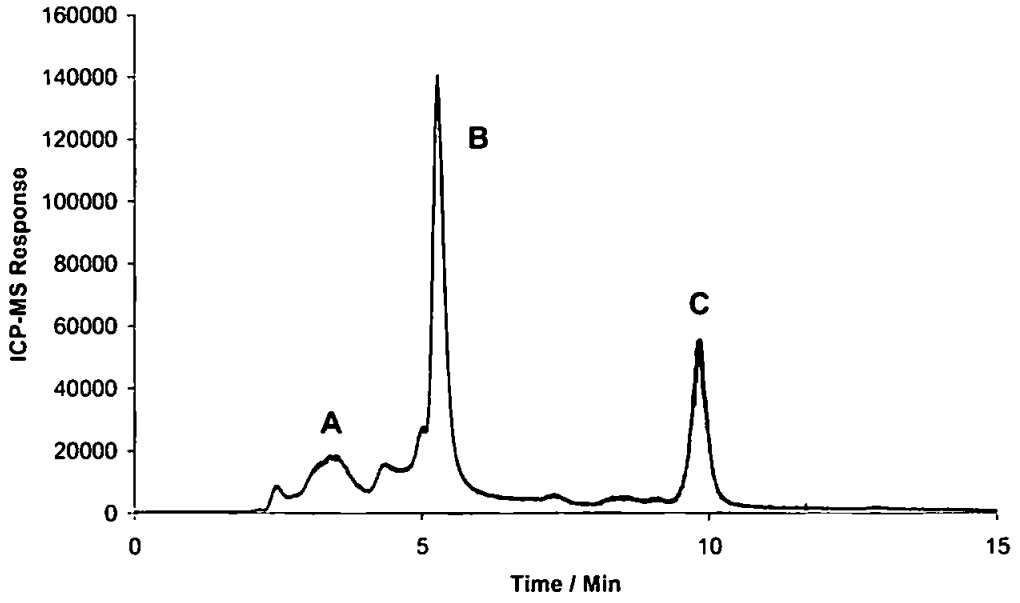

Figure 5-3, ICP-MS chromatogram at m/z 115 of isothiocyanate-benzyl-DTPA derivatised peptide mix with indium chelation. Peaks labelled; A, free In^{3+}; B, SCN-Bz-DTPA-In; C, indium labelled bradykinin.

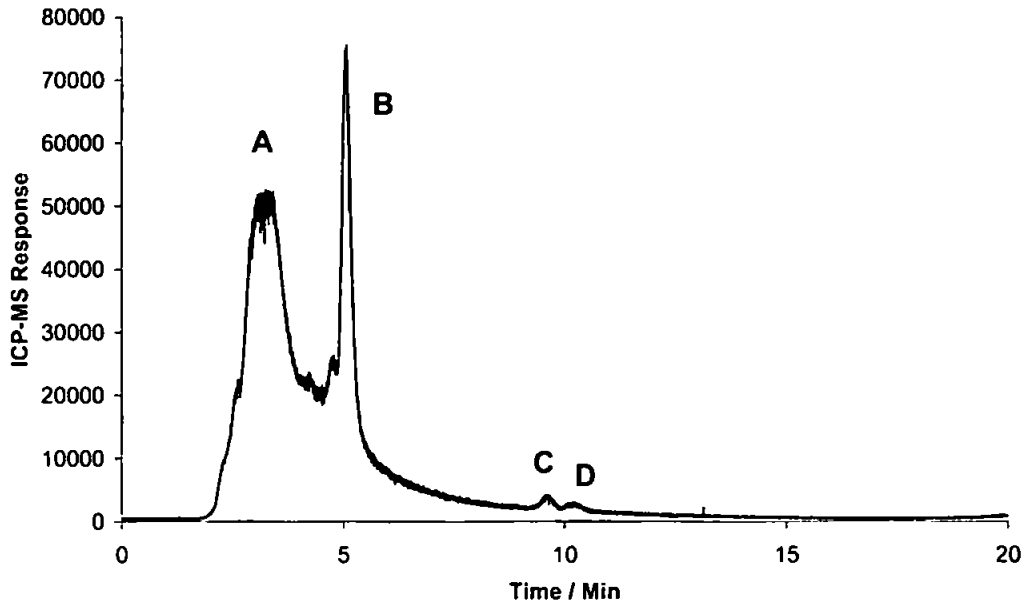

Figure 5-4, ICP-MS chromatogram at m/z 115 of isothiocyanate-benzyl-DTPA derivatised peptide mix with indium chelation. Peaks labelled; A, free In^{3+}; B, isothiocyanate-benzyl-DTPA-In; C and D, possible indium labelled peptides.

5.3.2 *Solid Phase Extraction of Europium Labelled Peptides*

Previous investigation for the use of the solid phase analytical derivatisation of peptides approach showed that is was not suitable for the derivatisation of peptides using isothiocyanate based BFCA's. This was primarily due to the time required for the reagent to be on the solid phase for derivatisation. However, solid phase extraction can be applied to method after derivatisation and chelation. The advantage of using solid phase extraction prior to LC-ICP-MS analysis is that derivatisation and chelation reagents can be used in a much greater excess hence, ensuring near complete derivatisation and chelation. In addition, excess reagents can be isolated and do not interfere in the subsequent analysis.

Figure 5-5 shows the LC-ICP-MS chromatograms of europium labelled bradykinin solid phase extracted. The sample was analysed by prior to SPE by LC-ICP-MS and the resulting chromatogram is shown in Figure 5-5a. The chromatogram contains for four resolved components: peaks A, Free Eu^{3+}; B, SCN-Bz-DTPA-Eu; C, bradykinin-SCN-Bz-DTPA-Eu; and D, SCN-Bz-DTPA-Eu by-product. The resulting SPE is shown in LC-ICP-MS chromatograms Figure 5-5b through to Figure 5-5j. It was shown that majority of the free Eu^{3+} was eluted in the 5% ACN fraction (Figure 5-5d).

In Figure 5-5f, the 15% ACN fraction all of the SCN-Bz-DTPA-Eu was eluted off the SPE tip, however, this fraction also contained the SCN-Bz-DTPA-Eu by-product and a fraction of the Eu labelled bradykinin. In contrast, Figure 5-5g 25% ACN fraction contained only the Eu-labelled-bradykinin. Subsequent fractions Figures 5-5h through to Figure 5-5j resulted in no discernible amount of respective

peaks being eluted. It was noted that SPE improved the chromatographic peak shape of the resulting eluted peaks.

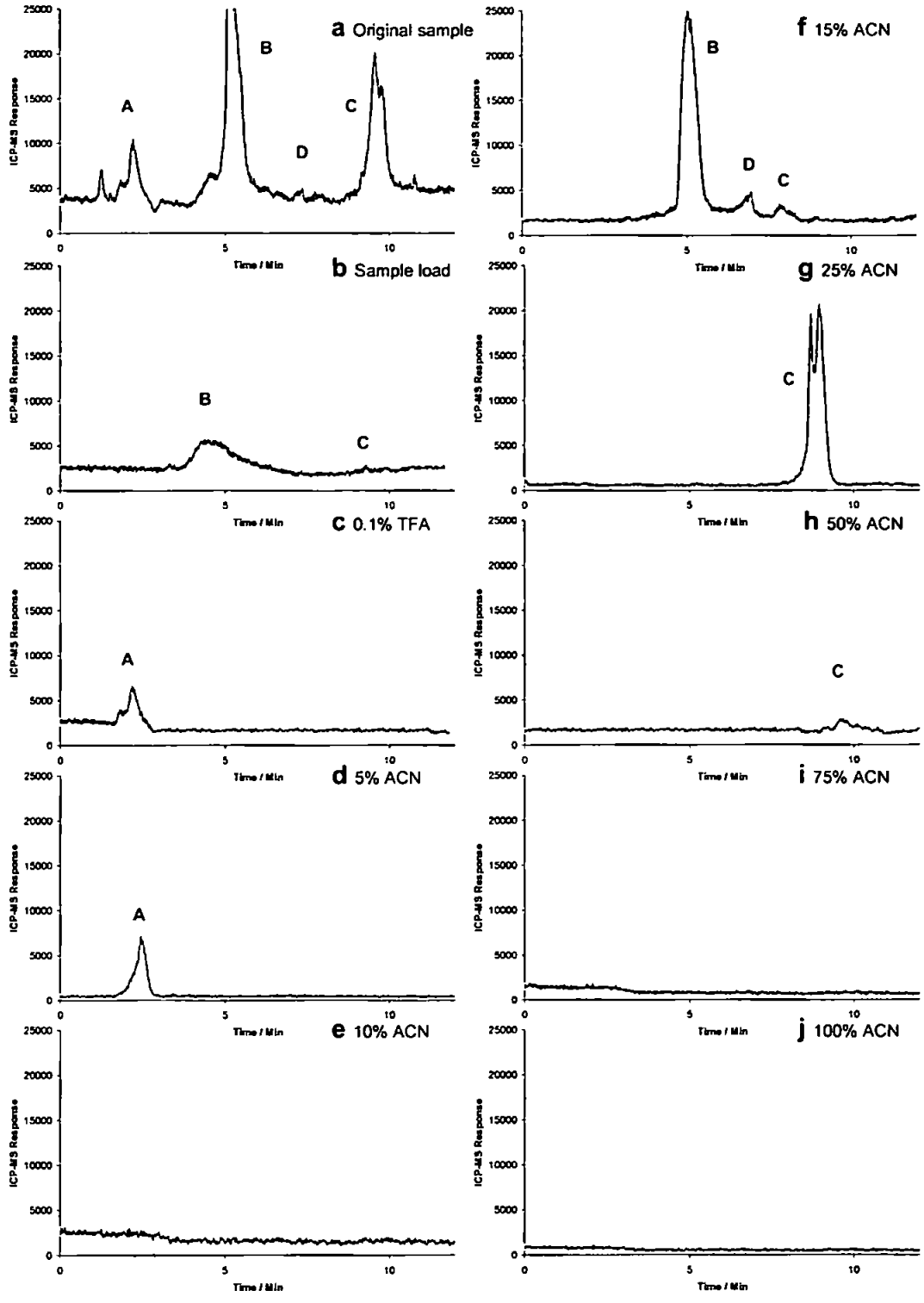

Figure 5-5 LC-ICP-MS chromatograms at m/z 151 detection of the solid phase extracted fractions from the derivatisation and chelation of bradykinin with SCN-Bz-DTPA and natural europium respectively. Peaks identified from retention times as: A, free Eu^{3+}; B, SCN-Bz-DTPA-Eu; C, bradykinin-SCN-Bz-DTPA-Eu; and D,SCN-Bz-EDTA-Eu by-products.

5.3.3 Solid Phase Extraction and Peptide Relative Quantitation

Relative quantitation of peptides was achieved with europium labelling and the selective isolation and extraction. Using the method described in section 5.2.3 samples were solid phase extracted and the only the 25% ACN fraction analysed by LC-ICP-MS. In addition, to validate the measured isotope ratio obtained through SPE the original sample prior to SPE was analysed by LC-ICP-MS shown in Figure 5-6.

Using the pseudo steady approach described in section 3.2.3 and subsequent isotope dilution mass spectrometry (IDMS) analysis the resulting peptide amounts in the sample were determined. Table 5-2 compares the theoretical peptide ratio expected to the measured IDMS peptide ratio with the respective relative errors and expanded uncertainty on the measured peptide ratio shown for either extracted or unextracted europium labelled bradykinin.

Samples 1-6 show the relative quantitation of bradykinin it was observed that there was a significant difference in the theoretical and measured peptide ratios approximately 50% or greater in some cases whether samples were solid phase extracted or not. In addition, a natural europium labelled sample was analysed and in those cases ratios measured and theoretical ratios did not agree. However it was noted that the there was good agreement in the measured ratios between extracted and unextracted samples. This suggested that SPE can be utilised for sample isolation and extraction.

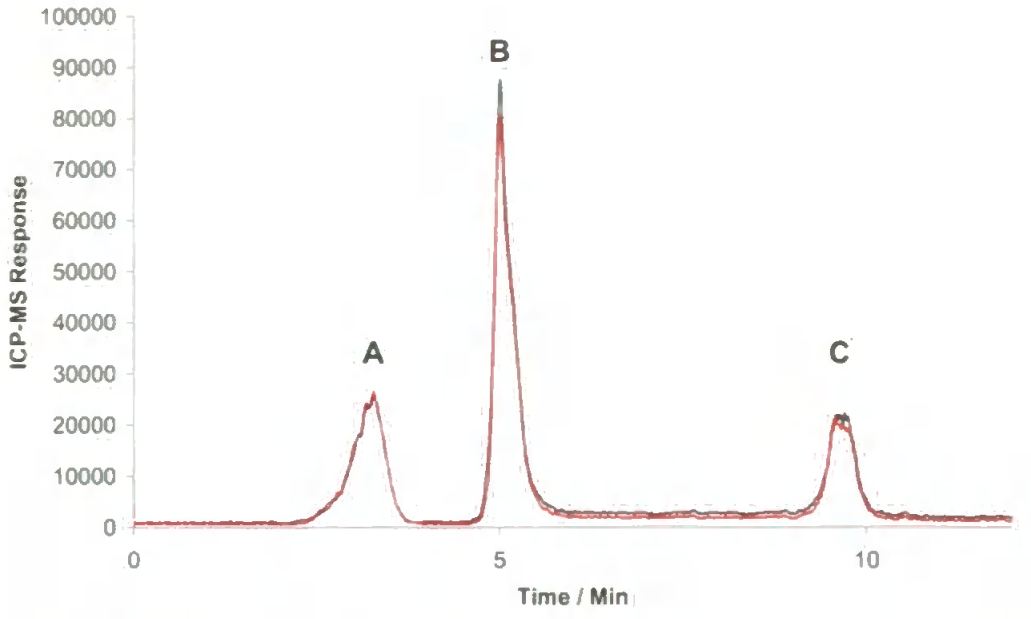

Figure 5-6 Multi-isotope ICP-MS chromatogram of a derivatised peptide mixture containing bradykinin and substance P chelated with Eu using gradient elution profile 1, ^{151}Eu black and ^{153}Eu red. Resolved peaks; A, free Eu^{3+}; B, SCN-Bz-DTPA-Eu^{3+}; and C, Bradykinin-SCN-Bz-DTPA-Eu^{3+}.

Table 5-2 Relative quantitation of peptides comparing theoretical and measured peptide ratios.

Sample	Peptide	Concentration (μM)	Theoretical	Measured		
			Peptide ratio nx/ny	Peptide ratio* nx/ny	± Relative error (%)	± Relative Expanded Uncertainty (%)
Natural	Bradykinin SPE	0.8	1.092	0.9382	23.284	8.695
Natural	Bradykinin*	0.8	1.092	0.9325	22.652	8.686
1	Bradykinin SPE	0.8	3.901	1.378	64.6	8.677
2	Bradykinin*	0.8	3.901	2.023	48.1	6.537
3	Bradykinin SPE	0.8	2.005	1.059	47.0	4.389
4	Bradykinin*	0.8	2.005	0.987	50.6	5.836
5	Bradykinin SPE	0.8	1.195	0.634	46.7	5.565
6	Bradykinin*	0.8	1.195	0.608	48.9	5.814

* represent the LC-ICP-MS analysis performed without SPE

Subsequent investigation into the cause of these m/z 151 biased peptide ratios suggested that the problem was either with the quadrupole analyser or the software (PlasmaLab, Thermo Elemental, Winsford, UK) was not integrating the 151 ion signal. The instrument was integrating one of the channels for m/z 151 twice as high as other the other channels, which results incorrect measured $^{153}Eu/^{151}Eu$ ratio and hence incorrect peptide ratio. This scenario is exaggerated when enriched ^{151}Eu is spiked into the sample as seen in Table 5-2 suggesting a bias towards m/z 151.

5.4 Conclusions

Relative quantitation of bradykinin has been achieved with the SCN-Bz-DTPA and isotopic labelling with europium with subsequent LC-ICP-MS detection. Solid phase extraction was applied to the derivatised and chelated samples to isolate and selectively extract the labelled, so that excess reagents do not interfere in the analysis. The resulting IDMS analysis, suggested that there was no significant difference in the peptide ratios obtained through extraction or not. However, the measured peptide ratio and theoretical peptide ratio were significantly with relative errors greater than 50% observed. However, investigations suggested that this was either due to instrumentation or software faults and not the labelling procedure.

This shows the potential that complex samples can be relatively quantified using differential isotopic labelling with selective isolation and extraction. However, suitable derivatisation reagent must be used and is considered in the next potential area of investigation. The SCN-Bz-DTPA reagent has shown the potential for monoreactive derivatisation, however, due to the reagents poor stability in solution and loss in reactivity at low analyte concentrations it cannot be considered as a viable reagent for relative quantitation.

CHAPTER 6 CONCLUSIONS AND FUTURE WORK

6. CONCLUSIONS AND FUTURE WORK

6.1 Conclusions

The aim of this work was to develop a method to relatively quantitate peptides using ICP-MS detection. Majority of naturally occurring peptides are not detectable by ICP-MS and require some form of chemical derivatisation to facilitate ICP-MS detection. ICP-MS peptide detection was made amenable through the derivatisation of the N-terminal amine group with various bifunctional chelating agent (BFCA), cyclic diethylenetriaminepentaacetic acid anhydride (cDTPA), isothiocyanate-benzyl-ethylenediaminetetraacetic acid (SCN-Bz-EDTA) and isothiocyanate-benzyl-diethylenetriaminepentaacetic acid (SCN-Bz-DTPA). Once derivatised with peptides these BFCA were then subsequently chelated with trivalent metals such as indium and europium respectively. The detection of these element labelled peptides has been achieved using LC coupled with ICP-MS via membrane desolvation which facilitated the use of gradient chromatography.

Using this derivatisation and labelling technique, biologically important peptides bradykinin and substance P were relatively quantified. This was achieved by derivatising two gravimetrically known amounts of peptide samples with cDTPA and subsequently chelating the samples with either natural Eu or enriched ^{151}Eu respectively. Once chelated, equal amounts of the samples are combined and analysed by LC-ICP-MS. The resulting 153/151 Eu isotope ratio is measured using the pseudo steady state approach. The blank and mass bias corrected 153/151 Eu ratio was used to calculate the relative peptide amount in the original peptide samples using a modified isotope dilution mass spectrometry (IDMS) equation.

The ICP-MS measured peptide ratio was calculated to be within 2.8% of the theoretical peptide ratio. However, it was noted that precision was relatively high and hence uncertainty was approximately 13%, which could be attributed to plasma instability.

The peptide-cDTPA and peptide-cDTPA-Eu complexes was characterised using ESI-MS and LC-ESI-MS-MS. However, to facilitate ESI-MS detection, derivatisation and chelation buffers were changed to ammonium bicarbonate and ammonium acetate respectively. The change in derivatisation buffer resulted in an additional conformer of the peptide complex being formed. This conformer had a different chromatographic retention, however, had the same m/z ratio and collision induced fragmentation to the original peptide complex. The original derivatisation method in sodium bicarbonate and sodium acetate did not result in the formation of the additional conformer. This was evident by the respective LC-ICP-MS chromatograms of the peptides in sodium and ammonium based buffers.

When the cDTPA labelling technique was applied to a relatively complex peptide sample, the resulting derivatisation reaction resulted in more than the expected number derivatised products being formed. This was due the cDTPA reagent containing two reactive groups, which can lead to the formation of intermolecular and intramolecular species with one or more peptides. However, once the derivatised peptide mixture was chelated, only five europium chelated peaks were chromatographically resolved. This suggests only the singly derivatised form of the peptides are detectable by LC-ICP-MS. However, the relative quantitation would

be inaccurate because it cannot be predicted how much of each individual peptide is complexed in the intermolecular and intramolecular species.

A monoreactive BFCA, isothiocyanate-benzyl-EDTA (SCN-Bz-EDTA) was used to overcome the formation of the additional by-products. The reagent was successful derivatising a complex mixture of peptides, which resulted in the no additional by products being formed, subsequently the derivatised peptide(s) was labelled with indium. However, chelation of the derivatised peptide(s) was not successful using indium and europium using different buffers and varying pH. It was also noted that the derivatisation efficiency was significantly lower when compared to the derivatisation with cDTPA by approximately 45%.

To improve derivatisation efficiency solid phase analytical derivatisation (SPAD), was employed for peptide derivatisation. The SPAD technique isolates peptides of a solid phase sorbent for derivatisation. The technique allows the use of excess and selective isolation of reagents that can potentially interfere with the analytes of interest. The derivatised peptides was isolated on a conditioned solid phase tip and eluted in fractions of acetonitrile increasing in elution strength. However, a decrease in derivatisation efficiency was noticed when comparing solid phase derivatisation to solution phase derivatisation. The reduction in derivatisation efficiency was due to the reaction time required for derivatisation, solution phase required between 2 to 24 hours for derivatisation, and the SPAD method the reagent was on column for no longer than two minutes. Although SPAD, did not result in the expected increase in derivatisation efficiency, the selective extraction of derivatised was implemented into solution phase derivatisation method of SCN-

Bz-DTPA. Solution phase extraction (SPE) allowed the isolation of derivatised and chelated peptides from excess reagents.

To afford chelation of the isothiocyanate derivatised peptides, SCN-Bz-DTPA was selected as an alternative BFCA. The reagent was chelated with indium and europium using the same method as cDTPA chelation. The labelled peptides (natural and enriched) were combined and solid phase extracted , the labelled peptide was isolated from the excess reagents used in derivatisation and chelation. The solid phase technique was used to relative quantitate bradykinin and compared to relative quantitation obtained from LC-ICP-MS of the original combined sample, however, measured isotope ratios were all biased in favour of m/z 151, hence rendering relative quantitation ineffective due to either instrumentation or software faults.

6.2 Future Work

During the course of this work a number of areas have been identified for further study. The objectives set out at the start of the research project was to develop a method for the relative quantitation of peptides using LC-ICP-MS. Currently the method has only been investigated using control situations, for this method to be a comparable to current relative quantitation techniques development in the tags, labels, and sample preparation of the method must be investigated.

6.2.1 Labelling / Tagging Reagent

Peptide relative quantitation by ICP-MS detection has been shown to afford high accuracy and precision. However, the labelling method itself requires additional research to become comparable to the other current techniques available, in a number of areas such as sample complexity, removal of excess reagents, and isolation of labelled analytes. To establish ICP-MS as a routine analytical tool in proteomics and relative quantitation, a concerted effort must be made in designing and evaluating tags and labels for specific proteomic techniques. This would involve several scientific disciplines to synthesise, characterise and analyse these tags and labels, rather than the current state of affairs where ICP-MS analysts have been evaluating methods for proteomic analysis. Currently tags and labels being used for proteomic analysis are ones that are available on the market though not designed for a particular application.

A possibility would be an investigation into the synthesis of specific tags which already contain a specifically enriched isotope; this would avoid additional steps after the derivatisation. In our laboratory, a tag brominated

tris(trimethoxyphenyl)phosphonium (Br-TMPP) was synthesised by Cartwright *et. al.*[98] to determine the degree of phosphorylation in peptides. The tag contained six bromine atoms and one phosphorous atom, which reacted with the carboxylic residue on peptides and carboxylic acids. The Br-TMPP tag was synthesised to increase the sensitivity of LC-ICP-MS detected carboxylic acids over the first generation TMPP tag. This compound could potentially be used as a template to design other similar tags to be used in this manner.

Considerations for tags and labels:

Reactive group

The reactive group must be monoreactive and stable in solution. The reactive group can either be amino acid specific such as thiol specific reagents (maleimide, iodoacetamide etc) or N-terminal amine specific such as anhydrides

Isotope/Elemental coding

The tag should contain four or more enriched isotopes of either ^{151}Eu or ^{153}Eu, this is because most mass spectrometers do not have sufficient sensitivity at unit mass resolution. Therefore if the tag contained four or more isotopes with a 2Da mass difference this would allow sufficient resolution of tagged peptides for identification using molecular mass spectrometry and detection using ICP-MS. In addition and once derivatised with the peptide, there is a potential to determine the extent of phosphorylation on peptides by monitoring the either ^{151}Eu or ^{153}Eu /P ratio using LC-ICP-MS.

6.2.2 Immunoassays

We have previously described the used of solid phase analytical derivatisation for sample isolation and preconcentration prior to derivatisation. However, proteomic samples can contain many thousands of peptides to isolate, derivatise and detect this could possibly overload any LC-ICP-MS system. A simple method to reduce the numbers of peptides to be analysed and to isolate key peptides if the use of immunoassays.

Immunoassays can be described as the detection and quantitation of antigens with specific antibodies such as Enzyme-Linked ImmunoSorbent Assay (ELISA), which are used to detect antigens and antibodies in biological samples. Performing an ELISA involves at least one antibody with specificity for a particular antigen. The antigen could be an biological complex, peptide, or protein. For example if antibodies can be cultivated that have a specificity for a specific group of peptides such as kinins, the peptides can be immobilised on a solid support. Firstly, the antibodies are immobilized on the solid support, with the sample passed through the wells, specific antigens bind to antibodies through non-covalent bonds and conformational fit. Typical ELISA protocol requires a detection antibody to be added forming a complex with the antigen which can be detected using optical detection by complexing the antigen with a labeled antibody.

6.2.2.1 Relative Quantitation by Immunoassay

The labeling of the peptide for relative quantitation can be achieved with three different approaches:

On-column labeling, derivatisation and chelation of peptides by bifunctional chelating agents are carried out *in situ*. This approach is similar to that to solid phase analytical derivatisation and the possible advantages of that approach can be maintained such as, excess reagent is removed, preconcentration effect can be maintained. However, a consideration to be made is the orientation of the peptide within the complex and that the site for derivatisation is obstructed or completely unavailable by the antibody. Once derivatised and chelated excess reagent is removed from the well and labeled peptides eluted from the immunoassay well and combined with the derivatised products from a corresponding immunoassay well and analysed by LC-ICP-MS. This approach is similar to the method developed by Wallac AutoDELFIA[92].

Solution phase labeling, the isolated peptides are eluted from the immunoassay and then derivatised and chelated. The labeled samples can then be isolated from the excess reagent using solid phase extraction. An advantage of this approach is that, dependant on derivatisation efficiency all peptides will be labeled and that derivatisation sites will not be obstructed.

Antibody Labeling, this approach is similar to that presented by Baranov *et.al.*[83], using the ELISA approach of two antibodies, the second antibody is tagged with natural europium and enriched europium and used to label antigens respectively. The Eu-labeled antigens are then eluted of the immunoassay and combined prior to analysis by LC-ICP-MS. Advantage of this approach is that it negates the derivatisation and chelation steps, and excess reagents can be re-used. A possible disadvantage is that the size of the Eu-antibody-antigen complex maybe

too large for standard LC columns and that another form of chromatography maybe required.

6.2.3 Proteomic Labeling

Current state of proteomics and ICP-MS either requires the biomolecules to contain heteroatom detectable by ICP-MS or tag or label biomolecules for elemental analysis. However, highlighted by Quinn *et al.*[85] no specific tags and labels have yet been developed for routine analysis. However, these tags and labels require derivatisation of some form which increases analytical time, sample handling and with the potential of unreacted reagent interfering in the analysis.

An alternative method such as metabolic labeling can be used to incorporate metals and heteroatoms in to biological samples for ICP-MS detection. Cell lines can be grown in media containing either a metal[75] or enriched isotopes such as carbon[17] and deuterium[15]. This method of isotope labeling has been previously to proteomic measurement using ICP-MS detection. A procedure described by Polatajka *et al.*[122] grew selenised yeast in $^{77}SeO_4$ rich culture and purified a specific tryptic peptide has an internal standard for isotope dilution analysis (IDA). Harrington *et al.*[75] applied a similar technique for the detection of copper-containing protein rusticyanin. Hence, two different samples can be grown in the natural or enriched form of a metal to allow relative quantitation. This approach as many advantages over chemical derivatisation labelling and tagging as there is no requirement for a label/tag therefore derivatisation chelation steps are not required and therefore sample isolation from excess reagent is not required. As the element labels are incorporated at the earliest possible stage they can be combined

immediately after extraction from the growth media. This is beneficial to IDMS analysis as any effect on the peptide ratio from subsequent sample treatment can be ignored as it would be equal for sample and spike.

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APPENDIX PAPERS PUBLISHED

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SHORT COMMUNICATION

Isotopic labelling of peptides and isotope ratio analysis using LC–ICP–MS: a preliminary study

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Abstract Bradykinin and substance P have been derivatised with cyclic diethylenetriaminepentaacetic anhydride (cDTPA) and subsequently labelled with natural and isotopically enriched Eu^{3+}. This enabled the detection and relative quantitation of the peptides using element-selective detection by high-performance liquid chromatography inductively coupled plasma mass spectrometry (LC–ICP–MS). Relative quantitation was achieved by differentially labelling two peptide sources, after derivatisation with cDTPA, using natural and enriched ^{151}Eu respectively. The ^{151}Eu:^{153}Eu isotope ratio was measured and used to calculate the original peptide ratio. The measured ratios came within 5.2% of the known ratio. Derivatisation and chelation reactions were additionally confirmed using LC–ESI–MS.

Keywords Relative peptide quantitation · Relative quantitation · Differential isotopic labelling · Isotope ratio · Eu tagging · Element-selective detection · Inductively coupled plasma mass spectrometry

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Introduction

The derivatisation of molecules to make them amenable to detection is a long-established technique in analytical chemistry [1]. The addition of a chemical or isotopic label allows a target molecule to be distinguished from other compounds in a system. The expanding fields of proteomics and genomics require simplified methods for the characterisation and quantification of biological macromolecules, and the development of reagents for the derivatisation of specific functional groups has played a vital role in the development of new instrumental methods. One well-known example of this approach is the isotope-coded affinity tag (ICAT) method [2] used to determine the relative expression of peptides from diseased and healthy states. In this method, the cysteine residues in proteins from two lysate samples are tagged using thiol-reactive labels, one of which is isotopically enriched with ^{2}H or ^{13}C, then mixed together in a tryptic digest and the resultant peptides are analysed by ESI–MS. The isotope ratio of the molecular ions yields the relative expression of the proteins.

Some work involving the elemental labelling of organic molecules has started to emerge [3–9], but there has not been a concerted effort to design and evaluate isotopically labelled tags for element-specific detection. Inductively coupled plasma mass spectrometry (ICP–MS) is a prime candidate for the application of isotopic labelling, and recent research has demonstrated the potential of this approach [7, 8]. In our laboratory, we have used the reagent tris(trimethoxyphenyl)phosphonium (TMPP) [10] to label organic carboxylic acids with phosphorus to make them amenable to detection using ICP–MS [8]. Bifunctional chelating agents such as diethylenetriaminepentaacetate (DTPA) have been used to label conjugate functional groups on peptides and proteins [11], and subsequently

chelated with ^{111}In or other radioactive isotopes to produce radiopharmaceuticals. Likewise, other DTPA derivatives with maleimide, bromoacetamide and pyridyldithio linkers have been used as luminescent probes when chelated with Eu^{3+} and Tb^{3+} [12]. Cyclic diethylenetriaminepentaacetate (cDTPA) is the anhydride of DTPA which will form an amide bond with the -NH_2 terminus of a peptide or protein [13]. The advantage of this compound is that it forms an amide bond without the need for any other reagents, and so it has been utilised for the derivatisation of amines and detection by ICP–MS [4].

Experimental

Instrumentation

A Luna C18(2) column (Phenomenex , Macclesfield, UK) was used for LC-UV/vis, LC–ICP–MS and LC–ESI–MS analysis. The LC system used with UV/vis and ICP–MS analysis was a HP1090 liquid chromatograph (Agilent Technologies, Stockport, UK), and a Waters 2695 Separation Module and Waters 2487 dual wavelength absorbance detector was used with LC–ESI–MS–MS. The LC mobile phase was a binary system consisting of: (A) 0.05% formic acid in water and (B) 0.05% formic acid in acetonitrile. The mobile phases were sparged with helium prior to analysis. All analyses were performed using 25-μL sample volumes and a 1.0 mL min^{-1} flow rate. Two gradient elution profiles were employed: (1) a linear gradient elution performed by increasing mobile phase B from 0% to 33% over 20 min; (2) a nonlinear gradient elution profile where mobile phase B was increased from 0% to 15% over 5 min and to 18.75% over the next 15 min. Element-specific detection was performed using quadrupole ICP–MS (VG PlasmaQuad 3, Thermo Elemental, Winsford, UK). The LC outlet was connected to the ICP–MS nebuliser by 2 m of PTFE tubing (1/16″×ID 0.18 mm). A membrane desolvator (Vestec Corporation, Houston, TX, USA) was connected between the spray chamber (the nebuliser) and the torch to remove organic solvent vapour. Electrospray ionisation mass spectrometry was performed in positive ion mode using LC–ESI–MS–MS (Micromass Quattro Ultima Pt, Micromass, Manchester, UK). The LC outlet was connected to an ESI–MS–MS and a UV detector via a 30-cm length of peek tubing (1/16″×ID 0.25 mm) connected in-line with a flow splitter (Upchurch Scientific, Oak Harbor, WA, USA). The flow of 1 mL min^{-1} from the LC column was split in the ratio 1:9, where 0.1 mL min^{-1} was sent to the electrospray ionisation source and 0.9 mL min^{-1} to waste. The molecular masses to be monitored were selected by direct infusion of the appropriate sample at 0.1 mL min^{-1} prior to analysis.

Reagents

Bradykinin acetate, substance P, cyclic diethylenetriaminepentaacetic anhydride (cDTPA), sodium bicarbonate, and sodium acetate were purchased from Sigma Aldrich (Poole, UK). Dimethyl sulfoxide (DMSO), ammonium acetate, and nitric acid (trace analysis grade) were purchased from Fisher Scientific (Loughborough, UK). Ammonium bicarbonate was purchased from Sigma Aldrich (Poole, UK). ^{151}Eu-enriched europium (Eu_2O_3 99.24%-enriched) was purchased from CK Gas Products (Hook, UK). Standard ICP–MS solutions of natural europium and samarium were purchased from Alfa Aesar (Heysham, UK).

Procedure

Isotopic labelling

The method used for derivatisation of peptides with cDTPA, and chelation with In^{3+} or Eu^{3+}, was modified from Hnatowich et al. [13]. A solution (0.2 g) of cDTPA (between 5–5000 μM) in anhydrous DMSO was added to a solution (0.2 g) of peptide (between 2.5 and 2500 μM) buffered to pH 8.3 with either 0.05 M sodium bicarbonate for ICP–MS analysis, or 0.05 M ammonium bicarbonate for ESI–MS analysis. The ratio of cDTPA to peptide was 2:1 to ensure an excess of cDTPA; however, too much cDTPA compromised the chromatographic separation efficiency. The derivatisation reaction was allowed to proceed for 30 min at room temperature. The resultant peptide-cDTPA conjugate solution (0.1 g) was labelled with In^{3+} or Eu^{3+} by adding a solution (0.1 g) of the appropriate metal (between 7.5–7500 μM) buffered to pH 6 with either 0.05 M sodium acetate for ICP–MS analysis, or 0.05 M ammonium acetate for ESI–MS analysis. The molar ratio of trivalent metal to cDTPA was 2:1 to ensure chelation with the cDTPA moiety. Chelation was achieved by agitating the solution at room temperature for 30 min.

Isotope ratio analysis and relative quantitation

A peptide solution, x, amount n_x, was labelled with natural-abundance Eu^{3+}, and a peptide solution, y, amount n_y, was labelled with isotopically enriched Eu^{3+}. The separate solutions were then combined and the ^{151}Eu:^{153}Eu isotope amount ratio was determined after separation and detection by LC–ICP–MS. The relative amounts of peptide in the two original solutions could then be calculated using the isotope

dilution equation (Eq. 1), after correction for mass bias using the ^{152}Sm:^{154}Sm isotope amount ratio.

$$\frac{n_x}{n_y} = \frac{(A_y - RB_y)}{(RB_x - A_x)} \quad (1)$$

where n_x is the amount of peptide in solution x labelled with natural Eu; n_y is the amount of peptide in solution y labelled with enriched Eu; A_x is the atom fraction of natural ^{151}Eu; B_x is the atom fraction of natural ^{153}Eu; A_y is the atom fraction of enriched ^{151}Eu; B_y is the atom fraction of enriched ^{153}Eu; and R is the (mass bias) corrected ^{151}Eu:^{153}Eu isotope amount ratio. The ratio n_x/n_y gives the amount ratio of the peptides in the two original solutions and, by extension, can be used to determine the relative amounts of peptide in two different cell states.

Results and discussion

Preliminary studies

Preliminary studies performed using LC–UV/Vis detection at 254 nm indicated that the derivatisation reaction efficiency was >94%; however, the efficiency was much poorer at lower concentrations when ICP–MS was used for detection. The chromatogram of derivatised bradykinin (data not shown) contained two peaks corresponding to the derivatised peptide, eluting at 14.6 and 14.8 minutes respectively. The presence of two separate peaks may have been caused by derivatisation with one or both of the cDTPA functional groups, thereby yielding more than one product. However, subsequent ESI–MS analysis indicated that the molecular ions at these retention times had identical *m/z*, so it is more likely that the two peaks were due to different conformers with slightly different elution times, but this was not confirmed.

Optimisation for ICP–MS and ESI–MS

The next step was to chelate the derivatised peptide with an appropriate elemental label and perform element-specific detection using ICP–MS. Europium was chosen as the isotopic label because it is relatively inexpensive, contains only two isotopes which can be readily corrected for mass-bias using a samarium spike, and is free from polyatomic interferences under the operating conditions used. It was also necessary to undertake complementary analysis using ESI–MS to confirm the expected derivatisation products. Positive ion mode ESI–MS was employed because it is the preferred and most sensitive mode of detection for multiply charged proteins and large biomolecules.

Derivatisation of bradykinin with cDTPA and chelation with Eu^{3+} was initially performed using sodium bicarbonate and acetate buffers respectively, however, these were changed to ammonium buffers for ESI–MS to eliminate the possibility that sodium adducts might form during the ionisation process. The ESI–MS mass spectra shown in Fig. 1, obtained by infusion of the derivatised products, confirmed that the reactions had successfully resulted in bradykinin–cDTPA and bradykinin–cDTPA–Eu conjugates. An isotopic pattern was not observed using positive ion mode ESI–MS due to insufficient mass resolution; however, separate negative ion mode ESI–MS showed the isotopic pattern for natural europium of the derivatised peptides.

The results of further complementary analysis using LC–ESI–MS–MS and LC–ICP–MS are shown in Fig. 2. Derivatisation using an ammonium acetate buffer (used for ESI–MS) yielded two peaks at 12.3 and 14.8 min (Fig. 2b–d) and confirmation that both of these peaks were due to bradykinin–cDTPA–Eu was provided by monitoring masses corresponding to the MRM (multiple reaction monitoring) transitions 1584>904 (Fig. 2c) and 1584>540 (Fig. 2d). These transitions were the result of loss of a cDTPA–Eu–arginine group and a bradykinin molecule from the bradykinin–cDTPA–Eu complex, respectively. The peak at

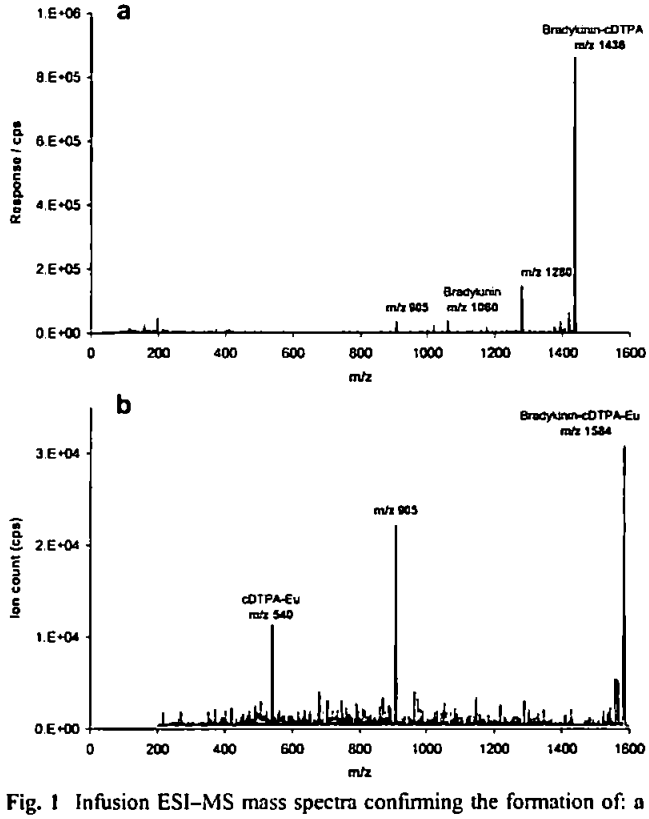

Fig. 1 Infusion ESI–MS mass spectra confirming the formation of: a bradykinin–cDTPA; b bradykinin–cDTPA-Eu conjugates

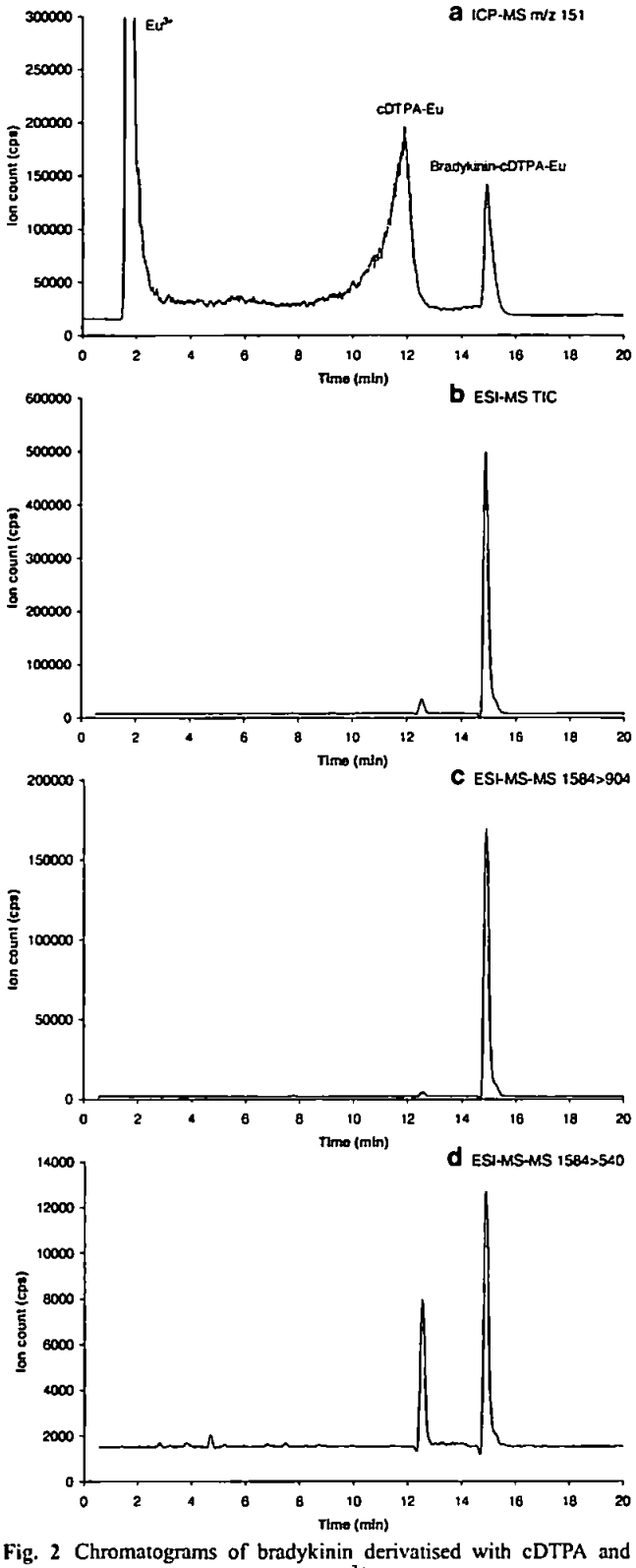

Fig. 2 Chromatograms of bradykinin derivatised with cDTPA and chelated with isotopically enriched Eu^{3+}: a ICP–MS detection at *m/z* 151; b ESI–MS total ion count; c ESI–MS–MS MRM transition 1586>904; d ESI–MS–MS MRM transition 1586>542

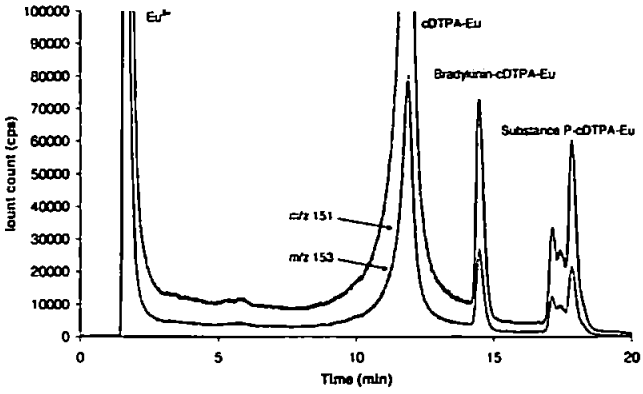

Fig. 3 Multi-isotope chromatogram of bradykinin derivatised with cDTPA and chelated with isotopically enriched Eu^{3+} obtained using ICP–MS detection at *m/z* 151 and 153

12.3 min was identified as bradykinin–cDTPA–Eu using ESI–MS–MS, but was not visible in the ICP–MS trace (Fig. 2a) when using sodium acetate as the buffer, either because it was not formed under these conditions or it was too close to the tail of the cDTPA–Eu peak to be observed above the noise. The presence of two well-resolved peaks in the chromatogram with identical masses is probably the result of the formation of two different products during the conjugation reaction.

Relative peptide quantitation

A pseudo steady-state approach was used to determine the isotope amount ratios from the multi-isotope chromatograms obtained by LC–ICP–MS [14]. In order to obtain the best accuracy, all data points were corrected by baseline subtraction, and the ^{151}Eu:^{153}Eu isotope amount ratio was mass-bias-corrected by interpolation using the ^{152}Sm:^{154}Sm isotope amount ratio and the Russell expression [14]. The

Table 1 Relative peptide ratios determined using LC–ICP–MS and the mass-bias-corrected $^{151}Eu/^{153}Eu$ isotope ratio

Sample	Peptide	Concentration (μM)	Peptide ratio, n_x/n_y	
			Theoretical	Measured[a]
1	Bradykinin	0.6	1.000	0.968± 0.073
2	Bradykinin	0.6	1.875	1.877± 0.244
3	Substance P	0.7	1.021	0.969± 0.116
4	Bradykinin	0.6	1.029	1.029± 0.206
	Substance P	0.7	1.027	1.018± 0.163

[a] Uncertainties are combined standard uncertainties calculated by combining all of the standard uncertainties in the measurement

Sm standard was added post-column via a low dead volume t-piece. The peptide ratio (n_x/n_y) was subsequently calculated using Eq. 1.

A LC–ICP–MS chromatogram showing the element-selective detection of ^{151}Eu and ^{153}Eu isotopes in the bradykinin and substance P, after derivatisation with cDTPA and chelation with natural and isotopically enriched Eu, is shown in Fig. 3. A comparison of the theoretical and measured peptide ratios for bradykinin, substance P and a mixture of the two peptides is shown in Table 1. As can be seen, the measured peptide ratios agreed extremely well with the theoretical ratios, with relative errors of between 0 to 5.2%, so, in principle, small differences in peptide expression should be detectable. In this particular case, the standard uncertainties associated with the measurements were quite high; however, it should be noted that these were combined standard uncertainties calculated by combining all standard uncertainties in the measurement using the method of Kragten [15]. Based on our experience of using isotope dilution analysis for the speciation of methylmercury [16], it should be possible to reduce the standard uncertainty to less than 6% relative. Additionally, the relative quantitation of substance P was hindered by the fact that this peptide forms disulfide bridges relatively easily, so it elutes as several peaks at ~17 min.

Detection limit

Two factors influence the lowest concentration of peptide which can be determined using this method: the efficiency of derivatisation; and the instrumental detection limit of the derivatised peptide. The lowest detectable concentration of derivatised bradykinin using LC–ICP–MS was 10 nM; however, in practice 10 μM bradykinin was the lowest concentration that could be derivatised in situ and detected using the current approach. For the method to be comparable to current ESI–MS methods for relative quantitation, an improvement to nM concentrations is required. This can be achieved by improving the derivatisation reaction efficiency, an area that is currently being investigated.

Conclusion

Derivatisation and isotopic labelling with Eu has enabled ICP–MS to be used for the detection and relative quantitation of bradykinin and substance P, which are not normally amenable to detection. This method has potential for the accurate relative and absolute quantitation of peptides using ICP–MS. A number of challenges still need to be overcome to make this a viable approach, and work is ongoing in our laboratory to improve the derivatisation reaction efficiency and remove excess reagents using solid-phase derivatisation.

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