QconCAT method development and applications in proteomics

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List of Abbreviations

QconCAT| Concatemer of standard Q-peptides
ELISA| Enzyme-Linked Immunosorbent Assay
AQUA| Absolute Quantification
TAP-tag| Tandem Affinity Purification tag
ICPL| Isotope-Coded Protein Label
ICAT| Isotope-Coded Affinity Tag
iTRAQ| Isobaric Tag for Relative and Absolute Quantification
TMT| Tandem Mass Tag
PSAQ| Protein Standard Absolute Quantification
SILAC| Stable Isotope Labelling by Amino Acids in Cell Culture
MS| Mass Spectrometry
RT| Retention Time
SRM| Selected Reaction Monitoring
MRM| Multiple Reaction Monitoring
m/z| Mass to charge ratio
MALDI| Matrix Assisted Laser Desorption Ionisation
ESI| Electrospray Ionisation
LC| Liquid Chromatography
HPLC| High Performance Liquid Chromatography
TOF| Time of Flight
Q-TOF| Quadrupole-Time of Flight
CID| Collision Induced Dissociation
AMRT| Accurate Mass Retention Time
AUC| Area Under the Curve
PLGS| ProteinLynx Global Server
CV| Coefficient of Variation
SD| Standard Deviation
SDS-PAGE| Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis
OD| Optical Density
Glufib| [Glu1]-FibrinopeptideB peptide
IPTG| isopropyl β-D-1-thiogalactopyranoside
BSA| Bovine Serum Albumin
ORF| Open Reading Frame
EDTA| Ethylenediaminetetraacetic acid
DTT| Dithiothreitol
IAM| Iodoacetamide

Abstract

Quantitative data is an excellent resource in any proteomics study but is essential in many. In recent years this area has expanded from relative to absolute quantification with a wide range of methods available for absolute quantitative proteomics. In general protein quantification is based on either label-mediated or label-free strategies. Common label-mediated approaches are isotope dilution strategies, such as AQUA, coupled with mass spectrometry, where analyte signal is compared to a stable isotope labelled standard added in known abundance. These methods are suited to small-scale studies but increasing demand for large-scale proteome quantification exposed the need for alternative quantification methodologies. The QconCAT technology, first published in 2005, is a label mediated approach which utilises the principle of surrogacy to quantify analyte proteins based on a signature peptide, or peptides, for each protein. QconCATs are concatenations of quantotypic peptides for a group of proteins, the QconCAT gene is designed *in silico* and expressed heterologously in *E.coli* with [$^{13}C_6$]arg and [$^{13}C_6$]lys to elicit a stable isotope labelled multiplexed absolute quantification standard.

In this thesis I describe several developments to the QconCAT production protocol. These developments reduce the production time from ~19d, using the initial method, to less than 7d. Time gains have been made across the whole workflow in the areas of protein expression, cell lysis, and product purification. Moreover verification of the QconCAT is delayed until the final product is synthesised, made possible by evidence of high quality reproducible expression. I explain how these alterations allow for production of several QconCATs in parallel, giving added efficiency. The success of the method is demonstrated through the use of multiple QconCATs. As a result of this work it is now possible to make at least eight QconCATs per week and the rate-limiting step of the quantification workflow has migrated from standard preparation to data processing. The final study in this thesis discusses methods for accurate quantification of the QconCAT protein and additional applications of QconCATs for testing mass spectrometer performance.

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1. Introduction

Proteomics is a wide ranging discipline, of which quantitative proteomics is just one aspect; QconCAT is one of many methods which can be used for quantification. In order to introduce the QconCAT methodology I will discuss some background to proteomics with an emphasis on quantitative proteomics. I will also introduce mass spectrometry as the major analysis tool most commonly used with QconCAT mediated quantification and discuss the instrumentation used.

1.1 Proteomics

The proteome (Wilkins *et al.*, 1996) (a) (Wilkins *et al.*, 1996) (b) of an organism describes the full complement of proteins expressed from its genetic material under a defined physiological and pathophysiological state. Proteomics is the study of the proteome, with an aim to characterise every protein expressed and map each protein to its cellular location, function and interactions. The field defines identification proteomics, where expressed proteins in a particular cell state are identified as present or absent, and quantification proteomics, which assesses the amount of protein expressed in a given cell state. This quantification can be given relative to other proteins in the cell or sample or as an absolute value. Disciplines such as genomics, a study of genes and their function, and transcriptomics, a study of mRNA in a genome, work in conjunction with proteomics. However the key advantage of proteomics is its ability to look at the changes in protein expression by directly analysing the levels of a protein present. The discrepancy between the level of mRNA produced within a cell and the resulting level of protein expression was first noticed during a study of human liver proteins (Anderson and Seilhamer, 1997), another study into the protein and mRNA in *Saccharomyces cerevisiae* cells (Gygi *et al.*, 1999) revealed a similar discrepancy. More recently a study combining transcriptome and proteome analyses on a number of *Escherichia coli* samples revealed that whilst many results from the transcriptome and proteome aligned well there were also frequent cases where the values were inconsistent (Yoon *et al.*, 2003). Vogel also found this to be true during a study on oxidative stress in *Saccharomyces cerevisiae* (Vogel *et al.*, 2011). This illustrates the importance of proteomics as an alternative to gene-focussed studies so that the full picture of cell dynamics can be understood.

As previously mentioned, there are many facets to proteomics; often analysis of a biological sample begins with identification of a target protein or proteins, the proteins can be sequenced and characterised to define the structure, post-translational modifications,

localisation within the cell, and associated proteins and function. To acquire all of this information a wide range of techniques can be employed. The proteins can also be quantified, either by relative abundance in changing cell or tissue states, or by absolute abundance in copies per cell, and again quantification can employ a variety of methodologies.

1.2 Protein quantification

An established and widely used approach to protein quantification is through the use of protein binding reagents. Applications range from specific protein targeting to analyses of entire proteomes; an atlas of more than 700 antibodies to human proteins has been generated, covering tissues in normal and disease states (Uhlen *et al.*, 2005). Two major affinity techniques are Enzyme Linked Immunosorbent Assay (ELISA) (Engvall and Perlmann, 1971) and western blotting (Towbin *et al.*, 1979). More recently the non-affinity based technique of mass spectrometry (MS) has also been used for protein quantification.

An ELISA uses antibodies to probe for specific proteins of interest. A simple ELISA involves the immobilisation of a sample, containing the “analyte” protein, onto a surface; a primary antibody is then used to bind the analyte. Often a secondary antibody is used to bind the primary antibody and this antibody is conjugated to an enzyme. To develop the assay the substrate for the enzyme is added, the enzyme catalyses the breakdown of the substrate to a product, this is usually detectable by fluorescence or absorbance. If the fluorescence or absorbance can be detected quantitatively then the technique can be used to quantify the amount of secondary antibody bound and to therefore infer the amount of analyte protein bound to the primary antibody. The quantification values obtained can be relative to other samples in the assay, or if a “standard” is included in the assay, where a known protein is measured in a range of defined amounts, this can be used to create a standard curve, from which the absorbance or fluorescence values for the analyte can be read off to give an absolute quantification value. This type of assay is specific and sensitive due to the use of antibodies which are targeted solely to the protein of interest. The use of an enzyme coupled to the secondary antibody allows for possible amplification of signal which increases the sensitivity of the assay.

The western blotting technique also involves the use of antibodies and often detection using enzymes, so like ELISA it can be specific and sensitive. For the blot the sample is initially separated using SDS-PAGE. The separated proteins in the sample are then transferred to a membrane (often nitrocellulose) using an electric field and the membrane is incubated with

an antibody to detect the protein of interest. As with ELISA the antibody can be conjugated to a radioactive, fluorescent or enzyme marker to allow detection of the bound antibody. When an enzyme is used often the product of catalysis will give off a chemiliminescence which can either be detected on an x-ray film or with a specialised camera. The resulting image (either from the developed film or the image from the camera) will show bands, reflecting where the analyte protein was present on the membrane. This image can be analysed using densitometry to compare the optical densities (ODs) of the protein bands. If a number of samples are present on the blot the relative ODs provide information about the relative abundance of the analyte protein between samples. Additionally a standard can be applied in a range of known concentrations to the same SDS-PAGE gel as the analyte protein. The ODs from the standard can then be used to create a standard curve as with the ELISA technique, and the OD of the analyte bands can be used to calculate the amount of analyte in the sample.

Another affinity technique involves tagging a protein of interest. The tagging of a protein was initially used as a purification technique to extract specific proteins and protein complexes from a solution. Two plasmids were developed which "tag" an inserted gene with the Staphylococcal protein A gene, when the inserted gene is transcribed the protein produced is tagged with protein A. The ability of protein A to bind immunoglobulin, particularly immunoglobulin G (IgG), is used to remove the protein and tag by interaction with immobilised IgG (Uhlen *et al.*, 1983). The idea was developed to produce TAP-tags – tandem affinity purification tags. In this design the tag consisted of protein A, as previously used, followed by a TEV protease recognition sequence and then the calmodulin-binding peptide (CBP). The TAP-tag was fused to a target protein, and then introduced into cells where the target protein-tag conjugate was expressed. The benefit of the dual tag is that there can be a two step purification to ensure that the final protein isolated is highly pure and that any further analysis of the protein can be as accurate as possible (Rigaut *et al.*, 1999). The TAP-tag idea was expanded to quantification through tagging each of the open reading frames (ORFs) in *S. cerevisiae.* A library of *S.cerevisiae* ORFs was created where, for each ORF, the gene was tagged with a TAP and inserted into the chromosome. The method was designed not to alter the expression levels of the tagged protein so, after isolation, quantification could be completed on each protein in the given cell state. Quantification was performed by quantitative western blotting (Ghaemmaghami *et al.*, 2003).

1.3 Mass spectrometry

Mass spectrometry (MS) is a key tool in proteomics; major applications of MS include protein identification, peptide or protein sequencing, and importantly for this thesis, protein quantification. The diversity of the instrumentation and sample preparation techniques available mean that MS can be used for studying a small number of proteins or peptides, up to large scale analysis of highly complex samples.

ELISA and western blotting techniques are typically performed on intact protein. MS can also be used to analyse an intact protein but in quantification it is more common to analyse peptides resulting from a digested protein or proteins. Within the mass spectrometer peptides can be fragmented through collision with an inert gas to give characteristic ion products which can be used to sequence the protein, and the use of peptides also gives a wider scope of possible methods for quantification. When a peptide is used in a quantitative experiment the principle of surrogacy is applied. In proteomics surrogacy presumes that the molar quantification of a peptide, arising from a proteolytically digested target protein, can be used to directly infer the quantification of that protein. The assumption is made that the peptide chosen is present in the same molar amount as the protein itself. The peptide is chosen as a "proteotypic" peptide, meaning that it is a sequence unique signature peptide for that protein, when the chosen peptide is used for quantification the term "quantotypic" can be used, meaning that the peptide is both proteotypic for the protein and also that it is suitable for quantification. The criteria which define a peptide as being suitable for quantification will be discussed later.

Typically trypsin is used to digest protein samples; this enzyme is most often chosen as it reliably and consistently recognises specific cleavage sites on the C-terminal side of arginine (arg) and lysine (lys) residues, except when they are followed by a proline. Tryptic cleavage produces predictable arg and lys terminated peptides, with the exception of the C-terminal peptide. Once the sample has been fully digested into peptides it is then ready to be analysed.

THE MASS SPECTROMETER

A mass spectrometer has three components; an ionisation source, a mass analyser and a detector. The role of the ionisation source is to deliver the peptides to the mass analyser in the form of charged ions in the gas phase. Once inside the instrument the ions travel through a vacuum in the mass analyser, the primary purpose of which is to separate the ions according to their mass (m) and charge (z) in the relationship m/z. It is the mass to charge ratio (m/z) of

an ion which determines how it will travel through the analyser. Once separated the ions collide with the detector, each event is recorded and measured. The output from the detector is plotted as a spectrum of ion intensity plotted against m/z. There are a range of ionisation techniques which are currently used. The two mostly commonly used in biological sciences, which will be discussed here, are both soft ionisation techniques and produce positively charged ions by addition of a proton or protons.

IONISATION

Matrix Assisted Laser Desorption Ionisation (MALDI), as its name suggests, uses a matrix in order to convert analyte (in our case peptides) into positive singly charged ions in the gas phase. Matrix ionisation techniques were developed as a way to ionise large intact masses of polymers and proteins (Karas and Hillenkamp, 1988) and are extremely effective for peptides. The principle of matrix assisted ionisation, (Figure 1) is that the analyte is embedded into crystals of a matrix which absorb energy from a laser, in this way energy can be delivered to the analyte to induce the non-volatile components into the gas phase without the molecules being damaged by extremely high levels of energy; matrix and analyte are ejected into the gas phase together (Fitzgerald *et al.*, 1993). It is thought that charge is delivered to the analyte, to create ions, via photoexcitation of the matrix, which leads to transfer of protons to the analyte molecules (Hillenkamp *et al.*, 1991); in MALDI-MS only singly charged peptide ions are produced. Ionisation is variable with MALDI-MS and bias can be seen towards arginine terminated peptides due to preferential ionisation of the arginine side chain (Krause *et al.*, 1999).

Many different matrices have been screened for their properties as useful compounds for the ionisation of a sample for MS (Fitzgerald *et al.*, 1993). We use α-cyano-4-hydroxycinnamic acid, this is a crystalline matrix which can be solubilised, mixed with the sample and then spotted onto a laser-target plate and allowed to dry before use in the solid state. Some are of the opinion that the solid-phase matrices give bias to an analysis (Tholey and Heinzle, 2006). It has been argued that regardless of how well combined the matrix and sample are, “hot spots” will form when the matrix dries; these are places within the overall sample spot that provide much more intense spectra, other areas of the spot may give much weaker spectra and so reproducibility between analyses is called into question. For this reason liquid matrices are sometimes considered more suitable. Liquid matrices are made by mixing the crystalline matrix of choice with an organic solvent, such as pyridine. Due to the fact that the liquid contains the same matrix material as in crystalline matrices the ionization of analyte is

thought to occur in much the same way, however it has been reported that the homogeneity of the spot is increased using liquid matrices, something which is beneficial for reproducibility of data.

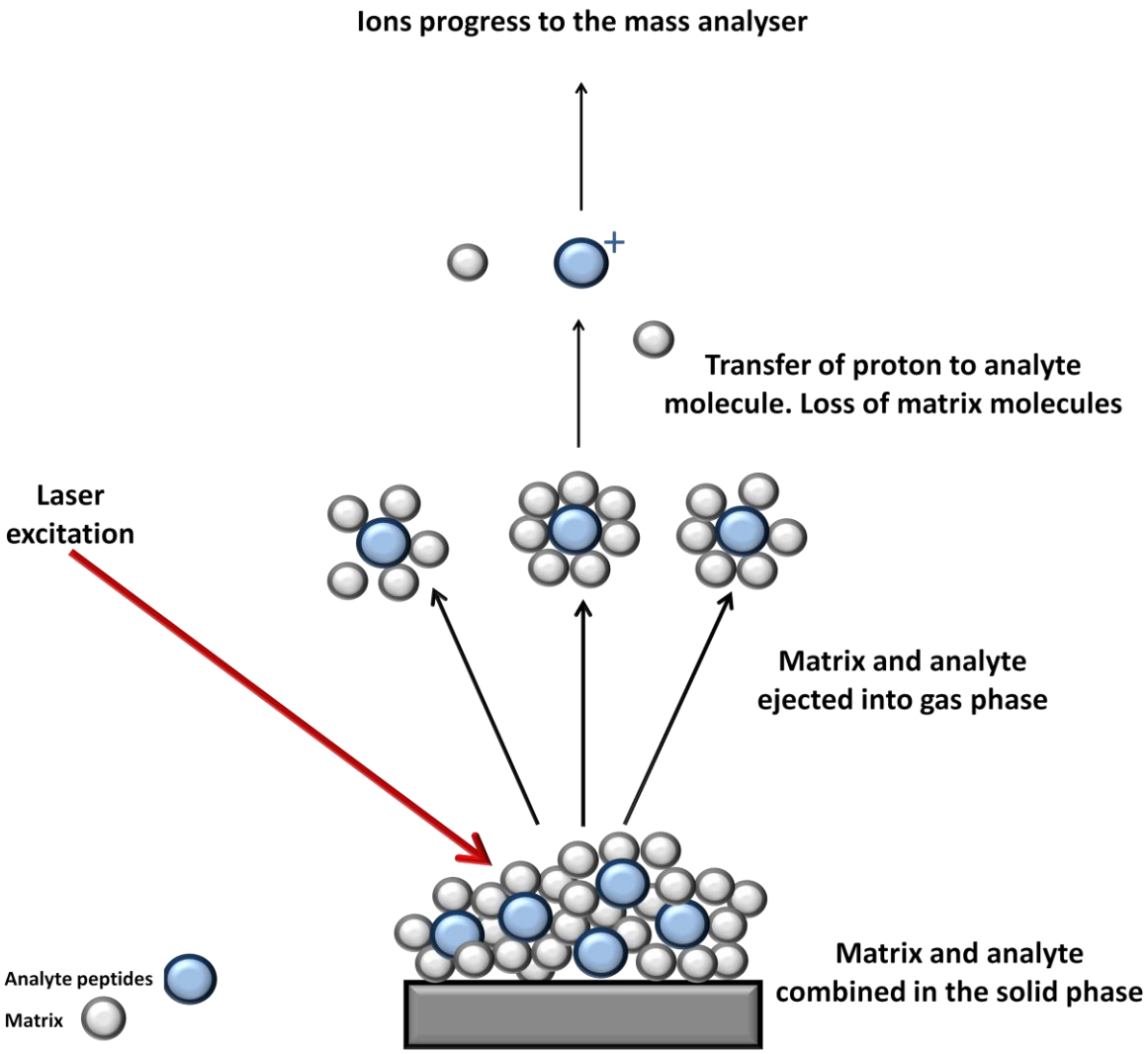

Figure 1| MALDI ionisation of peptides. Energy from laser excitation of the matrix induces expulsion of matrix and analyte peptides into the gas phase. Protons are transferred to the peptides and matrix ions are lost to create positively charged ions which progress into the mass analyser

Whilst MALDI ionisation is effective and has the benefit of being relatively tolerant to contaminants in the sample, it has been observed that the response factor of a peptide can be unpredictable with MALDI and equimolar peptides in a sample can give radically different ion signals such that one peptide might not even be detected. Couto *et al*. conducted an investigation into the effects of using different proteases to digest the sample. They found that changes in the positioning of a basic residue within a peptide, dictated by the location of the enzymatic cleavage, greatly altered the response of an ion in MALDI (Couto, 2011).

An alternative to MALDI ionisation is Electrospray Ionisation (ESI) (Figure 2). The end result of ESI is the same as MALDI; positively charged peptide ions are ejected into the gas phase and directed into the mass analyser. However the way in which the ions are created is very different and as a result ESI can produce ions with multiple positive charge states.

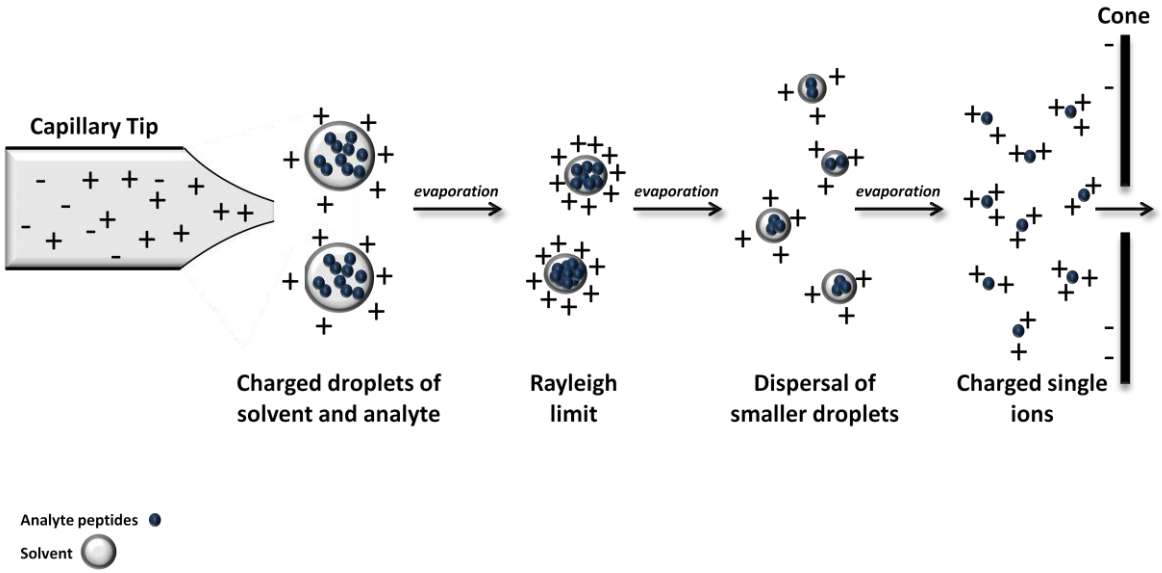

Figure 2| ESI ionisation of peptides. The sample is positively charged by electric current, a fine spray of charged droplets are ejected from the tip. The solvent evaporates until the Rayleigh limit is reached, whereby the droplets separate out into smaller droplets, this cycle repeats until charged ions remain, these move through the negatively charged cone into the mass analyser.

The sample, a solution of peptides, is delivered via a capillary tube to a tip from which the sample is sprayed into the mass spectrometer. In the case of positive ionisation an electric field, delivered to the tip, positively charges the liquid as it flows through. As the multiply charged liquid exits the tip, Coulomb (C) electric charge forces cause droplets to form which repel each other and disperse, creating a spray. The solvent carrying the peptides evaporates and the charge density on the droplet increases until the "Rayleigh limit" is reached. This limit is defined as the point where the charge on the surface of the droplet (C) reaches a density where the repulsion between charges is equal to the surface tension of the droplet; at this point the droplet separates out into smaller droplets. This cycle repeats for the smaller droplets and eventually leaves peptide ions with multiple positive charges which pass through a negatively charged sample cone and can be analysed in the mass spectrometer (Fenn *et al.*, 1989; Cech and Enke, 2001).

Due to the fact that ESI is compatible with liquid flow a significant proportion of MS analyses that use ESI as their ionisation technique are preceded by a Liquid Chromatography (LC) step

to reduce the complexity of the sample. This step is not part of the MS analysis but is often used in conjunction with MS to increase the resolution of the analysis. The sample to be analysed is taken up into an LC system (or nano-HPLC if very small volumes are being used). Depending on the technology an autosampler can be used to inject samples from pre-defined vial locations. The sample is then loaded onto a column; there are various columns which can be used which separate out peptides based on different properties, one example is a reverse phase column which separates out peptides based on their hydrophobicity. The elution from the column is pumped through capillary to the ESI source where it can be sprayed into the mass spectrometer. The elution time from the column becomes a factor in the MS analysis and adds another dimension to the MS spectra; peptide ions elute into the mass spectrometer according to their retention time (RT) so the m/z, intensity and RT of a peptide ion is measured.

THE MASS ANALYSER

Ions are separated according to their m/z in the mass analyser. The instruments used in this study have Time of Flight (TOF) mass analysers. The m/z value for an ion is based on the time it takes for the ions to travel along a TOF tube of specified length. In the simplest TOF instruments the ions enter the instrument in the gas phase and are accelerated by an electric field. This acceleration means that ions of the same charge should have the same kinetic energy as they enter the flight tube. After acceleration the ions travel along a linear tube, which is free from any electric field, until they hit the detector. The time taken for this flight is recorded and can be used to calculate the m/z of a peptide.

The longer the flight tube the greater the separation, or resolution, of the ions, therefore the TOF tube is ideally as long as possible. The tube is generally not longer than 1-2 meters, due to space restrictions in laboratories; however a way to increase the length of the flight without increasing the size of the instrument is to reflect the ions back along a different trajectory using ion mirrors (reflectron), therefore doubling the length of the flight. This increase in flight distance increases the separation between the ions and therefore improves resolution in the spectrum; this is termed reflectron TOF. Two instruments used in this study - the Waters™ MALDI-TOF and the Bruker Ultraflex MALDI-TOF, both use single reflectron TOF. The Waters™ Synapt G1 and Waters™ Synapt G2 and are also used in this study, these instruments have a TOF system which can be used in reflectron “V” mode with a single ion reflection or in “W” mode with three reflections to further increase the resolution.

In the case of the Synapt G1 and G2 their mass analysers differ to the standard TOF seen with the MALDI-TOF instruments. In these two instruments there is a quadrupole (Q) mass analyser placed before the TOF, the instruments are therefore described as being Q-TOF tandem mass spectrometers. A schematic of the G2 can be seen in Figure 3. The ions enter the mass spectrometer and are guided through to the quadrupole. The quadrupole is made up of four rods which conduct electric current; variations in the voltage applied to the rods will alter the field around them. As an ion enters the quadrupole the electric field can be used to conduct the ion through to the TOF, or the ion could be lost by altering the flight path in the electric field, resulting in a collision with one of the rods. The quadrupole can therefore be used to simply transmit ions through to the TOF or to select specific ions according to their m/z.

Use of the quadrupole to select specific ions is often coupled with another feature of the mass analyser in the Synapt instruments – the collision cell. In the G2 this is made up of the trap, ion mobility and transfer regions that can be seen on the schematic. The collision cell is continuously active however in normal MS mode ions simply pass through to the TOF as the collision cell is in a low energy state. When the collision cell is active it switches to high energy, and analyte ions are bombarded with atoms of an inert gas, this fragments the peptide (in terms of fragmentation the peptide is referred to as the precursor ion) into smaller fragments (referred to as the product ions) which can then be separated in the TOF and measured by the detector. This fragmentation process by collision induced dissociation (CID) is a form of MS/MS. There are two different types of MS/MS, data dependant and data independent. In data dependant mode ions are monitored and periodically the top 3 ions, or ions over a certain intensity threshold, are selected by the quadrupole and the instrument then switches to high energy mode and fragments the peptides chosen. The Synapt instruments are also able to perform data independent MS/MS (termed MS^E). In MS^E all of the ions present in the quadrupole are fragmented without selection of any kind. The purpose of MS/MS is to fragment the peptide ions to provide more detailed structural information about the peptide. A similar feature can be used on the Bruker Ultraflex MALDI-TOF instrument, termed "TOF/TOF". In MS/MS the range of fragments that can be produced can be characterised under the Biemann nomenclature (Johnson, 1988), we mainly use b and y ions as these are fragments at the peptide bond and are usually most abundant. An additional feature of the Synapt G2 is the ion mobility separation feature, which Waters™ term the "HDMS" mode. This delivers increased separation of ions in the MS mode; ions are separated according to their cross-sectional area using a Travelling Wave potential. This increases the temporal separation between different m/z ions and therefore increases resolution in the mass spectrum.

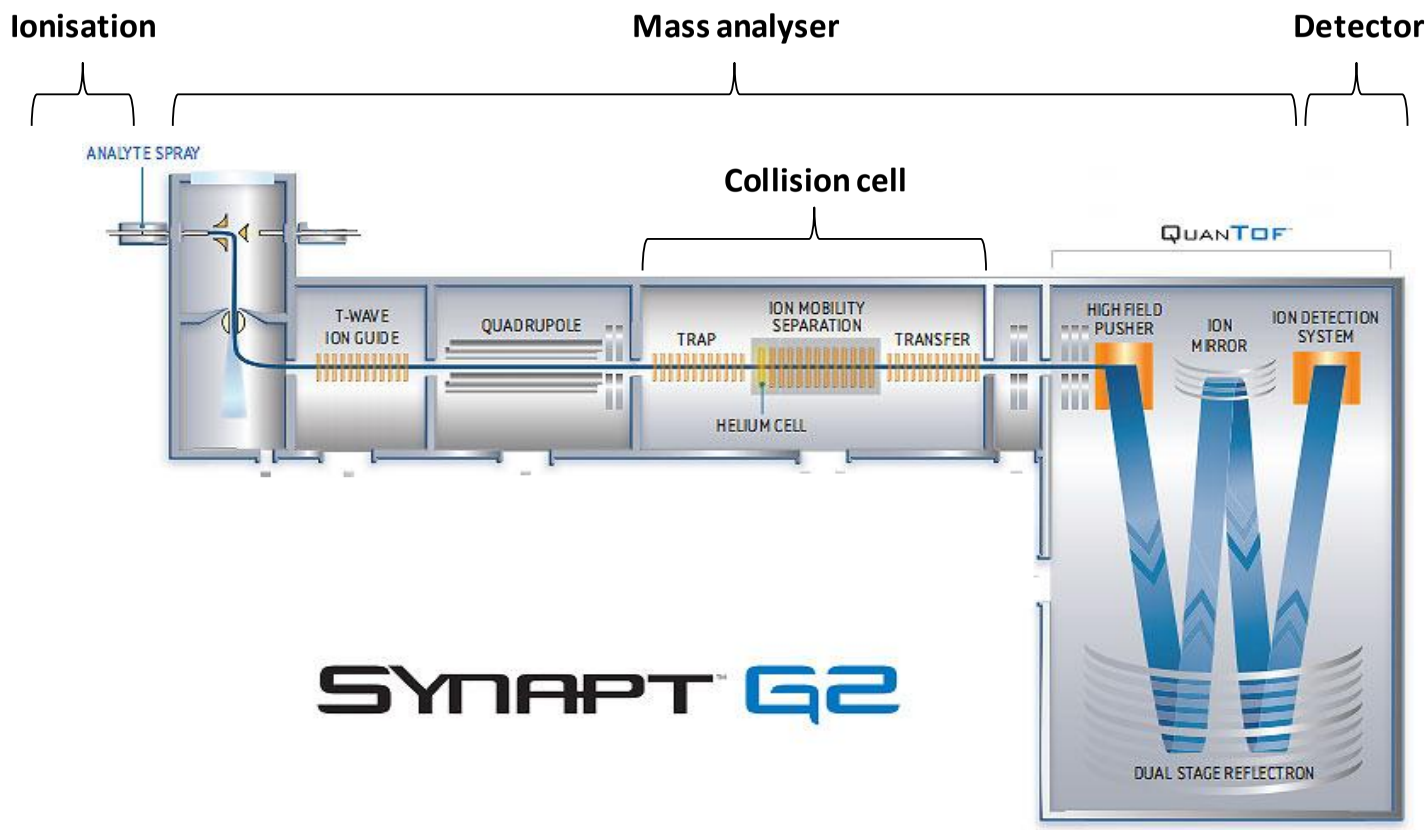

Figure 3| Schematic of Q-TOF instrument Synapt G2. Schematic from Waters™ publications, detail of the mass analyser is shown made up of the quadrupole, the collision cell and the TOF. TOF has "W" mode ion reflection to increase the flight path of ions for better resolution. MS/MS fragmentation occurs in the collision cell.

A method which utilises the selective abilities of the quadrupole and MS/MS fragmentation is Selected Reaction Monitoring (SRM). With SRM a particular ion in selected by the user, this ion is isolated and then fragmented and the product fragments recorded. The instrument will have the capability to monitor a specific product ion as well as the precursor; the precursor-product ion pair is a "transition". The instrument used determines whether the analysis can be termed an SRM or a pseudo-SRM. A true SRM is performed on a triple-quadrupole MS instrument; the mass analyser in a triple-quadrupole is made up of three quadrupoles that 1) select the precursor peptide ion to fragment 2) fragment the ion and 3) select product ions for the detector. In the case of a Q-TOF instrument the quadrupole is used to select the precursor ion, however once the ions are fragmented the lack of a second quadrupole means that product ions cannot be specifically selected, so all of the product ions are monitored; this is a "pseudo SRM".

Multiple SRMs can be used in a single MS analysis. Multiple SRMs can be referred to as Multiple Reaction Monitoring (MRM), although the term is less often used and SRM is commonly used to describe MRM experiments. The benefit to SRM or MRM is that the user selects the precursor ions to be monitored and these are programmed into the method prior to the analysis. MRM does not use Top3 or intensity threshold criteria to select ions for fragmentation, therefore the peptide of interest is always selected for MS/MS fragmentation. In MS based proteomic analyses a common obstacle is the limit of detection (LOD); this is the limit at which an ion can be detected and reliably distinguished from the background "noise". The LOD therefore becomes more of a challenge when analysing peptides in a complex background (Gallien *et al.*, 2011). SRM can help to pull the target peptide out of the background and acquire fragmentation data on lower abundance peptides, and in this way it can "dig deeper" into the proteome. SRM is well suited for use in quantification studies across a large dynamic range (Picotti *et al.*, 2009) however the method does require initial analyses, selection of the best transitions and correction of collision energies before it can be run (Campbell *et al.*, 2011), this means it can be a lengthy process. Picotti et. al. (Picotti *et al.*, 2010) have presented a higher-throughput method for SRM where they use proteomic data sets to select potential SRMs for target proteins and then synthesise all potential peptides using Spot Synthesis. The potential peptides are screened using SRM based MS assays and the most appropriate transitions are selected. Their workflow could make SRM more accessible and applicable to large scale studies.

1.4 Quantitative techniques utilising mass spectrometry

In a quantification experiment, as mentioned previously with ELISA and western blotting, the quantification can be relative, comparing the abundance of protein(s) between different cell states and monitoring changes in abundance under certain stimuli; or absolute, where analyte protein(s) are quantified to the level of copies per cell. The approaches taken for relative or absolute quantification can be classed according to whether the method is label-free, relying on MS data processing and in some cases unlabelled internal standards, or label-mediated, where a chemically or metabolically incorporated label is used to differentiate between standard and analyte (Figure 4).

LABEL-FREE QUANTITATIVE STRATEGIES

Initially, label-free quantification strategies were solely focused on relative quantification values. Techniques such as spectral counting can be used to estimate the relative abundance of a protein (Liu *et al.*, 2004); this approach involves counting the number of MS/MS spectra acquired for a peptide. It was discovered that the number of spectra acquired directly correlated to the abundance of that peptide and therefore the abundance of the parent protein in the sample. However spectral counting can become less reliable when looking at lower abundance proteins with fewer spectral counts (Lundgren *et al.*, 2010). One of the more simple relative quantification methods available is Accurate Mass Retention Time (AMRT) (Silva *et al.*, 2005). This method of quantification is based on the peptide ion signal intensity in the MS spectrum; by using the accurate mass measurement and corresponding retention time for a peptide signal, the ion intensity of the same peptide can be compared across samples and the relative abundance of the parent protein inferred from the intensity ratios. This technique relies on robust and reproducible sample preparation and LC-MS performance and can therefore be subject to inaccuracies.

Similar to AMRT, other quantitative techniques have been established which also utilise the peptide ion signal intensity or signal area. There are a number of different Area Under the Curve (AUC) label-free methodologies which compare the signal area and use this to infer quantification. In initial development tryptic digests of a range of concentrations of myoglobin were analysed using MS. The concentrations of myoglobin prior to digestion ranged from 10fmol to 100pmol and it was discovered that over this range the concentration of the myoglobin starting protein and the sum peak areas in the MS spectrum, for five selected peptides, showed a linear correlation (r^2=0.991) (Chelius and Bondarenko, 2002). In a later study, the molar range was decreased to cover 10fmol to 1000fmol for cytochrome *c* digest

and the target peptide peak intensities were normalized to four common internal standards, this was found to give an improved r^2=0.9978 (Bondarenko *et al.*, 2002).

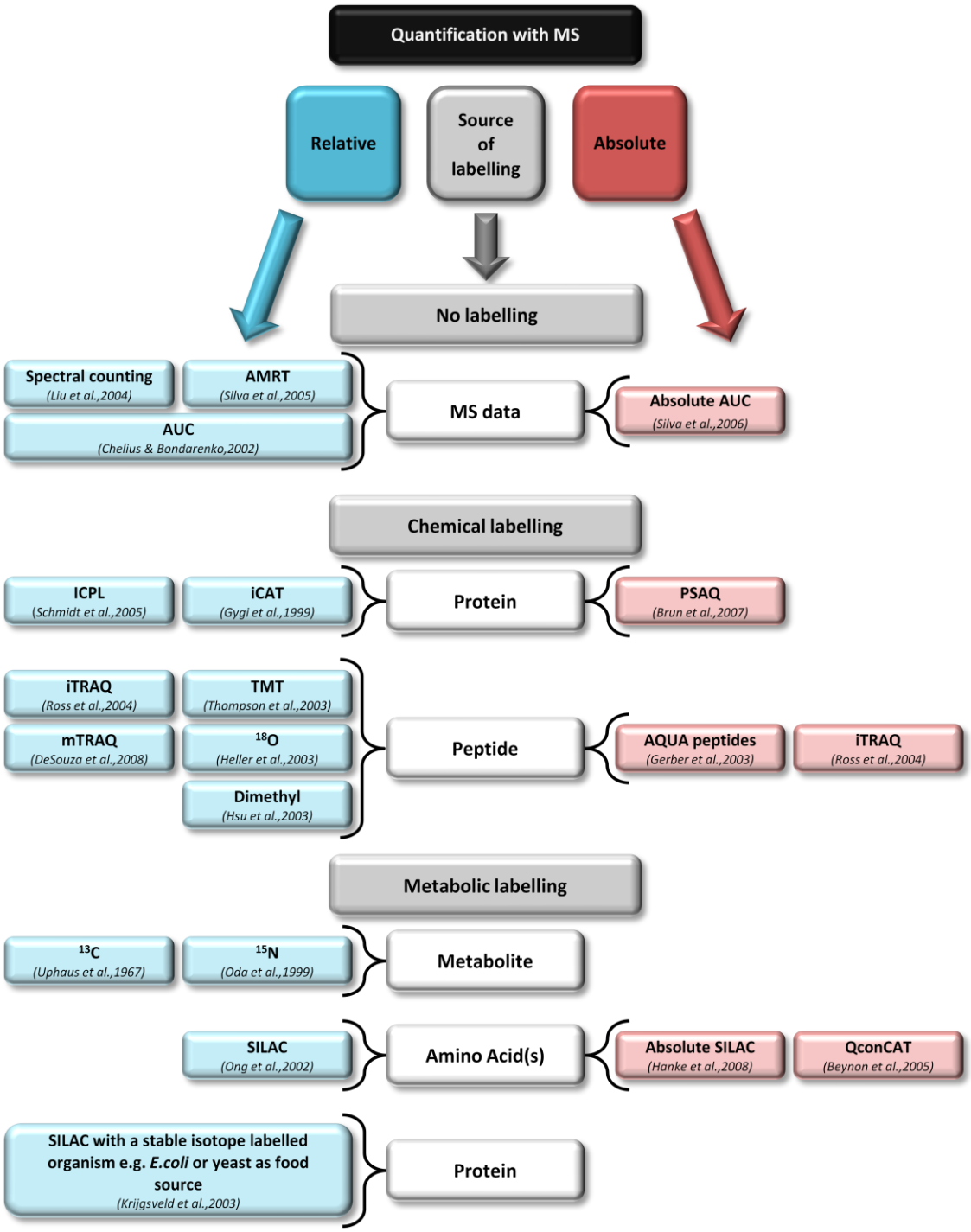

Figure 4|Quantitative methods. Summary diagram giving an overview of quantitative methods applicable for use with mass spectrometry, the methods are divided into relative and absolute quantification strategies and further subdivided according to whether label is used, and how the label is introduced.

Relative quantification data can give a broad overview of the expression changes within a cell and it is often more simple to set up a relative quantification experiment. However, whilst relative quantification is all that is necessary for many applications, if individual proteins within the same sample need to be compared to one another, or a more accurate view of the scale of an abundance change and its effects is required, absolute quantification is necessary (Bronstrup, 2004). More recently AUC label-free quantification has been developed to an absolute quantification strategy (Silva *et al.*, 2006). In this approach the top three most intense tryptic peptides for the protein of interest are used. A single unlabelled standard is also included in the assay, which can be added to the sample, in a known amount, prior to digestion and co-digested with the analyte proteins. After the sample is analysed using MS an average signal response for the top three peptides from the standard is taken and used to calculate the "universal signal response factor", which is measured in counts/mol protein. The absolute value of each of the proteins in the sample can then be calculated by dividing the average signal response, for the top three peptides for that protein, by the universal signal response factor. It was also demonstrated that this method was applicable to simple and complex samples.

Quantitative MS based proteomics, especially label-free quantification, relies heavily on the software used to analyse the data. There is a huge variety of software available for different applications. In this study ProteinLynx Global Server (PLGS) software from Waters™ is used, this performs label-free quantification based on the "top3" principle and comparison to a spiked in protein digest of known concentration acting as a standard. Another example software is MaxQuant, which started out as software for label-mediated SILAC quantification (Cox and Mann, 2008) but now also includes label-free quantification (Luber *et al.*, 2010).

Label-mediated quantitative strategies

In general, for label-mediated quantification, stable-isotope labels are used. The benefit of these isotopes is that they introduce a mass shift in the tagged peptide, distinguishing it from the untagged peptides but the tagged and untagged peptides still show similar behaviour; they have the same retention time, efficiency of ionisation and during MS/MS produce the same ion fragments (Pan *et al.*, 2009). They are also preferred over radioactive isotopes as they do not require stringent safe handling procedures and do not rapidly decay. There are many different stable isotopes available; common forms used are ^{13}C, ^{15}N, ^{2}H and ^{18}O. In order to have clear separation between tagged and untagged peptides it is helpful to have a mass shift of 3/4Da to reduce overlapping peaks affecting the quantification (Ong and Mann, 2005).

For relative quantification with stable isotope labels, the samples are differentially labelled to distinguish between them, they are then digested and analysed in parallel using MS. Analysis of the standard and analyte produces pairs of peaks on a spectrum – an unlabelled lower m/z peak and the higher m/z labelled peak. The intensity ratio between the two peaks echoes any changes in expression of that protein between the two cell states (Gouw *et al.*, 2010). For absolute label-mediated quantification the same principle of comparing standard and analyte peaks is used but an accurately quantified standard is added to give an absolute value to the quantification.

With the standard and analyte requiring differential labelling a consideration for label-mediated quantification is the level of error which could be introduced through the individual treatment of the samples. Ideally they should be combined as early on as possible in the workflow to minimise variation in treatment (Figure 5). If two cultures are differentially labelled they can be combined prior to cell lysis giving the greatest minimisation of variance. If this is not possible the next point to combine is prior to digestion; this still minimises variation in digestion efficiency and errors in pipetting. A greater level of variation is introduced if the peptides are pooled post-digestion.

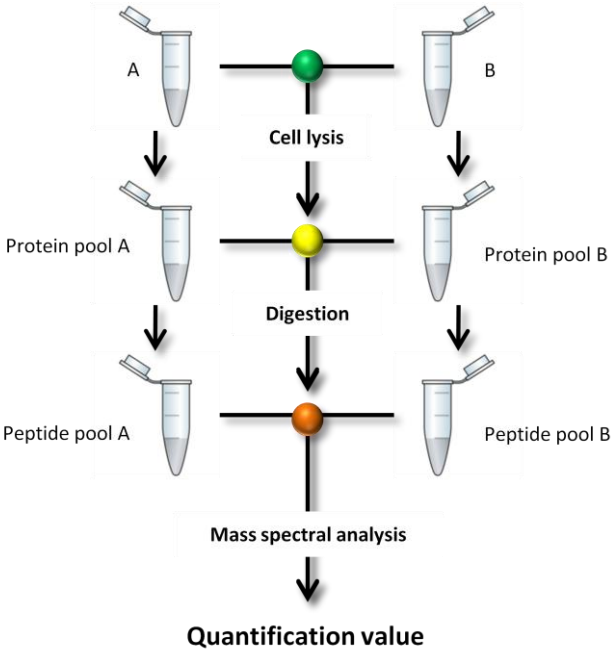

Figure 5| Stages in the quantitative workflow where samples can be combined. A and B represent samples to be analysed, ideally A and B would be combined as early on in the workflow as possible, prior to cell lysis. Alternatively proteins can be combined prior to digestion or, less desirably, prior to MS analysis. The greatest level of variance is introduced if samples are not co-analysed.

Label can be introduced to the standard, or to differentially labelled samples, by chemical or metabolic means. Chemical labelling involves tagging the target proteins prior to digestion, the target peptides post-digestion or using a standard synthesised in a cell-free environment. Isotope-Coded Protein Label (ICPL) is a chemical labelling method for relative quantification of proteins (Schmidt *et al.*, 2005). Two samples can be differentially labelled after cell lysis; the ICPL reagent is either a deuterium containing (heavy) tag or is deuterium free (light tag). The proteins are tagged at free amino groups and can then be combined for digestion. Signal intensity from the light and heavy tagged peptides can be used to infer relative quantification. Another labelling method for relative quantification is ICAT, where the protein is again targeted for tagging, however unlike ICPL, in ICAT it is the cysteine residues that are tagged. The tag can be deuterated to elicit an isotopically labelled reagent, and in a similar way to ICPL two samples can be differentially labelled with the light or heavy forms of the reagent. The samples can then be combined for digestion. The reagent is restrictive in that it only tags cysteine containing peptides so not all of the peptides are suitable for quantitative analysis. However the reagent contains a biotin molecule so post-digestion the tagged peptides can be isolated from the solution using avidin. Comparison of the heavy and light peptide pairs in an MS spectrum is used to infer relative quantification (Gygi *et al.*, 1999). The benefit of both ICPL and ICAT is that the proteins are tagged so co-digestion is possible, which helps to reduce variation in the treatment of samples and increases the accuracy of quantification.

For targeting of peptides, rather than proteins, Isobaric Tags for Relative and Absolute Quantification (iTRAQ) (Ross *et al.*, 2004) reagents are commonly used. The tags bind free amines at the N-terminus of the peptide and on lysine side chains. Every peptide in a digest is labelled, meaning that every peptide can be used as a standard or basis for comparison for relative quantification. Initially there were just four reagents however this has now increased to eight. Each reagent could be used to tag a different sample set, for example eight different growth conditions or disease states could be analysed and compared simultaneously. The reagents are isobaric so they do not differ in mass, therefore when the tagged peptides are analysed using MS there is no mass shift seen in the spectrum; however each of the four or eight tags releases a different “reporter ion” in an MS/MS spectrum. The intensity of the reporter ion can be used to relatively quantify the parent peptide and therefore the protein. iTRAQ is commonly used as a relative quantification technique, however it can be made absolutely quantitative with the use of accurately quantified, and subsequently tagged, surrogate peptides, at least one peptide per protein in the analyte mix would need a quantified surrogate. iTRAQ is an excellent technique to study multiple samples in parallel.

The method does call for extremely careful sample preparation, there are pitfalls which can compromise the accuracy of the data, such as the reactivity of the tags with amine groups, meaning that amine containing buffers such as Tris and ammonium bicarbonate cannot be used, and adding the tags to a peptide solution at low pH can cause the tags to react with tyrosine residues (Unwin, 2010). Additionally as the tag is applied to peptides it is introduced later in the protocol, which allows for a greater level of error due to variance between treatment of samples. A significant number of chemical labelling techniques tag peptides and hence they all have an increased propensity for variation in samples. However, with iTRAQ the late introduction of the tag can also be useful in some cases where *in vivo* labelling of the analyte is not possible due to the cost or scale of the labelling, for instance in the analysis of human tissue. As a variation to iTRAQ reagents there are also mTRAQ reagents available (Applied Biosystems, Inc., Foster City, CA) which work on a similar principle to iTRAQ but introduce a mass-shift with the tag (DeSouza *et al.*, 2008). There are fewer channels available with the mTRAQ tag – just three including an "unlabelled" tag so there is less scope for comparison between multiple cell states simultaneously. As with iTRAQ, the mTRAQ reagents can be used for relative quantification, or absolute if a standard is used as one of the tagged samples.

Another tagging technique is Tandem Mass Tags (TMT), which again can be used to differentially label two samples (Thompson *et al.*, 2003). Similarly to iTRAQ, the tags are designed to generate a reporter ion in an MS/MS spectrum which can be used for quantification. A benefit of the TMTs is that the reactive functionality can be altered to bind to different groups depending on the needs of the user.

Chemical labelling does not always involve tagging with a reagent. Amongst other labelling techniques is ^{18}O labelling. Trypsin is used to digest the analyte protein(s) in a buffer containing enriched ^{18}O water, which is incorporated into the peptides as the enzyme hydrolyses the peptide bond. Two ^{18}O molecules are included in each peptide, one at each terminus, which gives a 4Da mass shift (Heller *et al.*, 2003). This method is typically used for relative quantification, however if a standard protein of known abundance was ^{18}O labelled before combining the standard and analyte peptides, then absolute quantification could be possible. The label in this method is introduced at a later stage in the protocol, so there is a greater chance for error. However in a similar way to iTRAQ this method is widely applicable to studies where the samples cannot be SILAC or otherwise labelled. As a final example of chemical labelling of peptides, dimethyl labelling can be used (Hsu *et al.*, 2003), formaldehyde

is used to label the N-terminus and an amino group on lysine residues to differentially label a set of peptides.

Rather than labelling analyte proteins with reagents, labelled standards can be synthesised for quantification of target proteins. These standards can be produced in stable isotope labelled form in a cell-free environment and can subsequently be accurately quantified to enable them for use for absolute protein quantification. Protein Standard Absolute Quantification (PSAQ) is an example of a chemically synthesised protein standard which can be used for absolute quantification (Brun *et al.*, 2007). The full length protein can be combined and co-digested with the analyte protein, the resulting peptides can all be used for quantification of the target, giving greater reliability to the method as co-digestion with the analyte minimises variation in sample treatment. However, for each target protein a standard needs to be synthesised so this method does not lend itself to large scale quantification. Absolute Quantification peptides, or AQUA peptides (Gerber *et al.*, 2003) are chemically synthesised peptides that are stable isotope labelled and accurately quantified. They can be spiked into a sample in known abundance before mass spectrometric analysis in order to quantify the corresponding analyte peptide and therefore the analyte protein. Whilst quantification of the target protein is possible from a single standard peptide, a more reliable quantification value would be obtained from multiple peptides, in this way AQUA could be less reliable than PSAQ unless multiple peptides were synthesised for each protein. The peptides are also combined with the sample later on in the analysis as they do not need to be co-digested, and this has the potential to introduce variation. Production of AQUA peptides can be expensive, especially if a large scale study is considered. However a benefit to the chemical synthesis of the peptides is that post-translational modifications can be added to the peptide if desired (Kettenbach *et al.*, 2011).

As previously mentioned, to reduce error in quantification the analyte and standard samples should be combined as early on as possible in the protocol. In order to do this the label needs to be introduced as early as possible and metabolic labelling *in vivo* rather than chemical labelling *in vitro* can allow for early introduction of the label (Gouw *et al.*, 2010). As with chemical labelling, metabolic labelling can be performed with a number of different isotopes and in different forms. For algae, bacteria or yeast the growth media can be supplemented with a stable isotope such as ^{13}C and ^{15}N. Commonly ^{15}N can be supplied in the form of ammonium chloride and ^{13}C can be supplied in the form of glucose (Uphaus *et al.*, 1967; Oda *et al.*, 1999). The resulting proteins will be labelled with varying mass shifts depending on the

length of the protein; this can make processing more difficult. The labelled culture can be compared to an unlabelled culture for relative quantification; as the proteins are labelled *in vivo* the cells can be combined prior to cell lysis to minimise variation.

A widely used metabolic labelling strategy is Stable Isotope Labelling by Amino Acids in Cell Culture (SILAC). SILAC is an umbrella term which covers a wide range of techniques. Initially SILAC defined the labelling of a cell culture with a stable isotope labelled amino acid such as deuterated leucine (Leu-d3), a labelled culture and a non-labelled culture could be combined and the relative protein abundances quantified using MS by comparing the intensity of labelled and unlabelled peptide ion peaks (Ong *et al.*, 2002). This has the benefit of being able to compare an entire proteome from one labelled culture. Arginine and lysine are commonly used in SILAC experiments along with trypsin as the enzyme of choice. Trypsin reliably cleaves protein after Arg or Lys residues and so leaves peptides with a consistent single heavy amino acid at the C-terminus of the peptide, releasing a labelled peptide which will show a predictable m/z shift in a mass spectrum. However there are other possible amino acids and isotopes which can be used according to the particular needs of the study. With SILAC the label is introduced early in the workflow and so the analyte and standard can be mixed prior to digestion, this limits variation in digestion efficiency. As an alternative method SILAC has also been used to generate a heavy reference proteome which can then be "spiked in" to several light analyte samples for relative quantification (Geiger *et al.*, 2011). SILAC has recently been used to describe the metabolic labelling of larger invertebrates such as *Caenorhabditis elegans* up to mammals such as mice, additionally "absolute SILAC" has been developed.

It has become increasingly common to use labelling of larger organisms for relative quantification (Gouw *et al.*, 2010). As these organisms are not grown in culture the method of introduction of the label can be more complex. In the labelling of mice a synthetic, usually semi-synthetic, diet can be used which contains the stable isotope labelled amino acids. The end result of labelling is typically 50% which, if taken into account in analysis and data processing, still allows for effective quantification. For other organisms a two-step labelling technique can be used; C.*elegans* has been labelled in this way. This two step technique involves initially labelling *E.coli* with ^{15}N using ammonium chloride as the ^{15}N source (Krijgsveld *et al.*, 2003) or a supplemented labelled amino acid, lysine, (Fredens *et al.*, 2011)) and subsequently using the labelled *E.coli* as the food source for *C. elegans.* Quantification of proteins extracted from model labelled organisms such as *C. elegans* is relative.

For absolute quantification a SILAC labelled standard can be produced *in vivo* which is then spiked in with the analyte prior to digestion (Hanke *et al.*, 2008). The recombinantly expressed proteins are purified and accurately quantified before co-digestion, so comparison between the standard and analyte peptide signal intensities can be used to absolutely quantify the target protein. This method has the advantage of absolute quantification, however it is limited by the time and costs of producing multiple labelled protein standards.

When selecting a label-mediated quantification strategy the decision has to be made between relative and absolute quantification. If absolute quantification is to be used the two major classes of method use peptide or protein standards. Whilst the proteins standards offer the benefit of co-digestion with the analyte and a greater number of quantification peptides they can take a longer time to synthesise for each target protein and every peptide is not necessary for quantification of the analyte. A compromise between the two could be QconCAT.

1.5 QconCAT technology

A QconCAT is a concatenation of proteotypic peptides for a set of proteins of interest. A QconCAT is assembled as a protein and can be co-digested with the analyte to reduce variability. The benefit over other protein standards is that tryptic peptides for multiple proteins are included in a single QconCAT construct, so quantification can be multiplexed. QconCAT technology utilises the principle of surrogacy, which has been discussed previously. A QconCAT is a protein which is made up of carefully selected tryptic peptides; Figure 6 is an overview of the QconCAT design process preparation and use. The design of the construct occurs *in silico*; proteins of interest are selected and peptides from each are chosen, using a number of different criteria. The peptides are concatenated into a protein sequence, and certain peptides are added to the sequence for purification or quantification of the construct. Finally a gene is designed, which codes for the entire construct and this is inserted into a plasmid vector (Beynon *et al.*, 2005; Pratt *et al.*, 2006). The plasmid is inserted into *E.coli* and the QconCAT protein is expressed in media containing stable isotope labelled amino acids. The construct is purified from *E.coli* cell lysate using nickel affinity purification before co-digestion with the analyte proteins and MS analysis.

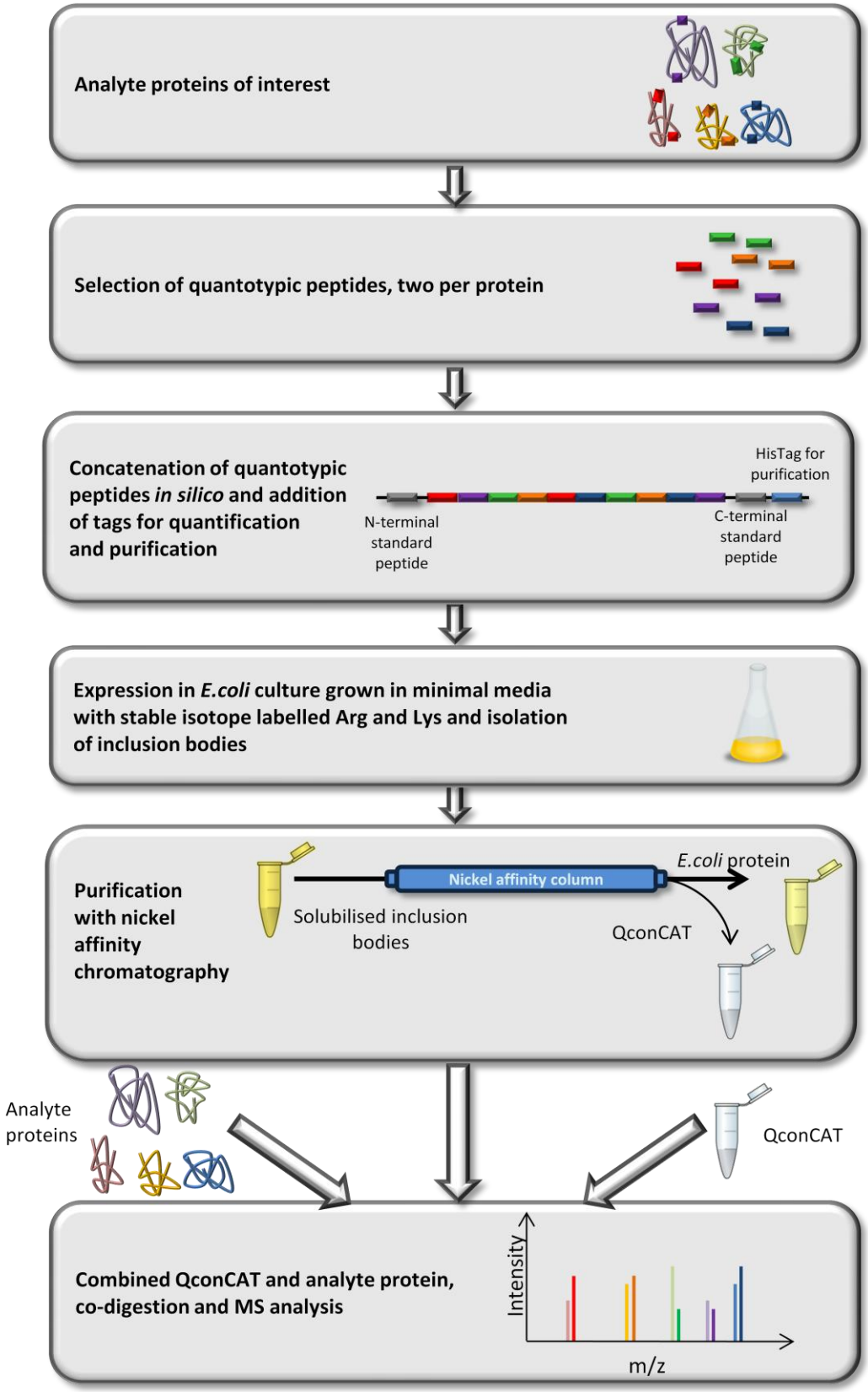

Figure 6 | QconCAT overview of production and use. Quantotypic peptides are selected from proteins of interest and assembled into a concatenation. Additional peptides are included in the sequence before the gene is synthesised and inserted into a plasmid vector. The QconCAT is expressed, purified and quantified before co-digestion with analyte proteins and MS analysis for quantification of analyte proteins.

SELECTION OF PEPTIDES

The terms proteotypic and quantotypic (section 2.3) are regularly used to describe peptides which are suitable, under a number of criteria, for analysis and quantification of proteins. Computational tools have been developed to predict proteotypic peptides for a given protein, especially the detectability of the peptide in an LC-MS/MS analysis (Mallick *et al.*, 2007; Li *et al.*, 2010). Properties of the amino acid residues affect the detectability of a peptide and it has become increasingly evident that a proteotypic peptide is not necessarily a quantotypic peptide; specific criteria need to be met for a peptide to be suitable for use in a quantification experiment (Brownridge *et al.*, 2011) such as:

- Charge on amino acid side chains
- Hydrophobicity of amino acid side chains
- Amino acid side chain length
- Peptide structure
- Must have a unique m/z value that does not overlap with another peptide in the analysis
- Ionisable to be able to be detected
- Must be reproducibly observed
- Avoid post-translational modification
- Avoid methionine residues

Post translational modification are avoided due to the fact that the peptide can exist in the modified and unmodified form in the analysis; the presence of both forms would split the signal for that peptide and so give a bias towards a weak quantification. Similarly methionine is avoided as it can be oxidised and again this would split the signal giving poor quality quantification.

Digestion efficiency is extremely important in a quantification experiment; for the quantification to be reliable all of the peptides, analyte and standard, must be released from the parent proteins. A problem which is often seen in proteomics is “missed cleavage”, where a digestion site, for example a tryptic cleavage site after an Arg or Lys residue, is not cleaved or only partially cleaved. This results in incomplete release of the peptide, and if that particular peptide is being used for quantification the value calculated with be biased. Recently prediction tools such as CONSeQuence include information derived from a missed cleavage predictor to avoid peptides that might not be completely cleaved (Eyers *et al.*, 2011). When the QconCAT method was first published in 2005 (Beynon *et al.*, 2005), then termed

"QCAT", the construct contained a single representative "Q-peptide" for each protein which was selected using much fewer criteria than are currently used. The QconCAT protocol has now been developed to include at least two Q-peptides per protein for more accurate quantification and CONSeQuence is regularly used to select appropriate quantotypic peptides.

SELECTING THE EXPRESSION STRAIN

For the last 40 years *E.coli* has been used as a tool in molecular biology. It lends itself well to this task as *E.coli* culture has a robust and high growth rate and cultures can be grown relatively cheaply (Baneyx, 1999; Wang *et al.*, 2010). It is also an extremely well characterised organism; the scientific community has accumulated a wealth of information on *E.coli* genes and proteins. Although the *E.coli* genome was not the first to be completely sequenced, it was one of the earliest with the complete genome sequence of K-12 published in 1997(Blattner *et al.*, 1997) and the complete BL21 (DE3) sequence following in 2009 (Jeong *et al.*, 2009). There are many different *E.coli* mutants available that are knockout strains for genes coding proteins such as proteases; crucially *E.coli* tolerates mutations well. The BL21 (DE3) strain has been used for many years for expression of recombinant proteins, the strain which we use for QconCAT expression is BL21 (λDE3). This BL21 strain is deficient in both *lon* protease (regulates gene expression by degradation of regulatory proteins such as transcription regulators) and *ompT* protease (protease located in the outer membrane) making it a more stable environment for recombinant proteins. The λDE3 indicates that the *E.coli* contains λDE3 prophage DNA, this means that the *E.coli* strain contains the T7 RNA polymerase gene and this is used during recombinant protein expression.

THE T7 EXPRESSION SYSTEM

Once the QconCAT gene has been synthesised it is inserted into the pET-21a plasmid vector. This vector is chosen for two key features; firstly the vector has a selectable marker for ampicillin resistance, meaning that *E.coli* cells which successfully take up the vector (transformed) will be resistant to ampicillin antibiotic, and growth of transformed cells on ampicillin-containing agar plates allows selection of only the correctly transformed cells. The exact mechanisms of transformation are not known; the use of $CaCl_2$ and heat shock aid transformation and it is thought that the calcium ions' positive charge may shield the negative charge on the DNA, or on the cell surface membrane (Watson, 2004) whilst the heat shock may induce pores in the membrane which would allow passage of the shielded DNA molecule into the cell. The transformation process is inefficient with only a small percentage of cells taking up the plasmid vector. The antibiotic selection of successfully transformed cells is

therefore very important. Secondly the inserted gene is placed under the control of a T7 promoter which means that expression of the gene can be controlled using the T7 expression system.

The T7 expression system, first used by Studier and Moffatt (Studier and Moffatt, 1986), is based on utilising the bacteriophage T7 RNA polymerase. The BL21 (λDE3) host contains the T7 RNA polymerase gene inserted into the bacterial chromosome under the control of the *lac* operon which keeps the polymerase from being transcribed under normal cell conditions. This polymerase is highly selective for the T7 promoter, which is not usually present in an *E.coli* cell. The pET21a vector, containing the T7 promoter, is transformed into the cell. After antibiotic selection of transformed colonies, the cells are cultured and once a suitable cell density is reached, isopropyl β-D-1-thiogalactopyranoside (IPTG) is added. The repressed *lacUV5* promoter in the *lac* operon prevents the T7 polymerase from being transcribed, however IPTG is a synthetic analogue of allolactose (the natural activator for the *lac* operon) and its presence within the cell switches off the *lac* repressor, activating the *lacUV5* promoter and allowing transcription of the T7 polymerase to occur (Lewis *et al.*, 1996). The newly synthesised T7 RNA polymerase recognises the T7 promoter on the plasmid vector and transcribes the QconCAT gene linked to it.

Another method has been developed to induce expression of a recombinant protein by autoinduction (Studier, 2005). This also uses the T7 expression system but does not require the addition of IPTG. BL21 (DE3) cells containing a recombinant gene on a pET21a plasmid vector are grown in autoinduction media specifically designed to use allolactose as the inducer. The media contains lactose and a restricted amount of glucose. The glucose is designed to sustain the culture only until it reaches a suitable density for induction. The presence of glucose prevents T7 induction by lactose as the cells use glucose preferentially as a carbon source. Once the glucose has been depleted the cells begin to use lactose as their carbon source. As the cells begin to metabolise lactose the metabolite allolactose is produced. Under normal cell conditions this would bind to the *lac* repressor, allowing transcription of the genes in the *lac* operon and so facilitating lactose metabolism. In a BL21 (DE3) cell the presence of allolactose removes the repression of the T7 *lac* promoter and so induces expression of the T7 RNA polymerase, leading to expression of the inserted gene on the pET21a vector. This method of induction can be extremely useful as the optical density of the culture does not need to be monitored to induce expression so a culture can be left to induce overnight.

QUANTIFICATION WITH QCONCAT

The QconCAT construct is typically expressed in minimal media containing stable isotope labelled arginine and lysine. The "heavy" labelled QconCAT is then purified using nickel affinity chromatography by virtue of the hexahistidine tag and the purified protein can be quantified using a protein assay. The QconCAT is added, in a known amount, to the analyte proteins and co-digested with trypsin to elicit peptide pairs for quantification – a heavy peptide from the QconCAT and a "light" peptide from the analyte. The digest is analysed using MS and the intensity ratios between heavy and light peak pairs can be used to quantify the target proteins. Quantification with QconCAT is applicable to all ionisation techniques and mass spectrometers including ESI-TOF and MALDI-TOF MS (Rivers *et al.*, 2007). Quantification experiments using QconCAT often involve MRM assays as a more targeted approach; SRMs are designed for each of the QconCAT transitions and its analyte counterpart.

A protein assay is commonly used to quantify the standard, however an alternative method for quantification of the QconCAT standard has been developed that involves the use of a quantification peptide that can be included in each QconCAT construct. Each QconCAT has the sequence for Glufibrinopeptide B (GluFib) located after the sacrificial peptide at the N-terminus of the construct and a variant of this peptide, known as Fib, at the C-terminus before the HisTag. The value of GluFib and Fib is two-fold; firstly they can both be used as quantification peptides, a known amount of unlabelled versions of each can be spiked into a digest to quantify the labelled QconCAT and verify the quantification by virtue of each other. The peptides are common to each QconCAT so quantification is comparable between QconCATs. Secondly the presence of GluFib and Fib at the N and C-termini respectively allows for verification that the full length construct is present in the digest (Brownridge *et al.*, 2011).

An advantage of QconCATs is that the construct can be co-digested with the analyte protein, therefore minimising variation in digestion efficiency. Additionally QconCATs are not designed with any structural considerations and it is highly likely that the higher order structure of the analyte proteins will be more complex than the QconCAT, so if the analyte is denatured to allow complete digestion it is likely that the QconCAT should also be fully denatured.

The QconCAT standard can be described as a multiplexed absolute quantification standard. The fact that standard peptides for several proteins can be produced and analysed simultaneously is both time and cost effective and is a major advantage of this method over other methods such as AQUA peptides as it facilitates easy multiplexing of quantification and

opens the possibility of much larger scale quantification projects. Once the QconCAT plasmid is created it can be used as many times as required without incurring great costs.

Finally the design of the QconCAT *in silico* means that there is huge scope for what is included in the construct. For example, traditionally each peptide is included in the sequence once, resulting in equimolar 1:1 ratios of each, however it is possible to include peptides a number of times if different molar amounts are required in the analysis. This highlights the additional properties of the QconCAT which can be exploited to act as a standard in MS to assess instrument performance and condition. An example of this is the QCAL QconCAT (Eyers *et al.*, 2008); QCAL was designed to contain 22 different tryptic peptides, some included a number of times, and peptide masses selected to cover a wide range of m/z for MALDI-TOF and ESI-MS. The standard can be used for instrument calibration and to assess performance, it can also act as a common standard between laboratories to facilitate better exchange and standardisation of data.

2. Aims and Objectives 35

2. Aims and Objectives

QconCAT technology was designed to be used as a tool in proteomics for multiplexed quantification of several target proteins. In this thesis I demonstrate how the QconCAT methodology can be streamlined and the production process can be multiplexed, ultimately leading to a significantly shorter and simpler workflow. Figure 7 shows the overall workflow which is taken from the protocol originally published in 2006 (Pratt *et al.*, 2006). The target boxes in green highlight the areas where the workflow can be developed in order to shorten and streamline the QconCAT protocol.

My first objective was to remove unnecessary stages from the production process in order to shorten the overall production time frame. I investigated if it was possible to remove the re-streaking of newly transformed QconCAT colonies and to eliminate the necessity for an initial trial induction of the QconCAT. The reproducibility of the QconCAT expression process was assessed to gauge whether the verification of the QconCAT construct could be delayed until after the stable isotope labelled protein was synthesised.

I investigated alternative methods of *E.coli* cell lysis and protein purification to establish whether more rapid methods could be used. Additionally I sought to find methods which would allow for multiplexing of QconCAT purification, improving the efficiency of the production workflow.

Finally I intended to demonstrate how QconCAT constructs can be used for more than quantification studies. With a study into 10 well characterised QconCAT proteins I considered the possible methods that could be used for quantification of the QconCAT standards and whether there are other potential applications for QconCATs as quality control standards.

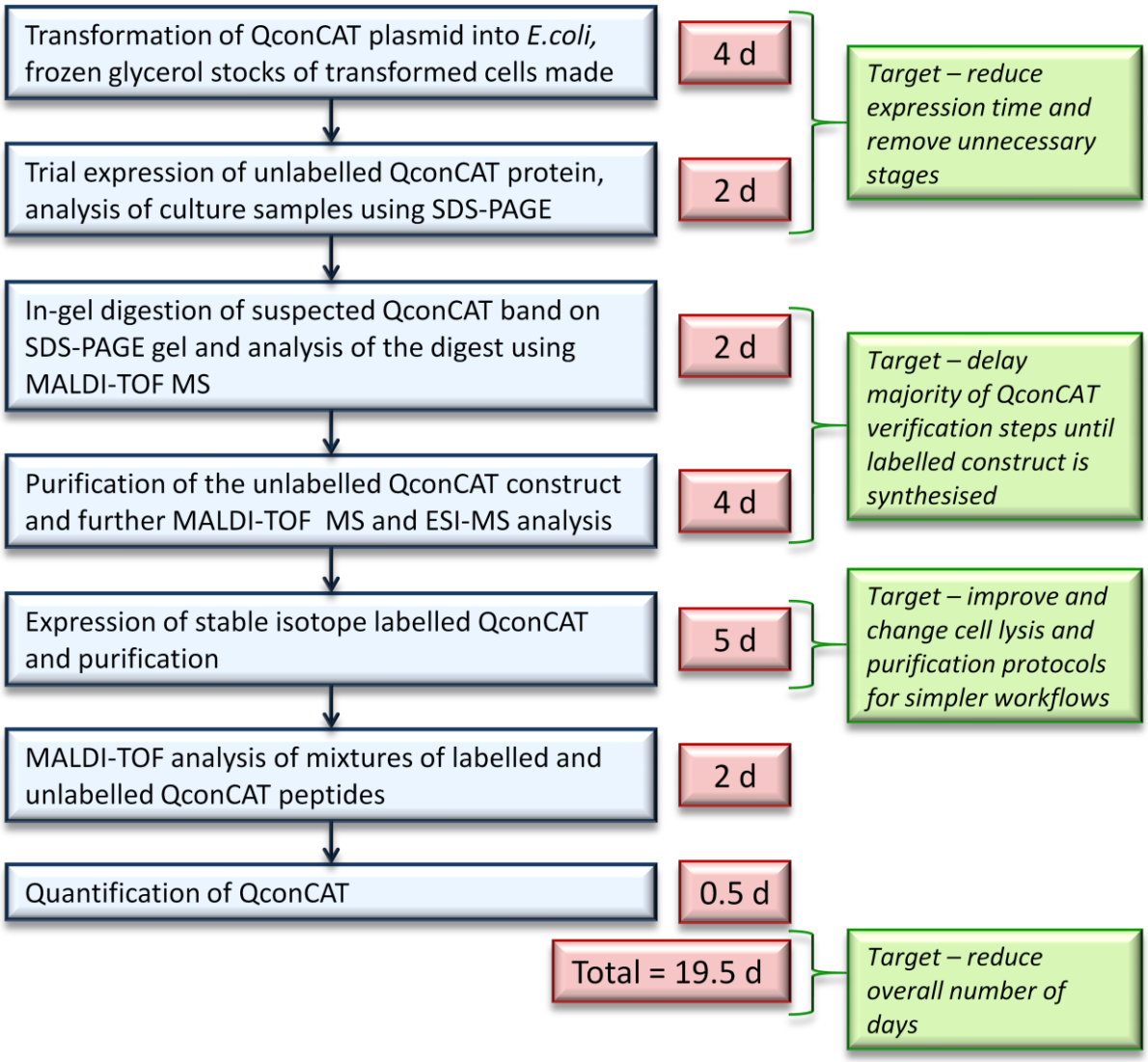

Figure 7| Aims and objectives. Summary flow diagram of the original QconCAT production protocol and the number of days allocated to each stage. The diagram is annotated with targets for where time reductions and simplification of the protocol can be implemented.

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3. Materials and Methods

Reagents used are purchased from Sigma Aldrich, Dorset, UK including: Amino acids, both unlabelled and stable isotope labelled [$^{13}C_6$]Arginine and [$^{13}C_6$]Lysine | Ampicillin salt | α-cyano-4-hydroxycinnamic acid | Guanidine hydrochloride | Isopropyl β-D-1-thiogalactopyranoside | Trypsin

Other reagents were acquired as detailed below:

Acrylamide (30% stock solution); Severn Biotech Ltd, Worcester, DY11 6TJ | Anti-mouse IgG; Cell Signaling Technology, Inc., Danvers, MA 01923 | Anti biotin IgG; Cell Signaling Technology, Inc., Danvers, MA 01923 | Anti his-tag monoclonal antibody; Novagen® from Merck KGaA, Darmstadt, Germany | Benzonase nuclease; Novagen® from Merck KGaA, Darmstadt, Germany | Biotinylated molecular marker; Cell Signaling Technology, Inc., Danvers, MA 01923 | BugBuster; Novagen® from Merck KGaA, Darmstadt, Germany | Complete EDTA free protease inhibitor tablets; F. Hoffmann-La Roche Ltd, Basel, Switzerland | Coomassie Plus™ Protein Assay reagent; Fisher Scientific UK Ltd, Loughborough, Leicestershire, LE11 5RG | Dithiothreitol; Melford Laboratories Ltd, Chelsworth, Ipswich, Suffolk, IP7 7LE | Generon midi spin tube; Generon Ltd., Maidenhead, Berkshire, SL6 1AP | Luria-Bertani broth (MILLER); Merck KGaA, Darmstadt, Germany | Ni-MAC columns; Novagen® from Merck KGaA, Darmstadt, Germany | Ni-Superflow Resin; Generon Ltd., Maidenhead, Berkshire, SL6 1AP | Protino® Ni-TED resin and disposable columns; Macherey Nagel supplied by Fisher Scientific UK Ltd, Loughborough, Leicestershire, LE11 5RG | QconCAT coding sequence; designed by Craig Lawless, synthesised and supplied by Entelechon/PolyQuant GmbH, Industriestr. 1, 93077, Bad Abbach, Germany | RapiGest™ SF; Waters, Elstree, UK | SDS-PAGE Molecular Weight Standards, Broad Range; Bio-Rad Laboratories Ltd., Hemel Hempstead, Hertfordshire HP2 7DX | StrataClean™ Resin; Stratagene supplied by Agilent, Stockport, Cheshire SK8 3GR | SuperSignal West Pico Chemiluminescent Substrate; Thermo Scientific, 3747 N Meridian Rd, Rockford, IL USA 61101 | Yeast extract; Merck KGaA, Darmstadt, Germany

NOTE: all of the solutions used in the methods 4.1-4.5 are sterilised before use either by autoclave or filter sterilisation

3.1 Transformation

The QconCAT was designed as a gene which was cloned into a plasmid vector, for expression in the host BL21 (λDE3) strain. The plasmid vector used for QconCAT expression was pET21a which has a selectable ampicillin resistance marker and the inserted gene is placed under the control of a T7 promoter, meaning that expression of the gene is controlled using the T7 expression system.

For transformation (Mandel and Higa, 1970) the cells were made competent, therefore receptive to DNA, by treatment with calcium chloride, and heat shock was used to allow the DNA to enter the cell. The supplied 5µg of plasmid was solubilised in TE buffer (10mM Tris 1mM EDTA) to a final DNA concentration of 1ng/ µl. 5µl (5ng) plasmid was combined with 100µl aliquot of competent cells, and 42°C heat shock was used. The cells were pelleted (1677 x g), resuspended in LB media and plated out onto ampicillin containing agar plates (50µg/ml) to allow for selected growth of only the transformed cells. Growth on the agar plates took place over 16hrs at 37°C.

3.2 Glycerol stocks

A single colony of transformed cells from the agar plate was used to inoculate 10ml of LB media (50µg/ml ampicillin), the broth was grown, at 37°C with 150rpm shaking to aerate the culture, to at least 0.6OD @600nm turbidity, before mixing 2:1 with 60% v/v sterile glycerol; glycerol stocks were stored at -80°C.

3.3 Expression via LB IPTG

A single transformed colony was used to inoculate 10ml of LB media, this was allowed to grow for 16 hours, 100µl of this culture was then used to inoculate a fresh flask of LB media. In the case of a trial induction (where expression of the QconCAT plasmid has not previously been verified) a small 10ml flask of LB was inoculated, in the case of a QconCAT already known to be expressed, 200ml of media was inoculated. The OD of the culture was measured at regular intervals until 0.6OD @600nm was reached; at this time IPTG was added to the culture at a final concentration of 1mM to induce expression of the QconCAT. Samples were taken throughout the culture time, both before and after induction, to measure the OD of the culture. These were subsequently centrifuged (1677 x g) and cell pellets resuspended in water to lyse the cells. Water was added in varying volumes calculated to make the concentration of the cell contents the same for all samples. Samples were then analysed by 1D SDS-PAGE to

assess QconCAT expression. The culture was harvested four/five hours later by centrifugation (1600 x g 15min).

3.4 Expression via minimal media IPTG

E.coli containing the QconCAT plasmid were grown in minimal media with amino acids [$^{13}C_6$]arginine and [$^{13}C_6$]lysine, to elicit a stable isotope labelled standard. Typically 0.1mg/ml of each labelled amino acid was used in a 200ml culture to ensure complete labelling of the product. All other amino acids were unlabelled. The culture was grown under the same conditions as above, and induced with 1mM IPTG at 0.6OD @600nm. All samples taken for OD were retained for 1D SDS-PAGE analysis.

3.5 Expression via autoinduction – unlabelled and stable isotope labelled cultures

Solutions and media used as detailed in (Studier, 2005) both for unlabelled and stable isotope labelled cultures. Baffled flasks were used to increase aeration of the culture and improve culture growth. 1ml samples of the culture were taken at inoculation and immediately prior to harvest for analysis with 1D SDS-PAGE.

3.6 Cell lysis using BugBuster

One cell pellet from 50ml of culture was solubilised in 2ml BugBuster solution (solution made up with 25µl Benzonase Nuclease and 1 x Complete EDTA-free protease inhibitor tablet added to 25ml BugBuster). The pellet was left to solubilise for 20 minutes then insoluble material pelleted (12,200rpm). The supernatant was retained for 1D SDS-PAGE analysis. The insoluble material was then re-suspended in 2.5ml BugBuster solution and lysozyme added to a final concentration of 0.7mg/ml, the suspension was incubated at room temperature for five minutes before the addition of 15ml 1 in 10 dilute BugBuster solution. Inclusion bodies were pelleted (12,200 rpm) and the supernatant again removed and retained for 1D SDS-PAGE analysis.

3.7 Cell lysis using sonication

Cell pellet from 50ml of culture was re-suspended in 2.5ml 50mM phosphate buffer (pH8.0) plus protease inhibitors and benzonase nuclease (25ml buffer made up with 1 x complete EDTA-free protease inhibitor tablet and 25µl benzonase nuclease). The suspension was

sonicated at 30% amplitude in 10 second pulses every minute for 13 minutes. Inclusion bodies were pelleted (6000 x g 8min) and washed three times with 50mM ammonium bicarbonate.

If sonicating a cell pellet prior to purification using Protino Ni-TED resin the pellet was resuspended in 5ml of LEW buffer (50mM NaH_2PO_4, 300mM NaCl, 1µl/ml benzonase nuclease, 1 x complete EDTA-free protease inhibitor tablet per 10ml, pH8.0) Once the pellet was re-suspended lysozyme was added to a final concentration of 1mg/ml and the suspension stirred on ice for 30min. After this time the suspension was sonicated as above and the inclusion bodies pelleted 10,000 x g 30min at 4°C.

3.8 Purification with the Ni-MAC column method

Ni-MAC nickel affinity columns are available in a kit containing columns and the four buffers. Buffers can also be made according to the standard recipe; bind, wash and elute buffers all contain 300mM sodium chloride, 50mM sodium phosphate, 6M guanidine hydrochloride and 10mM, 20mM or 250mM imidazole respectively (pH8.0). Up to 20ml bind buffer was used to solubilise the inclusion bodies (prepared from a 50ml culture cell pellet) containing the QconCAT. After 30min incubation at room temperature with agitation to solubilise the QconCAT the solution was filtered through a 1.2µm syringe filter to remove insoluble material. The column was prepared with ultrapure water and equilibrated with bind buffer before loading the solubilised material onto the column (the starting material); the flow through from loading was collected and retained for 1D SDS-PAGE analysis. The column was washed with bind and wash buffers before eluting the QconCAT from the column with the elute buffer. Washes and elutes were collected in 1ml fractions.

3.9 Preparation of purification fraction samples for SDS-PAGE analysis

Samples of 20µl were taken from representative column fractions; for example the flow through, the first three 1ml fractions of the bind, the first three 1ml fractions of the wash and all of the 1ml fractions of the elute. A volume of 10µl StrataClean resin suspension was added to each sample along with 0.5ml water. Each sample was vortexed for one minute to allow the resin to bind the solubilised protein in the sample. The resin was washed with water to ensure the buffer components, especially guanidine hydrochloride, were removed. Reducing sample buffer was then added to the resin to allow the bound proteins to be released and the samples analysed on a 1D SDS-PAGE gel. The elution fraction(s) which contained the majority of the QconCAT protein were selected for dialysis. The fractions were dialysed against 50mM

ammonium bicarbonate solution over a period of ~20hrs at 4°C. The QconCAT solution was then removed from dialysis and stored at -20°C.

3.10 Purification with the spin column method

Buffers used for this method are similar to those used for the Ni-MAC column purification method, however for spin columns the buffers do not contain sodium chloride and the amounts of imidazole are slightly different; 10mM imidazole for bind buffer, 30mM imidazole for wash and 300mM imidazole for elute (all three buffers pH8.0). Unlike the Ni-MAC column purification, either one or two pellets of inclusion bodies (arising from one or two cell pellets, each from 50ml of culture) can be purified at one time. Each inclusion body pellet was re-suspended in 1ml of bind buffer. The Ni-Superflow Resin was supplied as a slurry in ethanol, the ethanol was removed and the resin equilibrated in bind buffer before addition of the 1ml re-suspended inclusion bodies. A further 3ml or 6ml of bind buffer was added to the resin (depending on whether one or two inclusion body pellets are used) and the suspension was incubated with the resin for one hour at room temperature with agitation. The resin was then transferred to a midi spin column and the solution drawn through the resin with 200 x g centrifugation, the flow through was retained. In the same manner, the resin was washed using wash buffer and then the QconCAT eluted using elution buffer. The samples were treated with StrataClean resin, analysed using SDS-PAGE and dialysed as in method 4.9.

3.11 Purification with Protino Ni-TED resin

The Protino resin can be supplied in pre-packed columns, the method here used the free resin. A pellet of inclusion bodies (from a 50ml sonicated cell pellet) was resuspended in 10ml LEW buffer (recipe as above) to wash the inclusion bodies, the suspension was then centrifuged (10,000 x g 30min 4°C) and the resulting pellet was re-suspended in 2ml denaturing solubilisation buffer (50mM NaH_2PO_4, 300mM NaCl, 6M Gu.HCl pH8.0), the suspension was stirred on ice for 60 minutes. Remaining insoluble material was removed by centrifugation (10,000 x g 30min) and the supernatant retained. Protino resin was weighed out into the disposable column (1g) and equilibrated with denaturing solubilisation buffer before addition of the solubilised inclusion bodies; the column drained by gravity and the flow through was collected and retained. The column was washed with denaturing solubilisation buffer and then the QconCAT eluted using elution buffer (50mM NaH_2PO_4, 300mM NaCl, 6M Gu.HCl, 250mM imidazole pH 8.0). Method 4.9 was used to treat the fractions with StrataClean resin before analysis with 1D SDS-PAGE and dialysis.

3.12 Solubilisation in RapiGest as an alternative to purification

The inclusion bodies pellet resulting from sonication in 50mM phosphate buffer was taken and washed three times with 50mM ammonium bicarbonate. 500µl of 4mg/ml RapiGest solution (0.4% w/v solution) was added to the final pellet, which was then re-suspended and DTT added to the suspension (1mM final concentration). The suspension was incubated at 37°C with agitation for one hour to solubilise the QconCAT and then the insoluble material was pelleted (17136 x g). The supernatant was removed and diluted by the addition of 1.5ml 25mM ammonium bicarbonate (25ml solution plus 1 x complete EDTA-free protease inhibitor tablet, 1.5ml aliquot used); the resulting QconCAT solution was stored at -20°C.

3.13 Protein assay

For some purified QconCAT samples a protein assay was performed. Bovine serum albumin (BSA) was used as a standard for the assay; concentrations of 10mg/ml, 20mg/ml, 30mg/ml, 40mg/ml and 50mg/ml of the standard were used to construct a standard curve. Appropriate dilutions of the analyte protein solution were made so that the concentration fell in the range of the standard curve. Coomassie Plus^{TM} protein assay reagent was mixed 2:1 with standards and analyte samples in duplicate. The absorbance at 620nm was measured using a Labsystems Multiscan Ascent colourimetric analyser and by comparing the absorbance of the analyte samples against the BSA standard curve the concentration of the analyte solution was calculated.

3.14 1D SDS-PAGE gels

12% reducing gels and reducing SDS-sample buffer were used. BioRad Broad Range Molecular Markers added to each gel.

3.15 Western blotting

Western blotting was used to assess the expression of a QconCAT if the QconCAT band was not obviously present on a 1D SDS-PAGE gel. The standard method for western blot transfer of a 1D SDS-PAGE gel was used (Towbin *et al.*, 1979). To develop the western blot Anti-HisTag Monoclonal Antibody was used at a concentration of 0.05µg/ml, followed by Anti-mouse and Anti-Biotin antibodies (1 in 3000 dilution of supplied stock). In the dark room the nitrocellulose was then incubated for one minute in the mixed SuperSignal West Pico

Chemiluminescent Substrate solution before addition of x-ray film for up to five minutes. The nitrocellulose was stained using Ponceau S.

3.16 In-gel digests

1mm^3 plug was removed from a band of interest on a 1D gel. This plug was then destained in a 2:1 solution of 25mM ammonium bicarbonate/acetonitrile. The plug was treated with DTT and Iodoacetamide (IAM) before dehydration in 100% acetonitrile. 10µl of trypsin (12.5ng/µl) was added to the dehydrated plug and incubated for 16hrs at 37°C. After this time the digest was stopped by the addition of 1% v/v formic acid (final concentration).

3.17 In-solution digests

An appropriate amount of protein was selected and diluted to bring the volume up to 160µl. The protein was then treated with 0.05%w/v final concentration of RapiGest^{TM} at 80^{o}C for 10min, 3mM final concentration DTT 60^{o}C for 10min and 9mM final concentration IAM room temperature for 30 minutes before addition of trypsin in a 50:1 ratio to the protein content of the digest. The final volume of the digest is 200µl. An overnight incubation was performed at 37^{o}C after which TFA was added to a final concentration of 0.5%(v/v), this acidification step hydrolyses the RapiGest^{TM} and causes it to precipitate, it can then be removed from the solution by centrifugation leaving a clean digest ready for LC-MS analysis. During the course of its use the in-solution digest protocol was modified slightly and these changes are detailed in the results when used.

3.18 Preparation for MALDI-TOF analysis of digests

The sample, for analysis by MALDI-TOF, was mixed 1:1 with a 10mg/ml solution of α-cyano-4-hydroxycinnamic acid in 50% v/v acetonitrile/0.1% v/v trifluoroacetic acid in HPLC grade water (matrix solution). Calibrants for the instrument were also mixed in the same 1:1 ratio with matrix. 2µl of the calibrant and of each sample were spotted onto a target.

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4. Results and Discussion

4.1 Production of QconCAT EB1 with the original protocol

The EB1 QconCAT was synthesised for Dr Emøke Bendixen and Dr Stine L. Bislev (Bislev *et al.*, 2012). The construct consists of 40 peptides in a stoichiometric 1:1 ratio targeted to 20 proteins which are indicators of the bovine host response to mastitis pathogens and inflammation. A peptide map shows the 44 tryptic peptides included in the construct (Figure 8 (a)). There are two peptides targeted to each of the 20 proteins and the order of the peptides within the construct is optimised for maximum expression. At the N-terminus of the QconCAT there is a sacrificial peptide followed by a GluFib peptide which, as mentioned earlier, can be used for quantification. At the C-terminus there is another quantification and verification peptide, Fib, and finally the His-Tag sequence for purification.

EB1 was made using a slightly altered version of the original QconCAT production protocol (Beynon *et al.*, 2005; Pratt *et al.*, 2006). The production process requires a time allocation of approximately 16 days to produce a product which is highly pure, well verified and suitably concentrated. The QconCAT plasmid was transformed into BL21 DE3 *E.coli* and the transformed cells selected for by antibiotic resistance to ampicillin; the antibiotic resistance gene in the QconCAT plasmid vector selectively allows the growth of only transformed cells on the antibiotic-containing agar plate. In this method the transformed colonies were re-streaked for a second overnight growth. Re-streaking of colonies ensures correct selection of a colony containing the plasmid and allows the plates to be re-used for up to two weeks after initial overnight growth (Brown, 1991). Using a colony from the re-streaked plate, a small LB broth was inoculated and after another overnight growth at 37°C the turbid culture was used to make glycerol stocks of the QconCAT-plasmid-containing cells. The turbid culture was also used to inoculate a further LB broth for trial-expression. After induction and a suitable growth time to allow for the QconCAT to be expressed to sufficient levels, a final culture sample was taken and the remaining culture discarded. The samples were analysed using 1D SDS-PAGE (Figure 8 (b)).

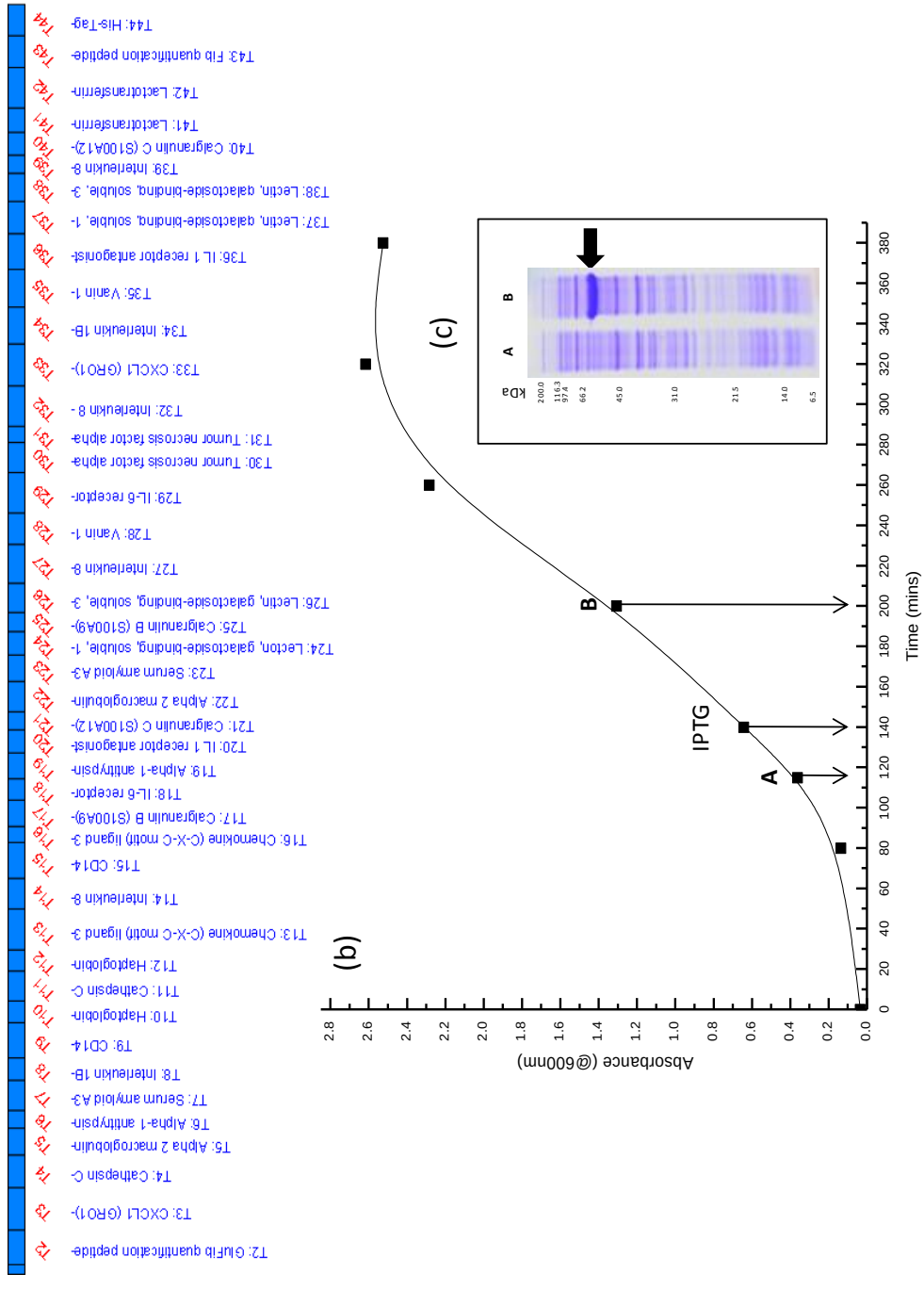


Figure 8| EB1 peptide map, *E.coli* culture and expression of the QconCAT. (a). Tryptic peptides in QconCAT EB1 annotated with the proteins to which the peptides are targeted. **(b).** Growth curve for EB1 QconCAT trail induction culture. Arrows A and B are time points where pre-induction and post-induction culture samples were removed for SDS-PAGE analysis, IPTG arrow indicates 140min time point of induction. **(c).** 1D SDS-PAGE, cell samples for the gel lysed in water to a volume which results in the concentration of cell contents being consistent for all samples. Arrow indicates the presence of a new band in the post-induction lane, suspected to be the expressed QconCAT.

Post-induction with IPTG a new protein band at ~56kDa is seen on the SDS-PAGE gel that was not visible pre-induction (Figure 8 (b)). This is the approximate mass for the QconCAT and the size of the band indicates a large amount of expressed product; over-expression is regularly seen with QconCATs. To verify the product was EB1, two $1mm^3$ spots were cut from the suspected QconCAT band and in-gel digestion performed. A sample of the digest was then mixed in a ratio of 1:1 with a solution of α-cyano-4-hydroxycinnamic acid matrix and analysed using MALDI-TOF MS (Figure 9).

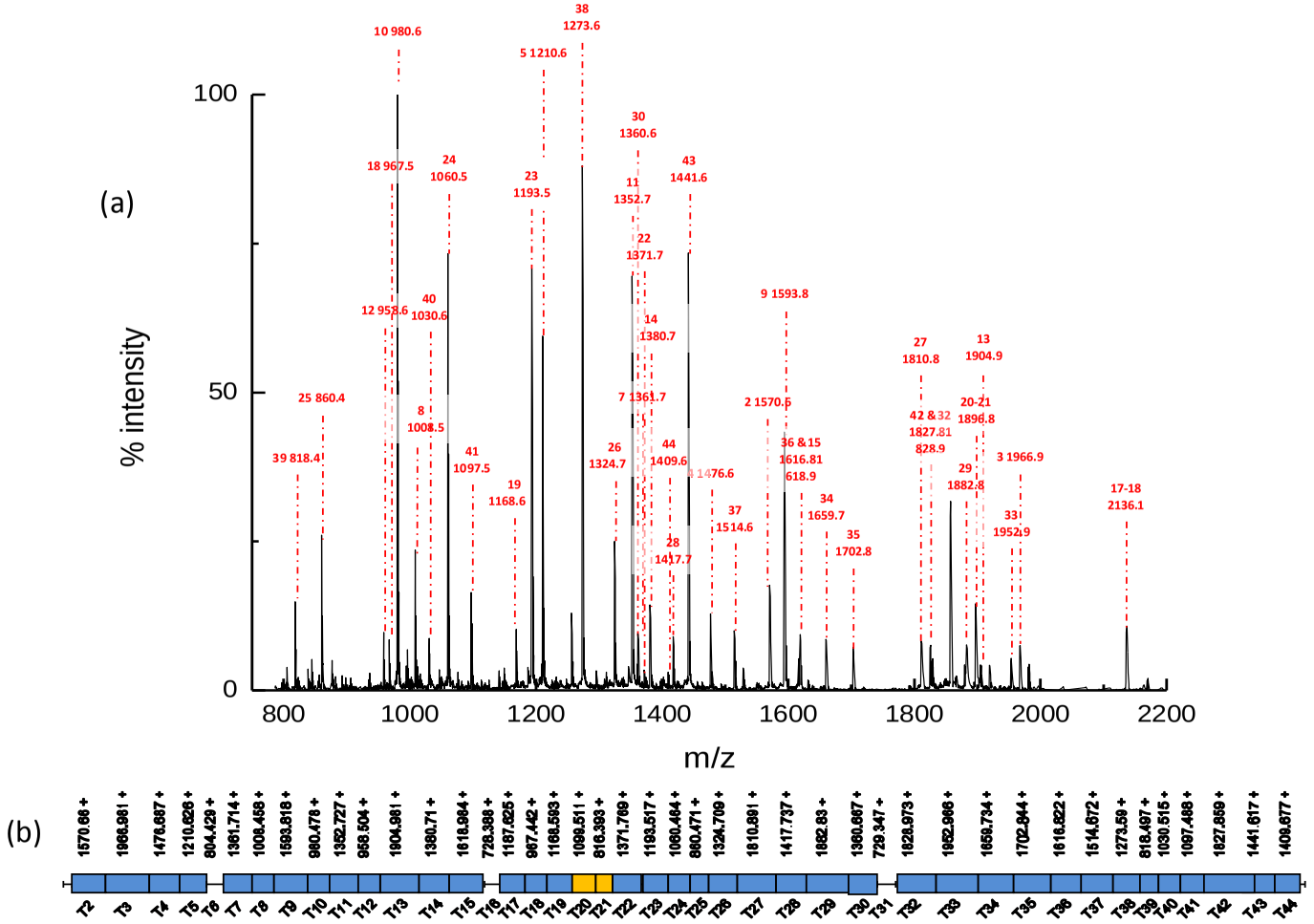
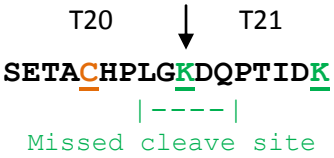


Figure 9| In-gel digestion of QconCAT EB1. (a). MALDI-TOF spectrum resulting from in-gel digest of QconCAT EB1 expression band (unlabelled culture). Significant peaks explained with label of peptide number and m/z value, apart from two peaks (1256.56 and 1856.8) where the mass could be attributed to a modification of a QconCAT peptide. **(b).** Peptide map shows in green the peptides seen in the MALDI-TOF spectrum. The yellow peptides appear together as a missed cleavage but are not identified individually.

The MALDI-TOF mass spectrum for the EB1 in-gel digest is complex and m/z values for all but three tryptic peptides are seen (Figure 9). It is expected that not all of the peptides will be seen in a MALDI-TOF spectrum, especially those at a lower m/z due to interference from matrix ion peaks. The high proportion of coverage of the QconCAT illustrated in the peptide map, coupled with the presence of peptides from the entire length of the sequence, confirm that the correct full length product has been made. The m/z for a missed cleavage between T20 and T21 has been identified; this suggests that digestion of that peptide bond was not complete. The sequence covering the missed cleavage site is:



Aspartic acid (D) is an acidic amino acid residue and the presence of this residue in the P1' location (first amino acid after the basic lysine cleavage site) could affect the cleavage of the peptide bond leading to incomplete digestion (Brownridge *et al.*, 2011).

No other missed cleave m/z values are seen however missed cleaves could be too large to appear in the mass range analysed in this MALDI spectrum. As a digest to show the presence of the full length QconCAT the data is sufficient but complete digestion of the QconCAT cannot be shown.

Once the correct QconCAT product was confirmed the stable isotope labelled QconCAT was made. An LB broth was again inoculated with a single colony. The turbid culture was used to inoculate a second starter culture of 10ml minimal media broth, this broth contains no amino acids; minimal media with [$^{13}C_6$]arg and [$^{13}C_6$]lys amino acids is inoculated using this second starter culture. The amino acids contained in the minimal media are incorporated into any new protein the *E.coli* cells synthesise, this includes the QconCAT. Expression of the QconCAT was induced and the culture harvested after the suitable growth time. Expression of the QconCAT is indicated with an arrow to the QconCAT band (Figure 10 (a)).

The culture was harvested into cell pellets resulting from 50ml of culture each. Because the culture produces more than is usually required in quantification studies only one cell pellet is processed at a time. For QconCAT EB1 the cells were lysed using BugBuster, which is a detergent reagent which acts to disrupt the cell membrane causing a release of the cell contents. Insoluble inclusion bodies (IBs) were recovered by centrifugation and SDS-PAGE was used to verify the presence of the QconCAT in the IBs. The IBs were then solubilised in a buffer

containing guanidine hydrochloride to denature the protein, and purified using the Ni-MAC purification protocol.

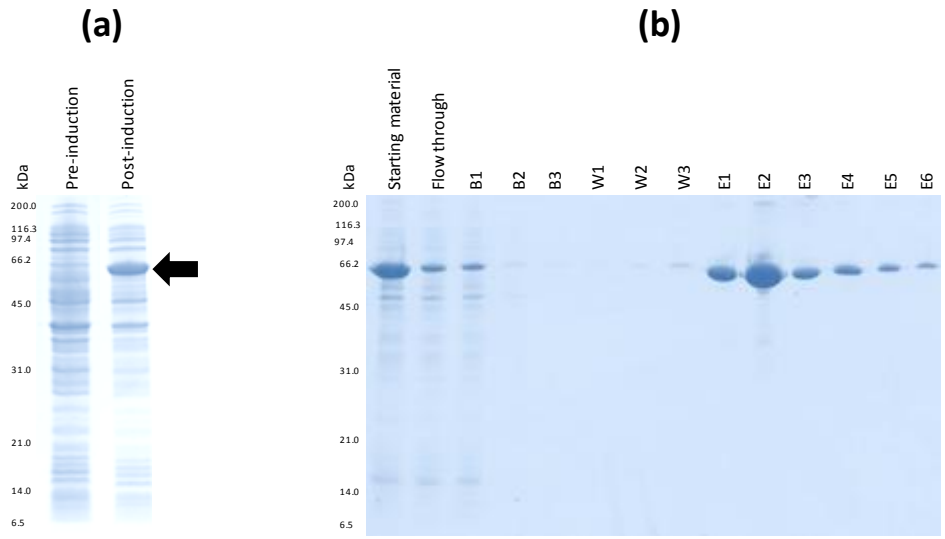


Figure 10| EB1 heavy isotope labelling and NiMAC purification. (a). Expression of the QconCAT indicated by the black arrow. **(b).** Starting material of solubilised IBs, the flow through, binding (B) washes (W) and elutions (E) from the column all sampled and analysed using SDS-PAGE. QconCAT band in E1-E6 is the majority or sole band present.

NiMAC Purification of the QconCAT elicits a highly pure product; this can be seen from the SDS-PAGE analysis (Figure 10 (b)). The QconCAT band is either the sole band in the elution fraction (E1, 3, 4, 5, 6) or the major band occupying the majority of the lane (E2). The elution buffer contains high levels of sodium chloride, guanidine hydrochloride and imidazole therefore dialysis is required to exchange the buffer to one more suitable for protein storage and compatible with MS analysis; the buffer usually used is 50mM ammonium bicarbonate. In the case of QconCATs that contain cysteines in their sequence there is a risk of di-sulphide linkages forming and causing the QconCAT to fold and precipitate. QconCAT EB1 contains seven cysteines which are shown in the QconCAT sequence below (orange). To prevent precipitation, DTT (a strong reducing agent) was added to the dialysis buffer to a final concentration of 1mM in order to reduce any disulphide bridges that might occur. A protein assay was performed on the dialysed QconCAT and the concentration calculated to be 1.14µg/µl, equivalent to 20.1pmol/µl.

QconCAT EB1 amino acid sequence

```
MGTKEGVNDN  EEGFFSARVT  TPGPHCDQTE  VIATLKTGNT  SENVNVNTAR
|Q1|---------Q2------|------------Q3----------|---------Q4-----|
LPPNVVEESA  RADLSGITKE  TIQGITDPLF  KGGQDITDFR  VQPQSLDLSH
|------Q5-----|---Q6--|---------Q7-------|----Q8--|-------Q9----
NSLRVGYVSG  WGRNVHGINF  VTPVRNQLVE  VEKVTPPGPH  CGQTEVIATL
----|-----Q10---|------Q11-----|-----Q12--|---------Q13---------
KNLENFLQFS  LRLGAAQVPA  QLLVAVLRAA  TAAAPRLGHY  DTLIQKEDHP
|------Q14-----|---------Q15------|----Q16---|-----Q17----|-Q18-
AGSVRLSISE  TYDLKSETAC  HPLGKDQPTI  DKIQHHTLLA  SPVRADQFAN
-----|------Q19---|-----Q20----|---Q21---|-------Q22----|--Q23-
EWGRDAGAWG  AEQRELPNFL  KIQVLVEPDH  FKVIESGPHC  ENSEIIVKNL
----|----Q24-----|---Q25---|------Q26----|--------Q27--------|--
DLLEGAVTSA  SKQVFQEPCQ  YSPESQRDVE  LAEEVLSEKA  GGPQGSRIAD
------Q28------|-------Q29-------|------Q30------|----Q31---|---
LITTPATNTD  LLEKVTTPGP  HCDQTEVIAS  LKNSAYAHVF  HDDDLRDSAP
-------Q31-------|-----------Q32---------|-------Q34------|----
NTLSDLTTQA  LRNNQLVAGY  LQGPNTKDDN  NLCLHFNPRG  NDVAFHFNPR
-----Q35-------|-------Q36-------|-------Q37-----|------Q38----|
VVQVFVKVGH  FDTLNKYYGY  TGAFRGEADA  LNLDGGYIYT  AGKGVNDNEE
--Q39--|-----Q40---|----Q41----|-------------Q42-------|---Q43--
GFFSARLAAA  LEHHHHHH
------|------Q44------|
```

The EB1 sequence follows the typical example set out in the introduction. Each peptide is indicated below the sequence. Q1 is the sacrificial peptide, Q2 the GluFib peptide (in purple) Q3-42 are the quantification peptides, Q43 is Fib (in purple) and Q44 is the hexahistidine tag (in blue) The location of the arginine and lysine residues, which denote the cleavage site for each peptide, are indicated with arginine in red and lysine in green.

The final verification step for the QconCAT is to establish that it is fully labelled. An in-solution digest was performed on the purified QconCAT and analysed using MALDI-TOF MS (Figure 11).

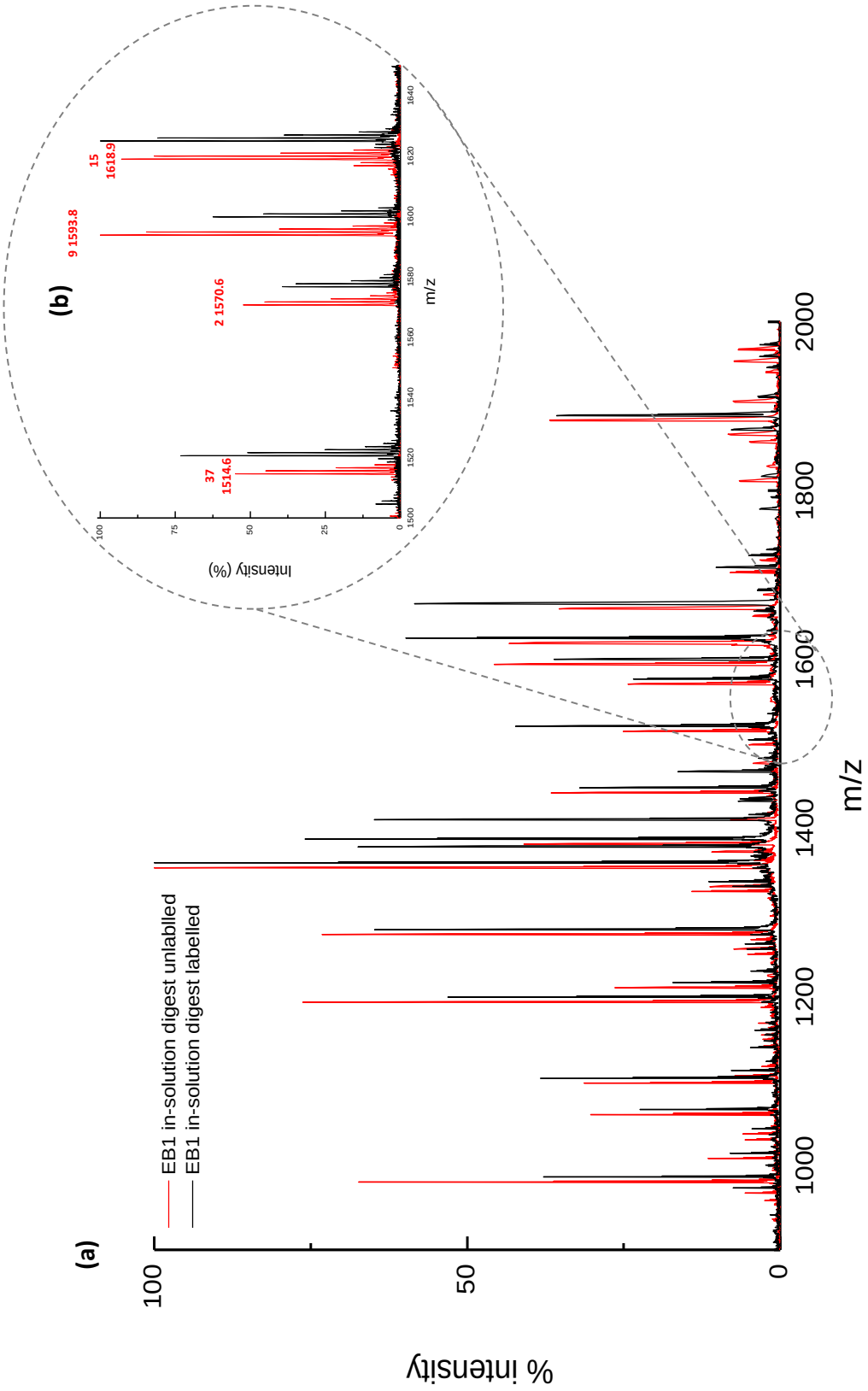


Figure 11 | Labelled and unlabelled EB1 QconCAT spectra. (a). Pair of MALDI-TOF spectra of stable isotope labelled QconCAT EB1 in-solution digest shown in black, overlaying spectrum of unlabelled EB1 in-solution digest shown in red. Peaks show a mass shift up the m/z scale for the stable isotope labelled QconCAT. This shift appears across the entire m/z range. **(b).** Zoomed in on section of the spectrum showing the +6Da mass shift for 5 EB1 tryptic peptides.

In Figure 11 the +6Da mass shift for each QconCAT peptide is clearly visible compared against the spectrum for the light QconCAT. The intensities for the peptides do not exactly match between heavy and light forms however there is good correlation and some level of error is expected due to inherent errors in pipetting and variability in MALDI ionisation. The increased mass for each peptide across the entire mass range shows that the QconCAT has been successfully labelled and indicates full labelling. The QconCAT is therefore suitable for use in quantification studies.

Section 1: modifying the QconCAT production protocol

In order to produce QconCAT EB1 a number of set protocols were used, these successfully resulted in the required standard and were originally chosen for a number of reasons; cost effectiveness, ease of use, most effective results or equipment available. However depending on the number of QconCATs required and the time scale being worked to, the process could be considered lengthy and it does not easily lend itself to multiplexing. Many of the methods used can be changed, and during the course of my work with QconCATs I have made developments to the protocols in order to reduce time and cost of production.

The QconCATs used during the rest of this study all pertain to the LOLA COPY project which is a BBSRC funded project aiming to quantify over 4000 proteins in the *S.cerevisiae* proteome, using QconCAT as the quantification strategy. QconCATs associated with this project are referred to as “COPYCATs” and are numbered in order of design date. The abbreviation “CC” is used for COPYCAT followed by a three digit number and the majority of further QconCATs will be referred to in this way.

4.2 Reducing expression time

The simplest step to consider removing from the protocol was re-streaking the transformed *E.coli* colonies before inoculation of an LB broth. In some cases it possible that a gene coding a recombinant protein can be excised from the pET 21a plasmid, the *E.coli* retains the plasmid so retains the additional properties, essentially the ampicillin resistance the plasmid confers, however the coding sequence for the protein is lost. If this occurs the *E.coli* colony will still grow on selective ampicillin agar plates, however expression of the recombinant protein from that colony is not possible. When a colony is re-streaked the morphology of the resulting colonies can be studied and a colony selected that has a typical morphology. In this way the probability of selecting a colony containing the full recombinant protein gene is increased.

However for QconCAT transformation the colony morphology from the first plating is regularly seen to be uniform which indicates universal uptake of the plasmid and retention of the QconCAT gene, therefore the re-streaking step was removed and inoculation with a freshly transformed colony seemed to have no detrimental effect on the culture. Removing this step reduces the production time by 1 day.

The QconCAT is usually expressed in unlabelled form initially to check for expression and to verify that the correct product is being synthesised. After production of a large number of QconCATs it became clear that expression was consistently successful (Figure 12), for each of these QconCATs we observed expression of the correct protein construct. We saw no evidence for an incorrect, truncated or otherwise altered QconCAT being synthesised and this indicated that verification of the QconCAT product was not necessary for the continuation to stable isotope labelling. The production protocol was altered to eliminate the initial unlabelled expression and MALDI-TOF verification of the QconCAT; transformed colonies were instead taken on to immediate expression in minimal media with stable isotope labelled arginine and lysine. This “straight to heavy” approach removes at least six days from the production time. In ~85% of cases this approach is highly successful, however in ~15% of cases a QconCAT construct has failed to express. In these situations the heavy amino acids used in the culture are wasted, however the infrequency of this occurrence means that the benefits of removing a number of days from production time outweigh the negatives.

Reduced expression time -6d and removed verification -6d

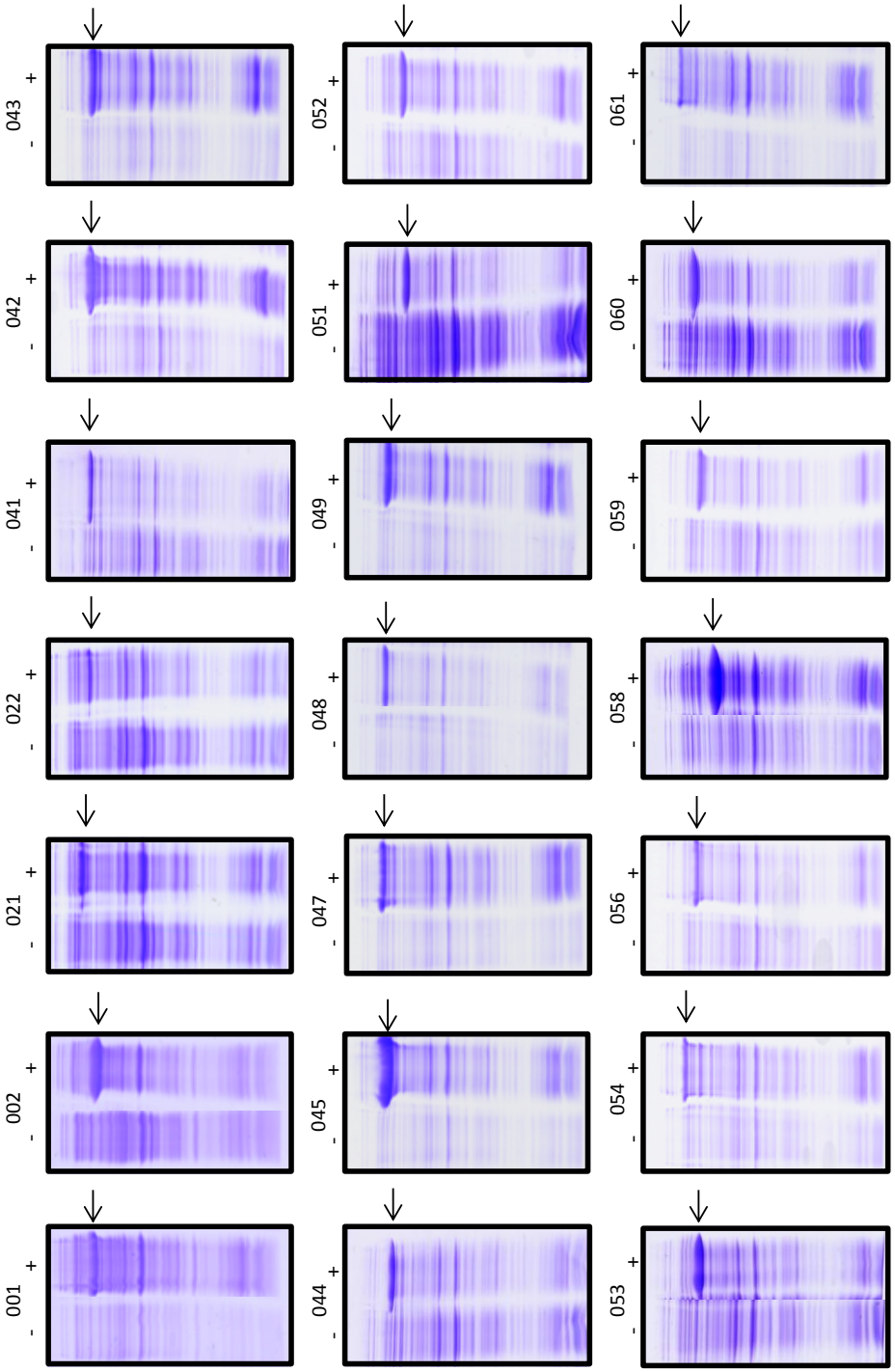


Figure 12 | Consistent successful expression of QconCATs. Data in this figure used in Brownridge et. al. 2011. Series of SDS-PAGE gel images of pre (-) and post (+) induction time point samples for 21 QconCATs. QconCAT bands indicated with an arrow.

4.3 Alterations to the cell lysis protocol

E.coli cells expressing EB1 QconCAT were lysed using BugBuster, a method which had been used a number of times in our lab successfully and which worked well for EB1. However with three QconCATs – CC001, CC002 and CC003 this method failed. CC001-003 expressed well in stable isotope labelled form (Figure 13 (a)), the cells were lysed using BugBuster, the inclusion body pellets solubilised and purified with nickel-affinity chromatography NiMAC cartridge. SDS-PAGE analysis of the fractions eluted from the Ni-MAC cartridge revealed a number of contaminant bands present in the elution lanes, in addition to the QconCAT band (Figure 13 (b)). The SDS-PAGE gel for CC001 was selected and in-gel digestion was performed on the contaminant bands to establish the nature of the contaminants. Two potential sources were considered, either fragmentation of the QconCAT had produced these bands, or inefficient purification could have left *E.coli* proteins from the inclusion bodies in the eluate. The in-gel digests was analysed using MALDI-TOF MS and the results, although giving low coverage of the QconCAT, clearly indicated that the bands had resulted from QconCAT fragments rather than *E.coli* proteins (Figure 13 (c)).

Next, the stage at which the QconCAT fragmented needed to be identified; the likely points could have been either immediately, as the QconCAT was expressed, during cell lysis, or during purification. An anti-Histag western blot was performed on the end-time-point samples from culture. This would show whether any fragments containing the HisTag were present during production and therefore whether the QconCAT was being fragmented immediately after synthesis. The western blot (Figure 14 (a)) shows a single his-tagged band for each time point sample; this shows a strong chance that the QconCAT was not fragmented in the intact cell. There is a possibility that the QconCAT had fragmented but that the fragments had lost their HisTag and so were not visible via western blot, but as the in-gel digests showed peptides which all came from the HisTag end of the QconCAT this is less likely.

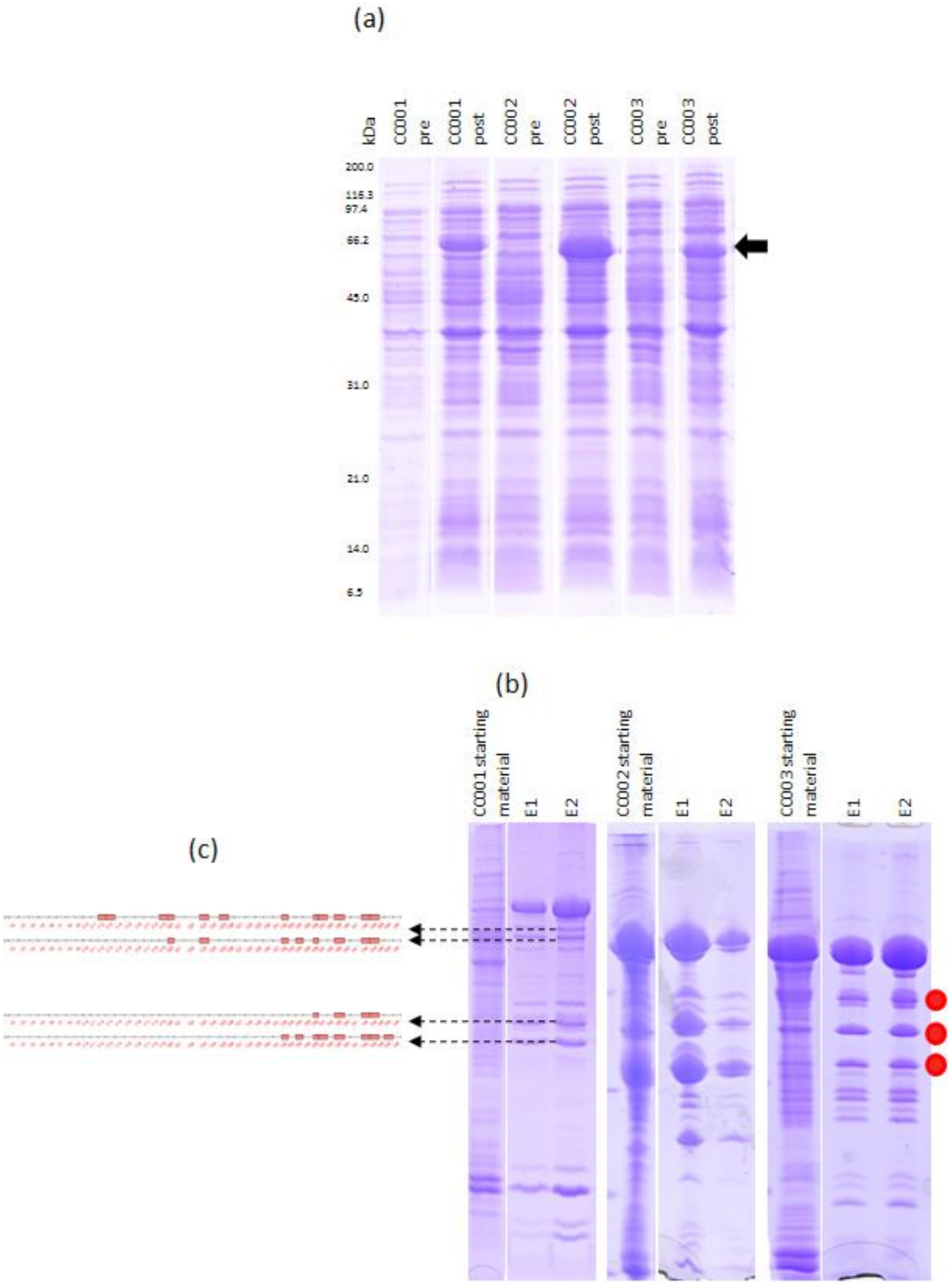


Figure 13| Expression and purification of CC001-003. (a). SDS-PAGE, pre and post induction time point samples, whole cell lysate from *E.coli* culture, QconCAT band is seen in the post-induction lane for each indicated by a black arrow. **(b)**. SDS-PAGE showing the starting material and eluted lanes 1 and 2 from purification of the expressed QconCAT, large number of bands present in the elution lanes in addition to the desired QconCAT band. **(c).** Peptide maps shows the coverage of QconCAT CC001 from the in-gel digest of the highlighted bands.

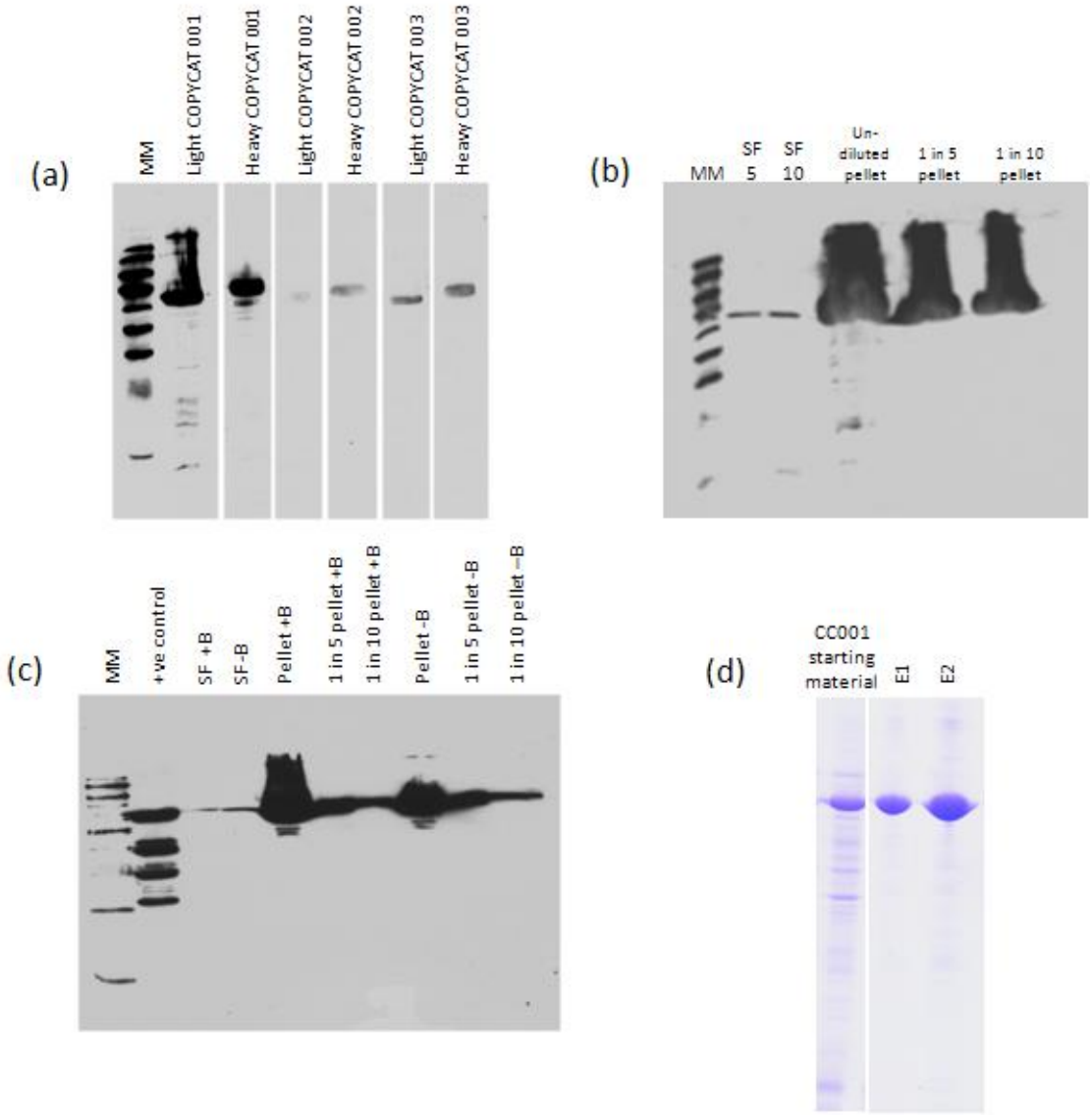


Figure 14| Western blots tracking the fragmentation of CC001-003. (a). Whole cell lysate end time-point samples from unlabelled (light) and labelled (heavy) culture. QconCAT band visible, very small or no fragment bands seen. **(b).** Sonicated inclusion bodies and soluble fraction, very small or no fragment bands seen.. **(c).** Soluble fraction and inclusion bodies blot with and without Benzonase Nuclease (+/-B). **(d).** NiMAC purification of CC001 inclusion bodies. Starting material contains the QconCAT band and additional bands, E1 and E2 contain QconCAT the elution as the sole band.

It was observed that some of the bands in the elute lanes of the purification gels can also be seen in the starting material, for example with CC003 as indicated on the gel image by ret dots (Figure 13 (b)). This suggests that the QconCAT was already fragmented before it was loaded on to the column. This left two possibilities – either the QconCATs were fragmenting during

cell lysis or fragmenting during solubilisation of the inclusion bodies. To test if the fragmentation was occurring during cell lysis the method of lysis was changed to sonication. After sonication, the soluble fraction and the insoluble pelleted material were analysed using SDS-PAGE and western blot performed to look for HisTag bands. In the western blot (Figure 14 (b)) some QconCAT is visible in the soluble fraction but the majority is in the insoluble pelleted material. Although there is clear overloading of QconCAT material there are no signs of fragmentation. It therefore looked likely that the source of the fragmentation was during BugBusting.

There should not be any enzymes in the BugBuster solution, BugBuster is reported to disrupt cell membranes without damaging proteins (Novagen® product information) and it is a reagent which has been used successfully in our lab for quite some time therefore it was considered that the source of the fragmentation could be added during BugBusting. Benzonase nuclease is added during BugBusting and it was considered that this might be a source of proteases which could cause the fragmentation. This hypothesis was tested with COPYCAT CC002. A cell pellet was resuspended in phosphate buffer for sonication, the suspension was sonicated using the standard protocol and then two samples of this material were taken – to one sample buffer containing Benzonase nuclease was added, to the other just sample buffer was added. This was allowed to incubate for a short time before centrifuging to pellet the insoluble material. The supernatant was decanted and samples of the pellet and supernatant were analysed using western blot. Figure 14 (c) shows that the Benzonase nuclease appeared to have no fragmentation effects.

The source of the fragmentation was still unknown, contaminants in that particular batch of BugBuster could have introduced proteases to the solution, however the alteration of lysis method solved the fragmentation problem. A comparison was drawn between sonication and BugBuster (Table 1).

Criteria	BugBuster	Sonication
Time per sample	76m • 2m resuspension • 22m solubilisation • 1m transfer • 20m centrifugation • 5m resuspension • 5m incubation • 1min vortex • 20min centrifugation	19m • 1m resuspension • 10m sonication • 8m centrifugation
Suitability for multiplexing	Centrifugation, solubilisation and incubation steps can be multiplexed	Centrifugation step can be multiplexed
Time increase for multiple	9m per sample	10m per sample
Time for typical 8 samples	139m	89m

Table 1| Comparison between BugBuster and sonication methods of cell lysis

Sonication takes less time than BugBusting whether processing a single QconCAT or 8 QconCATs in parallel. The change of cell lysis method reduced production time, making it possible for cell lysis and purification protocols to be easily completed in one day. The insoluble inclusion body material of each QconCAT CC001-003, resulting from sonication, was taken on to nickel affinity purification and the eluted QconCAT contained no fragment bands (Figure 12 (d)). Sonication was adopted as the standard cell lysis protocol.

Reduced cell lysis time up to -½d

4.4 Alterations to the purification protocol

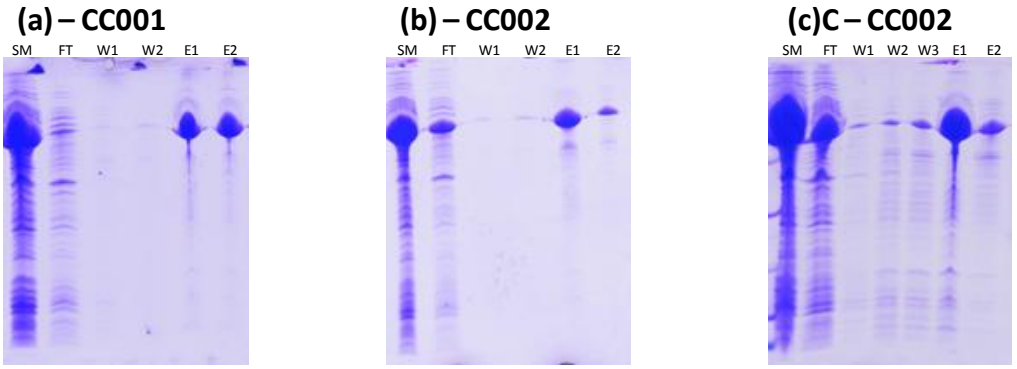
SPIN COLUMN

Purification of the QconCAT from the whole-cell lysate is achieved by nickel affinity chromatography using the HisTag, which is included in each construct. The standard purification method used for EB1 was Ni-MAC cartridge. The cartridges come ready prepared, containing nickel immobilised on resin, 1ml of which is packed into a column. Solubilised QconCAT is loaded onto the column at a rate of about 0.5ml/min, the column is washed and then the QconCAT eluted off. This method produces a pure product but is time consuming and difficult to multiplex without the use of a peristaltic pump or simultaneous delivery method. For this reason other methods were considered. The first of these was spin column purification using resin from Generon. This method uses the same principles as Ni-MAC purification, where immobilised Ni^{2+} ions bind to HisTagged QconCAT and the pure QconCAT is eluted after washing of the resin. In the case of spin columns the resin is packed into a column within a centrifuge tube and rather than pumping the liquid through the column at a set flow rate, the column of resin is centrifuged to bring the liquid through the resin. This method of flow means that a number of columns can have QconCAT applied at any one time and all centrifuged simultaneously, thereby multiplexing the purification process.

In order to refine the method, I varied and monitored the volume of resin used, the amount of IBs used and the volume of binding buffer in which the IBs were added to the resin. The results of varying these three factors are shown in Figure 15. For the first purification trial (gel (a)) one IB pellet of CC001 (arising from the sonication of a cell pellet from 50ml of culture, which should contain ~7.5×10^{10} cells) was solubilised in 4ml of bind buffer and incubated with 1ml of resin. The reported binding capacity of the resin is up to 15mg/ml resin depending of the protein. The protein assay of the starting material (shown in Table 2 in Figure 15) gave the total protein content of the 4ml solubilised IBs to be 3.4mg, it would be expected that the IBs do not contain just the QconCAT protein, therefore the actual amount of QconCAT would be less than 3.4mg, however as the majority protein in the solution (see starting material (SM) band to see the overwhelming presence of the QconCAT) 3.4mg is given as an estimate. As this amount of protein is less than the reported binding capacity of the resin it was presumed that 1ml resin should be more than adequate to bind all of the QconCAT. The results showed that some QconCAT had not bound to the column and had come through in the flow through but it seemed from the gel that the majority of the QconCAT was present in fractions E1 and E2. However, the values from the protein assay, in the table, do not reflect the gel; the assay

indicates 30% of the QconCAT was recovered, therefore a large amount either did not bind to the column and was washed off, or still remained on the resin. A sample of the resin was analysed using SDS-PAGE by washing the resin with water before adding reducing sample buffer and loading approximately 10µl of beads onto the gel; this analysis did not reveal any protein bands removed from the beads (results not shown). It was therefore likely that a significant proportion of the QconCAT protein did not bind to the resin in the first instance and was washed off the column in the flow through.

We have found that, although the amount of QconCAT produced varies between constructs, on average a 200ml labelled culture (~3 x 10^{11} cells) provides a minimum of 1.5mg protein, which is ~25nmol (based on average molecular weight of 60,000Da). Each culture therefore provides enough material for ~1000 quantification studies if 20-30pmol of QconCAT is used in each study. The loss of QconCAT during the purification method is therefore not necessarily a problem, a compromise can be drawn to use a method which is lossy but rapidly generates enough useable material for the required studies.



Purification	Starting material total amount	Dialysed material total amount	Percentage of QconCAT material recovered
A	56.0nmol/3488ug	17.08nmol/1067ug	30.5%
B	58.2nmol/3547ug	6.84nmol/416.6ug	11.75%
C	58.9nmol/3589ug	10.3nmol/626.6ug	17.5%

Table 2| Protein assay results

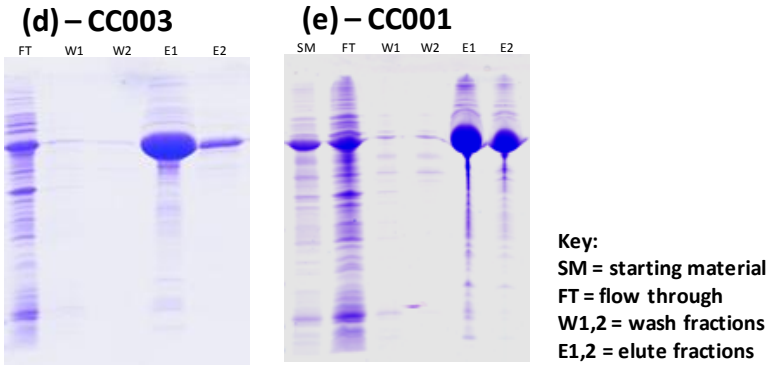


Figure 15| SDS-PAGE of fractions from purifications using spin columns. (a). Purification of QconCAT CC001, 1 IB pellet added to 1ml resin in a 4ml volume. **(b).** Purification of QconCAT CC002, 1 IB pellet added to 0.5ml resin in a 4ml volume. **(c).** Purification of QconCAT CC002 1 IB pellet added to 0.25ml resin in a 0.5ml volume **Table 2| Protein assay results** Comparing the recovery from the different conditions of purification. The largest recovery comes from purification A. **(d).** Purification of QconCAT CC003, 2 IB pellets added to 2ml resin in a 5ml volume. **(e).** Purification of QconCAT CC001, 2 IB pellets added to 2ml resin in a 5ml volume.

It was possible that the ratio between the starting material volume and the amount of resin was not optimal for complete binding; two more purification conditions were screened, one with half the amount of resin with the same volume of QconCAT (Figure 15 gel (b)) and the other with a quarter the amount of resin in a quarter volume of QconCAT (Figure 15 gel (c)). In gel (b) there is a large amount of QconCAT seen in the flow through lane, the protein assay also reports that much less QconCAT is recovered – 12%. In (c) again a large amount of QconCAT is seen in the flow through. Additionally there is also QconCAT seen in the washes, and a number of additional bands in the elute lanes. Although the protein assay shows the

amount of recovered QconCAT is not as low as in (b) (17.5% recovered) it is still lower than the recovery for (a). Lowering the volume of resin used was therefore concluded to be ineffective. The next investigation was based on the hypothesis that the concentration of the QconCAT in the starting material was not optimal for complete binding. For (d) and (e) two inclusion body pellets were used, with 2ml of resin in a volume of 5ml. For CC003, Gel (d), 14.5nmol of material was recovered. When the conditions were repeated for CC001 there was good recovery, however the elution contained a number of additional proteins shown by bands seen in the elute lanes on the gel. Moreover, when 1mM DTT was added to the buffer during dialysis of CC001 the QconCAT solution turned gradually brown over a period of ~2hr (previous QconCAT solutions had been uncoloured). This occurred for a number of different QconCATs under the same purification conditions, the image below(Figure 16) shows an example of the QconCAT solution after DTT addition:

Figure 16 | Brown QconCAT solution post-dialysis in 50mM ammonium bicarbonate with 1mM DTT

It is likely that the brown solution was an indication that the nickel in the affinity purification was leaching off the column. Other purification methods had been suggested and the spin column purification method was abandoned in favour of alternative methods where this discolouration was not observed.

It was observed that the intensity of the protein bands on the purification gels do not necessarily reflect the quantities stated in the protein assay; this is likely due to the use of StrataClean resin. The fractions eluted from the affinity purification column contain imidazole and guanidine hydrochloride, which need to be removed before running a sample of the fraction on a gel. In order to do this StrataClean resin is used to bind the protein in the sample and the resin is then washed to remove unwanted chemicals before loading the resin onto a gel. Variations in the binding capacity of the resin, the amount of sample loaded onto the resin in a set volume, the ability of the sample buffer to remove the protein from the resin and the number of beads that are able to be loaded onto the gel all affect the final amount of protein seen and could account for the disparity between the two assays.

FLOW THROUGH STUDY

During the purification process it was noted than QconCAT protein is invariably seen in analyses of the buffer which flows off the column during loading of the QconCAT (known as the "flow through"). The binding capacity of NiMAC column resin was reported to be variable depending on the protein being bound, with an upper limit of 30mg/ml. The presence of QconCAT in the flow through could be attributed to:

1. The resin being fully saturated therefore unable to bind all of the QconCAT
2. The resin not being incubated with the QconCAT for a long enough period of time
3. The resin not being incubated with the QconCAT in sufficient volume of buffer to allow complete mixing
4. The QconCAT may have lost the HisTag and therefore be unable to bind to the resin

To investigate which of these was correct a small study was undertaken. QconCAT CC050 was selected at random for this study. Three cell pellets of CC050, each produced from 50ml of culture, were used. The pellets were resuspended and sonicated individually. After sonication the three suspensions were combined and mixed well before removal of 3 x 2ml volumes (the rest of the solution was retained but not used), the three 2ml volumes were therefore identical. They were centrifuged for 8min 6000 x g to pellet the inclusion bodies in each; the pellets were washed once in 25mM AmBic and were then solubilised and purified using NiMAC cartridge, each pellet taken through a different method.

Method 1: Standard purification; the QconCAT was loaded onto the column followed by bind buffer, wash buffer and finally elute buffer passed through the column, the QconCAT was eluted and retained. Samples of each fraction were analysed by SDS-PAGE.

Method 2: Re-applying the flow through; the QconCAT was applied to the column and the flow through collected as usual (FT 1 sample). The flow through was then immediately passed through the column a second time and the flow through was collected again (FT2 sample) – flow through 2 was collected in small fractions with a sample of each fraction removed for SDS-PAGE analysis, this was to allow for monitoring of the elution profile of the QconCAT if any remained in FT2. Bind, wash and elute buffers were passed through the column, the QconCAT was eluted and samples analysed by SDS-PAGE.

Method 3: Two sequential purifications; the specifications for the NiMAC cartridges recommend only purifying one protein per column but that the column is useable for multiple rounds of purification of that protein, the column can be washed and stored in buffer at 4˚C to

be used up to 10 times. For this method the QconCAT was loaded onto the column, flow through 1 was collected and the purification proceeds according to the standard protocol, the QconCAT was eluted off the column, the column was then washed in storage buffer, water and then bind buffer to re-equilibrate the column. Flow through 1 from the first round of purification was then re-applied to the column; the resin had been washed so should be able to bind any QconCAT present in the flow through 1. Samples were again collected in fractions from flow through 2, the purification was then completed according to the usual protocol and samples analysed by SDS-PAGE.

The results of the study are seen in Figure 17. For gel (c) the QconCAT band can be seen in the flow through as expected. In gel (d), where the flow through was immediately re-applied to the column, there is a slight reduction of QconCAT material between FT1 and FT2 but not significant, there is still a QconCAT band in FT2. In gel (e) QconCAT material can be seen in the FT1. This material was reapplied to the column after elution and washing. FT2 (gel (f)) still shows some material is not binding to the column however QconCAT bands can be seen in E1, E2,E3 and E4 lanes, showing that QconCAT has bound to the column from FT1. The protein assay results for the purifications do not seem to reflect the gels. In the 3ml recovered after each purification 1-3, from **method 1** 0.39mg was recovered, for **method 2** 0.082mg was recovered and for **method 3** 0.36mg was recovered in the first round and 0.18mg in the second round giving a total of 0.53mg. There is an obvious anomalous result for purification with method 2. The amount of material recovered is much lower than the other purifications with no obvious reason as to why this would be; the QconCAT is visible in the elution lanes and the eluted fractions were all handled and dialysed in the same way for each purification; this result cannot be used in concluding the results of the study. The results of purification method 3 show that the QconCAT which comes through in the flow through can be re-bound to the column and the total amount of protein recovered is greater than with a single purification round. Tt can therefore be concluded that the QconCAT has not lost the HisTag, and that this cannot be the reason why the full amount of QconCAT does not bind to the column. It can be speculated that the likely cause for the QconCAT in the flow through is that the resin is saturated under the current conditions. However, the sampling through the flow though 2 for methods 2 and 3 (gels (a) and (b)) showed that the QconCAT elutes into the flow though continuously rather than saturating the column and then running off, therefore it could be speculated that the binding conditions are also affecting the amount of QconCAT which binds the column. A sure solution to acquire more material, as shown by this study, is to use two rounds of purification.

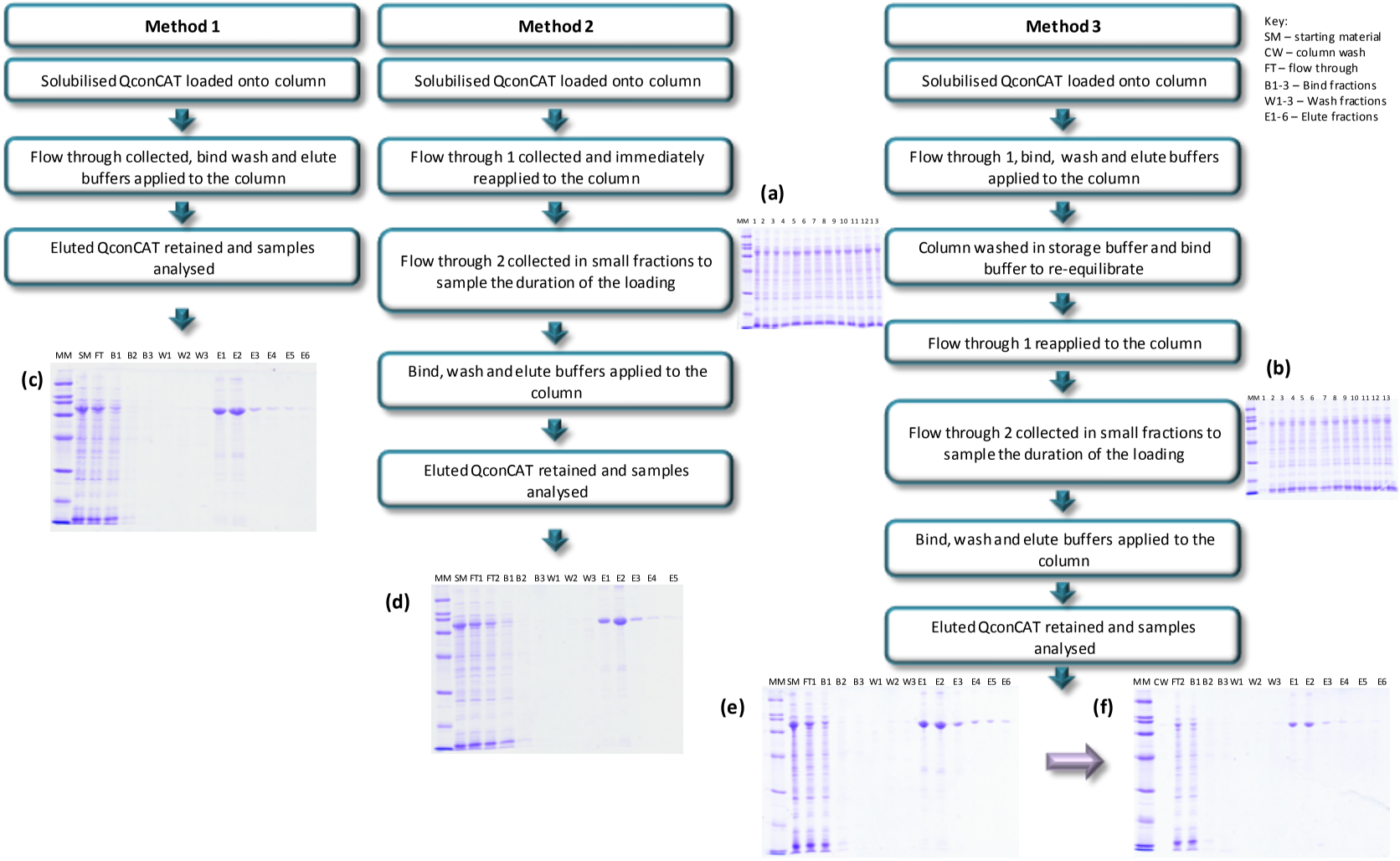


Figure 17 | Flow through study SDS-PAGE gels. Method 1. The standard protocol and results in gel (c). **Method 2.** The immediate re-application of the flow through (FT), gel (a) showing sampling through the flow through, results in gel (d) **Method 3.** Dual rounds of purification with reapplied flow through. Gel (b) showing sampling through the flow through, results in gels (e) and (f).

RapiGest solubilisation

The results from the flow-through study suggest that further optimisation of the purification method would lead to more complete recovery of the QconCAT. Purification is already a relatively time-consuming process and in a high-throughput workflow could be a significant rate limiting factor. Whilst the amount of QconCAT recovered is usually adequate for the quantification studies we perform, depending on the study and the type of analysis more QconCAT may be required and a "lossy" method could be costly. Further optimisation of purification would be time-consuming and possibly add further time to the protocol. For this reason other options were considered. It was observed that in the starting material for a purification the overwhelming majority protein is QconCAT. The starting material is simply solubilised IBs, therefore QconCAT is the majority protein in the IBs. Given this fact a possible alternative to purification was therefore to try using the un-purified IBs; with the QconCAT being the majority protein the other proteins present would be diluted to a level where they would become insignificant during use. Additionally the presence of the two internal quantification peptides means that pure protein is not required for the accurate quantification of the standard.

For the first trial the IBs of CC001 were washed in 50mM AmBic three times before solubilising in 6M guanidine hydrochloride and 1mM DTT; this is the same concentration of guanidine hydrochloride as used in the Bind Buffer for purification. Solubilisation took place over five hours with the solution agitated on an end-over-end mixer, before centrifuging to pellet the insoluble material, dialysing it against 50mM AmBic and analysing the soluble fraction via SDS-PAGE. A comparison was drawn between a sample of Ni-MAC purified CC001 and the washed CC001 (Figure 18 (a)). There are contaminant bands present in the lane for washed CC001, however the QconCAT protein is the majority band. Whilst each contaminant band individually is significantly weaker than the CC001 band it was considered the other methods of solubilisation might increase the concentration of CC001 in solution and so increase the percentage relative to other contaminant proteins.

Guanidine hydrochloride was used for the initial trial to solubilise the QconCAT as it is used in the purification buffers to denature the QconCAT protein to solubilise it. Other solutions to solubilise the QconCAT were considered, one of which was RapiGest solution. RapiGest is a standard component of our digest protocols. It is a detergent which is used to disrupt the structure of the proteins in the digest, allowing trypsin access to the entire length of the protein to ensure complete digestion. As it disrupts the structure of proteins it was considered

that RapiGest could help to solubilise the QconCAT, additionally as it is part of our standard digest protocol the RapiGest would not need to be dialysed out of the solution prior to use of the QconCAT. An IB pellet of CC001 was sonicated and for ease of use, to give better washing and for further investigation, the 2.5ml of sonicated material was split between two 2ml tubes before centrifuging. The soluble fraction was removed (SF1) and a sample retained. The two IB pellets were then washed three times in 50mM AmBic, samples taken of each soluble fraction (SF2-4). Finally the two pellets were solubilised in RapiGest – one pellet was solubilised in 250µl of 0.4% RapiGest solution and the other solubilised in 250µl of weaker 0.1% solution. Each pellet was left for 30 minutes to solubilise, the insoluble material centrifuged and the soluble fraction removed and retained. The gel image (Figure 18 (b)) shows the more concentrated RapiGest method results in a greater amount of QconCAT in solution and this was decided as the best method for solubilisation. The final gel (Figure 18 (c)) shows that if the 0.4% solubilised QconCAT is diluted to a level which is normally seen for an average protein on a gel, no other protein bands are visible.

For storage the solubilised material was diluted by the addition of 750µl of 25mM AmBic containing protease inhibitors before aliquotting and storage at -20˚C. On thawing, some aliquots of different QconCATs developed a precipitate. To prevent this from occurring in further solubilisations 1mM DTT was added to the RapiGest during solubilisation. Finally, during the digest protocol the digest is heated to 80˚C after addition of the RapiGest, this level of heat is not suitable for the QconCAT solubilisation but gentle warming to 37˚C for 1 hour was added to the protocol to increase the efficiency of the RapiGest solubilisation.

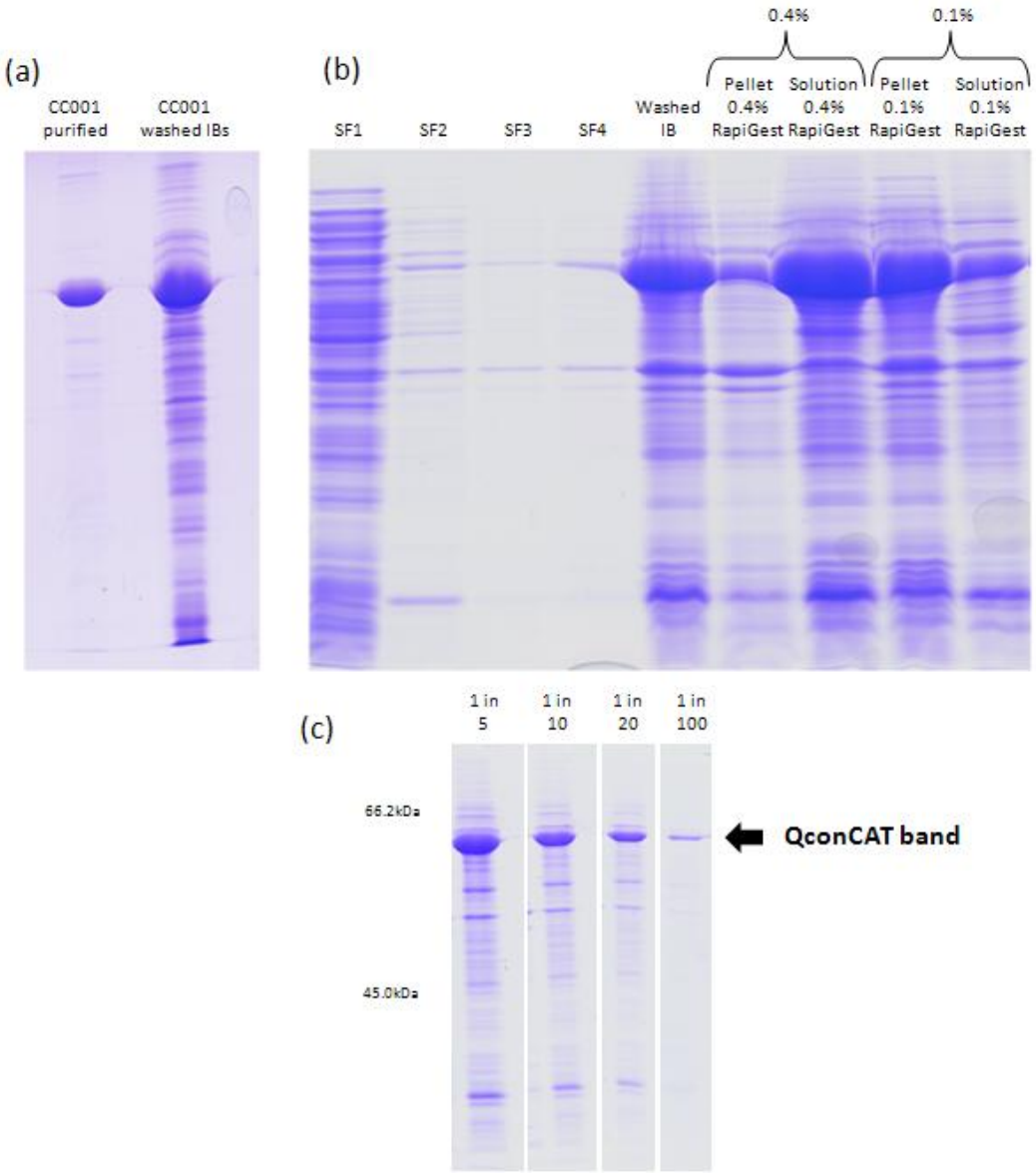


Figure 18| RapiGest solubilisation development. (a). Comparison of washed and purified CC001 material. **(b).** Different concentrations of RapiGest solution used to solubilise IB material, with 0.4% being most effective. **(c).** Diluted RapiGest solubilised CC001 shows a single QconCAT band.

To verify that the QconCAT was the majority protein in the solubilised material and also that any additional proteins present in the QconCAT solution would not interfere with using the QconCAT, two further investigations were performed. First, dilutions of the solubilised QconCAT were analysed by SDS-PAGE and densitometry performed on the protein bands to analyse the percentage content of each major band in the soluble fraction. The results show that the solubilised QconCAT occupies 69-80% of the lane on the gel, with an average of ~75%. In Figure 18, gel (c) shows that increasing dilutions of the QconCAT solution eventually lead to a lane where only the CC001 band is visible (1 in 100 dilution). This shows that, whilst the

contamination from *E.coli* proteins cannot be removed, the solution can be diluted to an extent where the contaminants are not significant. In a typical digest the QconCAT is diluted ~1 in 35; at a 1 in 20 dilution contaminant bands are still visible and although the QconCAT is diluted more than this for analysis there was therefore a concern that, even though the QconCAT was the majority protein in the solubilised material, the lack of purification would mean the introduction of other proteins into a digest and could affect the MS data and quantification. To check this, one aliquot of purified CC001 and one aliquot of non-purified RapiGest solubilised CC001 were digested separately. RapiGest is part of our usual digest protocol so was used with the purified CC001 for digestion, the key difference between the two CC001 samples is that one is purified and one is solubilised. The digests were analysed using LC-MS/MS and the results were compared against an *E.coli* protein database, the protein hits for each can be seen in the tables below.

Accession	Description	mW (Da)	Coverage (%)
B1X9B6	Small heat shock protein ibpB	16083	27.46
B1X9B7	Small heat shock protein ibpA	15764	18.97
B1XEK3	Methylmalonyl CoA decarboxylase biotin independent	29153	4.59
B1XBY2	Elongation factor Tu	43286	19.54
B1X6E8	30S ribosomal protein S4	23454	17.47

Table 3| RapiGest solubilised CC001, protein hits from the *E.coli* database

Accession	Description	mW (Da)	Coverage (%)
B1XEK3	Methylmalonyl CoA decarboxylase biotin independent	29153	4.59
B1X6I9	Elongation factor Tu	43256	15.98

Table 4| Purified CC001, protein hits from the *E.coli* database

The protein hits found, as expected, are some of the most abundant proteins in *E.coli* (Ishihama *et al.*, 2008). The fact that two *E.coli* proteins were found in the purified QconCAT digests (Table 4) indicates that whichever method is used for the preparation of IBs, contaminant proteins cannot be completely removed from the QconCAT solution. The small increase of protein hits from two, in the purified, to five in the RapiGest solubilised preparation (Table 3) suggest that there is not a great level of purity lost in simply solubilising the QconCAT.

RapiGest solubilisation is only effective if the QconCAT is over-expressed to an extent that the ratio between QconCAT protein and contaminant proteins is high. The majority of QconCATs are over-expressed, however some are not expressed to the levels which are required and these still need to be purified. The QconCAT is not quantified until after solubilisation or purification, so protein content cannot be easily assessed, and therefore cannot be used as a deciding factor for how the QconCAT is prepared for use. As a deciding rule the *E.coli* protein band indicated by the arrow (Figure 19), an *E.coli* protein that is present in every expression culture whole cell lysate, is compared to the QconCAT band (protein identified by Dr Amy Claydon as outer membrane protein F, PhD thesis "Approaches to understanding the dynamic proteome", September 2009). If the QconCAT band is as strong as, or stronger, than the *E.coli* protein band then the QconCAT is RapiGest solubilised; if the band is weaker than the *E.coli* band then the QconCAT is purified.

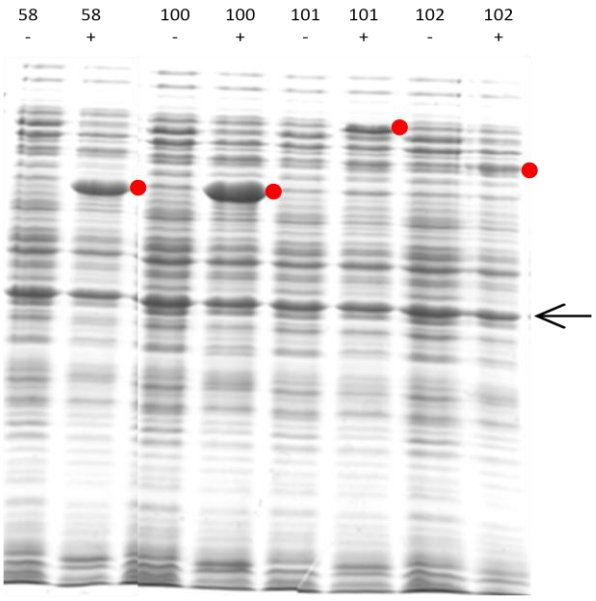


Figure 19 | QconCAT selection for purification or RapiGest solubilisation . Whole cell lysate samples from expression cultures, the red dot indicates the expression band. QconCATs CC058 and CC100 were RapiGest solubilised, CC101 and CC102 were purified.

Reduced purification time -1d and enabled multiplexing therefore reducing purification time further depending on the number of QconCATs being processed

There is an optimal QconCAT production workflow for the majority of "well behaved" QconCATs, easily multiplexed and high throughput which involves the use of all of the protocol improvements I have set out so far in this thesis. There are also troubleshooting and alternative methods for QconCATs which show atypical behaviour. The overall flow diagram for the optimised methods can be seen in Figure 20.

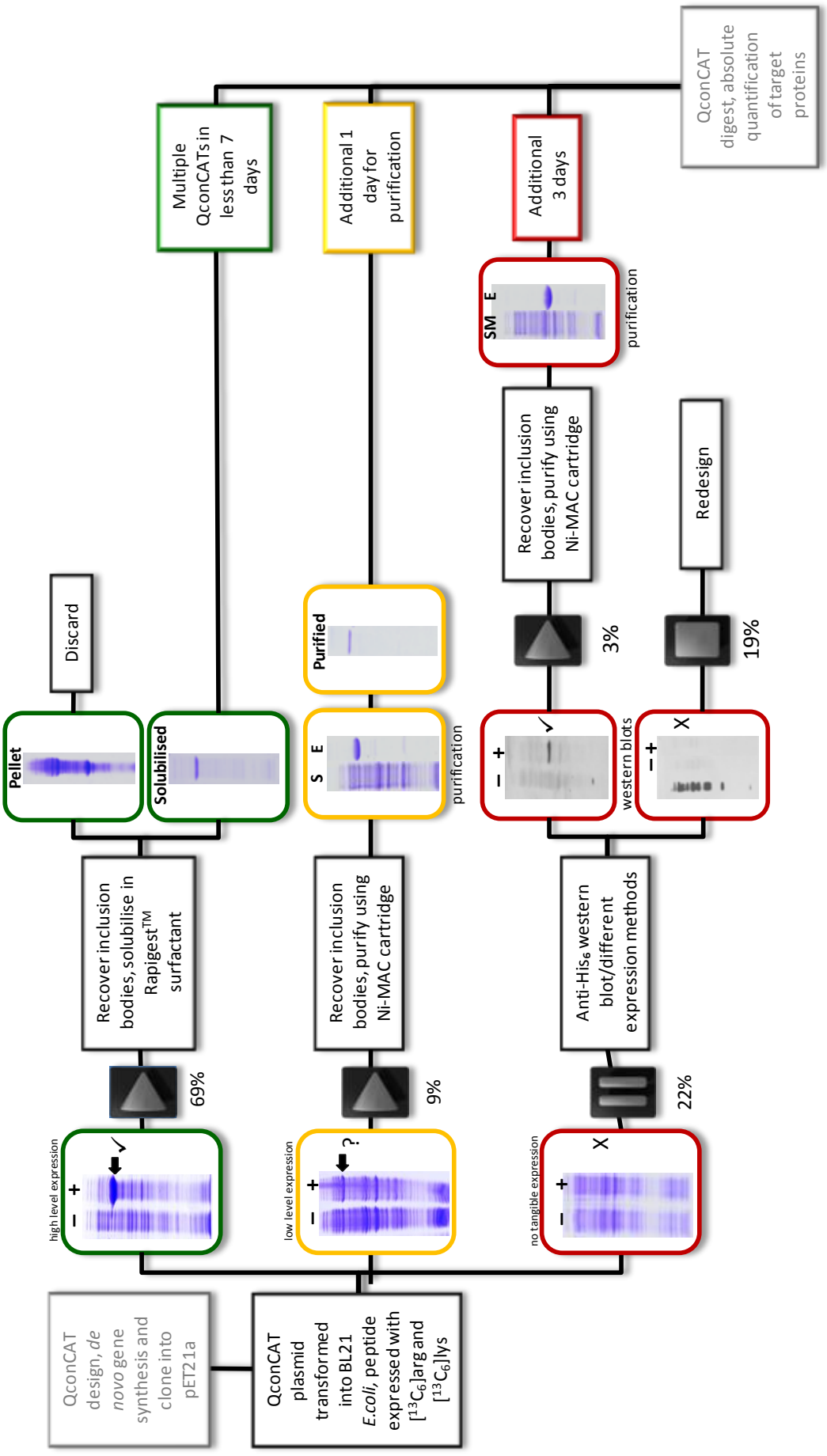


Figure 20 | The overall QconCAT production workflow. Following the ideal production protocol in green and modified protocols in yellow and red for problem QconCATs

4.5 The optimised method and troubleshooting

In Figure 20, the ideal optimised method is shown. The green top line represents the ideal workflow where the QconCAT expresses well and the inclusion bodies can be solubilised in a RapiGest solution for use. This happens in ~70% of cases. In ~10% of cases the QconCAT expresses to a lower level and needs to be purified, occasionally with western blot verification prior to purification to verify expression has occurred, this is shown in yellow. In ~20% of cases the QconCAT fails to express and needs to be redesigned, this is shown in red (data from COPY project COPYCATs).

So in the majority of cases the optimised, high throughput method works well. However there have been QconCATs where the protein did not express well, or did not express at all, and occasionally although the QconCAT expresses, it is more difficult to solubilise. In these cases troubleshooting protocols need to be used. The mostly commonly used tool when troubleshooting QconCATs, specifically very low expression of the QconCAT protein, is western blotting. Each QconCAT is designed with a HisTag at the C- terminus for purification and this tag can also be used to identify the presence of the QconCAT with a western blot; an anti-HisTag antibody is used along with a chemiluminescent substrate, x-ray film is used to develop the blot and the presence of the QconCAT is indicated by a black band.

Less common is where a QconCAT does not express well in a certain type of media. QconCAT Ribo4 was one of four QconCATs, designed for a study into yeast ribosomal proteins, which I expressed. Ribo4 showed low levels of expression in LB media (Figure 21 (a)), even with elevated levels of IPTG. To try to improve expression two other culture conditions were tried; culture in autoinducing media and IPTG induction in minimal media with light amino acids (Figure 21 (b)). The SDS-PAGE analysis of time points from these cultures show that in the minimal media and autoinduction media Ribo4 seems to express to improved levels. As minimal media is used for expression of the stable isotope labelled protein a heavy culture was then attempted. The heavy Ribo4 protein was expressed to adequate levels and was purified to yield a clean product (Figure 21 (c)).

Some QconCATs do fail to express entirely. The exact cause for this is unknown. A possibility is that the QconCAT produced could be toxic to the *E.coli* cells in some way, however this might be indicated by a lack of culture growth and this is not usually observed; it may be more likely that the QconCAT is not being synthesised. The usual solution in these cases is to change the

order of the peptides in the QconCAT gene and order a new construct, re-expression is then attempted.

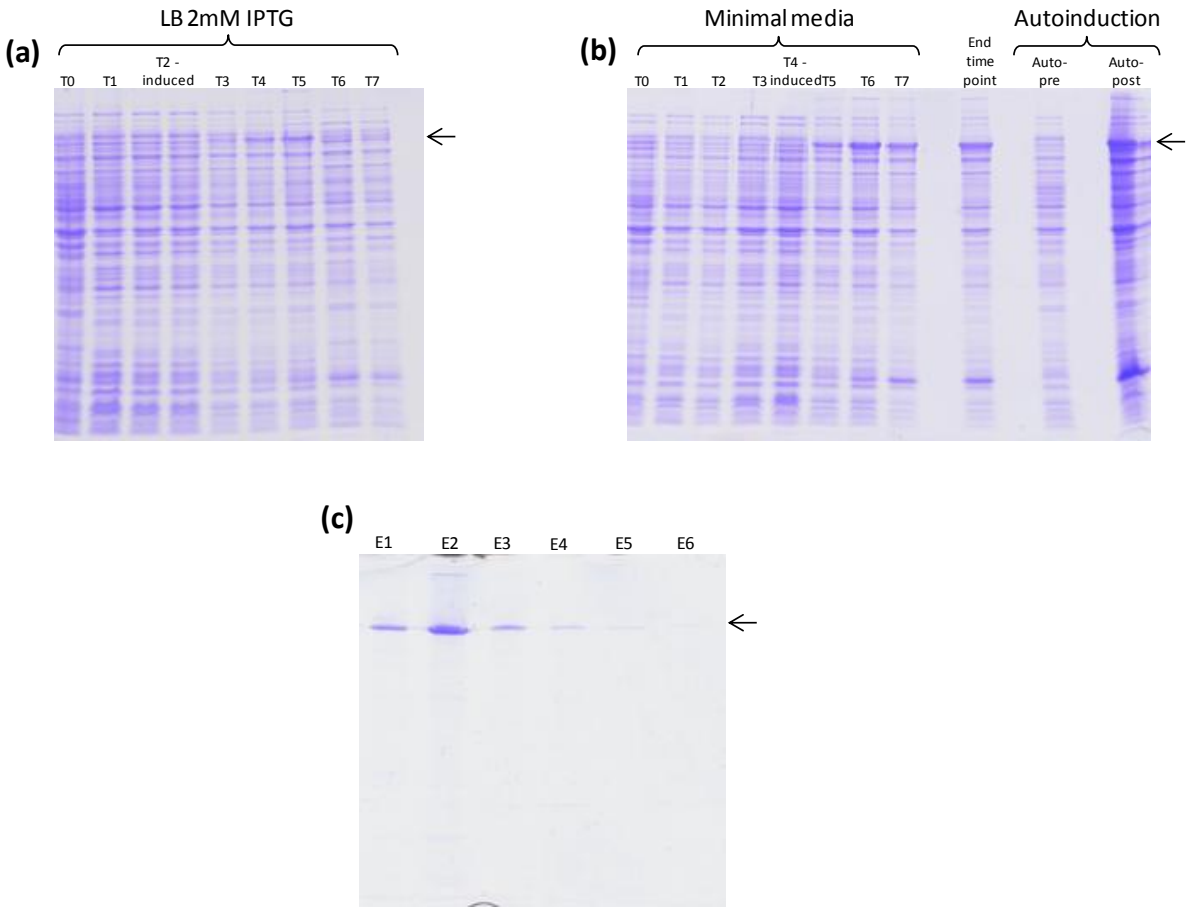


Figure 21 | Ribo4 QconCAT expression in different media. (a). Expression in LB with 2mM IPTG induction. Time point samples T0-T7 assessed. Low level expression seen faintly band in T4 and T5. **(b).** Minimal media and autoinducing media with improved QconCAT expression. **(c).** After stable isotope labelled culture Ribo4 is successfully purified.

Section 2: QconCAT protein as a quality control standard

The design of a QconCAT is inevitably selective. The peptides included in the construct are carefully chosen using the criteria previously discussed; one of these criteria is how well the peptide will behave during the mass spectral analysis. This means that each QconCAT that is produced is not only a quantification standard, it is also a protein consisting entirely of peptides designed to behave predictably in a mass spectrum. It was therefore considered that these proteins could be used for more than protein quantification; they could also be used to assess instrument performance under a number of criteria including quantitative performance. This idea is not new, as previously discussed Eyers *et al.* (Eyers *et al.*, 2008) designed their own QconCAT to use as a MS standard, however the approach taken here was different to QCAL in that the focus was to explore a different aspect of instrument performance - the ability of the instrument to detect and quantify peptides over a large dynamic range in a more complex sample than a single protein. Sigma-Aldrich® have also made a Universal Proteomics Standard (UPS1) which is an equimolar mix of 48 human proteins and a Proteomics Dynamic Range Standard (UPS2) which is a dynamic range mix of the same 48 human proteins ranging from 500amol to 50pmol in concentration; both formulations are designed to assess chromatographic and instrument performance. The purpose of this study was to assess whether QconCATs could be used in a similar way to the UPS1 and 2 standards with the addition of assessing quantitative performance and the benefit of the flexibility that comes from a "designer" set of proteins where the peptides are as well characterised as the protein itself.

Ten COPYCATs were selected to cover a dynamic range of 0.5fmol on column up to 0.5pmol on column with 8 concentration points between. Each of these was known to express well, no other selection criteria were used. The COPYCATs selected were 001, 002, 042, 043, 045, 052, 053, 058, 059 and 060. For this study each COPYCAT was expressed in unlabelled form; as the COPYCATs were not going to be used for quantification it was not necessary to distinguish them from an analyte. The time point samples for each COPYCAT culture before (-) and after (+) addition of IPTG show variable levels of expression for the 10, the expression bands, indicated with a green dot, are visible for each to indicate successful expression (Figure 22 (a)). A cell pellet from each COPYCAT culture was then purified using NiMAC cartridge and the purified material was analysed in a 1 in 2 dilution using SDS-PAGE to verify no contaminant bands could be seen (Figure 22 (b)).

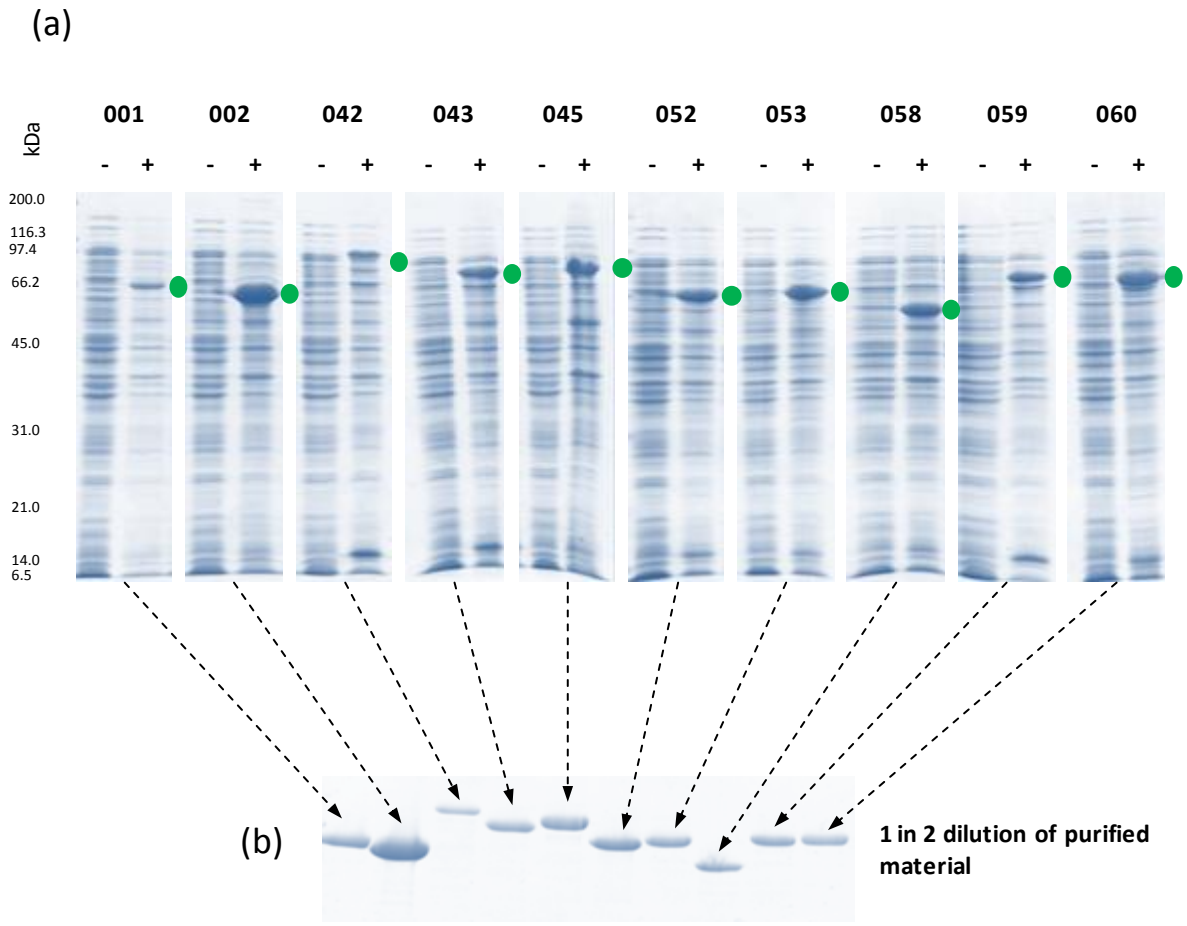


Figure 22| Expression and purification of 10 unlabelled COPYCATs. (a). SDS-PAGE of pre (-) and post (+) induction samples for COPYCAT expression in LB media. **(b).** The diluted purified material for each COPYCAT with a clean band for each, arrows indicate which band pertains to which COPYCAT.

In order to use the COPYCATs effectively they first needed to be accurately quantified. The first study using these 10 QconCATs was to assess the variability in quantification of the QconCATs using various methods.

4.6 Methods for quantification of the QconCAT standards

The accuracy of quantification using a standard is largely dependent on how well the standard itself is quantified. It is therefore extremely important in quantitative proteomics to select the most appropriate method for quantification of the standard and to be sure that this quantification is reliable. With respect to the study of 10 COPYCATs, to get the proteins into a dynamic range each COPYCAT needs to be accurately quantified. There are a number of different ways to quantify a standard and I investigated the use of a few of these.

The methods used for quantification:

Method	Merits	Demerits
Densitometry	▪ Whole protein quantification ▪ Visual check for contaminants possible	▪ Reliant on staining of a different protein to act as a standard, variation in dye binding behaviour could bias results
Protein assay	▪ Whole protein quantification	▪ Reliant on staining of a different protein to act as a standard, variation in dye binding behaviour could bias results
Quantification via the GluFib peptide on the QconCAT, using MALDI-TOF-MS	▪ Standard and analyte target peptide are almost identical, increasing accuracy of the quantification ▪ Sample and analyte are combined early on in the workflow, decreasing the possibility of error introduced during sample preparation	▪ Digestion required which may introduce error if incomplete ▪ Comparison of a single peptide to a standard, needs to be a quantotypic peptide ▪ MALDI-bias towards Arg containing peptides ▪ Single QconCAT protein quantified in each analysis
Quantification via the GluFib peptide, using ESI-QTOF-MS	▪ Standard and analyte target peptide are almost identical, increasing accuracy of the quantification ▪ Sample and analyte are combined early on in the workflow, decreasing the possibility of error introduced during sample preparation	▪ Digestion required which may introduce error if incomplete ▪ Comparison of a single peptide to a standard, needs to be a quantotypic peptide ▪ Single QconCAT protein quantified in each analysis
Label-free quantification, using ESI-QTOF-MS	▪ Rapid quantification of multiple standards simultaneously from MS data processing ▪ Multiple proteins can be quantified in a single analysis	▪ Digestion required which may introduce error if incomplete ▪ Comparison of top3 peptides from the analyte to the top3 standard peptides from an entirely different protein

Table 5| Merits and demerits of five different quantification methods for the QconCAT standard

These methods for quantification differ in the target; for the protein assay and densitometry it is the entire protein which is being used in the assay. In the case of quantification using GluFib it is a single target peptide within the digested COPYCAT, and for label-free a selected number of "top" intensity peptides are used, in this case the top three. In these methods, except for label-free, the COPYCAT is quantified by comparison with a standard which is present in a known amount.

DENSITOMETRY

For the densitometry, bovine serum albumin (BSA) was used as the standard for quantification. Several SDS-PAGE gels were run to include a range of dilutions of BSA in order to obtain the best quantification for each COPYCAT. A BSA standard graph was drawn for each gel and the equation for the best-fit line was only applied to COPYCATs from the same gel. The densitometry gels can be seen in Figure 23 along with their corresponding BSA-standard plots. The value for the absorbance of each band is given as a "volume", for the BSA standard this volume can be equated back to the amount of protein loaded on to that lane of the gel by plotting the corresponding values in a graph; this results in the BSA standard plots seen with the line of best-fit being used to calculate the quantification of the analyte COPYCATs. The amount of COPYCAT, in ng, on the gel was calculated. For CC001 a number of different dilutions were run to assess the consistency of the readings; an average ng on gel value for CC001 was therefore calculated. It should be noted that for CC002 the band was stronger than the highest amount of BSA for that gel, however the value was still included as the line-of best fit gave a good Pearson's r value of 0.96 and an adjusted R^2 value of 0.89 (values from OriginLab programme 8.5) and it was decided that the error introduced by extrapolating from the line would not be significant. The results can be seen in Figure 26.

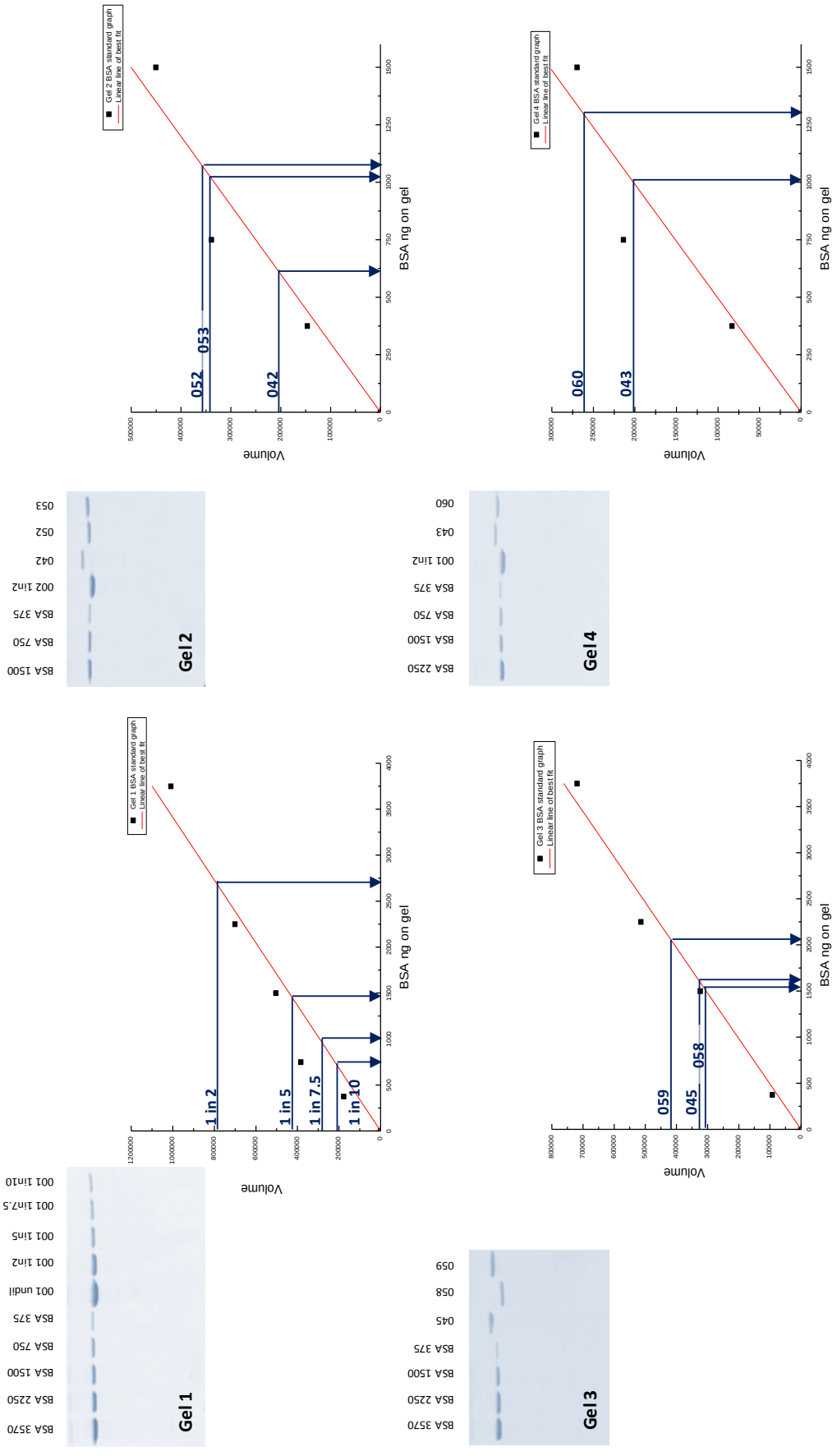


Figure 23 | Densitometry quantifications. The four gels used for quantification of the 10 light COPYCATs, with their corresponding BSA standard plots and arrow indicating the value taken from the graphs for quantification of the COPYCATs.

PROTEIN ASSAY

The protein assay was performed, as explained in the materials and methods section, on each COPYCAT immediately prior to digestion to minimise error. The protein assay relies on a very similar principle to densitometry, it is performed on the intact protein and the absorbance of a BSA standard, stained with Coomassie Plus™ protein assay reagent, is compared to the absorbance of a COPYCAT sample (Figure 26).

A recent application note released by Amersham Biosciences (Application Note #6 ImageMaster® VDS 80-6383-43) highlighted a key point to consider in the reliability of densitometry. The optical density of a Coomassie stained protein band on a gel was plotted against the concentration of that protein in the band, for two different proteins – Aldolase and GPDH. Their results indicate that the slope of the line of best fit for these two proteins was markedly different. This calls into question the reliability of a densitometry assay if the BSA standard used has a different slope of optical density/protein concentration to the analyte protein. A similar principle can also be considered concerning the reliability of the protein assay. The Coomassie dye used for protein assays is known to bind only selected amino acids at specific sites, if the number of these sites is not the same for the standard and analyte protein it stands to reason that the staining of the proteins will be different. Congdon et. al. showed that different proteins stained with Coomassie in a protein assay had different absorbances (Congdon *et al.*, 1993) and this calls into question the reliability of the quantification from a protein assay.

QUANTIFICATION USING THE GLUFIB PEPTIDE

For quantification using the GluFib peptide, with either MALDI-TOF or Q-TOF MS, digests of each COPYCAT were used. Ordinarily in our lab unlabelled (light) GluFib is used, in a known amount, to quantify a labelled (heavy) QconCAT. In this case the COPYCAT is light, therefore it needs to be quantified with heavy GluFib. Accurately quantified heavy GluFib peptide was not available as a stand-alone reagent, therefore the quantification needed to involve two steps. First heavy CC002 was made and quantified using light GluFib. The heavy CC002 was then used as a source of heavy GluFib, in a known amount, to quantify the light COPYCATs.

Each light COPYCAT was digested individually, the heavy CC002 was digested under two different conditions – one with light GluFib spiked into the digest mix (“heavy with” (HW)) and one without light GluFib in the digest (“heavy only” (HO)). The two heavy digests were performed in order to assess whether the quantification was affected by the stage at which the GluFib was added to the analysis; with the HW digest the GluFib is already present and the

sample can be directly analysed with MS to quantify the COPYCAT, with HO the GluFib needs to be spiked into the digest after digestion is complete, prior to MS analysis. This later stage of introduction could introduce more variance to the quantification.

There is also a second aspect when considering which heavy digest to use – with HO the only GluFib present in the digest is heavy, therefore when the heavy is spiked in with a light COPYCAT for quantification there can be a direct comparison drawn between the heavy and light GluFib peaks. However with HW there is already light GluFib present in the digest, it is at a known amount so the quantification of the light COPYCAT can be adjusted accordingly but the presence of this extra GluFib could affect the quantification accuracy.

The two heavy digests were analysed individually; HO was first spiked with a known amount of GluFib for quantification. Each light COPYCAT digest was quantified twice, once with HO and once with HW using MALDI-TOF MS and ESI-Q-TOF MS, an average of the resulting light COPYCAT quantification values was taken as the HO and HW were found to give very similar quantifications. The quantification was performed using MassLynx software to give an extract ion chromatogram for the GluFib peaks. To first quantify the heavy CC002 digests the peak areas for the light and heavy GluFib in the samples were calculated and the resulting values compared in a ratio which was then used to calculate the fmol of heavy GluFib in the sample from the known amount of light GluFib. Once the amount of heavy GluFib was known this was used to quantify the 10 light COPYCATs. So that the quantification values can all be compared against the protein assay and densitometry, where the amount of COPYCAT in the original undigested aliquot is quantified, the GluFib quantifications were then calculated back to give a final value for pmol/µl concentration of COPYCAT in the original aliquot. The resulting quantifications can be seen in Figure 26.

At this stage of the assessment the methods were compared to judge how well they correlated. Figure 24 shows the alignment between the different quantification methods so far.

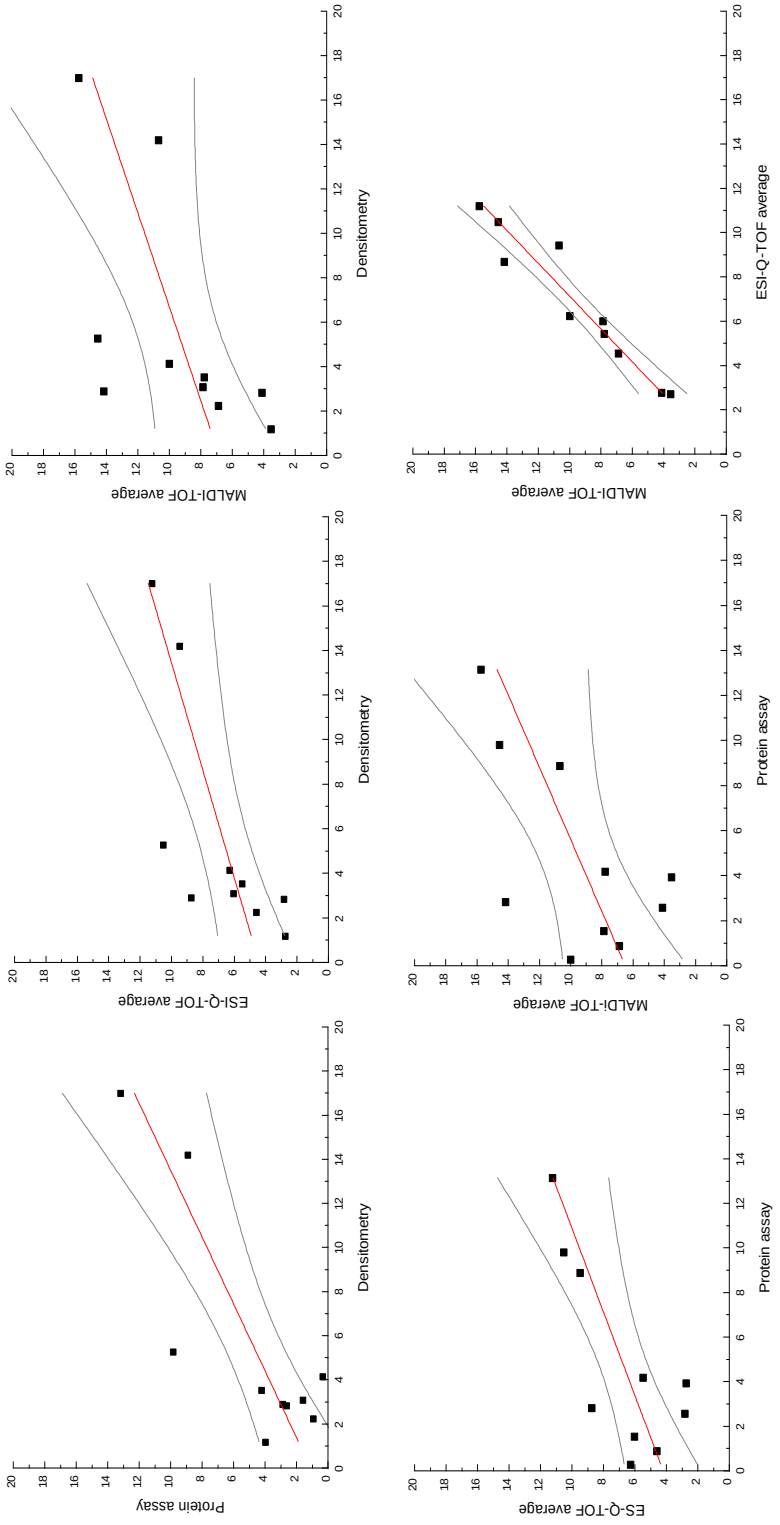


Figure 24 | Comparisons between the quantification values with different methods. The output quantification values for four different quantification methods are plotted against one-another. The line of best fit in red and the 95% confidence limits in grey.

Visually the MALDI-TOF and ESI-Q-TOF quantifications correlate better than the others with the data points distributed close to the lines of best fit. This is to be expected as the only difference here is the instrumentation, the method of quantification is still the same. Table 6 below shows the Pearson's-r values from each graph:

Y Axis pmol/ul value	X Axis pmol/ul value	Pearson's r
Protein assay	Densitometry	0.82528
ESI-Q-TOF average	Densitometry	0.73251
MALDI-TOF average	Densitometry	0.59659
ESI-Q-TOF average	Protein assay	0.75239
MALDI-TOF average	Protein assay	0.62927
MALDI-TOF average	ESI-Q-TOF average	0.96214

Table 6| Pearson's r values for comparisons between quantification method results

Looking at the values above there seems to be good correlation between the densitometry and the protein assay quantification values, this again is to be expected as both methods use a similar principle of measuring the staining of the intact protein and comparing it to a standard to acquire the quantification value. There is worsening correlation between the two MS GluFib based quantifications and the intact protein quantifications, with the worst correlation between MALDI-TOF and Densitometry. However the degree of correlation is still no indication as to which method provides the most accurate quantification.

The MS quantifications are only accurate if the GluFib quantification peptide is released from the COPYCAT entirely. If there is a miss-cleavage either side of the GluFib peptide then a reduced level of peptide will be available for quantification and this will bias the results. To assess this a time course digest was performed on three COPYCATs (all used in the 10 light study) these three COPYCATs are stable isotope labelled to facilitate the use of unlabelled GluFib to monitor the release of the heavy GluFib peptide from the COPYCAT.

The three COPYCATs chosen for the time-course were CC042, CC053 and CC060. Two digest conditions were set up for each, the first contained the COPYCAT plus 107.5fmol/ul concentration light GluFib and a yeast background. The second contained the same amount of COPYCAT and GluFib but no yeast. The two conditions were chosen to see if the complex yeast background affected the digestion efficiency. The digests were reduced with DTT and blocked with IAM before adding the trypsin. Time points were taken at 1 min, 2 min 5 min, 10 min, 15 min, 30min, 60min, 120min, 240min and a final overnight time point. For each time point a

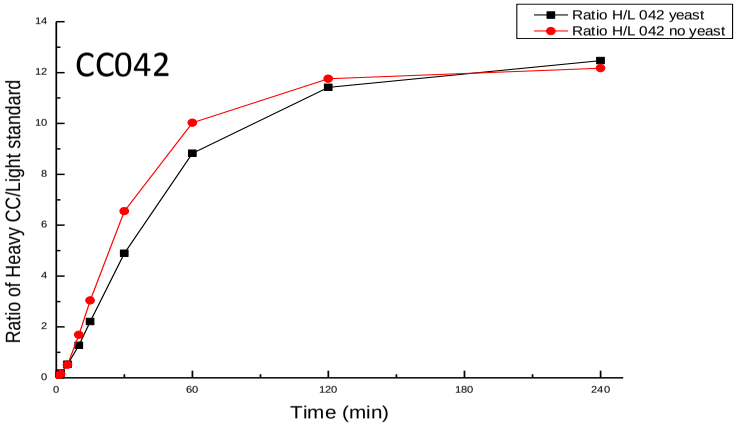
10µl sample was pipetted into 10µl of TFA, 1% final concentration, and placed on ice to stop digestion. The light GluFib is already present in each sample as it was added in the digest mix, meaning that the amount of light GluFib is constant for each sample. The time points were analysed using LC-ESI-Q-TOF MS. The peak areas for the heavy and light GluFib were extracted for each time point in each digest and the ratio between the two values calculated.

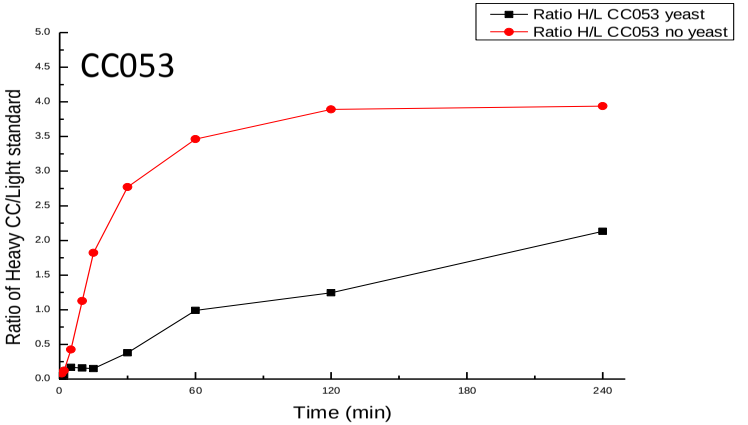
Plots of the changing ratios over time reveal that the QconCATs have very different digestion profiles (Figure 25). Additionally for each COPYCAT the behaviour is different dependant on whether the digest is performed in a yeast background. For CC042 the digestions profiles are very similar with or without the background however digestion does seem to be slightly faster without the yeast present. In the sequence context there is an acidic residue in the P1' location which could affect digestion and lead to a miss-cleave at that site however it does not seem to have affected digestion in this case. Neither of the two other sequence contexts for 053 and 060 indicate a problematic digestion site. The difference between digestion efficiency in the two conditions is most apparent for CC053 where digestion with the yeast background is significantly slower than without. However both digests do eventually reach the same end point. For CC060 we see a very rapid release of the GluFib peptide without the background and again a slower release with yeast present. For this plot the two digests do not reach the same ratio end point. The final ratio for the yeast-background digest is higher. Looking at the curve of the lines for CC060 the digest without yeast plateaus after approximately 120min and the ratio at the plateau is lower than the highest ratio achieved by the background digest at 240min. This might suggest that although the digest with yeast background is slower, more GluFib is released compared to the non-yeast digest. The difference in digestion profiles cannot be explained through the sequence context of the GluFib peptide. It is possible that another peptide released from the QconCAT is inhibiting the performance of the trypsin but there is no clear reason why this would affect the digest in yeast background more than without the background (CC053).

For CC042 the ratio of heavy CC/light standard reached 12, for CC052 and CC060 the maximum ratios seen were 4 and 4.5 respectively (Figure 25). The difference in maximum ratio seen can be explained by the fact that CC042 was prepared differently to CC052 and CC060 prior to digestion. CC042 was prepared for digestion using NiMAC purification whereas CC053 and CC060 were RapiGest solubilised, the difference in the two preparation methods will result in differing concentrations of prepared COPYCAT solution; the COPYCATs were not diluted prior to the time course digestion so the starting concentration of each COPYCAT will

have varied. The same concentration of light GluFib was used for all digests however, meaning that the concentration of heavy COPYCAT, and therefore heavy GluFib, varied in relation to the light GluFib for each COPYCAT and this explains the differences seen in the final H/L ratios.

In addition to the time course digests I also looked for missed cleavages around the GluFib peptide for each COPYCAT. I did this in two ways, I looked for any missed cleaves that were picked up in PLGS and I also viewed extracted ion chromatograms to look for the m/z for the sequence of the first sacrificial peptide coupled to GluFib - *MAGREGVNDNEEGFFSAR*. I could not find clear evidence of any missed cleaves in any of my 10 light COPYCAT digests from either method.





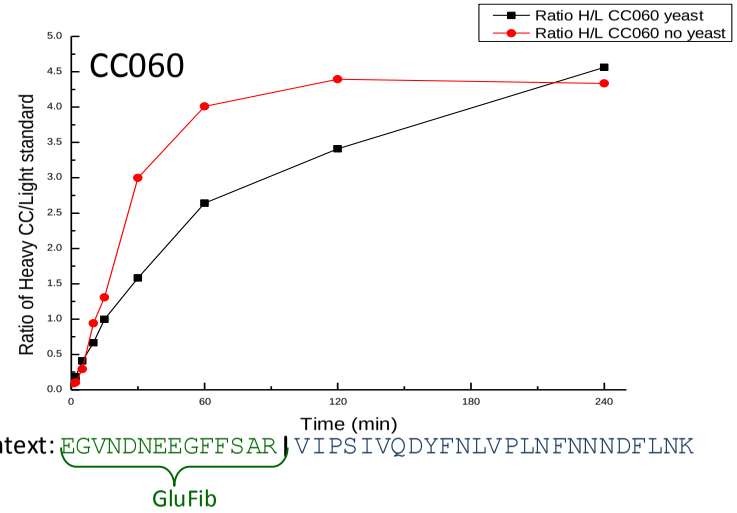


Figure 25 | Time course digest plots for three COPYCATs monitoring GluFib release. The time course digests conducted on three COPYCATs, COPYCATs digested individually and with a yeast background, time points removed and digestion stopped with acidification with TFA (1%). Samples were analysed using LC-ESI-Q-TOF MS , the peak areas for heavy and light GluFib were measured and a ratio between the two calculated. The changing ratio is plotted against time. Each plot represents a QconCAT with curves tracking the release of GluFib in a digest with a yeast background (black line) and without a yeast background (red). The sequence context of the GluFib peptide in each COPYCAT is seen below the plot.

LABEL-FREE QUANTIFICATION

The final method used for trial quantification of the COPYCATs was label-free quantification. For this it was decided that samples of each COPYCAT digest would be run at the same concentration. In order to do this it first needed to be decided which previous quantification value to use to calculate the dilutions required for each COPYCAT digest. As label-free quantification is an MS based approach it was decided that the other MS based quantification values would be most appropriate to use as a basis for dilutions. An average was taken of all of the GluFib quantification values (ESI-Q-TOF and MALDI-TOF MS data); these values were used to calculate dilutions for each COPYCAT digest. The samples were analysed using ESI-Q-TOF (Synapt G1), samples of each digest were run twice – once individually and once in a ratio of 1:1:1:1:1:1:1:1:1:1 mix with each other. The digests were diluted to 500fmol/µl concentration, the 10 COPYCATs were mixed equally and then an equal volume of 50fmol/µl Phosphorylase B (PhosB) was added as the standard. Sample (2µl) was injected onto the column to give, what should be, 50fmol of each COPYCAT on column and 50fmol of PhosB. Each COPYCAT digest was also diluted further to give 50fmol/µl concentration of each individually and, after mixing 1:1 with 50fmol/µl PhosB, 2µl was injected of each onto the column. The data was processed using the label-free quantification method in PLGS. The values given for fmol on column from the label-free quantification were calculated back to give a final value of pmol/ul in the original undigested COPYCAT aliquot (Figure 26).

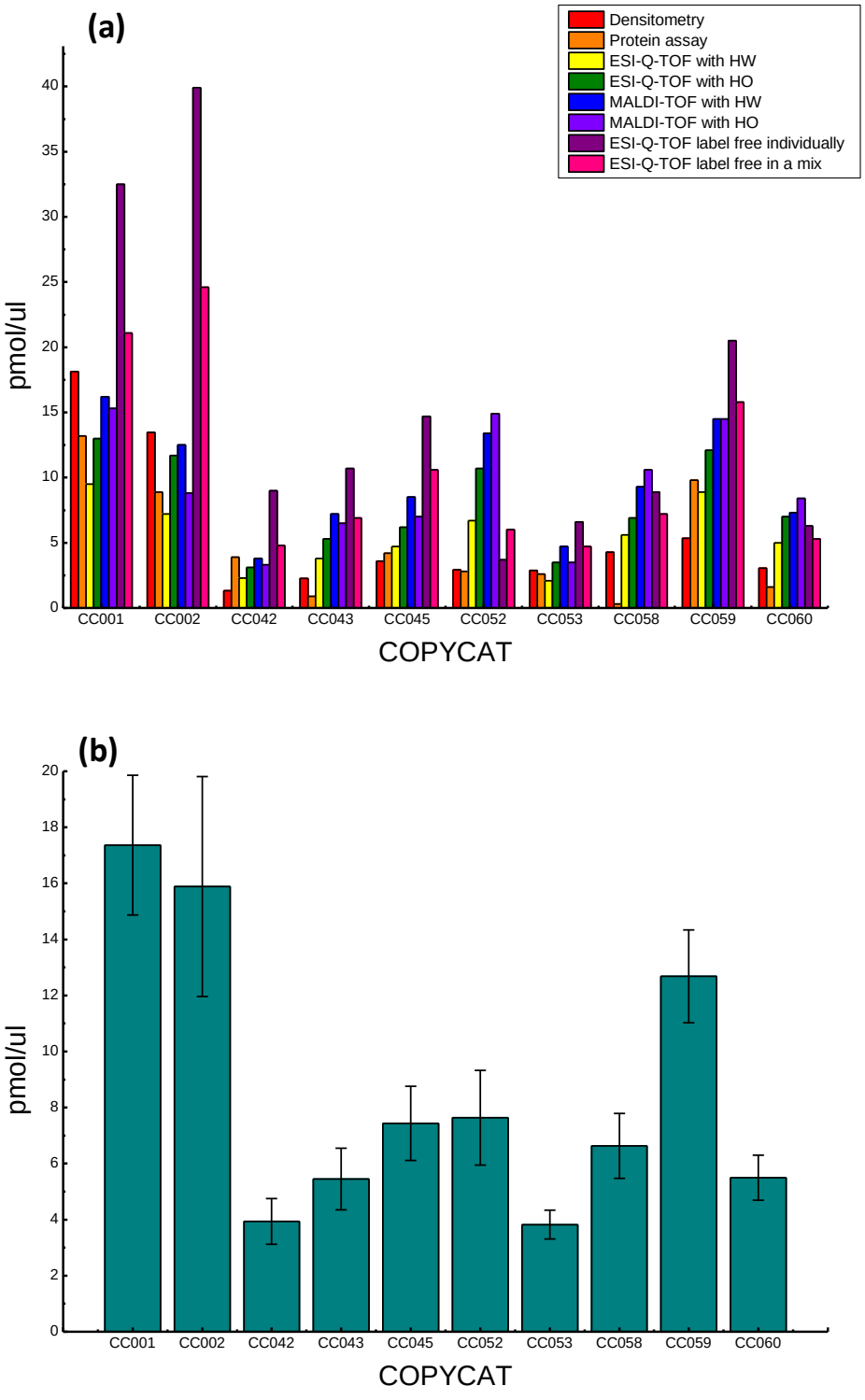


Figure 26 | Quantification values for the 10 COPYCATs. (a). The quantification values obtained for pmol/µl in original aliquot of each QconCAT, using 8 different methods, plotted as a column chart showing each individually. **(b).** The mean pmol/µl value for each QconCAT taking an average of the 8 different quantification methods, error bars showing the standard error for each mean.

The error bars for the average quantification values, for CC001 and CC002 particularly, reflect the variability in quantification values for the COPYCATs (Figure 26 (b)). This was expected given the range of values seen in Figure 26 (a). The label free quantification values tend to give higher pmol/µl concentrations than other methods (Figure 26 (a)). It can also be seen that the label free quantification when the COPYCATs are run individually gives a higher value than when the COPYCATs are analysed in a 1:1 mix. The reason for this difference was investigated and it was observed that the peptides selected as the top3 for quantification were not the same for the individual and the mix. This might suggest that the peptides ionise differently depending on the digest background, however further study and an increased number of technical replicates is necessary to analyse this further. As explained previously PLGS uses the "top3" rule to quantify proteins based on a comparison between the intensity of the top three peptides for a standard of known amount and the top 3 intensities for the analyte peptide. This method of quantification was developed using naturally occurring proteins as the analyte proteins. One explanation for the unusual quantification values given by the label-free analysis could be that the QconCAT peptides do not show the "normal" behaviour seen in naturally occurring proteins. These 10 COPYCATs were picked for this study because they are well characterised proteins containing specifically selected peptides, chosen by virtue of their reliable behaviour in a mass spectrometer. It could be that in selecting well behaved peptides for these proteins we have unconsciously increased the intensity of the "top3", giving bias to the quantification.

Given the above theory a brief comparison between the amino acid composition of the *S.cerevisiae* proteome and the amino acid composition of our current set of COPYCATs was performed (Figure 27). Some amino acids such as Leucine and Threonine are equally represented in the yeast and COPYCAT proteins, however there are several amino acids where there is a difference between the two protein groups. The greatest difference calculated was for methionine where there was an 86.97% drop in occurrence in the COPYCAT proteins compared to the yeast proteome. The average difference between the percentage occurrence of an amino acid in the yeast proteome and the percentage occurrence of an amino acid in a COPYCAT is 20%. This difference could be significant and could contribute to why label-free quantification of QconCATs may not be suitable.

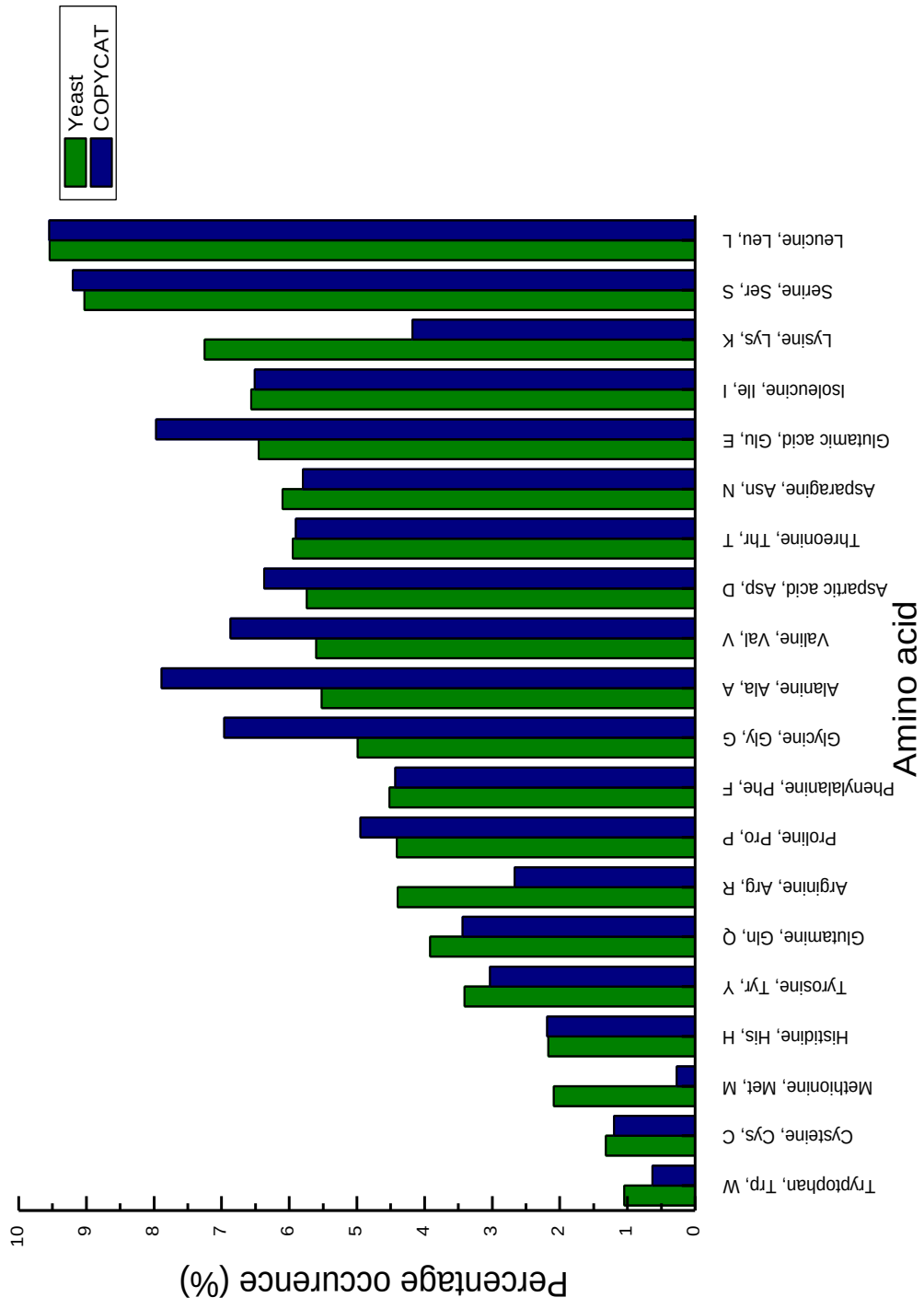


Figure 27 | Amino acid composition analysis. The comparison between the amino acid composition of the yeast proteome and of a collection of COPYCATs. Average difference reported as 20%.

The difference in average amino acid composition of COPYCATs compared to naturally occurring *S.cerevisiae* proteins could mean that the ionisation of COPYCAT peptides does not follow the same pattern as naturally occurring proteins, this could affect the reliability of label free quantification. The intensities of the top3 peptides chosen for the 10 light COPYCAT label free quantifications were compared to the intensities of the top3 peptides selected for label free quantification of 10 abundant *S.cerevisiae* proteins. The COPYCAT intensity values were taken from PLGS processing for the mix of COPYCATs; the mix was chosen as the *S.cerevisiae* for comparison was analysed in a complex background so the more complex sample was compared. The *S.cerevisiae* intensity values are taken from PLGS processing of a label free analysis of a *S.cerevisiae* whole cell lysate digest sample; the proteins listed as the 10 most abundant were selected. Two comparisons were made; first individual peptide intensities for the top 1, 2, and 3 were plotted for COPYCAT and *S.cerevisiae,* and secondly the average intensity for each of the top3 peptides for COPYCAT and *S.cerevisiae* were plotted for comparison (Figure 28). Comparing the top3 intensity distributions for each protein, the spread of curves in graphs (a) and (b) indicate that peptide ionisation behaviour varies greatly from protein to protein; variation appears to be present for both COPYCAT and *S.cerevisiae* proteins. The graph (c) suggests that for peptides 2 and 3 the percentage intensity is lower for COPYCAT proteins than for S*.cerevisiae* proteins. This does suggest a difference in behaviour between the COPYCAT proteins and the naturally occurring *S.cerevisiae* proteins, however this was not as originally anticipated. The label free quantification values for the COPYCATs were higher than the other quantification methods, in the majority of cases (Figure 26 (a)), and so it was suggested that the intensities of the top3 peptides chosen may be higher, on average, than for naturally occurring proteins. The results here show the converse and therefore do not explain the higher label free quantification values for the COPYCATs.

An absorbance assay (A_{280nm}) was considered for quantification of the QconCAT proteins, however the accuracy of the assay can vary widely depending on the accuracy of the estimated molar absorption coefficient for the protein *(Pace et al., 1995)*, which varies depending on the number of aromatic residues and cysteines, in the sequence. The amino acid composition analysis revealed that the composition of QconCATs is very different from the yeast proteome, and specifically the aromatic residues tryptophan and tyrosine, are present in significantly lower amounts in the COPYCATs compared to the yeast proteome (39.68% and 11.26% differences respectively). This is likely to affect the accuracy of the assay and for this reason it was not used.

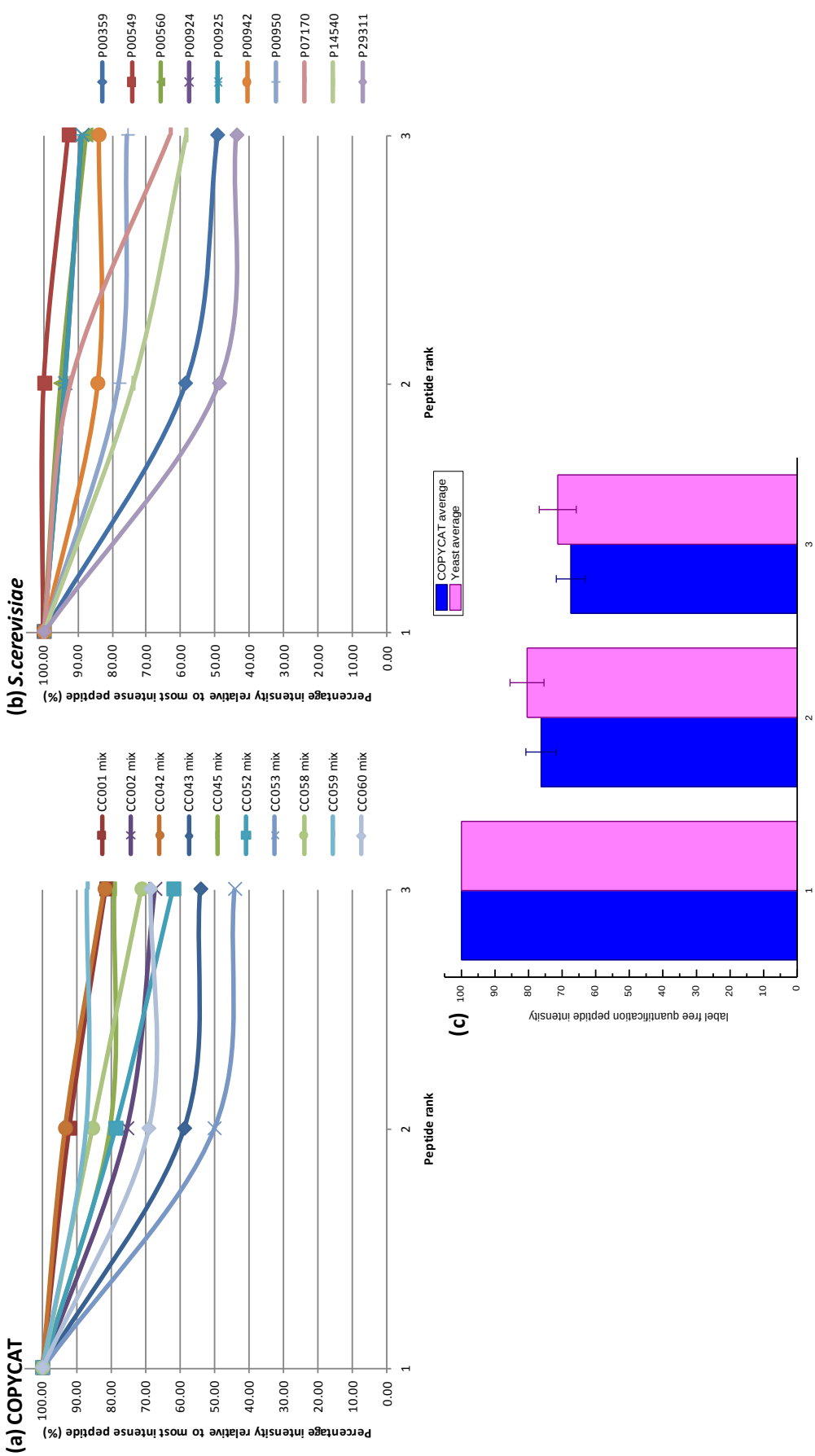


Figure 28 | Label free selected peptide intensities comparison for COPYCAT and *S.cerevisiae*. **(a)** and **(b)** top3 peptides intensities (top3 plotted as peptide rank 1, 2 and 3) for each individual protein from the COPYCAT and *S.cerevisiae* protein groups. **(c)** comparison between the average peptide intensity for the top3 for COPYCAT and *S.cerevisiae*.

SUMMARY

This work highlights the dependence of quantification on the method chosen for quantification of the standards. The wide range of values obtained here presents a common problem of deciding which method is the most accurate to give a reliable figure. Densitometry gives a visual result, contaminant proteins are immediately visible from the SDS-PAGE analysis giving more certainty to the fact that just the target protein is quantified; however the possible variation in staining makes the method unreliable. Protein assay works on a similar principle to densitometry and has the added disadvantage of not being able to assess any contaminants during the assay. With the previously mentioned potential for inaccuracy with label free quantification, in my opinion, quantification of the QconCAT using the GluFib peptide is the most reliable. GluFib has two major advantages over other methods. The first is that, like densitometry, contaminant proteins cannot interfere with the analysis as only GluFib heavy and light peptides are targeted. The second advantage is that, unlike any other method, the standard used to quantify the QconCAT resembles the QconCAT GluFib as closely as possible; a direct comparison is drawn between the heavy and light counterparts, there is no potential for differential behaviour due to the fact that, except for the m/z, the peptides behave identically in a mass spectrometer. I consider this similarity between standard and analyte to be of key importance to the accuracy of quantification. Amino acid analysis has been suggested as the gold standard for quantification of protein and this method could be used to compare to the other quantification methods and assess which aligns best with the amino acid analysis values.

4.7 QconCATs to assess instrument performance

Ideally to assess instrument performance the 10 COPYCATs would be combined in a dynamic range and this mixture used to assess instrument performance both in detection and quantification. The unexpected issue highlighted by the difficulty in standard quantification presents a problem in selecting a reliable quantification method. If the quantification of each COPYCAT is unreliable, we cannot be certain that the COPYCATs are in the correct ratio to create the dynamic range. We could take an average of the values and then simply take it on face value that the COPYCATs are present at the given amounts. MS performance could then be assessed by the ability to detect each COPYCAT to a suitable level of coverage. Hitting a certain level of coverage for each COPYCAT could be used as a quality control measure and comparisons could be drawn between the coverage obtained on different instruments to

assess instrument-to-instrument or lab-to-lab consistency. However a quality control tool that assesses quantitative as well as qualitative performance would be much more useful given the current trend in proteomics towards quantitative studies.

There are a number of potential studies that could be conducted further to this work. A particular study I would have liked to have looked at would have been to assess whether the outcomes of the quantification studies would have been the same had naturally occurring proteins been used rather than the artificial QconCAT proteins. Recombinant proteins could be synthesised with two additional peptides – a HisTag for purification and peptide for quantification. They could therefore be purified and quantified in much the same way as the COPYCATs. However, in contrast to COPYCATs, the rest of the protein would be the same as the naturally occurring protein so may perform better with label-free quantification, this may mean that quantitative as well as qualitative MS performance parameters could be assessed on the instrument.

5. Conclusions **98**

5. Conclusions

The primary aim of this work was to shorten the QconCAT production process in terms of the number of days taken for QconCAT production, and also to streamline the production process making it simpler and applicable to multiplexing. The overall aim was to make it a more time efficient and accessible technology. I have discussed the processes which led to changes in the protocol, and how they have been implemented. The flow diagram in Figure 29 recapitulates the Figure 7 for the Aims and objectives section and summarises the areas where the protocol has been altered.

I first addressed the cell culture and expression section of the protocol and reduced production time by removing the re-streaking of *E.coli* colonies on agar plates; this reduced the protocol by 1d. Secondly by eliminating the trial unlabelled culture the protocol was shortened, expressing the QconCAT in a "straight-to-heavy" approach reduced the production time by 5d. The reason for the straight to heavy approach was that we have never seen evidence for an incorrect QconCAT being synthesised, QconCATs are produced reliably and consistently as shown in this thesis. Repeated expression of QconCATs has lead to a greater understanding of the behaviour of these proteins; wide variability in expression levels has been observed and this impacts on the further processing of the protein. The consistent expression of the correct QconCAT construct lead to removal of the verification step, verification of the QconCAT is now delayed until after the final labelled QconCAT is produced. Removal of the early verification step reduced the production time by 6d.

I set out to investigate alternative methods of *E.coli* cell lysis and protein purification and to establish whether more rapid methods were available. The use of sonication rather than BugBusting for cell lysis has removed 0.5d from the protocol. Sonication also allows for multiple cell pellets to be sonicated in a short period of time therefore facilitating multiplexing of this step in the protocol. Alternative methods of QconCAT protein purification were investigated and ultimately the purification of the QconCAT protein was eliminated for adequately expressing QconCATs, replaced by simple solubilisation. RapiGest solubilisation is now used in place of purification; the unpurified inclusion bodies are quickly solubilised in a reagent which is compatible with protein digestion and mass spectrometric analysis. Removing the purification step reduces the production time scale by 1d. RapiGest solubilisation is also easily multiplexed. Overall the improvements to the QconCAT production protocol has reduced the time scale for preparation of the protein by 13.5d. The actual time

gains are greater than this as the improved protocol facilitates multiplexing, routinely 8 QconCATs are synthesised in parallel.

The final study of 10 QconCATs highlighted the difficulty in quantifying a standard. There were a number of different methods used for quantification; each method produced significantly different values. Every quantification method had merits and demerits and there is a significant difficulty in selecting the “correct” value in order to proceed. It was discussed that GluFib peptide based quantification may be the more reliable quantification method but that amino acid analysis should be considered for a gold standard quantification value. A potential further study was outlined to assess the behaviour of naturally occurring proteins in the same workflow.

The QconCAT production protocol has been adapted for use as a high throughput, multiplexed technique applicable to large scale quantification studies. However the applications of QconCATs reach beyond label-mediated quantification and this aspect of their use has yet to be fully explored.

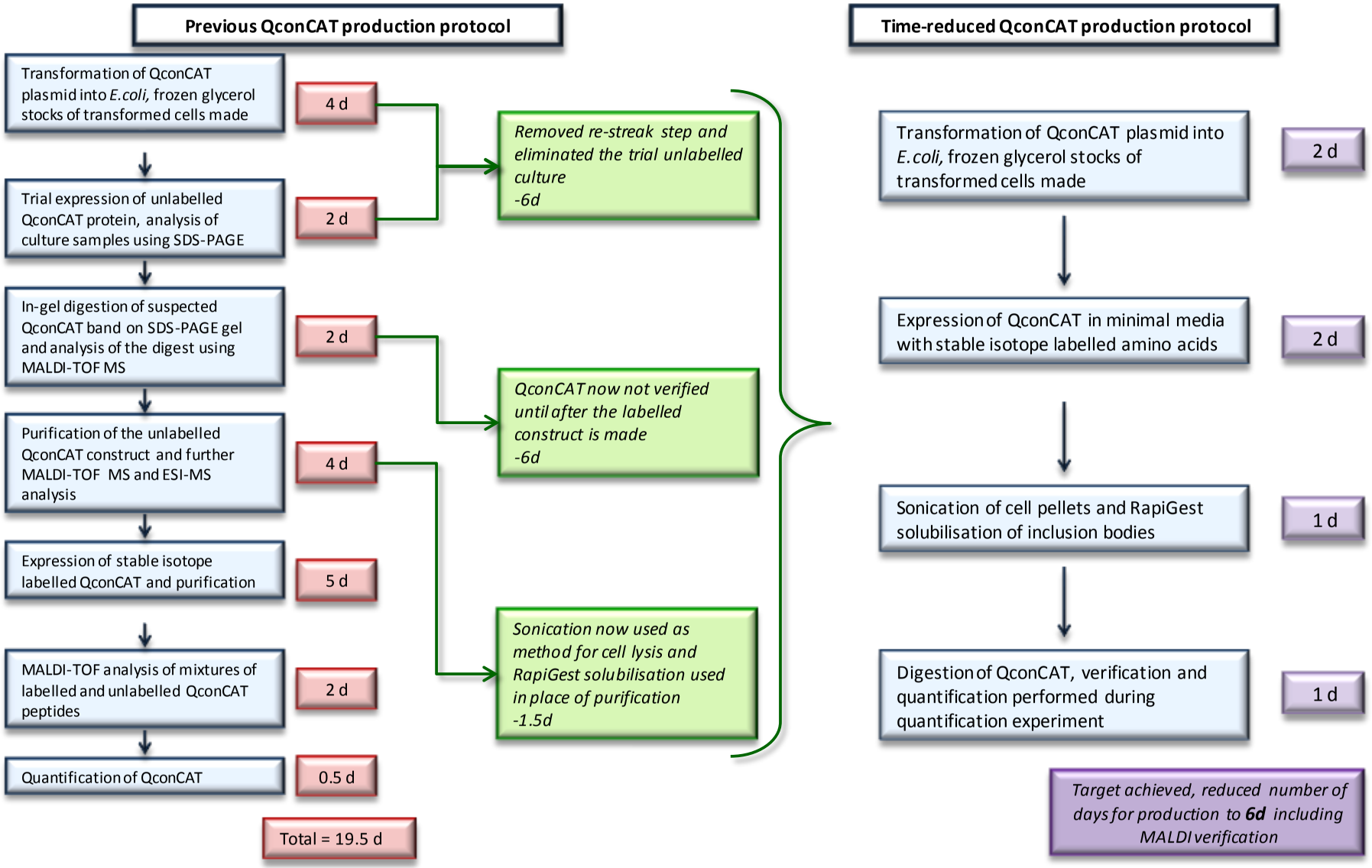


Figure 29| Conclusions. Echoing the figure in the Aims and Objectives, the original QconCAT production protocol is shown with the number of days for each step. The stages where days have been removed from the protocol are highlighted in green and the new time-duration for QconCAT production is now 6d.

6. References 102

6. References

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7. Publications containing work from this thesis

- Philip J. Brownridge, **Victoria M. Harman**, Deborah M. Simpson, and Robert J. Beynon (2012) *'Absolute Multiplexed Protein Quantification Using QconCAT technology'* Methods in Molecular Biology - Quantitative Methods in Proteomics, 893(3), 267-293 http://www.springerlink.com/content/j5753tj4g6761171/fulltext.pdf?MUD=MP

  For this book chapter I contributed protocols for QconCAT production, illustrated by figure Fig. 5 in the chapter, showing a growth curve and corresponding expression SDS-PAGE gel for an example QconCAT.

- Philip Brownridge, Stephen W Holman, Simon J Gaskell, Christopher M Grant, **Victoria M Harman**, Simon J Hubbard, Karin Lanthaler, Craig Lawless, Ronan O'cualain, Paul Sims, Rachel Watkins and Robert J Beynon (2011) *'Global absolute quantification of a proteome: Challenges in the deployment of a QconCAT strategy'* Proteomics, 11(15), 2957-2970
  http://onlinelibrary.wiley.com/doi/10.1002/pmic.201100039/pdf

  I contributed to this publication by providing expression and purification/solubilisation of the QconCATs utilised in each aspect of the study. I received plasmid DNA for each QconCAT and expressed and purified the proteins, initially using the primary production protocol and later using modified versions of the protocol as it was developed. Additionally I acquired and contributed the SDS-PAGE images displayed in Figure 5.

- Stine L. Bislev, Ulrike Kusebauch, Marius C. Codrea, Robert J. Beynon, **Victoria M. Harman**, Christine M. Røntved, Ruedi Aebersold, Robert L. Moritz and Emøke Bendixen (2011) *'Quantotypic properties of QconCAT peptides targeting bovine host response to Streptococcus uberis."* J Proteome Res, 11(3), 1832-1843
  http://pubs.acs.org/doi/pdf/10.1021/pr201064g

  The QconCAT used in this study was EB1, which is referred to in section 5.1. I received the QconCAT plasmid DNA for EB1 and expressed the protein in unlabelled and stable

isotope labelled forms. I verified the QconCAT through MALDI-TOF MS before it was handed to Stine Bislev and Emøke Bendixen for use in their quantification study.

- Some of the work in this thesis was also presented as a poster at the 8th British Society for Proteome Research - European Bioinformatics Institute joint meeting in Cambridge July 2011 and a report of the work was published in the November 2011 edition of Mass Matters – the official publication of the British Mass Spectrometry Society Edition 66. The poster was also presented at the ProteoMMX 2.0 Strictly Quantitative meeting in Chester, March 2012.