CHAPTER 2

Proteomic profiling of *P. falciparum* through improved, semi-quantitative two-dimensional gel electrophoresis

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"Two D, or not two D: that is the question:
Whether 'tis nobler in the mind to suffer
The streaks and blobs of intractable proteins
Or to take chips against a sea of genes
And by comparing, find them that
hold the bitter taste of disease and death."

(Fey & Larsen, 2001)

2.1 Introduction

Proteomics enables the direct study of the proteome in which sets of proteins occur together in a particular biological state at a particular time. One of the workhorses for proteomic applications has been bottom-up proteomics that include the use of differential expression detected on two-dimensional gel electrophoresis (2-DE) gels followed by mass spectrometry (MS) identification. Bottom-up proteomics is the process in which proteins and their post-translational modifications (PTM's) are identified and characterised by separating the proteins first, followed by proteolytic digestion prior to MS analysis. 2-DE was first introduced in the mid 1970's by O'Farrell (O'Farrell, 1975). In recent years the technology has gone from strength to strength and is now widely employed to assess proteomes of various organisms in a variety of applications that include proteome mapping, differential regulation of perturbation studies and detection of PTM's. Application of 2-DE technology has several visible properties which is irreplaceable and include good resolution of abundant proteins, information on quantity, detection of PTM's, immediate information on approximate pI and molecular weight values (Lopez, 2000). Despite these advantages the reality is that 2-DE is limited to high abundance proteins while the dynamic proteome within a cell range from 7-12 orders of magnitude. Furthermore, 2-DE also has bias towards soluble proteins and mid-range molecular weight and pI proteins (Ong & Pandey, 2001).

2.1.1 Minimum information about a proteomics experiment

To avoid discrepancies in the reporting of proteomic data minimum information about a proteomics experiment (MIAPE) (Taylor *et al.*, 2007) was established similar to minimum information about a microarray experiment (MIAME) (Brazma *et al.*, 2001) for transcriptomic data. The general criteria for reporting of data and the collection of metadata include sufficiency and practicality. Basically, sufficient information should be given to allow the reader to understand and to critically evaluate the data and repetition of experiments should be achievable to most laboratories (Taylor *et al.*, 2007). For 2-DE, guidelines exist on study design and sample generation, in which the origin of the samples together with sample processing and number of replicates should be reported (Gibson *et al.*, 2008). For the separation of samples and sample handling, fractionation, manipulation, storage as well as sample transport should be discussed. For gel electrophoresis the separation methods, stain, visualisation and image acquisition methods should be specified as well as all the information regarding image analysis (Gibson *et al.*, 2008). Spot identification by mass spectrometry require information on the generation of the peak list, sample handling, the informatics used, the search engine, spectra submitted, peptide matching, database used for identification purposes and quality control measures (Binz *et al.*, 2008, Taylor *et al.*, 2008). Considering the huge amount of proteomic data that is published each year, it is of utmost importance that data that are being reported in the public domain are standardised.

2.1.2 Liquid chromatography mass spectrometry and protein arrays used for proteomics

Liquid chromatography mass spectrometry (LC-MS) has an advantage of being able to analyse complex peptide mixtures that include soluble proteins as well as membrane-, trans-membrane-, and integral proteins. Commonly used MS based methods for quantification include isotope coded affinity tags (ICAT) and isobaric tags (iTRAQ) (Shiio & Aebersold, 2006, Aggarwal *et al.*, 2006). ICAT is dependent on the number of cysteine residues, which is of relative low abundance in the Plasmodial proteome (Sims & Hyde, 2006, Nirmalan *et al.*, 2004a) and would thus not be ideal to use. Labelling of peptides with iTRAQ targets primary amines and enables the simultaneous analyses and identification as well as quantification of proteins. iTRAQ uses 4 specific amine tags enabling the simultaneous detection of up to 4 different samples (Aggarwal *et al.*, 2006). Using iTRAQ, all types of proteins can be determined but it may have a slight bias against the more acidic proteins due to fewer arginine and lysine residues (Aggarwal *et al.*, 2006). Another setback of iTRAQ is the delayed sample mixing (Sims & Hyde, 2006). Metabolic labelling techniques has proved to be superior for Plasmodial proteins (Nirmalan *et al.*, 2004a). The method employed the use of labelled isoleucine added to *in vitro* cultures, with the added advantage that cultures could be

mixed immediately in equal ratios, but unfortunately a major setback is that the labeled isoleucine is extremely expensive. Overall, a major disadvantage with regard to MS-based methods is the lack of effective search algorithms and databases that may complicate and increase analysis time of data (Aggarwal *et al.*, 2006, Sims & Hyde, 2006, Nesvizhskii *et al.*, 2007).

Other technologies that can be applied to the analysis of the proteome include protein microarrays, which have been applied for identification, quantification and functional analysis in basic and applied proteomics (MacBeath, 2002, Poetz *et al.*, 2005). There is no absolute correlation between the mRNA expression level and the corresponding protein expression (Gygi *et al.*, 1999). Similarly it is impossible to correlate the protein state purely by investigation of the protein expression level (Poetz *et al.*, 2005). Protein arrays are able to analyse the function of the proteome by investigating binding partners and target proteins therefore providing a functional classification of the protein and its interacting partners. Surface-enhanced laser desorption/ionisation-time-of-flight/mass spectrometry (SELDI-TOF/MS) is able to employ a surface-based fractionation of proteins therefore separating protein mixtures and their binding properties (Gast *et al.*, 2006). Basically, proteins are captured on surfaces and then separated based on their biophysical properties which is then followed by TOF/MS to identify the proteins and expression profiles (Weinberger *et al.*, 2000, Merchant & Weinberger, 2000).

2.1.3 Plasmodial and parasite proteomics

The Plasmodial proteome is multifaceted and stage-specific, indicating a high degree of specialisation at the molecular level to support the biological and metabolic changes associated with each of the life cycle changes (Shock *et al.*, 2007, Sims & Hyde, 2006). Post-translational modifications are employed as a mechanism to regulate protein activity during the parasite's life cycle (Nirmalan *et al.*, 2004a) and certain proteins are predicted to act as controlling nodes that are highly interconnected to other nodes and thus results in a highly specialised interactome (Wuchty *et al.*, 2009, Birkholtz *et al.*, 2008b). These enticing properties motivate studies focused on in-depth characterisation of the Plasmodial proteome including regulatory mechanisms and the ability to respond to external perturbations. Analysis of the schizont stage proteome reinforced the notion that both post-transcriptional and post-translational mechanisms are involved in the regulation of protein expression in *P. falciparum* (Foth *et al.*, 2008).

Due to the >80% A+T-richness of the Plasmodial genome (Gardner *et al.*, 2002), the resultant Plasmodial proteome contains proteins in which long hydrophobic stretches and amino acid repeats (notably consisting of lysine and asparagine) are found. Moreover, the proteins from this parasite

are comparatively large, non-homologous and highly charged with multiple isoforms within the parasite (Birkholtz *et al.*, 2008a). These properties have confounded analyses of the Plasmodial proteome, including the recombinant expression of Plasmodial proteins (Mehlin *et al.*, 2006, Vedadi *et al.*, 2007). Few studies attempted to describe the Plasmodial proteome, which is predicted to have about 5300 proteins of which ~60% are hypothetical and un-annotated (Foth *et al.*, 2008, Gelhaus *et al.*, 2005, Makanga *et al.*, 2005). The last decade has experienced an explosion in proteomic studies with an exponential growth in proteomic publications, unfortunately it seems that Plasmodial proteomics has been left behind (Figure 2.1).

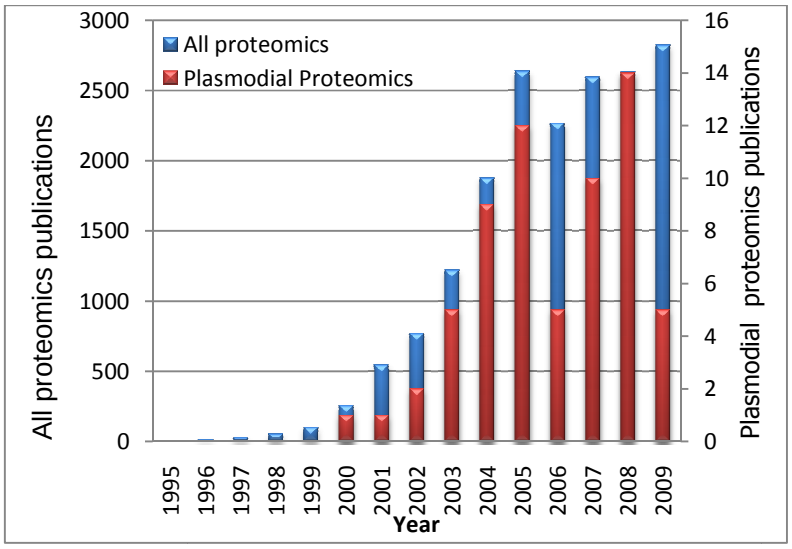

Figure 2.1: The state of proteomic publications per year as on ISI Web of Science.

Search criteria was according to title (proteom* AND (plasmodium* or malaria*) and publication year. Date last searched 02/12/2009.

Proteomics of most protozoan parasites is a fast evolving field. Early in the development of the 2-DE methodology for *Leishmania*, it was recognized that this parasite needs an efficient lysis buffer for 2-DE for optimal spot detection of the Leishmanial proteins (Acestor *et al.*, 2002). Five years later *L. amazonensis* proteins were used to demonstrate the efficiency of liquid phase isoelectric focusing (IEF) in combination with 2-DE to improve proteins detected in the acidic and basic ranges (Brobey & Soong, 2007). The 2-DE proteomic map of the protozoan parasite *Trypanosoma cruzi*, which is responsible for Chagas disease in humans, include 26 identified spots that corresponded to 19 unique protein groups accounting for 27% isoforms (Paba *et al.*, 2004).

A striking feature was that the majority of the spots remain similar throughout all the life stages and therefore the progression of the parasite is due to the expression of a limited number of proteins

(Paba *et al.*, 2004). Similarly, another protozoan parasite *T. brucei,* which causes sleeping sickness, was investigated with 2-DE. A large scale 2-DE proteomic study of the procyclic form of *T. brucei* identified 2000 spots that related to 700 proteins which included various isoforms due to PTM's (Jones *et al.*, 2006). Uncommon protozoan parasites characterised with 2-DE include the first 2-DE reference map of *Trichomonas vaginalis*, in which 116 spots that related to 67 different proteins, representative of 42% isoforms were identified (De Jesus *et al.*, 2007). The importance of PTM's was demonstrated for this parasite, since PTM's may regulate protein function in the cells by altering their localisation, interaction or activity. N-terminal acetylation was seen for actin, while deamidation of certain proteins has been associated with protein turnover, development and aging (De Jesus *et al.*, 2007). The yeast *(Saccharomyces cerevisiae)* proteome map has been in progress for 10 years, with a total of 716 proteins successfully identified that consists of 32% isoforms (Perrot *et al.*, 1999, Perrot *et al.*, 2009).

Compared to other protozoan parasites, the reported efficacy of 2-DE to analyse the Plasmodial proteome is relatively poor since only a low number of protein spots could be detected with various protocols and stains (Makanga *et al.*, 2005, Gelhaus *et al.*, 2005, Panpumthong & Vattanaviboon, 2006, Radfar *et al.*, 2008, Wu & Craig, 2006). The highest number of spots detected to date on Plasmodial 2-DE gels with silver staining is only 239 (Panpumthong & Vattanaviboon, 2006) and recently, a total of 345 spots were detected for 4 time points in the Plasmodial schizont stage using two-dimensional differential gel electrophoresis (2-D DIGE) (Foth *et al.*, 2008), of which only 54 protein spots were identified. This clearly illustrates the need for an optimised protocol including extraction, quantification and detection methods. This chapter details such an optimised 2-DE protocol, which was applied to the analysis of the Plasmodial proteome in the ring and trophozoite stages. Firstly, established methodology was optimised with regard to protein extraction, quantification, detection and finally MS identification is described. Once the protocol was established, it was applied to the analyses of the soluble Plasmodial proteome.

2.2 Methods

2.2.1 Blood collection

Type O^+ blood was collected in a blood bag (Fenwal Primary container with citrate phosphate glucose adenine anticoagulant, 70 ml anticoagulant for the collection of 500 ml blood, Adcock Ingram) which was left overnight at 4°C in the bag after collection. The following morning the blood was transferred to a sterile plastic container and kept for use at 4°C for 4-5 weeks. Erythrocytes were collected from the bottom of the container and washed by adding an equal amount of phosphate buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10mM phosphate, pH 7.4) to the erythrocytes and centrifugation at 2500×*g* for 5 min. The supernatant were aspirated and the step repeated at least another 4 times until there was no visible buffy coat left. The washed erythrocytes were then resuspended in an equal volume of culture media (RPMI 1640 media (Sigma), supplemented with 0.4% (w/v) D-glucose (Sigma), 50 mg/l hypoxanthine (Sigma), 48 mg gentamycin (Sigma), buffered with 12 mM HEPES (Sigma) and 21.4 mM sodium bicarbonate (Merck) per litre of MilliQ water (double distilled, de-ionised, 0.22 µM filter sterilised) and finally the addition of 0.5% (w/v) Albumax II (Gibco) for complete culture media) for use in all experimental procedures to follow.

2.2.2 Thawing of parasites

The chloroquine-sensitive *P. falciparum* 3D7 (*Pf*3D7) parasites were thawed from parasite stock solutions stored at -180°C in liquid nitrogen. Parasites were thawed at 37°C for 5 min after which 0.2 ml of 12% (w/v) NaCl was added, mixed, followed by the addition of 1.8 ml of 0.6% (w/v) NaCl. The parasites were then centrifuged at 2500×*g* for 5 min and resuspended in 30 ml culture media and 1.5 ml packed erythrocytes was added to obtain a 5% hematocrit. The resuspended parasites were finally gassed using a special gas mixture containing 5% CO_2, 5% O_2 and 90% N_2 (Afrox), before being placed in a shaking incubator at 37°C and 58 revolutions per minute (rpm). Thawed parasites were never used for longer than 2 months to prevent possible genetic alterations.

2.2.3 Daily maintenance of parasites

*Pf*3D7 parasites were maintained *in vitro* in 75 cm^3 Cellstar culture flasks (Greiner bio-one) in human O^+ erythrocytes in culture media (Trager & Jensen, 1976). The culture media of the parasites were changed daily by transferring the cultures to a sterile 50 ml tube which was then centrifuged at 2500×*g* for 5 min. The culture media was then aspirated and the remaining parasite-containing pellet was resuspended in pre-heated fresh culture media. The resuspended parasites were then transferred back into a 75 cm^3 Cellstar culture flask and gassed for 30 s with the special gas

mixture. The flasks were sealed air-tight before being placed back into the 37°C incubator. On every second day, when the parasites were in the trophozoite stage the parasite culture were either divided into several flasks or parasites were removed from the original flask in order to maintain the parasitemia at 5%. Fresh erythrocytes were also added to maintain the hematocrit at 5%. Parasites were monitored daily through light microscopy of Giemsa stained thin blood smears. Giemsa's Azur Eosin methylene blue solution (Merck) was diluted 1:5 in proprietary buffer for staining blood smears pH 6.4 (Merck). Slides were incubated for 3 min before investigation by light microscopy to determine the parasitemia. Slides were analysed using a Nikon light microscope at 1000× magnification under oil immersion. At least 10 fields of 100 erythrocytes each were examined for the determination of parasite progression.

2.2.4 Synchronisation

Synchronisation was done using a modified sorbitol method of Lambros and Vanderberg (Lambros & Vanderberg, 1979). Parasites mostly in the ring stage, were centrifuged at 2500×*g* for 5 min, after which the supernatant were aspirated. Three volumes 15% (w/v) sorbitol were added to the parasite pellet, resuspended and incubated at 37°C for 5 min. This was followed by the addition of 6 volumes of 0.1% (w/v) glucose, mixed, and incubated for 5 min at 37°C. After incubation the mixture was centrifuged at 2500×*g* for 5 min, the supernatant removed and the synchronised parasite pellet resuspended in culture media and a 5% hematocrit. Parasites were always synchronised for 3 consecutive cycles (6 times in total, always 8 h apart once in the morning and later in the afternoon). The morning synchronisation is done to remove parasites that are still schizonts and the afternoon synchronisation is to remove trophozoites. This is done to ensure that the parasites that fall out of the ring stage window is removed thus resulting in better synchronisation with a smaller window.

2.2.5 Culturing of parasites for proteomics

*Pf*3D7 parasites were maintained *in vitro* in human O^+ erythrocytes in culture media and monitored daily through light microscopy of Giemsa stained thin blood smears as described in section 2.2.3. Before treatment could commence the parasites were always synchronised for 3 consecutive cycles (6 times in total, always 8 h apart once in the morning and later in the afternoon) as described in section 2.2.4. Thirty millilitres of *Pf*3D7 parasite cultures at 8% parasitemia and 5% hematocrit were used per gel to establish the proteomics methodology. Saponin was added to a final concentration of 0.01% (v/v) followed by incubation on ice for 5 min to lyse the erythrocytes. Parasites were collected by centrifugation at 2500×*g* for 15 min at room temperature, and washed in PBS at 16 000×*g* for 1 min at 4°C. This step was repeated at least 4 times until the supernatant was

clear instead of 3 times as previously reported (Nirmalan *et al.*, 2004a). The parasite pellet was stored at –80°C until use, but never stored for longer than 30 days. For the analyses of proteomes of different developmental stages of the parasites, parasites were harvested from 60 ml cultures at 16 hours post invasion (HPI) (late rings) and 20 HPI (early trophozoites).

2.2.6 Protein preparation

Parasite pellets were suspended in 500 µl lysis buffer as described by Nirmalan *et al.* (8 M urea, 2 M thiourea, 2% CHAPS, 0.5% (w/v) fresh DTT and 0.7% (v/v) ampholytes, pH 3-10 linear) (Nirmalan *et al.*, 2004a). Samples were pulsed-sonicated on a Virsonic sonifier with microtip for 20 s with alternating pulsing (1 s pulse, 1 s rest) at 3 W output with 1 min cooling steps on ice (to prevent foaming and carbamylation) and repeated 6 more times (Table 2.1).

Table 2.1: Program settings used for Virsonic sonifier

Process time	10 s
Pulsar on	1 s
Pulsar off	1 s
Power	3 W
Total time	20 s
Microtip	Yes
Pulsed	Yes

Sonication was followed by centrifugation at 16 000×*g* for 60 min at 4°C, after which the protein-containing supernatant was used in subsequent 2-DE.

2.2.7 Protein quantification

Four different protein quantification methods were tested on the samples obtained using 2 BSA standard curves in each of the methods: firstly, BSA in 0.9% saline, and secondly, BSA in the Plasmodial lysis buffer, each containing the same amount of protein for analysis.

2.2.7.1 Bradford method

The Bradford method is based on the principle that the dye binds mainly to basic and aromatic amino acids. Upon binding of the dye to the protein the dye is converted into the stable un-protonated blue form that can be detected at 595 nm (Bradford, 1976). The Quick Start™ Bradford dye method (Bio-Rad) was used for protein determination at an absorbance of 595 nm with a Multiskan Ascent spectrophotometer (Thermo Labsysytems).

2.2.7.2 Lowry

The Lowry method is based on the Biuret reaction in which peptide bonds react with Cu^{2+}. Under alkaline conditions the copper will react with the Folin Ciocalteau reagent giving a blue colour that can be detected at 660 nm. The reaction is also partially dependent on aromatic amino acids (Lowry *et al.*, 1951). The Lowry method used a reaction mixture containing solution A (2% (w/v) $NaCO_3$, 2% (w/v) NaOH, 10% (w/v) Na_2CO_3), solution B (2% (w/v) $CuSO_4.5H_2O$), and solution C (0.5% (w/v) potassium tartrate). Two hundred microlitres of the reaction mixture was added to each protein sample, mixed and incubated for 15 min at room temperature. Six hundred microlitres of Folin Ciocalteau reagent (1:10, FC reagent and H_2O) were added and incubated at room temperature for 45 min in the dark. Absorbance was measured at 660 nm.

2.2.7.3 Protein quantification by the BCA method

The BCA method uses bicinchoninic acid (BCA) as the detection reagent for Cu^{+} which is formed when Cu^{2+} is reduced by protein in an alkaline environment. A purple coloured reaction product is formed by the chelation of 2 molecules of BCA with one Cu^{+} ion, and can be measured at 562 nm. The colour formation is due to the macromolecular structure of the protein, the number of peptide bonds and the presence of 4 amino acids (cysteine, cystine, tryptophan, tyrosine) (Smith *et al.*, 1985). The commercially available Micro BCA™ Protein assay kit (Pierce) was used. In short, a working solution was prepared and added to the protein standards and then incubated for 2 h at 37ºC. The plate was left to cool to room temperature for approximately 30 min, before the absorbance was measured at 550 nm.

2.2.7.4 Protein quantification by 2-D Quant kit

The 2-D Quant kit quantitatively precipitates protein, leaving the interfering substances in solution. It is based on the specific binding of copper ions to proteins. The precipitated proteins are resuspended in a copper containing solution of which the unbound copper is then measured with a colorimetric agent at 480 nm. The colour density is inversely related to the protein concentration. The commercially available 2-D Quant Kit (GE Healthcare) was used according to the manufactures instructions with a few modifications. In short, a standard curve containing 6 dilutions (0, 10, 20, 30, 40, 50 µg) was prepared using the 2 mg/ml BSA stock solution provided by the kit. Varying volumes of Plasmodial proteins (2.5, 5, 7.5, 10, 15 µl) were used to determine the protein concentration of each Plasmodial sample. 500 µl precipitant were added to each tube, vortexed and left to incubate for 3 min at room temperature, followed by 500 µl of co-precipitant and mixed by

inversion immediately upon addition. Samples were centrifuged at 16 000×*g* for 15 min at 4°C. The supernatants were decanted and centrifuged for 3 min at 16 000×*g*, 4°C. The remaining supernatant was removed by pipette, before the addition of 100 µl of a copper containing solution followed by 400 µl MilliQ water and mixing each tube. This was followed by the addition of 1 ml working solution to each tube, which was mixed immediately upon addition to ensure rapid mixing, before proceeding to the next tube. The tubes were then incubated for 20 min at room temperature, before the absorbance was measured at 492 nm.

2.2.8 SDS-PAGE gels

Low molecular weight markers (GE Healthcare) were diluted in reducing buffer (0.06 M Tris-glycine, 2% (w/v) SDS, 0.1% (v/v) glycerol, 0.05% (v/v) β-mercaptoethanol and 0.025% (v/v) bromophenol blue, pH 6.8), to provide a total protein concentration range of 1250 ng to 9.7 ng and individual protein concentrations ranging from 100 ng to 0.6 ng. Equal amounts of markers were loaded onto 4 different 12.5% SDS-PAGE gels and the gels were subsequently stained with either Colloidal Coomassie, silver, SYPRO Ruby (Molecular Probes) or Flamingo Pink (Bio-Rad) stains. The gels were scanned on a Versadoc 3000 and analysed using Quantity One 4.4.1 (Bio-Rad). The Rf values and the intensities of each band were compared, and used to determine the limit of detection and linearity.

2.2.9 Two-dimensional gel electrophoresis (2-DE)

For 2-DE, the protein concentration was determined with the 2-D Quant kit. Two hundred micrograms of protein in rehydration buffer (8 M urea, 2 M thiourea, 2% (w/v) CHAPS). 0.5% (w/v) DTT and 0.7% (v/v) IPG Buffer (pH 3-10 Linear) was applied to a 13 cm IPG, pH 3-10 L strip. First dimensional isoelectric focusing (IEF) was performed on an Ettan IPGphore Isoelectric Focusing Unit (GE Healthcare), and commenced with a 10 h active rehydration step. Isoelectric focusing time followed an alternating gradient and step and hold protocol and was always allowed to proceed to a total of 18 500 Volt-hours, that completed within 15 h. The complete IEF focusing steps is given in Table 2.2.

Table 2.2: The IEF focusing steps used for the 13cm IPG, pH 3-10 L strips.

Step	Voltage limit (V)	Time or Volt hour (h) or (V-h)	Gradient
1	30 V	10:00 h	Step and hold[a]
2	200 V	0:10 h	Gradient[b]
3	200 V	0:15 h	Step and hold
4	500 V	0:15 h	Gradient
5	500 V	0:15 h	Step and hold
6	2 000 V	0:15 h	Gradient
7	2 000 V	0:30 h	Step and hold
8	8 000 V	0:30 h	Gradient
9	8 000 V	14 500 V-h	Step and hold
Total		18 500 V-h	

[a] Step and hold $V\text{-}h = h \times V$ Equation 2.1

[b] Gradient $V\text{-}h = h \times \frac{(V\ previous\ step + V\ new\ step)}{2}$ Equation 2.2

Following IEF, the IPG strips were equilibrated for 10 min each in SDS equilibration buffer (50 mM Tris-glycine, pH 6.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 0.002% bromophenol blue) containing 2% DTT, and then incubated in 2.5% iodoacetamide. Finally, the strip was placed in SDS electrophoresis running buffer (0.25 M Tris-HCl, pH 8.3, 0.1% SDS, 192 mM glycine) for 10 min as a final equilibration step. Second dimensional separation was performed by placing the IPG strips on top of the 10% SDS PAGE gel (Hoefer SE 600), covered with 1% agarose dissolved in SDS electrophoresis running buffer. Separation was performed at 80 V at 20°C until the bromophenol blue front reached the bottom of the gel. The gels were then fixed in the appropriate fixing solution for each specific stain (see below). For proteomic analyses of the different developmental stages of *P. falciparum*, 400 µg protein was applied to 18 cm IPG strips for separation and subsequently stained with Flamingo Pink.

2.2.10 Staining of 2-DE gels

Fluorescent stains often present with problematic background as the sensitivity of the stain also enables staining of dust particles and any impurity present within the gel or solutions used during the preparation and staining of the gel. For this reason, special care was taken during 2-DE preparation to avoid dust, to wash all glassware with special care and take extra special precaution to avoid contamination of the sample. The buffers and the stain used were filtered to ensure good quality gels and data.

2.2.10.1 Flamingo Pink staining of 2-DE gels

Flamingo Pink is a fluorescent stain that is a dilute alcoholic solution of an organic dye that binds to denatured protein. It is non-fluorescent in solution, but becomes strongly fluorescent when bound to protein. Gels were fixed overnight in 40% (v/v) ethanol, 10% (v/v) acetic acid, and subsequently in 200 ml Flamingo Pink working solution (diluted 1:9 with Milli-Q water as per the manufacturer's instructions) and incubated with gentle agitation in the dark for 24 h, to increase the sensitivity of the stain. The gels were washed in 0.1% (w/v) Tween-20 for 30 min to reduce background. Finally the gels were rinsed in Milli-Q water twice before scanning on the Versadoc 3000. All gels were stored in Flamingo Pink at 4°C until use for MS.

2.2.10.2 Silver staining of 2-DE gels

Silver binds to the amino acid side chains usually the sulfhydryl and carboxyl groups, in which the silver is reduced to metallic silver on the protein. The silver is then deposited on the gel to give a black and brown colour. Gels were fixed in 45% (v/v) methanol, 5% (v/v) acetic acid overnight, followed by sensitising for 2 min in 0.02% (w/v) sodium thiosulfate, and rinsing with Milli-Q water twice. 200 ml ice cold 0.1% (w/v) silver nitrate was added and incubated at 4°C for 30 min, rinsed twice with Milli-Q water and developed in fresh 2% (w/v) sodium carbonate with 0.04% (v/v) formaldehyde. Development was stopped by adding 1% (v/v) acetic acid (Jensen *et al.*, 1999). All gels were stored in 1% (v/v) acetic acid at 4°C in airtight containers until use for MS.

2.2.10.3 SYPRO Ruby staining of 2-DE gels

SYPRO Ruby is a fluorescent stain that consists of an organic and ruthenium component that binds non-covalently to the proteins (Berggren *et al.*, 2000). Gels were fixed in 10% (v/v) methanol, 7% (v/v) acetic acid overnight. The fixing solution was replaced with 200 ml SYPRO Ruby stain (used undiluted as supplied by the manufacturer) and the gels were incubated with agitation for 24 h in the dark, to increase sensitivity. After staining, the gels were washed for 60 min with 10% (v/v) methanol, 7% (v/v) acetic acid to reduce fluorescent background. Finally, the gels were rinsed twice with MilliQ water before scanning on the Versadoc 3000. Gels were stored in SYPRO Ruby at 4°C until use for MS.

2.2.10.4 Colloidal Coomasie Blue (CCB) staining of 2-DE gels

Colloidal Coomassie Brilliant Blue G250 stock solution (2% (v/v) phosphoric acid, 10% (w/v) ammoniumsulfate, and 0.1% (v/v) Coomassie Brilliant Blue G250) was diluted (4:1) with methanol just before use. The gels were immersed in the Colloidal Coomassie solution and left shaking overnight. Gels were rinsed with 25% (v/v) methanol, 10% (v/v) acetic acid before destaining with 25% (v/v) methanol, until the background was clear (Neuhoff *et al.*, 1988). Gels were then scanned on the Versadoc 3000, and stored in 1% (v/v) acetic acid at 4°C until use for MS.

2.2.11 Image Analysis of 2-DE gels by PD Quest

All the gels were scanned using the VersaDoc 3000 image scanner (Bio-Rad) and the appropriate software from the PD Quest™ 7.1.1 Software package (Bio-Rad). Scan settings for each of the 4 stains is given in Table 2.3.

Table 2.3: Scan settings used on PD Quest and the Versadoc 3000 for the 4 stains used

Stain	CBB	Silver	SYPRO Ruby	Flamingo Pink
Light application	Clear white TRANS	Clear white TRANS	520 LP UV TRANS	520 LP UV TRANS
Gain	0.5× Gain	0.5× Gain	4× Gain	4× Gain
Bin	1 × 1 Bin	1 × 1 Bin	1 × 1 Bin	1 × 1 Bin
Total exposure	3 s	3 s	30 s	120 s
Start exposure	0.5 s	0.5 s	5 s	30 s
Nr of exposures	6 (1 image taken every 0.5 s)	6 (1 image taken every 0.5 s)	6 (1 image taken every 5 s)	6 (1 image taken every 15 s)

For the method optimisation protocol, gel image analysis was performed using PD Quest 7.1.1 (Bio-Rad). All 8 gels were filtered using the Filter Wizard. Spot detection was performed on the gels by automated spot detection. The display of the gels stained with SYPRO Ruby and Flamingo Pink was inverted for easier comparisons with the gels stained with CCB and silver. Additional manual settings for spot detection were sensitivity (2.22), size scale (5) and min peak (1244). For proteomic analyses of the different developmental stages of *P. falciparum*, 400 µg protein was applied to 18 cm IPG strips for separation and subsequently stained with Flamingo Pink and scanned using the Versadoc 3000 as described below. PD Quest 7.1.1 was used to identify the number of spots on each of the gels that were done for the ring and trophozoite 2-DE proteomes (8 gels for each stage). First, all images were cropped to the same dimensions (1.59 Mb, 933 × 893 pixels, 303.7 × 290.7 mm) and filtered using the salt setting (light spots on dark background) of the Filter Wizard. The Spot Detection Wizard was used to automatically detect spots on the selected master image by manual identification of a small spot, faint spot and large spot. Additional settings

for spot detection were manually selected for sensitivity (5.31 for rings and 4.35 for trophozoites), size scale 5.0 (both), min peak (808 for rings and 4712 for trophozoites). After automated matching of all the gels, every spot was manually verified to determine correctness of matching.

2.2.12 2-DE spot identification by tandem mass spectrometry

MS is an analytical technique that measures the motion of charged particles (usually +1 for MALDI-TOF) in an electric field. The particle or peptide is ionised and is then separated according to its mass:charge ratio (m/z) which is then compared to a database containing theoretical mass values for the peptides of specific proteins. Unfortunately, it is possible that the mass of a particular peptide may be similar to another peptide of an unrelated protein, and therefore the use of MS/MS to obtain partial amino acid sequences are of utmost importance. The PMF are analysed in the first chamber and then one peptide at a time is allowed into the second collision chamber where it is fragmented with nitrogen gas to produce daughter ions which are then used to obtain an amino acid sequence. During this MS/MS fragmentation, low collision energy is used to fragment the peptide ion at each amide bond along the peptide backbone, hence yielding a peptide sequence. Upon fragmentation of the peptide two complimentary ion series can be obtained that include the b-ion series and the y-ion series (Roepstorff & Fohlman, 1984). The b-ion series will contain the N-terminal amino acid and is therefore the total residue mass of the amino acid, while the y-ion series will contain the C-terminus of the amino acid and is the total mass with an additional mass of 19 (18 for the presence of water and +1 Da for the ionising proton). Since a tryptic digestion was done the y_1-ion will always be either Arg with a mass of 175.1 Da or Lys with a mass of 147.1 Da.

For comparative purposes mostly the same 39 spots (154 in total) covering a wide range on the gels as well as low molecular weight markers were cut from each of the 4 differently stained gels, dried and stored at -20°C. The silver stained samples were first destained with 30 mM potassium ferricyanide and 100 mM sodium thiosulfate to remove the silver before proceeding to wash steps (Gharahdaghi *et al.*, 1999). All gel pieces were cut into smaller cubes and washed twice with water followed by 50% (v/v) acetonitrile for 10 min each. The acetonitrile was replaced with 50 mM ammonium bicarbonate and incubated for 10 min, repeated twice, except for CCB samples, which had an additional wash step to ensure complete removal of the dye. All the gel pieces were then incubated in 100% acetonitrile until they turned white. This was followed by another ammonium bicarbonate, acetonitrile wash step as above, after which the gel pieces were dried *in vacuo*. Gel pieces were digested with 20 µl of a 10 ng/µl trypsin solution at 37°C overnight. Resulting peptides were extracted twice with 70% acetonitrile for 30 min, and then dried and stored at -20°C. Dried peptides were dissolved in 10% (v/v) acetonitrile, 0.1% (v/v) formic acid and mixed with saturated

alpha-cyano-4-hydroxycinnamic acid before being spotted onto a MALDI sample plate. Experiments were performed using Applied Biosystems QSTAR-ELITE, Q-TOF mass spectrometer with oMALDI source installed. Laser pulses were generated using a Nitrogen laser with intensities between 15 and 25 µJ depending on sample concentration and whether single MS or MS/MS experiments were performed. First, single MS spectra were acquired for 15-30 s. The 50 highest peaks from the MS spectra were automatically selected for MS/MS acquisition. Tandem spectra acquisition lasted 4-8 min depending on sample concentration. Argon was used as cooling gas in Q0 and collision gas in Q2. The collision energy was first optimised using a 9 peptide mixture covering the scan range of 500–3500 Da and then automatically set during MS/MS experiments using the Information Dependent Acquisition (IDA) function of the Analyst QS 2.0 software. The instrument was calibrated externally, in TOF-MS mode, via a two point calibration using the peptides Bradykinin 1-7 and Somatostatin 28 ($[M+H]^+$ = 757.3992 Da and 3147.4710 Da, respectively).

2.2.13 Submitting MS/MS data to the MASCOT database

Data was submitted in MASCOT (www.matrixscience.com). The list of PMF's and the peptide sequence data (amino acid sequences for the 50 highest peptide peaks) was submitted to MASCOT using the MS/MS ion search utility that uses uninterpreted MS/MS data from one or more peptides for identification of the protein. The National Centre for Biotechnology Information non-redundant (NCBInr database, April 2009) was selected for protein identification and is a composite non-identical protein and nucleic acid database. Taxonomy was set to search all entries, using the NCBI database (April 2009). The enzyme used to obtain peptides was specified as trypsin, and allowed 1 missed cleavage. Fixed modifications were specified as carbamidomethyl (C) due to the use of iodoacetmide during sample preparation, and variable modifications were selected as oxidation (M) for possible methionine oxidation. Peptide tolerance was set to 50 parts per million (ppm, determined by MS calibration) and the MS/MS tolerance was set at 0.6 Da. The peptide charge was set to +1 since the MS used was a MALDI-TOF and would thus usually generate only singly charged ions. Finally, the instrument was selected as a MALDI-TOF-TOF and the data format was selected as Mascot generic. The final ion score is the probability that the observed match is a random event. Protein scores of more than 45 was considered as significant for identification of the protein ($p<0.05$).

2.3 Results

A: Optimisation of Plasmodial proteins for 2-DE

2.3.1 Protein concentration determination of Plasmodial proteins

Semi-quantitative proteomic analysis requires highly specific protein quantification procedures, to ensure the application of equal amounts of material in all downstream applications. In this study, 4 different methodologies were evaluated in their accuracy to determine Plasmodial protein concentration. The standardly used Bradford method achieved high correlation (R^2 = 0.9971) for proteins dissolved in a saline buffer, but was not compatible with the composition of the lysis buffer (Figure 2.2 A).

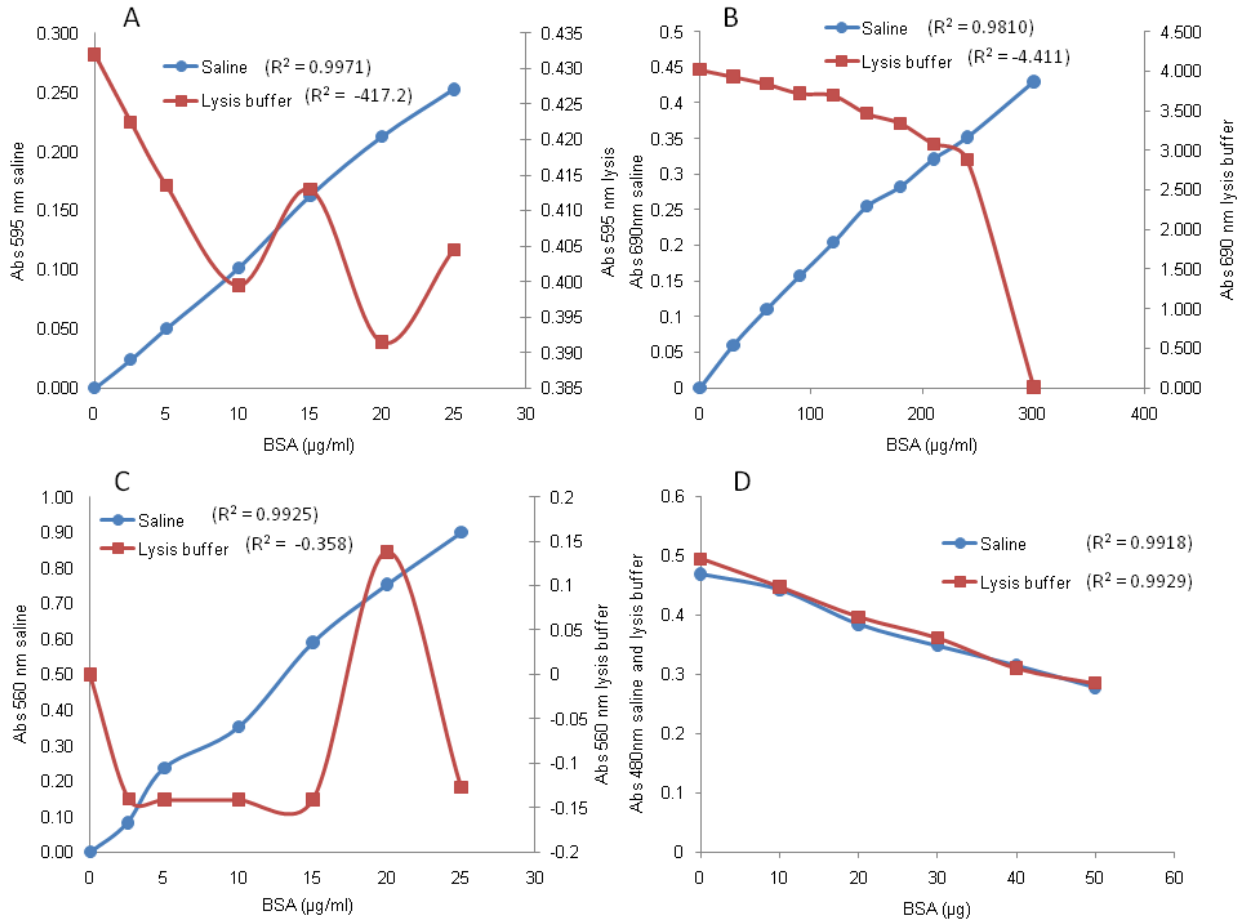

Figure 2.2: Comparison of 4 different protein concentration determination methodologies

(A) Bradford method, R^2 = 0.9971 for saline (——●——), R^2 = -417.2 for lysis buffer (--- ---), (B) Lowry method, R^2 = 0.981 for saline (——●——), R^2 = -4.411 for lysis buffer (--- ---), (C) BCA method R^2 = 0.9925 for saline (——●——), R^2 = -0.358 for lysis buffer (--- ---), (D) 2-D Quant kit, R^2 = 0.9918 for saline (——●——), R^2 = 0.9929 for lysis buffer (--- ---). (——●——) Saline standard curve, (--- --) lysis buffer standard curve. No secondary axis is necessary for 3.2 D from the 2-D Quant kit since both the saline and lysis buffer standard curves gave similar results.

Similar results can be seen for Lowry and the BCA method (Figure 2.2 B and C). The 2-D Quant kit was able to give both similar as well as accurate data for the saline (R^2 = 0.9918) and lysis buffer (R^2 = 0.9929) standard curves (Figure 2.2 D). The 2-D Quant kit was used as the method of protein quantification in all determinations to follow.

2.3.2 Stain performance on SDS-PAGE using standard protein markers

In order to determine the sensitivity and performance of various protein stains, a 2-fold serial dilution was made of a standard molecular weight marker, and then loaded quantitatively onto 4 different SDS-PAGE gels and subsequently stained with 4 different stains: Colloidal Coomassie Blue (CCB), silver stain, SYPRO Ruby and Flamingo Pink (Figure 2.3). The 4 gels were compared by using Quantity One to determine the sensitivity and linear regression constant of each individual stain (Table 2.4).

Table 2.4: Comparative stain analysis for Plasmodial proteins analysed with 1-D SDS PAGE.

Stain	LOD[a] (ng)	R^2
CCB	25-90	0.89
Silver	10-90	0.83
SYPRO	1-90	0.97
Flamingo	1-90	0.97

[a]Limit of detection (LOD) is defined as the minimum amount of protein that could be detected on the SDS-PAGE gel with a specific stain.

Both Sypro Ruby and Flamingo Pink achieved similar results, as both were able to detect as little as 1 ng of protein, and were linear with R^2 = 0.97. CCB was the least sensitive of the 4 stains with a detection limit of 25 ng and linearity of R^2 = 0.89. Silver stain was able to detect a minimum of 10 ng but has a very poor linear range of R^2 = 0.83, and would thus not be ideal to use for quantitation.

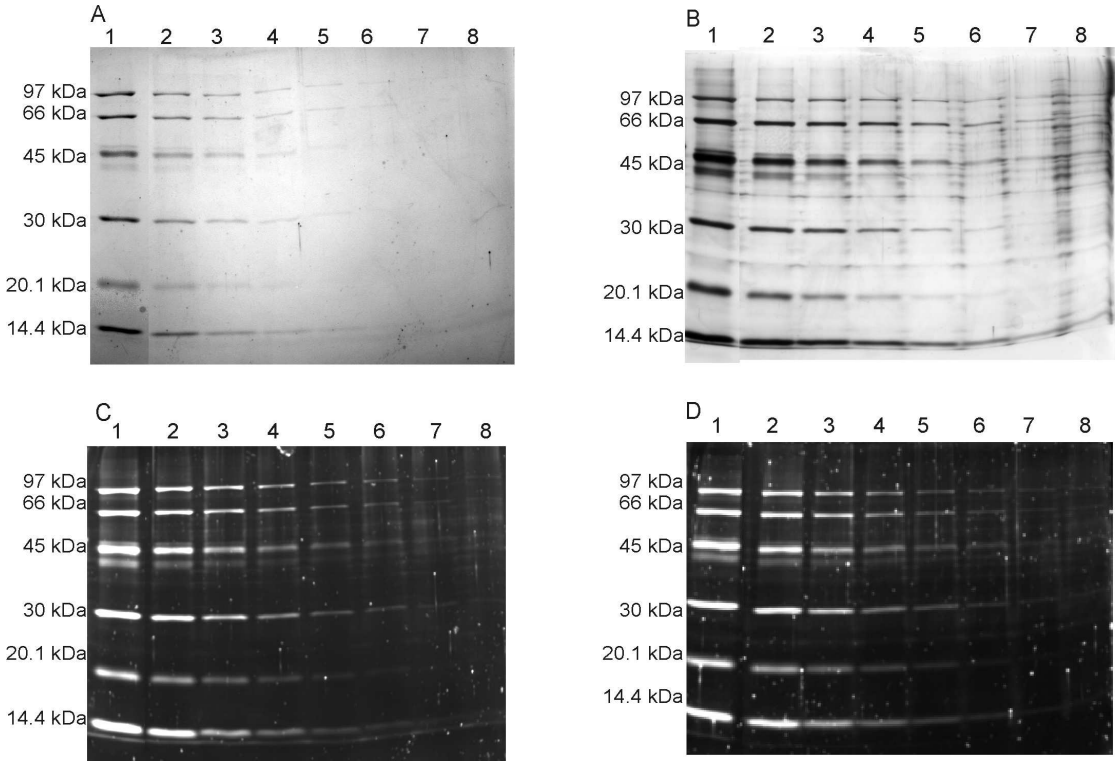

Figure 2.3: Comparison of standard proteins on SDS PAGE gels using 4 different stains.

Two fold dilutions of a standard molecular weight marker were loaded similarly onto each gel. (A) Colloidal coomassie blue, (B) MS-compatible silver stain, (C) SYPRO Ruby, (D) Flamingo Pink. The total protein per lane is: lane (1) 1250 ng, (2) 625 ng, (3) 312.5 ng, (4) 156.3 ng, (5) 78 ng, (6) 39 ng, (7) 19 ng, (8) 9.7 ng. Bands from the top to the bottom are: Phosphorylase b, 97 kDa, Albumin, 66 kDa, Ovalbumin, 45 kDa, Carbonic anhydrase, 30 kDa, Trypsin inhibitor, 20.1 kDa, Alpha-lactalbumin, 14.4 kDa

2.3.3 Stain performance on 2-DE using Plasmodial proteins

These 4 stains were subsequently tested on the proteome of Plasmodial proteins after 2-DE. All the samples were pooled to one sample and used for all 8 gels that were run. This is to ensure that gels are only judged on staining performance and not on possible sample differences. The concentration was determined using the 2D Quant kit as above (Figure. 2.2 D). Two hundred microgram protein was loaded onto each 13 cm IPG strip (pH 3-10 L). Duplicate 2-DE analysis were performed for all 4 stains used (n = 2 per stain, n = 8 in total) and spot analyses were performed with PD Quest. The CCB stain performed poor in detection with an average of 126 spots detected, markedly less than any of the other 3 stains tested (Table 2.5).

The MS-compatible silver stain is a highly sensitive stain able to detect proteins in their low nanogram levels (Berggren *et al.*, 2000) and was also superior within this study in terms of sensitivity with an average of 420 spots detected (Figure 2.4). However, the poor linearity and spurious artefacts associated with silver staining of 2-DE could lead to unreliable results when

groups of gels with differentially expressed proteins are compared (Table 2.6) (Berggren *et al.*, 2000).

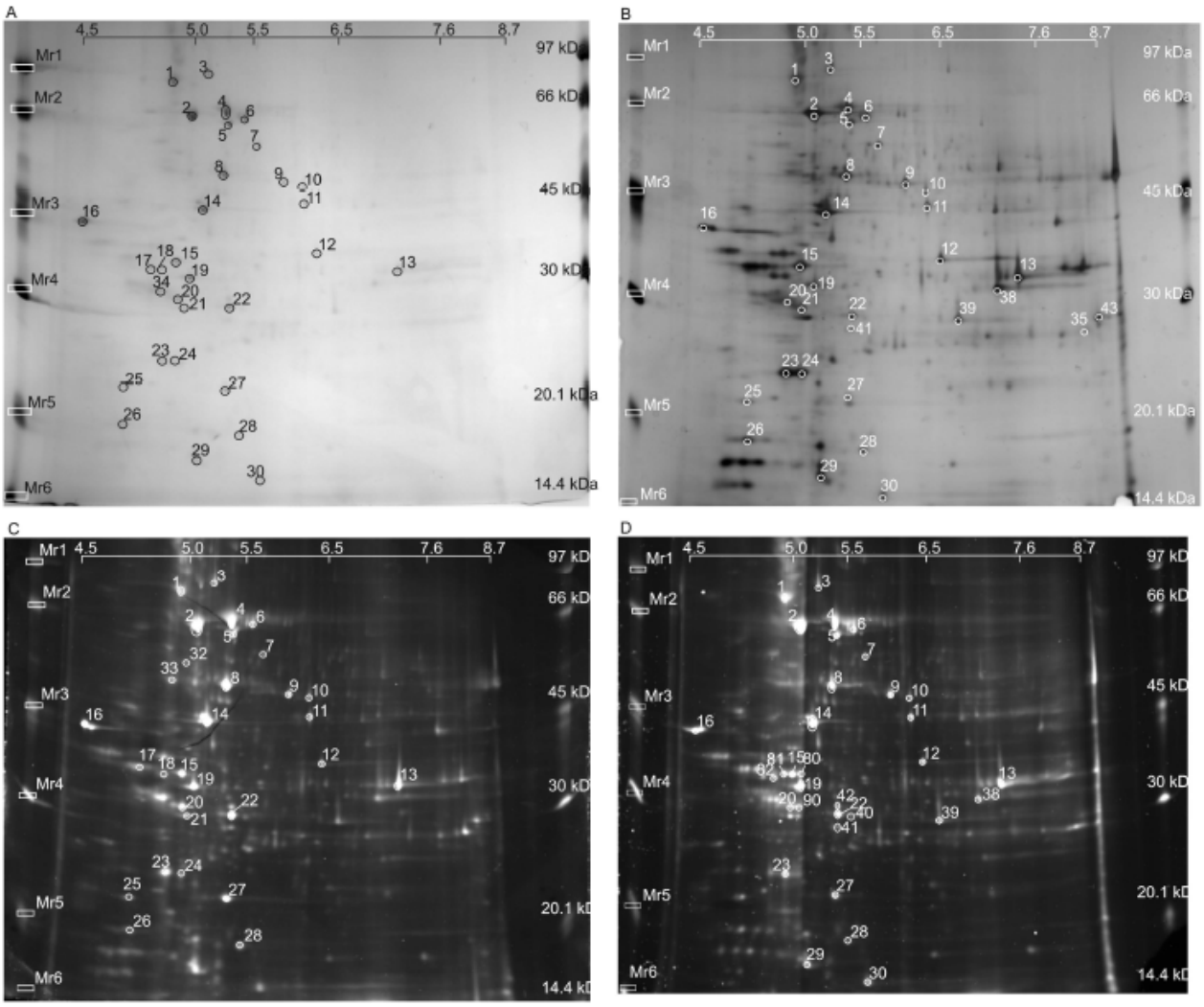

Figure 2.4: Comparison of Plasmodial proteins on 2-DE gels using 4 different stains.

Two-hundred micrograms of *Pf*3D7 proteins were loaded onto 13 cm IPG pH 3-10L strips for 2-DE analysis. After electrophoresis, the gels were stained with (A) Colloidal Coomassie Blue, (B) MS compatible silver stain, (C) SYPRO Ruby, (D) Flamingo Pink. The number of spots was determined using PD Quest 7.1.1 with n = 2 for each individual stain. About 39 similar spots were cut from each of the stained gels to determine the MS efficiency. The identified spots are marked on the gels. All MS data for the identified spots can be obtained in Appendix A as supplementary tables A-D.

SYPRO Ruby only detected 235 spots on the 2-DE gels. This loss in sensitivity is in sharp contrast to the results that were obtained for SYPRO Ruby when tested on the molecular weight markers when it had similar sensitivity to Flamingo Pink. It has also been shown that Flamingo Pink is highly consistent in the number and array of spots detected, and has little gel to gel variability (Harris *et al.*, 2007). In this study Flamingo Pink was able to detect 349 spots.

2.3.4 Filtering of trophozoite data

The total Plasmodial trophozoite proteome is predicted to contain 1029 proteins (Florens *et al.*, 2002, Aurrecoechea *et al.*, 2008) (PlasmoDB 6.0), which spans a wide molecular weight range and pI with different degrees of solubility. Filtering of this dataset to represent the conditions used in this study for 2-DE resulted in the identification of 443 Plasmodial trophozoite proteins that should be detectable on a standard 2-DE gel in the molecular weight range of 10-110 kDa with a pI range of 4-9. Unfortunately, these 443 Plasmodial proteins that should be detectable on 2-DE out of the total 1029 trophozoite proteins accounts for only 41% of the total trophozoite proteome (Figure 2.5). Silver detected 420 protein spots which accounts for 95% (420 out of 443) of the 2-DE detectable proteome as per our calculations. However, this does not take the possibility of protein isoforms being present within these protein spots. Similarly, Flamingo Pink detected 79% (349 out of 443), SYPRO Ruby 53% (235 out of 443) and CCB 28% (126 out of 443) of the detectable 2-DE proteome as with our calculations.

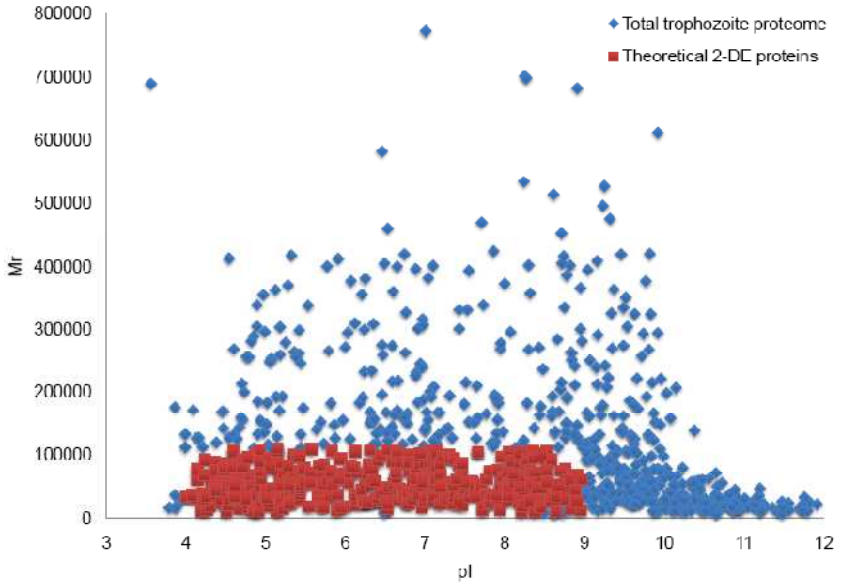

Figure 2.5: Plot of the total trophozoite proteome

The diamond shapes (blue) represent a computer generated plot of the total trophozoite proteome as given in PlasmoDB 6.0. The squares (red) are proteins that are detectable on 2-DE gels, within the range of 10-110 kDa, and a pI of 4-9 as per our calculations.

2.3.5 Compatibility of the 4 stains with MALDI-TOF MS/MS

In order to assess the overall MS-compatibility of the 4 staining methods, approximately 39 spots of each of the 4 individual gels were selected, consisting of 33 Plasmodial proteins each (Figure 2.4, 1-33) and 6 standard molecular weight marker proteins (Figure 2.4, marked Mr1 to Mr6), summarised in Table 2.5 (2-DE trophozoite analysis of stains). The spots were prepared for MS as described in the methods section, with the exception that for CCB samples an additional wash step was

incorporated to ensure that the dye is washed out, although some of the very highly abundant spots still had a faint blue colour despite this extra wash step. The silver stained samples were first destained to remove all the silver from the gel pieces (Gharahdaghi *et al.*, 1999). Proteins were identified when a significant Mascot score was obtained and further criteria of at least 5 peptides and sequence coverage of at least 10% was achieved (Appendix A). This was done to increase the MS/MS identification confidence. A summary of the precise number of spots that were cut for each of the different stains and the number of spots identified by tandem MS for each stain as well as the success rate for each stain and overall success rate is shown in Table 2.5.

Table 2.5: Comparative stain analysis for Plasmodial proteins analysed with 1-D as well as 2-DE SDS PAGE. Spot detection and MS identification rates are included for each of the 4 different stains, analysed on duplicate gels each (n=2).

Stain	Spots detected (PD Quest)	Nr cut for MS	Nr identified by MS	Identification success rate (%)
CCB	126	37	35	95
Silver	420	39	33	85
SYPRO	235	39	33	85
Flamingo	349	39	37	95
Total	**1130**	**154**	**138**	**90[a]**

[a] =average

Silver staining resulted in the least number of positive identifications (33 out of 39 selected spots). Similar to silver staining SYPRO Ruby resulted in the identification of 33 out of 39 spots. The best results were obtained with CCB (35 out of 37 tested) and Flamingo Pink (37 positive identifications out of 39 tested). The high success rate was due to the fact that tandem MS were performed on all of the samples.

B: Application of 2-DE optimised method on the Plasmodial ring and trophozoite stages

2.3.6 2-DE analysis of the Plasmodial proteome

After the successful establishment of a reliable protein quantification method, linear staining and good MS identification, the methodology could now be applied to the Plasmodial early trophozoite proteome as proof-of-principle. The parasites were harvested in the late ring and the early trophozoite stages and 400 µg of the protein containing supernatants were applied to 18 cm IPG strips pH 3-10 L. Linear IPG strips were used since this would enable similar increments between the pH values, and therefore give an overall view of the proteome spanning a wide pI range. Spots were analysed using PD Quest after which the spots were manually cut and prepared for MS analysis. The spots selected for analysis of the ring and trophozoite proteomes included spots of various intensities covering the whole 2-DE range (pI 4-9, and Mr 13-135 kDa). The normalised intensities of these spots ranged from 58 to a maximum of 9734 with 1963 as the average intensity per spot. Normalisation was done to correct for inconsistencies that may occur between gels that are not due to differential expression of spots but are rather due to experimental errors like inconsistency in staining and pipetting. Normalistion is of utmost importance for the determination of differentially regulated spots. The normalisation method entails removing starurated spots (flagged as invalid) and then averaging the intensities of a single spot between the comparative technical repeated gels. This is done for every single valid spot for all technical repeated gels. For the ring stage proteome analysis, 77 spots were selected for MS identification and 63 spots were selected for the trophozoite stage. The spots that were positively identified are marked in Figure 2.6 and the MS data is given in Table 2.6 A and B. The identified proteins all had significant MASCOT scores, at least 5 peptides identified, and sequence coverage of at least 10% each (Table 2.6 A and B).

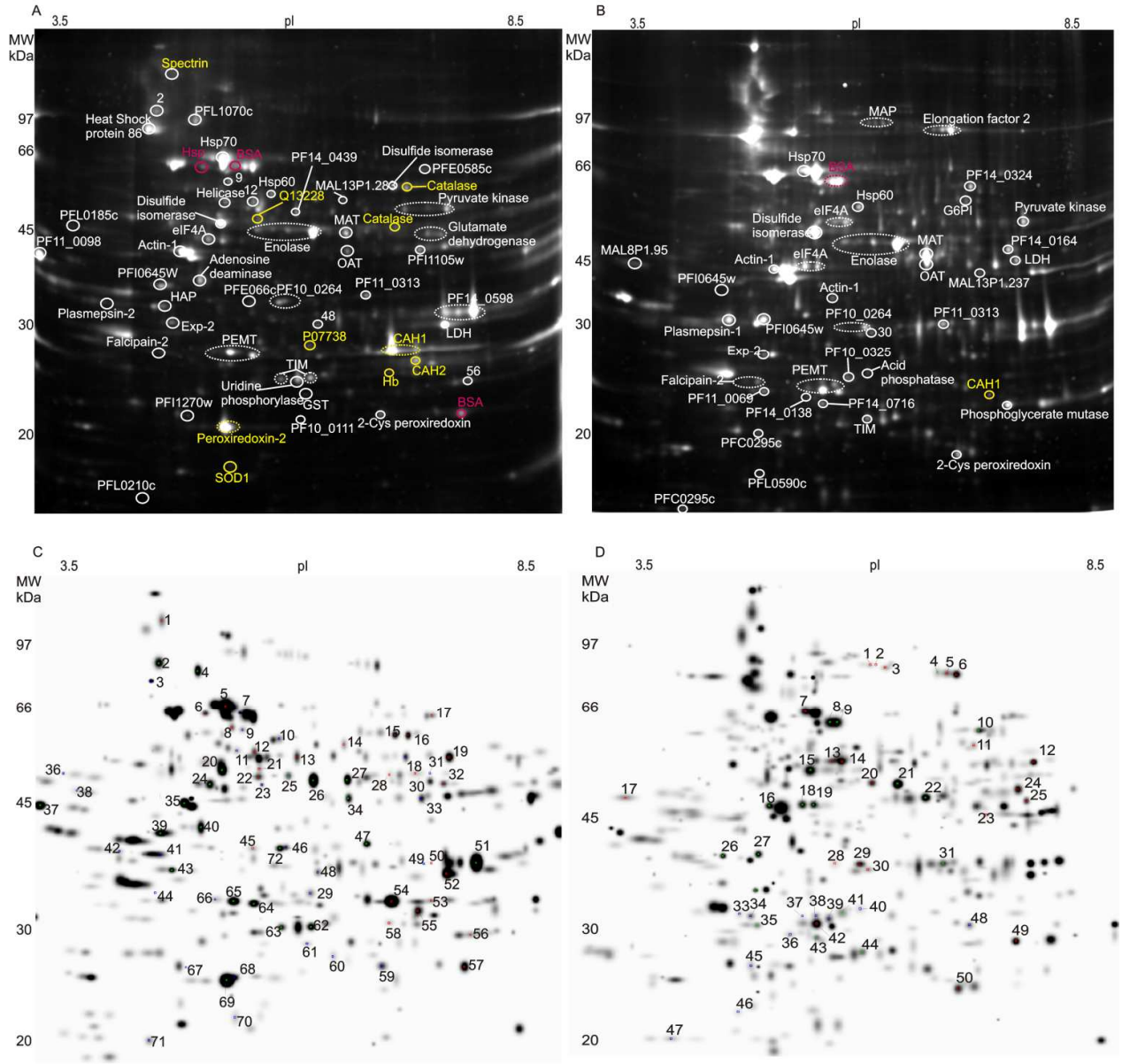

Figure 2.6: 2-DE of the rings and trophozoites stage *P. falciparum* indicating identified proteins.

2-DE of Plasmodial ring-stage proteome (A) and its master image (C) compared to the 2-DE of early trophozoites stage proteome (B) and its corresponding master image (D). Master images were created by PD Quest as representative of all the 2-DE gels performed for each of the time points and contains spot information of a total of eight 2-DE gels. Plasmodial proteins are marked in white, human proteins are marked in yellow and bovine proteins are marked in red. Isoforms are encircled with dotted lines. The representing master images are also marked with identified proteins and all positively identified proteins are listed in Table 1 A and B.

Table 2.6: List of proteins identified by tandem mass spectrometry for late rings and early trophozoites

Spot nr[a]	Transcript trend[b]	PlasmoDB ID	Name	Mr (obtained) Da	pI (PlasmoDB)	Mascot Score MS/MS[c]	Seq Cover[d]	Match[e]
(A) Proteins identified for late rings								
60	Up	□□□□□□□□	20S proteasome beta subunit, putative	30862	5.18	150	9	4
59	↔	PF14_0368	2-Cys peroxiredoxin	21964	6.65	540	59	8
46	↔	PF10_0264	40S ribosomal protein, putative (1)	30008	5.91	152	11	3
72	↔	PF10_0264	40S ribosomal protein, putative (2)	30008	5.91	146	14	4
35	↔	PFL2215w	Actin-I	42022	5.27	627	33	10
40	Up	PF10_0289	Adenosine deaminase, putative	42895	5.41	573	38	15
29	—	—	Bisphosphoglycerate mutase *(Homo sapiens)*	30027	6.1	441	46	10
53	—	—	Carbonic anhydrase 1 *(Homo sapiens)*	28778	6.63	531	50	8
54	—	—	Carbonic anhydrase 1 *(Homo sapiens)*	28620	6.65	845	58	11
55	—	—	Carbonic anhydrase 2 *(Homo sapiens)*	28802	6.63	320	30	7
16	—	—	Catalase *(Homo sapiens)*	59816	6.95	659	29	15
28	—	—	Catalase *(Homo sapiens)*	59816	6.95	425	22	9
15	Up	MAL8P1.17	Disulfide isomerase, putative (1)	55808	5.56	693	35	15
20	Up	MAL8P1.17	Disulfide isomerase, putative (2)	55808	5.56	1005	41	17
6	—	—	dnaK-type molecular chaperone hsc70 *(Bos Taurus)*	71454	5.37	579	20	11
24	—	PF14_0655	eIF4A	45624	5.48	580	36	16
11	↔	PFB0445c	eIF4A-like helicase, putative (1)	52647	5.68	589	26	10
12	↔	PFB0445c	eIF4A-like helicase, putative (2)	52647	5.68	251	13	6
37	Up	PF11_0098	Endoplasmic reticulum-resident calcium binding protein	39464	4.49	1135	59	17
4	Up	PFL1070c	Endoplasmin homolog, putative	95301	5.28	298	14	10
22	Up	PF10_0155	Enolase (1)	48989	6.21	313	18	7
23	Up	PF10_0155	Enolase (2)	48989	6.21	373	18	7
25	Up	PF10_0155	Enolase (3)	48989	6.21	414	27	11
26	Up	PF10_0155	Enolase (4)	48989	6.21	1000	40	16
71	↔	PFL0210c	Eukaryotic initiation factor 5a, putative	17791	5.42	159	27	4
43	↔	PF14_0678	Exported protein 2	33619	5.1	379	26	8
44	↔	PF11_0165	Falcipain 2	56405	7.12	212	12	6
30	Down	PF14_0164	Glutamate dehydrogenase (NADP+) (1)	53140	7.48	283	17	8
31	Down	PF14_0164	Glutamate dehydrogenase (NADP+) (2)	53140	7.48	212	15	6
32	Down	PF14_0164	Glutamate dehydrogenase (NADP+) (3)	53140	7.48	497	30	13
61	—	PF14_0187	Glutathione s-transferase	24888	5.97	47	11	2

49	Up	PF14_0598	Glyceraldehyde-3-phosphate dehydrogenase (1)	37068	7.59	302	25	7
50	Up	PF14_0598	Glyceraldehyde-3-phosphate dehydrogenase (2)	37068	7.59	131	11	3
51	Up	PF14_0598	Glyceraldehyde-3-phosphate dehydrogenase (3)	37068	7.59	810	47	14
56	—	PF11_0183	GTP binding nuclear protein Ran	24974	7.72	485	55	12
41	Down	PF14_0078	HAP protein	51889	8.05	645	34	13
5	↔	PF08_0054	Heat shock 70 kDa protein	74382	5.51	1378	34	23
3	↔	PF07_0029	Heat shock protein 86	86468	4.94	1153	25	24
58	—	—	Hemoglobin subunit beta *(Homo sapiens)*	16112	6.75	294	43	6
10	Up	PF10_0153	Heat shock protein 60 kDa	62911	6.71	870	37	19
13	↔	PF14_0439	Leucine aminopeptidase, putative	68343	8.78	172	14	7
52	↔	PF13_0141	Lactate dehydrogenase	34314	7.12	611	43	12
14	↔	MAL13P1.283	MAL13P1.283 protein	58506	6.09	261	10	6
17	↔	PFE0585c	Myo-inositol 1-phosphate synthase, putative	69639	7.11	454	25	14
36	Down	PFL0185c	Nucleosome assembly protein 1, putative	42199	4.19	293	16	7
34	Up	PFF0435w	Ornithine aminotransferase	46938	6.47	589	27	11
68	—	—	Peroxiredoxin-2 *(Homo sapiens)*	21918	5.67	515	41	10
69	—	—	Peroxiredoxin-2 *(Homo sapiens)*	21918	5.67	664	43	11
64	Up	MAL13P1.214	Phosphoethanolamine N-methyltransferase, putative (1)	31309	5.43	871	50	14
65	Up	MAL13P1.214	Phosphoethanolamine N-methyltransferase, putative (2)	31309	5.43	935	50	14
66	Up	MAL13P1.214	Phosphoethanolamine N-methyltransferase, putative (3)	31309	5.43	252	22	5
33	Up	PFI1105w	Phosphoglycerate kinase	45569	7.63	214	15	5
42	↔	PF14_0077	Plasmepsin 2	51847	5.35	72	6	3
48	↔	MAL8P1.142	Proteasome beta-subunit	31080	6.00	212	22	7
2	—	PFF0940c	Putative cell division cycle protein 48 homologue, putative	90690	4.95	303	10	7
18	Up	PFF1300w	Putative pyruvate kinase (1)	56480	7.50	633	28	15
19	Up	PFF1300w	Putative pyruvate kinase (2)	56480	7.50	732	37	16
67	—	PFI1270w	Putative uncharacterized protein PFI1270w	24911	5.49	327	26	6
47	↔	PF11_0313	Ribosomal phosphoprotein P0	35002	6.28	430	36	9
27	Up	PFI1090w	S-adenosylmethionine synthetase	45272	6.28	863	40	14
21	—	—	Selenium binding protein 1 *(Homo sapiens)*	52928	5.93	140	12	6
7	—	—	Serum albumin *(Bos Taurus)*	71274	5.82	620	24	15
57	—	—	Serum albumin *(Bos Taurus)*	71274	5.82	510	16	10
38	—	—	Solute carrier family 4, anion exchanger, member 1 *(Homo sapiens)*	101987	5.13	189	7	4
1	—	—	Spectrin alpha chain *(Homo sapiens)*	282024	4.98	889	24	9
70	—	—	Superoxide dismutase *(Homo sapiens)*	16154	5.70	219	37	4

39	↔	PFI0645w	Translation elongation factor 1 beta	32121	4.94	208	24	7
62	↔	PF14_0378	Triosephosphate isomerase (1)	27971	6.02	490	43	10
63	↔	PF14_0378	Triosephosphate isomerase (2)	27971	6.02	430	38	9
45	Up	PFE0660c	Purine nucleoside phosphorylase, putative (1)	27525	6.07	315	31	8
63	Up	PFE0660c	Uridine phosphorylase, putative (2)	27525	6.07	572	35	10
8	—	PF13_0065	V-type proton ATPase catalytic subunit A (1)	69160	5.51	291	19	10
9	—	PF13_0065	V-type proton ATPase catalytic subunit A (2)	69160	5.51	184	13	7
			(B) Proteins identified for late rings					
50	↔	PF14_0368	2-Cys peroxiredoxin	21964	6.65	504	72	11
45	Down	PFC0295c	40S ribosomal protein S12, putative (1)	15558	4.67	85	14	2
47	Down	PFC0295c	40S ribosomal protein S12, putative (2)	15558	4.67	217	36	5
28	↔	PF10_0264	40S ribosomal protein, putative (1)	30008	5.91	27	11	3
29	↔	PF10_0264	40S ribosomal protein, putative (2)	30008	5.91	267	24	8
40	Up	PF14_0036	Acid phosphatase, putative	35972	6.3	63	5	2
51	↔	PFL2215w	Actin-1 (1)	42272	5.17	81	36	12
16	↔	PFL2215w	Actin-1 (2)	42022	5.27	455	36	9
38	↔	PFL2215w	Actin-1 (3)	42022	5.27	225	14	5
48	—	—	Carbonic anhydrase 1 *(Homo sapiens)*	28620	6.65	70	20	4
15	Up	MAL8P1.17	Disulfide isomerase precursor, putative	55808	5.56	883	38	16
18	Up	PF14_0655	eIF4A (1)	45624	5.28	353	30	12
19	Up	PF14_0655	eIF4A (2)	45624	5.48	326	23	12
13	↔	PFB0445c	eIF4A-like helicase, putative (1)	52647	5.68	320	23	8
14	↔	PFB0445c	eIF4A-like helicase, putative (2)	52646	5.68	62	42	14
5	↔	PF14_0486	Elongation factor 2 (1)	94546	6.36	91	4	4
6	↔	PF14_0486	Elongation factor 2 (2)	94546	6.78	657	26	18
20	↔	PF10_0155	Enolase (1)	48989	6.21	408	32	10
21	↔	PF10_0155	Enolase (2)	48989	6.21	949	36	12
30	—	PFD0615c	Eryhrocyte membrane protein 1 (fragment)	13608	6.96	51	38	7
33	↔	PF11_0165	Falcipain 2 (1)	56481	7.9	47	23	10
34	↔	PF11_0165	Falcipain 2 (2)	55928	7.49	56	24	11
11	↔	PF14_0341	Glucose-6-phosphate isomerase	67610	6.78	61	28	14
24	Down	PF14_0164	Glutamate dehydrogenase (NADP+)	53140	7.48	336	28	11
39	↔	PF10_0325	Haloacid dehalogenase-like hydrolase, putative	33220	5.62	180	27	6
7	↔	PF08_0054	Heat shock 70 kDa protein	74382	5.33	861	33	18
52	Up	PF10_0153	Heat shock protein 60 kDa	62911	6.71	128	38	21
35	Up	PF11_0069	Hypothetical protein	32112	4.91	55	13	3

36	Up	PF14_0138	Hypothetical protein	23889	5.49	53	9	2
23	Up	MAL13P1.237	Hypothetical protein MAL13P1.237	42475	7.14	574	37	13
17	Down	MAL8P1.95	Hypothetical protein MAL8P1.95	37933	4.13	385	25	8
10	↔	PF14_0324	Hypothetical protein, conserved	66415	6.63	66	7	4
25	Up	PF13_0141	Lactate dehydrogenase	34314	7.12	100	12	3
1	↔	MAL13P1.56	M1 family aminopeptidase (1)	126552	7.3	102	26	23
2	↔	MAL13P1.56	M1 family aminopeptidase (2)	126552	6.68	124	26	25
3	↔	MAL13P1.56	M1 family aminopeptidase (3)	126552	7.3	107	27	23
22	Up	PFF0435w	Ornithine aminotransferase	46938	6.47	637	29	12
37	Up	MAL13P1.214	Phosphoethanolamine N-methyltransferase, putative (1)	31043	5.28	69	9	2
38	Up	MAL13P1.214	Phosphoethanolamine N-methyltransferase, putative (2)	31043	5.28	261	26	6
41	Up	MAL13P1.214	Phosphoethanolamine N-methyltransferase, putative (3)	31309	5.28	177	22	5
42	Up	MAL13P1.214	Phosphoethanolamine N-methyltransferase, putative (4)	31309	5.28	722	48	13
49	↔	PF11_0208	Phosphoglycerate mutase, putative	28866	8.3	401	36	10
26	Down	PF14_0076	Plasmepsin-1	51656	6.72	540	35	12
43	↔	PF14_0716	Proteosome subunit alpha type 1, putative	29218	5.51	268	31	6
46	—	PFL0590c	P-type ATPase, putative	135214	6.13	54	18	16
12	Up	PFF1300w	Putative pyruvate kinase	56480	7.5	101	51	16
31	↔	PF11_0313	Ribosomal phosphoprotein P0	35002	6.28	121	13	3
22	Up	PFI1090w	S-adenosylmethionine synthetase	45272	6.28	480	32	10
8	—	—	Serum albumin *(Bos Taurus)*	71274	5.82	466	24	15
9	—	—	Serum albumin *(Bos Taurus)*	71274	5.82	822	36	21
27	↔	PFI0645w	Translation elongation factor 1 beta	32121	4.94	488	35	9
44	Up	PF14_0378	Triosephosphate isomerase	27971	6.02	183	22	6

Proteins identified are sorted alphabetically according to name with isoforms grouped together and the number of isoforms per protein is marked in brackets. [a]Spot number corresponds to marked spots on the master image of ring stage parasites. [b]Trend of transcripts regulation from 16-20 HPI as acquired from the IDC database (http://malaria.ucsf.edu/comparison/index.php) for each of the identified proteins. (↔) indicates unchanged transcript levels and (—) is indicative that result is not applicable. [c]Mascot scores are based on MS/MS searches and is only taken when the score is significant ($p<0.05$). [d]Sequence coverage is given by Mascot for detected peptide sequences. [e]Matched is the number of peptides matched to the particular protein

In this study any spot on the 2-DE gel that was cut out and identified by MS is referred to as a protein spot. Unique Plasmodial protein groups represent Plasmodial proteins that may contain more than one isoform but are still grouped into one unique protein group. A protein isoform is when more than one spot was identified as the same protein as a result of PTM's. For example in the ring stage 4 different spots on the 2-DE gel were identified as enolase due to the presence of various PTM's. Therefore, this will be representative of 1 unique protein group which is enolase, but 4 protein isoforms. This nomenclature will be used throughout this chapter as well as in Chapter 3. For the ring stage proteome 73 protein spots were positively identified out of 77 spots subjected to MS/MS, while for the trophozoite proteome 57 protein spots were positively identified out of 63 spots subjected to MS/MS (Table 2.7 A and B). Of the 73 protein spots identified in the ring stage proteome, 57 protein spots were from Plasmodial origin, and consisted of 41 unique Plasmodial protein groups and protein isoforms were representative of an additional 28% (16 isoforms) of these Plasmodial protein spots. The trophozoite proteome consists of 52 protein spots identified by MS of which 49 protein spots were from Plasmodial origin. Of these, 29% (14 protein spots) additionally accounted for isoforms from the 35 unique Plasmodial protein groups.

2.3.7 Comparison of ring, trophozoite and schizont proteome

The earlier release of the schizont proteome by 2-DIGE (Foth *et al.*, 2008) prompted investigation of the late ring and early trophozoite stage proteomes with 2-DE. A total of 54 protein spots were identified in the schizont proteome (Foth *et al.*, 2008). Upon filtering of the schizont protein identifications it was observed that only 24 unique Plasmodial protein groups were identified. The ring and trophozoite data from this study was compared to the schizont data and it was determined that only 9 unique Plasmodial protein groups were shared between all 3 life stages of the parasite (Figure 2.7 and Table 2.7). Nineteen unique Plasmodial protein groups were shared between the ring and trophozoite stages, 14 unique Plasmodial protein groups shared between the ring and schizont and 11 shared between the trophozoite and schizont stages.

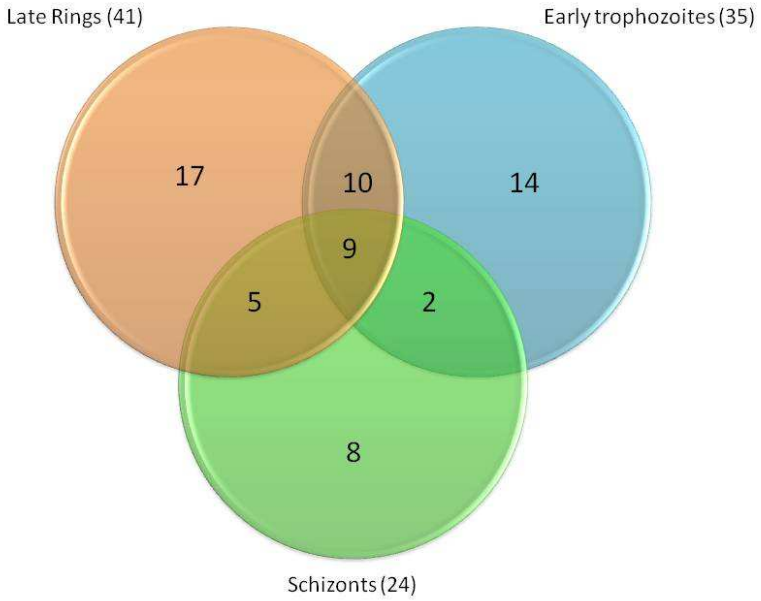

Figure 2.7: Venn diagram of 3 stages investigated by proteomics in *P. falciparum*.

Seventeen ring stage proteins, 14 trophozoite proteins and 8 schizont stage proteins were not shared in any way between the 3 life stages. The unique Plasmodial protein groups shared between the ring, trophozoite and schizont life cycle stages are given in Table 2.7. A total of 26 proteins are shared which consist of 24 ring proteins shared, 21 trophozoite proteins shared and only 16 schizont stage proteins that are shared.

Table 2.7: Table of the proteins shared between each of the 3 life stages.

Unique Plasmodial protein groups only (occurrence in more than one of the stages) excluding human proteins and isoforms.

PlasmoDB ID	Annotation	R	T	S
MAL13P1.214	Phosphoethanolamine N-methyltransferase, putative	Y	Y	Y
MAL13P1.56	M1-family aminopeptidase		Y	Y
MAL8P1.17	Disulfide isomerase precursor, putative	Y	Y	
PF08_0054	Heat shock 70 kDa protein	Y	Y	Y
PF10_0153	Hsp60	Y	Y	Y
PF10_0155	Enolase	Y	Y	Y
PF10_0264	40S ribosomal protein, putative	Y	Y	
PF10_0289	Adenosine deaminase, putative	Y		Y
PF10_0325	Hypothetical protein, conserved		Y	Y
PF11_0165	Falcipain 2 precursor	Y	Y	
PF11_0313	Ribosomal phosphoprotein P0	Y	Y	
PF13_0141	L-lactate dehydrogenase	Y	Y	
PF14_0164	NADP-specific glutamate dehydrogenase	Y	Y	
PF14_0368	2-Cys peroxiredoxin	Y	Y	Y
PF14_0378	Triose-phosphate isomerase	Y	Y	Y
PF14_0655	RNA helicase-1, putative	Y	Y	Y
PF14_0678	Exported protein 2	Y		Y
PFB0445c	Helicase, putative	Y	Y	Y
PFE0660c	Uridine phosphorylase, putative	Y		Y
PFF0435w	Ornithine aminotransferase	Y	Y	
PFF1300w	Pyruvate kinase, putative	Y	Y	
PFI0645w	EF-1B	Y	Y	
PFI1090w	S-adenosylmethionine synthetase, putative	Y	Y	
PFI1270w	Hypothetical protein	Y		Y
PFL0210c	Eukaryotic initiation factor 5a, putative	Y		Y
PFL2215w	Actin	Y	Y	Y
Total		**24**	**21**	**16**

2.3.8 Comparison of proteomic data with transcript levels

Comparison of the protein levels from the ring and trophozoite proteomes to the IDC transcript profile demonstrated distinct similarities between transcript production profiles (obtained from PlasmoDB 6.0 www.plasmodb.org)(Aurrecoechea *et al.*, 2008) and protein levels (Table 2.6 A-B). Proteins that increased in abundance from rings to trophozoites mostly exhibited a corresponding increase in transcript level when compared to IDC data (Figure 2.8, Table 2.6). Enolase, S-adenosylmethionine synthase (AdoMet synthase), ornithine aminotransferase (OAT), uridine phosphorylase (PNP) and disulfide isomerase all demonstrated an increase in abundance of both the transcript and protein expression levels. Similarly, eIF4A-like helicase and ribosomal phosphoprotein P0 all exhibited unchanged transcript and protein expression levels from ring to trophozoite stage parasites. Actin-1 was one of the few exceptions in which transcript levels remained constant from ring to trophozoite stage parasites whilst protein levels were increasing. Similarly, the transcript levels of 2-Cys peroxiredoxin remained constant over the two time points whilst the protein was decreased.

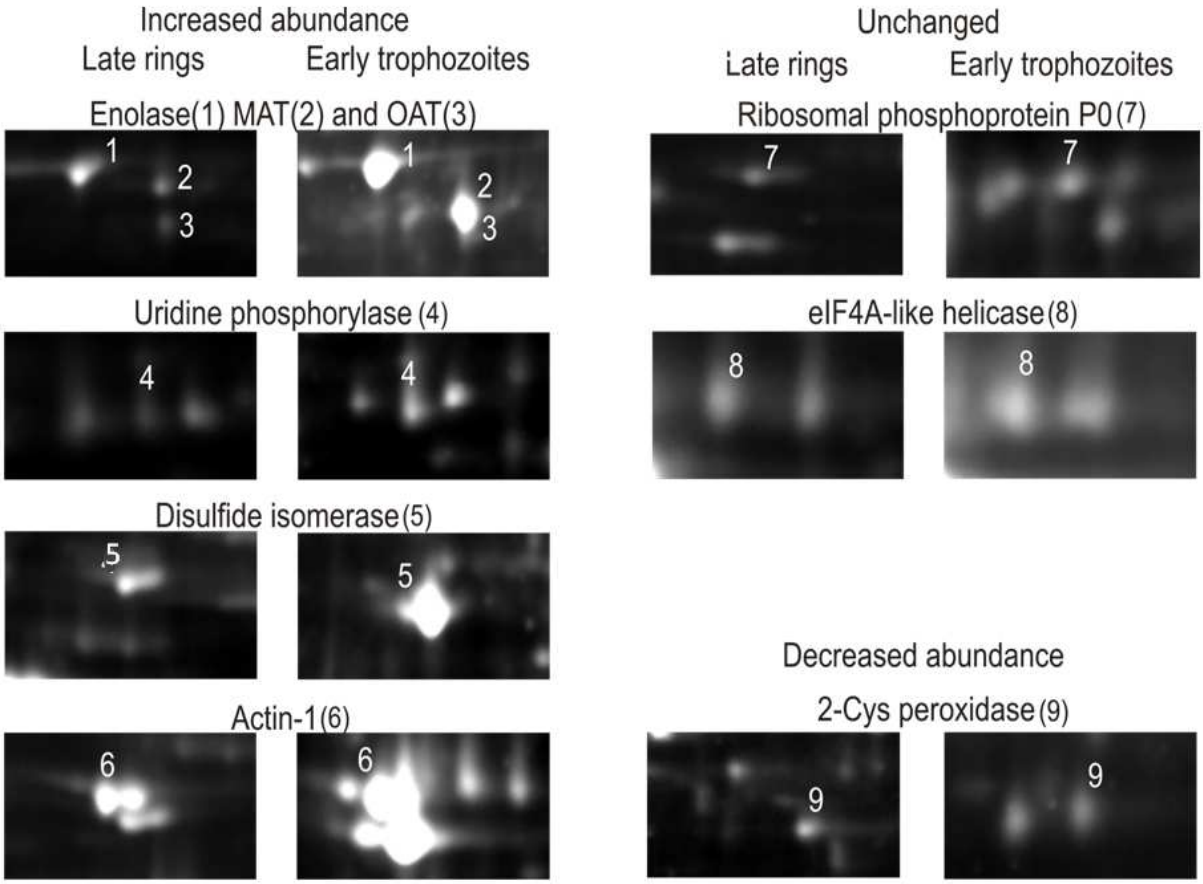

Figure 2.8: Proteins that are differentially regulated in the *P. falciparum* ring and trophozoite stage proteomes.

Numbers are indicative of protein spot that is indicated. MAT: S-adenosylmethionine synthase, OAT: ornithine aminotransferase

2.3.9 Differential expression of isoforms

Of the nineteen identified Plasmodial proteins shared between the ring and trophozoite stages of the parasite, several proteins appear as isoforms (Figure 2.9, isoforms are also marked in Figure 2.6 and Table 2.6 A-B). Moreover, some of these protein isoforms display differential regulation from the ring to trophozoite stages (Figure 2.9). An increase in both transcript as well as protein expression levels were determined for the 4 enolase and phosphoethanolamine N-methyltransferase (PEMT) isoforms and the 3 glyceraldehyde-3-phosphate dehydrogenase (G3PDH) isoforms. The transcript levels of pyruvate kinase (2 isoforms) increased over the specified period, but the protein expression levels for both isoforms declined. The transcript levels for both triosephosphate isomerase (TIM, 2 isoforms) and eIF4A (2 isoforms) remained constant during this period but the corresponding proteins increased in abundance. For glutamate dehydrogenase (3 isoforms) the transcript level decreased but the protein level remained constant from the ring to the trophozoite stages. Unchanged transcript and protein levels were detected for eIF4A-like helicase (2 isoforms).

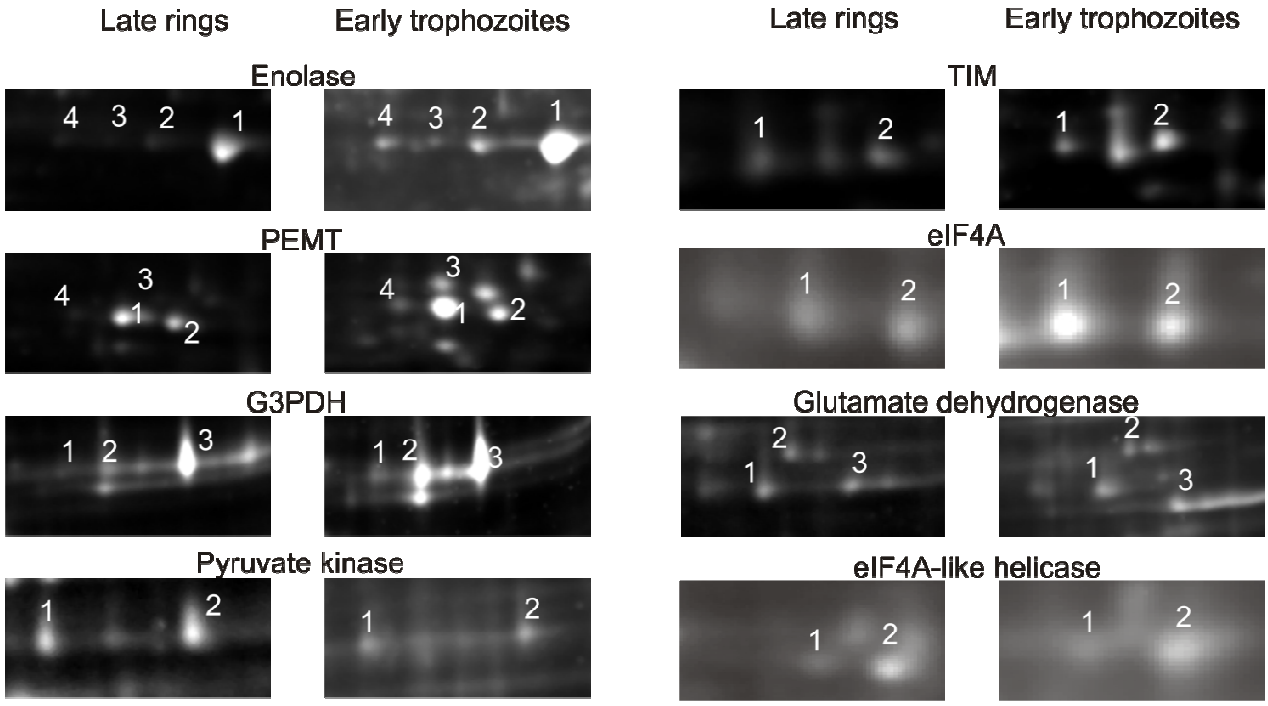

Figure 2.9: Isoforms of proteins that are differentially regulated in the *P. falciparum* ring and trophozoite stage proteomes.

The numbers are indicative of the number of isoforms per protein that were detected. Enolase, PEMT, and G3PDH, TIM and eIF4A all increase in protein abundance from the ring to the trophozoite stage. Pyruvate kinase decreased in protein abundance from rings to trophozoites, while glutamate dehydrogenase and eIF4A-like helicase remained unchanged over the specified time in protein expression levels. PEMT: phosphoethanolamine methyltransferase, TIM: triosephosphate isomerase, G3PDH: glyceraldehyde-3-phosphate dehydrogenase.

2.4 Discussion

2.4.1 Optimisation of Plasmodial proteins for 2-DE

The ability of 2-DE to provide a snapshot of the proteome at any particular time, is a distinct advantage for a multistage organism such as *Plasmodium*. The 2-DE technique remains the most widely used for proteomic investigation techniques (Wang *et al.*, 2009) due to several advantageous properties such as good resolution of abundant proteins as well as information on protein size, quantity and isoforms with post-translational modifications or different pIs (Lopez, 2000). However, 2-DE gels are biased to the detection of relatively high abundant proteins as well as soluble and mid-range molecular weight proteins (Ong & Pandey, 2001). Besides the visual advantages of 2-DE in comparing protein levels, proteins are differentially stained due to their specific chemical and physical properties, which necessitates careful selection of the staining method in terms of its sensitivity, reproducibility, ease of use and cost-effectiveness. Most importantly, the stain should be compatible with downstream applications such as MS. This chapter describes an improved protocol for the detection and identification of Plasmodial proteins separated by 2-DE, which was then also subsequently applied to identify the proteome of the Plasmodial ring and trophozoites stages.

The analysis of the Plasmodial proteome by 2-DE has been hampered by numerous technical constraints. Plasmodial proteins are notoriously insoluble, comparatively large, non-homologous and highly charged (Birkholtz *et al.*, 2008a) and therefore necessitates the use of optimised lysis buffers to ensure maximal solubility of these proteins for 2-DE. The lysis buffer described by Nirmalan *et al.*, is able to solubilise a large proportion of Plasmodial proteins. In this study, the combination of using 5-fold less saponin, increased washing steps and shorter sonication cycles (with prolonged cooling in between cycles), contributed to the absence of hemoglobin on the 2-DE gels in the 14 kDa range and enabled the detection of proteins in the range of pH 8-9 that was previously cumbersome in Plasmodial 2-DE. The use of this lysis buffer however, precludes the use of traditional methods of protein concentration determination.

A two-pronged approach was used in this study to determine the most effective and reproducible detection and staining method for Plasmodial proteins. Firstly, the effect of the extraction medium on standard protein determination methods was established as well as the sensitivity of staining methods to detect gel-separated molecular weight standards and secondly, for comparative purposes the sensitivity and reproducibility of these staining methods to detect Plasmodial proteins on 2-DE gels. Four different methodologies were evaluated to determine Plasmodial protein concentrations in the lysis buffer used for the protein extraction. The standard Bradford method as well as the

Lowry and BCA methods was found to be incompatible with the lysis buffer. The 2-D Quant kit conversely provided reproducible and comparable data for both the saline (R^2 = 0.9918) and lysis buffer (R^2 = 0.9929) standard curves, most likely due to the quantitative protein precipitation step by which any other interfering substances in the lysis buffer are also removed. Although various Plasmodial proteomic studies have employed the Bradford method (Makanga *et al.*, 2005, Panpumthong & Vattanaviboon, 2006), the present study confirms recent reports of the reliability of the 2-D Quant method (Foth *et al.*, 2008, van Brummelen *et al.*, 2009).

A second caveat in semi-quantitative proteomics is the sensitivity of the staining method used for the detection of protein spots after 2-DE. The sensitivity, performance, and linear regression constants of 4 different staining methods were compared in this study with quantitative 1-D analyses of standard molecular weight markers. Four different SDS-PAGE gels were individually stained with Colloidal Coomassie Blue (CCB), MS-compatible silver stain, SYPRO Ruby and Flamingo Pink, and compared by using Quantity One 4.4.1 to determine the detection limits. CCB was the least sensitive of the 4 stains and had relatively poor linearity (R^2 = 0.89). The MS-compatible silver stain was able to detect a minimum of 10 ng but has a very poor linear range (R^2 = 0.83). The fluorescent stains, SYPRO Ruby and Flamingo Pink, thus seem superior to CCB and silver in both sensitivity and dynamic linear quantification range of standard protein molecular weight markers. Coomassie Brilliant Blue is one of the most commonly used stains for detection of highly abundant proteins, and has been widely employed since its discovery in the 1960's. The more sensitive Colloidal Coomassie Blue (CCB) stain used here is a enhanced modification of the Coomassie Brilliant Blue stain and has a detection limit similar to that of silver staining but with the added advantage of limited background noise (Neuhoff *et al.*, 1988). It has an average linear dynamic range, is easy to use, cheap, has little protein to protein variation and is MS compatible (Berggren *et al.*, 2000). Silver staining is labour-intensive and can easily saturate during staining, and is generally not MS compatible due to the addition of cross linkers and fixatives such as gluteraldehyde and formaldehyde. Silver staining relies on salt or complex formation involving sulfhydryl and carboxyl groups of amino acid side chains. The formaldehyde and gluteraldehyde (not used in this case) can attach covalently to the proteins and alkylate alpha and epsilon amino groups of proteins, thus limiting down-stream applications and reducing the MS quality and the amount of peptides that can be obtained (Lin *et al.*, 2008). In this study a good identification rate (85%) were obtained, which may be due to the ferricyanide destaining step that reacts with the sodium thiosulfate to form a water soluble complex, that can be removed from the gel pieces, hence reducing background interference (Gharahdaghi *et al.*, 1999). Various MS compatible silver stains have been developed which omits gluteradehyde, but unfortunately this usually results in reduced

sensitivity (Gharahdaghi *et al.*, 1999, Shevchenko *et al.*, 1996). Another problem with silver quantitation of spots is the formation of a donut effect on the gel image, with the edges of the spot darker than the middle and ultimately creates problems during analysis of spots (Winkler *et al.*, 2007). This effect was not seen in the silver stained gels here.

Fluorescent stains have been developed with seemingly similar sensitivity to silver as well as being MS-compatible, which include the earlier SYPRO Orange and SYPRO Red (Steinberg *et al.*, 1996b, Steinberg *et al.*, 1996a), and the currently commonly used SYPRO Ruby stain (Berggren *et al.*, 2000). SYPRO Ruby is a fluorescent ruthenium-based stain that binds non-covalently to proteins in gels, and can be used to stain refractory proteins like glycoproteins and lipoproteins without staining nucleic acids. SYPRO Ruby has good photo-stability, cannot over stain proteins, and has a good detection limit and linear dynamic range, as well as being MS-compatible (Berggren *et al.*, 2000, Yan *et al.*, 2000 (a)). Despite several advantages that are associated with SYPRO Ruby (Berggren *et al.*, 2000, Yan *et al.*, 2000 (a)), SYPRO Ruby was only able to detect 235 Plasmodial protein spots after 2-DE with a MS identification rate of 85% (33/39). These results are in sharp contrast to those obtained with standard protein molecular weight markers and indicate that SYPRO Ruby is not an appropriate stain to use with Plasmodial proteins. It may be due to the fact that SYPRO Ruby dye binds to the proteins in such a way that it interferes with ionisation and identification and hence reduces the chance of a positive identification (Lanne & Panfilov, 2005). New generation fluorescent stains such as Flamingo Pink are reported to be able to detect proteins across the full range of molecular weights and isoelectric points separated on 2-DE with little gel-to-gel variability (Harris *et al.*, 2007), good linear dynamic range and MS-compatibility. These properties seem to be supported by the results of this study since 79% (349/443) of the Plasmodial trophozoite proteome predicted by our calculations were detected on 2-DE. The most promising results concerning protein identification were obtained with CCB and Flamingo Pink, which both had MS/MS success rates in excess of 90% (CCB had positive identification for 35 out of 37 proteins subjected to MS/MS and Flamingo Pink had positive identification for 37 out of 39 proteins subjected to MS/MS). The MS-compatibility of CCB is well documented (Winkler *et al.*, 2007, Lauber *et al.*, 2001), but literature evidence for the MS-compatibility of Flamingo Pink is still lacking. However, for the Plasmodial proteins investigated here, Flamingo Pink was superior to the other standard stains regarding its ability to provide excellent MS/MS identification rates (95% success). This suggests that Flamingo Pink may the preferable stain as far as Plasmodial proteomics are concerned but this may also be generally true for proteome analyses due to its superior detection and identification of proteins after 2-DE.

2-DE based analyses of the Plasmodial proteome is hampered by contaminating hemoglobin derived products (HDP) (Nirmalan *et al.*, 2007), possibly as a result of the thiourea/sonication steps during the extraction of Plasmodial proteins, and the resultant destabilization of hemozoin. Typically, these HDPs are observed as an intense smear focused around pI 7-10 with varying molecular weights. The less harsh sonication steps used in this study combined with extensive wash steps (to remove hemoglobin) and 5-fold less saponin, resulted in discrete spots identified in the 2-DE based Plasmodial proteome described here. Very little background and smearing were observed here compared to other Plasmodial proteome studies (Nirmalan *et al.*, 2004a, Makanga *et al.*, 2005, Panpumthong & Vattanaviboon, 2006, Aly *et al.*, 2007) enabling the identification of several proteins in the pI 7.5-9 and 14 kDa range (Figure 2.4, e.g. LDH, G3PDH, Adenylate kinase). Moreover, the protocol used here makes it unnecessary to use additional fractionation steps to remove contaminating high pI fractions (Nirmalan *et al.*, 2007) or two-step extraction procedures (Panpumthong & Vattanaviboon, 2006). Furthermore, the use of the 2-D Quant kit provided the only means of protein concentration determination for Plasmodial proteins in the lysis buffer. Finally, Flamingo Pink proved to be superior with regard to sensitivity as far as detection of spots on 2-DE is concerned and provided excellent MS/MS compatibility for Plasmodial proteins.

2.4.2 Application of 2-DE optimised method on the Plasmodial ring and trophozoite stages

The successful establishment of an optimised 2-DE method allowed the comprehensive analyses of the Plasmodial proteome during its IDC. Due to the just-in-time nature of transcript production per life cycle stage in the parasite, and little delay between transcript and protein production, the majority of this parasite's proteins are relatively life cycle specific (Le Roch *et al.*, 2004). Proteins are therefore expressed over 0.75 to 1.5 times of a life cycle (Bozdech *et al.*, 2003). Highly synchronized parasites were used where proteins were isolated from either >98% pure ring stage or conversely trophozoite stage proteins. For the ring-stage parasite proteome, an average of 328 spots were detected on 2-DE with Flamingo Pink staining, and of these spots, 73 protein spots were identified by MS/MS. An average of 272 spots were detected on 2-DE with Flamingo Pink staining for the trophozoite proteome, of which 52 protein spots were positively identified by MS/MS, resulting in a total of 125 protein spots identified (out of 140 analysed) in the late ring and trophozoite proteomes. These results confirmed the high MS success rate (90%) that was achieved by applying the optimised methodology to the analyses of the Plasmodial proteome. Of the 73 proteins spots identified in the ring stage proteome, 57 proteins spots were from Plasmodial origin, and consisted of 41 unique Plasmodial protein groups, where some groups contained multiple

isoforms of the same protein. The trophozoite proteome consists of 52 protein spots identified by MS of which 49 protein spots were from Plasmodial origin. Therefore, protein isoforms represented ~28% of the total number of Plasmodial protein spots identified. From this data, it is clear that protein isoforms are prominent within both the ring and trophozoite stages and may play an important role in Plasmodial protein regulation. Similarly, this has also been demonstrated on 2-DE proteome maps for other protozoan parasites that also highlighted the importance of isoform detection and PTM's that regulate protein function (De Jesus *et al.*, 2007, Brobey & Soong, 2007, Jones *et al.*, 2006). The significance of isoforms is further exemplified in a 2-DE proteomic study of *T. brucei* where the absence of a single protein isoform was associated with drug resistance (Foucher *et al.*, 2006).

Comparison of the positively identified proteins groups from the ring (41 Plasmodial proteins) and trophozoite (35 Plasmodial proteins) stage proteomes to those of the schizont stage proteome (24 Plasmodial proteins) (Foth *et al.*, 2008) revealed only 9 proteins (~9%) which were shared between all three stages. These include proteins involved in a variety of biological processes such as glycolysis, protein folding, oxidative stress and the cytoskeleton. Nineteen (19) proteins are shared between the ring and trophozoite stage whilst only 11 proteins were shared between the trophozoite and schizont. However, 14 proteins are shared between the ring and schizont stage parasites suggesting differentiation of the schizont stage proteins in preparation for the next round of invasion by the merozoites and the formation of the subsequent ring stage parasites. The remaining 39% of the proteins (39 proteins, 31 proteins from ring and trophozoite stage and 8 from schizont stage) were not shared between the different life stages of the parasite, consistent with stage-specific production of proteins (and their transcripts) due to tightly controlled mechanisms within the parasite (Bozdech *et al.*, 2003).

Comparison of the protein levels from the ring and trophozoite proteomes to the IDC transcript profile demonstrated distinct similarities between transcript production profiles (obtained from PlasmoDB 6.0 www.plasmodb.org) and their corresponding protein levels as determined in our study. Proteins that were up-regulated from rings to trophozoites mostly exhibited a corresponding increase in transcript level when compared to IDC data, with only a few exceptions illustrated, that could indicate possible differential regulation of these proteins at a post-transcriptional/translational level. Mostly the results emphasised the general observation of correspondences between transcript and protein levels in *P. falciparum* (Le Roch *et al.*, 2004). Several isoforms were also detected that displayed differential regulation from the ring to trophozoite stages. These examples, demonstrate

the complexity of post-transcriptional and post-translational regulation in the *P. falciparum* proteome.

Post-translational modification of proteins in *P. falciparum* has also been observed in the schizont stage proteome (Foth *et al.*, 2008) similar to what has been detected within this study. Post-translational modifications of Plasmodial proteins include at least phosphorylation (Pal-Bhowmick *et al.*, 2007, Wu *et al.*, 2009), glycosylation (Davidson *et al.*, 1999, Gowda & Davidson, 1999, Yang *et al.*, 1999, Davidson & Gowda, 2001), acetylation (Miao *et al.*, 2006) and sulfonation (Medzihradszky *et al.*, 2004). The lateral shift of the eIF4A-like helicase isoforms in this study suggests phosphorylation or sulfonation as potential modifications (Kinoshita *et al.*, 2009, Thingholm *et al.*, 2009). However, only 2 isoforms of this protein were observed in the trophozoite stage compared to five in the schizont stage, indicating additional regulatory mechanisms e.g. increased phosphorylation in later stages of the parasite (Wu *et al.*, 2009) consistent with the proposed involvement of this protein in controlling developmentally regulated protein expression. Enolase seems to undergo post-translational modifications to produce 5 isoforms in *P. yoelii*, 7 isoforms in the *P. falciparum* schizont stages (Foth *et al.*, 2008, Pal-Bhowmick *et al.*, 2007) and 4 isoforms as described here. However, enolase phosphorylation was not reported in the *P. falciparum* phospho-proteome (Wu *et al.*, 2009). Some of these enolase-isoforms have also been detected in nuclei and membranes in *P. yoelii* and therefore suggests moonlighting functions including host cell invasion, stage-specific gene expression (*Toxoplasma*), stress responses and molecular chaperone functions (Pal-Bhowmick *et al.*, 2007). The biological significance of these isoforms is not yet fully understood, but it clearly emphasises the need for further in-depth investigations of post-transcriptional and post-translational modifications to further our understanding of the biological regulatory mechanisms within the Plasmodial parasite.

This is the first Plasmodial proteome study in which the 2-DE proteomic process was optimised in detail, from sample preparation through to spot identification with MS/MS. This resulted in a more detailed description of the Plasmodial proteome due to the removal of some contaminating hemoglobin without additional fractionation steps or extraction procedures. The fluorescent stain, Flamingo Pink, proved superior to the other stains tested and resulted in the detection of 79% of the predicted trophozoite proteome after 2-DE and achieved exceptional protein identification by MS. The reproducibility of the methods described here makes it highly expedient for the analysis of differentially expressed Plasmodial proteins. The application of the optimised 2-DE method allowed the characterisation of 2-DE proteomes of the ring and trophozoite stages of *P. falciparum*, which showed that some proteins are differentially regulated between these life cycle stages and included

the identification of a significant number of protein isoforms. These results emphasise the importance of post-translational modifications as regulatory mechanisms within this parasite. Application of this methodology will be demonstrated in Chapter 3 where the proteome of AdoMetDC inhibited parasites will be investigated.