The Characterisation and Identification of Body Fluid Proteins for Forensic Purposes

Thesis submitted in partial fulfilment of the requirements of the Robert Gordon University for the degree of Doctor of Philosophy.

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Declaration

This thesis has been written entirely by myself and has not been presented previously for any degree award. It is a record of work carried out by myself unless otherwise stated. All sources of information have been acknowledged.

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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 and Larsen 2001)

Abstract

Advances in DNA technology have led to the extremely sensitive and rapid analysis methods used in forensic science. It can often be crucial to a criminal case to unequivocally identify the body fluid source of DNA. This is of particular importance in rape cases where the defence may argue that the source of a female DNA profile might be from a casual touch or from saliva.

In this study, proteomics has been employed in an attempt to identify potential biomarkers that are specific to a range of body fluids. Many publications cite the use of proteomics to identify biomarkers of disease such as cancer. In these reports, diseased and healthy tissues or tissues that have been treated or not treated with a drug are compared and the expressed proteins are compared by 2D electrophoresis. Human body fluids differ in function, composition and protein expression. Proteomics therefore seemed an ideal application to isolate the proteins that are characteristic of and specific to, different fluid types.

Both saliva and vaginal fluid proteomic methodologies were optimised for sample preparation, IPG strip pH range and protein load, and post-electrophoretic staining.

Seventeen isolated protein spots from saliva and vaginal fluid samples were submitted for LC-MS/MS analysis. Nine saliva spots and eleven vaginal spots were identified as known proteins on the MASCOT database. Of those thought to be specific to saliva or vaginal fluid six candidate biomarkers were tested further against a panel of body fluids for specificity using ELISA or Dot Blot.

Zinc-α-2 glycoprotein (ZA2G) was detected and present in all body fluid samples thus could be used as a human body fluid positive control in a future assay. SCC (Squamous cell carcinoma) ELISA was capable of distinguishing samples of vaginal origin by detection of SCCA (Squamous cell carcinoma antigen). This antigen could be used in conjunction with a menstrual blood marker to distinguish between vaginal fluid and menstrual blood. Antibody specificity was a limiting factor in the success of the dot blots performed and hence the analysis of Cystatin SA, Cystatin SN and SERPIN B1 was inconclusive.

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Abbreviations

1D	One Dimensional
2D	Two Dimensional
2DE	Two Dimensional Electrophoresis
3D	Three Dimensional
ABO	A, B and O Alleles for Blood Typing
ACN	Acetonitrile
ALAS	Erythroid δ-aminolevulinate synthase
Amu	Atomic Mass Unit
AMY1	Salivary Amylase
AMY2	Pancreatic Amylase
AP	Acid Phosphatase
APS	Ammonium Persulphate
BSA	Bovine Serum Albumin
CBB	Coomassie Brilliant Blue
CCD	Charged Coupled Device
cDNA	copy Deoxyribonucleic Acid
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
CID	Collision Induced Dissociation
CK	Cytokeratin
Cps	counts per second
CVF	Cervical Vaginal Fluid
DAB	Diaminobenzidine
DIGE	Difference Gel Electrophoresis
DNA	Deoxyribonucelic Acid
DSM	Biodegradable Starch Microspheres
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme Linked Immunosorbent Assay
ESI	Electrospray Ionization
ESR	Oestrogen Receptor
FN	False Negative
FP	False Positive
GE	Gel Electrophoresis
GPA	Glycophorin A
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
HBA	Haemoglobin Alpha Locus
HBB	Haemoglobin Beta
HBD	Human Beta-Defensin
HCl	Hydrochloric Acid
HPLC	High Performance Liquid Chromatography
HRP	Horseradish Peroxidise
HSA	Human Serum Albumin
HTN	Histatin
HUPO	Human Proteome Organisation
IEF	Isoelectric Focusing
IFI	Independent Forensics of Illinois
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M

IPG	Immobilised pH Gradient
kDa	kilo Dalton, 1000 amu
KLK	Kallikrein
KM	Kastle-Meyer
LC-MS/MS	Liquid Chromotography – Tandem Mass Spectrometry
LMG	Leucomalachite Green
MALDI	Matrix Assisted Laser Desorption Ionization
MCSP	Human Mitochondrial Capsule Selenoprotein
MMP	Matrix Metalloproteinase
MOWSE	Molecular Weight Search
mRNA	Messenger Ribonucleic Acid
MS	Mass Spectrometry
MSMS	Tandem Mass Spectrometry
MUC	Mucin
MW	Molecular Weight
m/z	Mass:Charge
NDNAD	National DNA Database
NL	Non Linear
NPIA	National Policing Improvements Agency
OFN	Oxygen Free Nitrogen
PAGE	Polyacrylamide Gel Electrophoresis
PAS	Periodic Acid-Schiff
PBGD	Porphobilinogen Deaminase
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PDE	Phosphodiesterase
p*I*	Isoelectric Point
PRP	Proline-Rich Protein
PRM	Protamine
PS	Pooled Saliva
PSA	Prostate Specific Antigen
PTM	Post Translational Modifications
PV	Pooled Vaginal Fluid
PVDF	Polyvinylidene Fluoride
Q	Quadrupole
QTOF	Quadrupole Time of Flight
RBC	Red Blood Cells
RCDC	Reducing Agent Compatible Detergent Compatible
RNA	Ribonucleic Acid
RSID	Rapid Stain Identification
RT-PCR	Reverse Transcription- Polymerase Chain Reaction
SA	Salivary Acid
SCC	Squamous Cell Carcinoma
SCCA	Squamous Cell Carcinoma Antigen
SD	Standard Deviation
SDS	Sodium Dodecyl Sulphate
SEMG	Semenogelin
SERPIN	Serine Proteinase Inhibitor
SPTB	β-Spectrin
SPM	Spermine

SN	Salivary Neutral
STATH	Statherin
STR	Short Tandem Repeats
SVSA	Seminal Vessicle Specific Antigen
TBS	Tris Buffered Saline
TBST	Tris Buffered Saline with TWEEN
TCA	Trichloroacetic Acid
TE	Tris EDTA
TEMED	Tetramethylethylenediamine
TGM	Human Prostate Transglutaminase
TIC	Total Ion Count
TN	True Negative
T-RFLP	Terminal – Restriction Fragment Length Polymorphism
tRNA	Transfer Ribonucleic Acid
TOF	Time of Flight
TP	True Positive
UPPA	Universal Protein Precipitation Agent
UV	Ultraviolet
VEGh	Von Ebners Gland Protein
v/v	Volume:Volume
w/v	Weight:Volume
ZA2G	Zinc-α-2-Glycoprotein

Chapter 1
Introduction

Chapter 1. Introduction

In forensic science it is often of vital importance to know the source of evidence such as a body fluid. For instance, it can make a huge difference if DNA (Deoxyribonucleic acid) has come from saliva or vaginal secretion. However, there is a lack of a good biochemical test for the identification of body fluids. The work described in this thesis sets out to discover proteins that may provide unequivocal evidence for the source of an unknown fluid.

1.1 Forensic Analysis

DNA is unique to each individual (except for identical twins). For this reason forensic science has exploited DNA as a method to identify and associate victims, suspects and pieces of evidence found at crime scenes. Short tandem repeat (STR) analysis is the method used to obtain DNA profiles, where short repeated sequences of non-coding, highly variable DNA are used to differentiate between individuals (Butler 2005). The number of criminal cases relying on DNA evidence has dramatically increased over the past 10 years. In 1999 the number of crimes with a DNA match on the national DNA database (NDNAD) was 21,239 whereas in 2008/09, there were 36,727 crimes with suspect-to-crime-scene DNA matches, (National Policing Improvement Agency (2009a)). The increase in matches is partly due to the expansion of the NDNAD. Figures as of the 30th September 2009 show that the number of individuals retained on the database is 4,762,033 and 351,461 crime scene samples to the database. The NDNAD may hold more than one profile from a given individual as samples could be taken from the same individual on more than one occasion. This may happen as a result of a person giving different names, or different versions of their name, on separate arrests (National Policing Improvement Agency (2009b)).

DNA analysis has become routine in forensic laboratories and sample submission increases have led to some of the steps of DNA profiling being automated to reduce time and labour. Although this has decreased turnaround times for DNA analysis it has meant that most of the time on a case is spent performing body fluid screening on exhibits, searching for potential stains to sample for DNA analysis. Without a confirmatory test for the different biological fluids on exhibits, the time involved and associated costs have led to an increase in samples bypassing body fluid identification prior to STR typing. DNA profiles are loaded to the NDNAD as quickly as possible.

DNA evidence can be in the form of body fluid or tissue samples, either swabs (from an individual or item taken at the scene) or on a physical article. Although the generation of a DNA profile from a piece of evidence implies that biological material is present, it is often important to a case to know from which biological fluid the DNA profile was obtained. It may also be of importance to identify individual stains. If multiple stained areas are present on an exhibit – they may have originated from more than one individual and identifying the type of stain could assist in the investigation of the case.

The most common body fluids encountered in forensic laboratories are blood, semen and saliva but others such as vaginal fluid, urine and sweat (Virkler and Lednev 2009) can also be considered important sources for DNA within certain case circumstances. A case submission may also yield a mixed DNA profile (consisting of DNA from more than one person) derived from a mixture of body fluid samples. It may be important to the case to ascertain the body fluid source for each DNA profile within the mixture for example, semen and victim's blood in a rape case. Many body fluids are difficult to identify visually as they are colourless and therefore not easily discriminated from the background material.

It is important to use a method to identify body fluid samples prior to STR profiling, to limit the number of submissions being analysed unnecessarily. It is important to ascertain whether a submission is likely to yield a DNA profile, and to ask if there is a body fluid stain present and if so if it is of human origin. As well as obtaining a DNA profile it could also be of value to prove the identity of the source of the DNA profile or even to exclude a particular fluid in any given forensic case.

1.2 Body fluids

Body Fluids have a varied composition related to their function and site of secretion or action within the body. The functions of body fluids include transport (blood), protection and lubrication (vaginal fluid), digestion (saliva) and excretion (sweat and urine). The different components of body fluids provide the basis for current identification tests. Frequently encountered forensic body fluids and their main components are shown in Table 1.1.

Table 1.1: The main constituents of human body fluids (*adapted from Virkler and Lednev 2009 and Wilson 2005*).

Blood	***Saliva***	***Semen***	***Urine***	***Vaginal fluid***
Haemoglobin	Glycoprotein	Acid phosphatase	Urea	Mucins
Fibrinogen	Bicarbonate	PSA	Sodium	Albumin
Erythrocytes	Chloride	Spermatozoa	Chloride	Ig
Albumin	Potassium	Choline	Potassium	Transferrin
Glucose	Sodium	Spermine	Ammonium	Lactoferrin
Ig	cAMP	Putrescine	Phosphate	Glucose
	Amylase	Spermidine	Calcium	Glycogen
	Ig	Cadaverine	Creatinine	Mannose
	Urea	Semenogelin	Uric acid	Glucosamine
	Phosphate	Zinc	Bicarbonate	Fructose
	Lysozyme	Citric acid	Chlorine	Neutral lipids
	Calcium	Lactic acid	Glucose	Phospholipids
	Amino acids	Fructose		Urea
	Ammonia	Urea		Lactic acid
	Uric acid	Ascorbic acid		Acetic acid
	Lipid	Ig		Butanoic acid
	Thiocyanate			Propanoic acid
	Glucose			Amino acids
	Peroxidise			Sodium
	Citrate			Potassium
				Chloride

Saliva and vaginal fluid were analysed in this study for their particular relevance in ascertaining whether a rape or sexual assault has been committed. The components of each of these fluids relate to their function within the human body. The body fluids of focus within this study are saliva and vaginal fluid. The composition of these two fluids have been described in more detail. Both saliva and vaginal fluid contain epithelial cells originating from the oral cavity and vagina respectively and suspended in a liquid matrix. It is necessary to know a little about these fluids in order to predict potential markers and interpret the importance of characterised proteins.

1.2.1 The Epithelium

The human body is protected from microbes by a continuous tissue known as the epithelium, which is present on all surfaces of the body in contact with the external environment. There are two types of epithelial surfaces, the dry epithelia of the skin (epidermis) and the moist epithelia (mucosae) covering the internal body surfaces. The focus of this section will be on mucosal epithelia as it is found in the oral cavity and the genital tract. The apical surface of the epithelium is in contact with the external surface and the basal surface is in contact with the basal membrane and the underlying tissues. The cells of the oral cavity and the vagina are both stratified squamous epithelia (Figure 1.1) which is characteristic of the mucosae. Stratified epithelial surfaces consist of more than one layer of cells and are characteristic of areas subjected to mechanical abrasion.

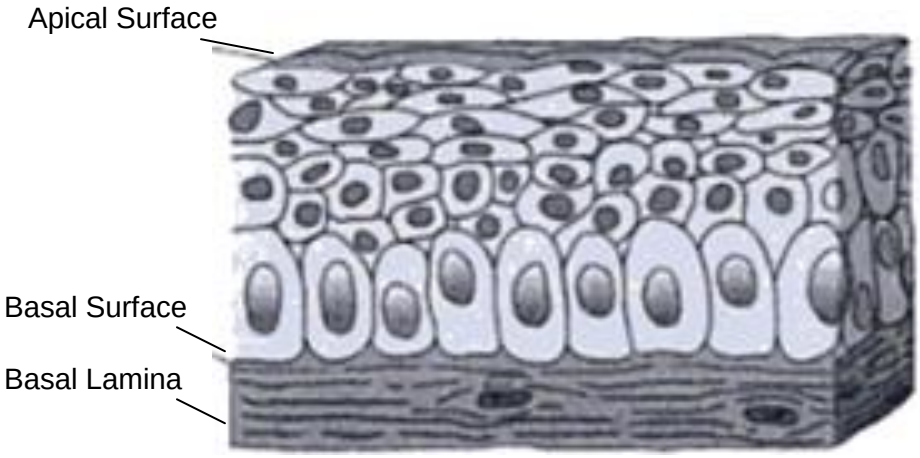


Figure 1.1 A Schematic diagram of stratified squamous epithelium (Wilson 2005).

The epithelium acts as a physical barrier to microbes preventing their penetration to the underlying tissues. The epithelial surface contains receptors for the microbe adhesion molecules thus trapping them in the mucus. Epithelial cells are continually produced and shed along with trapped microbes in the mucus and expelled from the body. The epithelium secretes antimicrobial compounds such as, enzymes and antibodies capable of either killing microbes or inhibiting their growth. These antimicrobial compounds become concentrated in the mucus layer which enhances their affectivity.

1.2.2 The Physiology of Saliva

Saliva is an aqueous fluid with a number of functions including lubrication, digestion, and host defence. It also acts as a buffer preventing pH fluctuations within the oral cavity as well as enhancing the remineralisation of teeth.

Saliva is a dilute fluid containing of more than 99% water (Humphrey and Williamson 2001) and is produced by both major (90%) and minor (10%) salivary glands. The major saliva glands, the parotid, submandibular and the sublingual are located outside the oral cavity (Figure 1.2). The parotid glands are located in front of the ears with ducts opening next to the upper second molar teeth. The submandibular glands are located beneath the tongue with their ducts opening behind the incisors. The sublingual glands are located in front of the submandibular glands and open into the oral cavity below the tongue. There are an estimated 450-750 minor salivary glands which are located in the buccal glands of the oral mucosa, the palate and the tongue (Aps and Martens 2005).

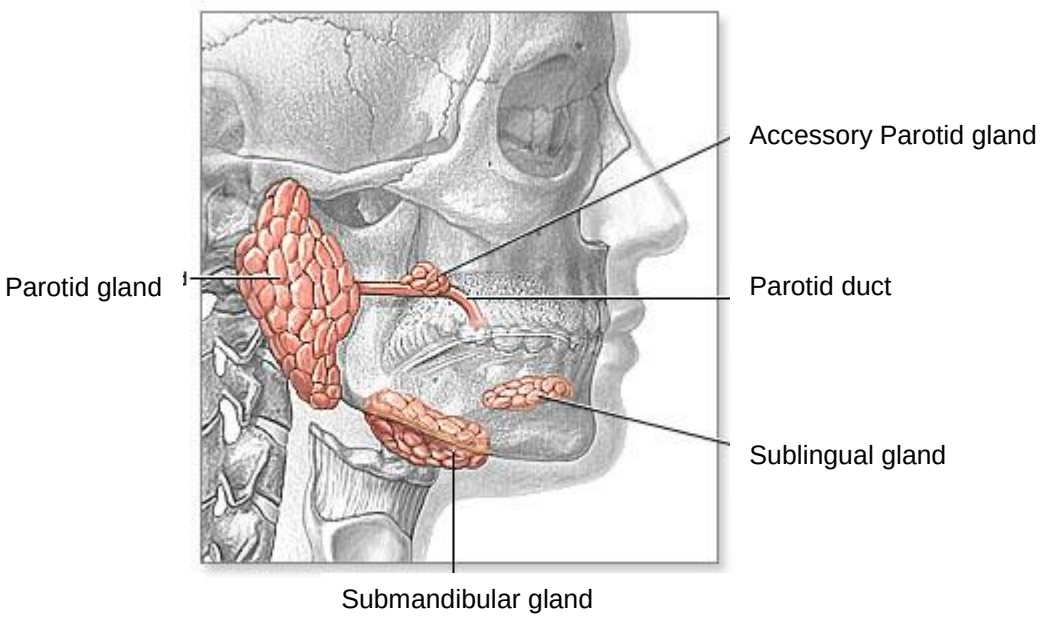


Figure 1.2 A diagram showing the position of the human salivary glands adapted from The Medline Encyclopedia 2009 [online].

Salivary glands are composed of acini where the initial isotonic saliva is produced. The acini are connected by ducts and the secreted saliva is drained into the oral cavity where there are changes in electrolytes giving saliva its hypotonic character. The hypotonic nature of saliva allows the taste buds to perceive different tastes without

being masked by normal plasma sodium levels (Humphrey and Williamson 2001). The pH of saliva is subject to fluctuations and can range from 5.30 (with low flow) to 7.80 (high flow) (Humphrey and Williamson 2001). The composition of saliva originating from these glands differs. The saliva produced by the submandibular and sublingual gland has a higher mucus content than that produced by the parotid glands.

The flow and secretion of saliva is dependent on numerous factors ranging from the time of day to the smell or sight of food as well as eating itself. The autonomic nervous system plays an important role in salivation, both sympathetic and parasympathetic stimulation are capable of causing salivation although the constituents of each secretion differs. Sympathetic stimulation leads to secretions containing more protein whereas parasympathetic secretions are more watery in composition (Humphrey and Williamson 2001).

There are two types of saliva, resting saliva (in the absence of a stimulus) and stimulated saliva (produced in response to a stimulus). The composition of these types of saliva also varies (Table 1.2). Stimulated saliva contributes between 80-90% of saliva produced daily. Stimulated saliva production is quicker than resting production for this reason; the faster speed that saliva passes through the salivary ducts means that there is less time for electrolyte exchange to occur thus a higher bicarbonate concentration is produced. This results in a higher buffering capacity of the saliva (Aps and Martens 2005).

Table 1.2: The glandular composition and flow rate of resting and stimulated saliva (*adapted from Wilson 2005*).

Type of saliva	***Composition***	***Flow Rate***
Resting (high viscosity and protein rich)	20% Parotid 65% Submandibular 7% Sublingual 8% minor salivary glands	0.30ml/minute
Stimulated	Parotid Submandibular Sublingual Minor salivary glands	2.50-5.00 ml/minute

The main components of saliva are proteins and glycoproteins with salivary mucins comprising approximately 25% of the total protein complement of saliva. Amylase, IgA (immunoglobulin A) and lysozyme are also major contributors to saliva.

The concentration of protein in saliva varies according to the abundance of the protein, and ranges between 140-640 mg/100ml (Wilson 2005)

Saliva also contains electrolytes, nitrogenous compounds and proteins, which play a role in the five different functions of saliva (Table 1.3). Although amylase and lipase are present in saliva the short time that food remains in the oral cavity may mean that limited digestion occurs. Amylase remains active up to 15 minutes after being swallowed whereas lipase is able to withstand the high gastric pH. Thus digestion of starch and fats may proceed with the aid of salivary enzymes after being swallowed. The oral cavity can contain approximately 1.1ml of saliva before the swallowing reflex is initiated (Wilson 2005). Swallowing only removes approximately 25% of saliva allowing all the surfaces of the oral cavity to remain covered with saliva. Saliva also acts as the main source of nutrients for microbes in the oral cavity.

Table 1.3: The constituents of saliva and how they relate to the different functions of saliva (data *source Wilson 2005*).

Function	***Substance***
Lubrication	Mucins
Digestion	Amylase Lipase
Buffer	Bicarbonate Phosphates Urea Amino acids Proteins
Tooth mineralisation	Calcium Phosphates Proteins
Host defence	Immunoglobulins Proteins Mucins

1.2.3 The Physiology of Vaginal Fluid

To understand the components of vaginal fluid it is important to understand the structure of the vagina and its function.

The vagina is a muscular organ comprised mainly of smooth muscle with a mucosal epithelial lining. The three functions of the vagina are; a childbirth canal, an outlet for menstrual blood and a receptacle for the penis during sexual intercourse. The folded epithelial lining is lubricated by mucus produced by the cervix. The vaginal mucosa has three main layers. The inner layer consists of basal cells active in cell division, the intermediate layer has glycogen producing cells with intracytoplasmic glycogen granules and there is a superficial layer with fewer glycogen granules and microfilaments than the intermediate layer which provides rigidity and protection for the other layers. The mucosa also contains lymphocytes and Langerhans cells (Wilson 2005).

There are two cycles that make up the commonly described menstrual cycle; the ovarian cycle and the menstrual cycle. The ovarian cycle is associated with changes leading to ovulation (Figure 1.3B). The menstrual cycle is associated with changes in the endometrium leading to menstruation (Figure 1.3D). Both cycles are under hormonal control (Figure 1.3A and C).

Menstrual cycle changes occur in the epithelium stimulated by the hormone Oestrogen, involving the proliferation, maturation and desquamation of the cells. Conversely progesterone inhibits the maturation of vaginal epithelial cells. The vaginal epithelium prior to the onset of menstruation and after menopause is thin due to the low Oestrogen levels at these stages.

The mucus layer, although an important component of the host defense mechanism is also a major source of nutrients for microbes in the vagina. The mucus secreting cells of the cervix are affected by hormones; the quantity of mucus produced increases 10 fold and peaks at ovulation when the levels of Oestrogen are at their highest. The viscosity of the mucus also changes throughout the menstrual cycle. Prior to ovulation mucus viscosity is low, following ovulation when the level of progesterone rises less mucus is produced and it is much more viscous in composition than during the follicular phase.

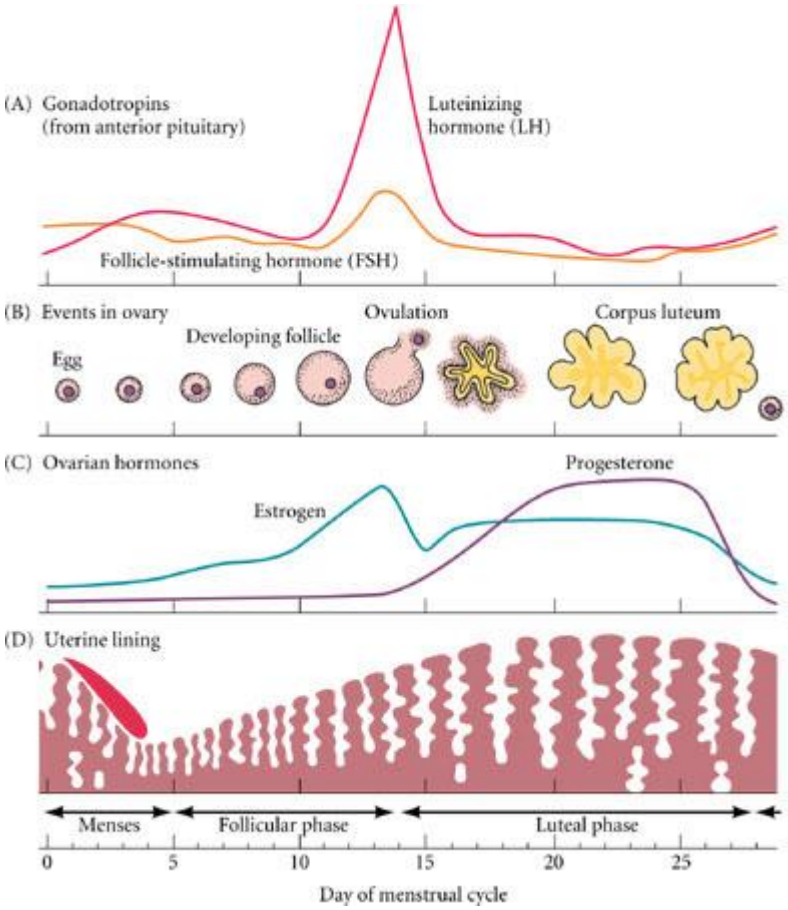


Figure 1.3 The menstrual cycle and its relationship to the ovarian cycle. Reference.Queen Mary University of London [online].

The vaginal mucosa contains stores of glycogen which are metabolised anaerobically releasing organic acids e.g. lactic acid producing a pH in the vagina of approximately pH4. This acidic environment has an inhibitory affect on the growth of many bacterial species. Unlike other mucosal fluids the predominant class of antibody within vaginal fluid is IgG (Immunoglobulin G) rather than IgA (Wilson 2005).

Vaginal fluid is a mixture of fluids derived from a number of different sources; transudate from the vaginal mucosa, cervical mucus, endometrial fluid secretions of the bartholins glands, desquamated vaginal epithelial cells and leukocytes. Between 1-3 g of vaginal fluid are produced daily by women of reproductive age. It contains water, carbohydrates, inorganic ions, proteins and amino acids (Dasari *et al*. 2007) providing the main source of nutrients for vaginal microbiota. Table 1.4 shows the principle components of vaginal fluid of pre-menopausal women.

Table 1.4: The main components of vaginal fluid (*adapted from Wilson 2005*).

Type of molecule/ion	***Examples***
Protein	Mucins, albumin, Immunoglobulins (IgG, IgA, IgM), trasferrin, lactoferrin
Carbohydrate	Glycogen (1.50g/100g) Glucose (0.62g/100g)
Lipid	Neutral lipids Phospholipids
Low molecular weight organic compounds	Urea Lactic acid Acetic acid Butanoic acid Propanoic acid
Amino acids	
Inorganic ions	Sodium Potassium Chloride

1.3 Current Body Fluid Identification

Body fluid identification relies on two distinct method types. These are physical methods such as irradiation at different wavelengths and biochemical methods such as chemical presumptive tests. Presumptive tests are used for preliminary identification of the fluid but in order to unambiguously characterise the fluid, a confirmatory test is needed. Although presumptive tests are used routinely there is a need for a confirmatory test for a range of body fluids. The current identification methods for major body fluids encountered at a crime scene or submitted to a forensic laboratory are described in further detail below.

1.3.1 Blood

Blood is one of the few fluids for which both presumptive and confirmatory tests are available.

Ultraviolet (UV) light can be used as a presumptive test to enhance blood stains present on dark background fabrics. However, overexposure to UV light is known to degrade DNA (Pang and Cheung 2007a) so caution must be used.

Many of the biochemical tests for blood rely on the peroxidase - like properties of haemoglobin reacting with a substrate to create a colour change reaction. The most sensitive of presumptive tests currently used is the chemo-luminescent test reagent Luminol (Webb *et al*. 2006) which has been used for the past 40 years (Barni *et al*. 2007). Luminol is oxidised in the presence of hydrogen peroxide to an unstable excited state which upon decomposition liberates light. The reaction is catalysed by iron present in haemoglobin within blood samples (Figure 1.4).

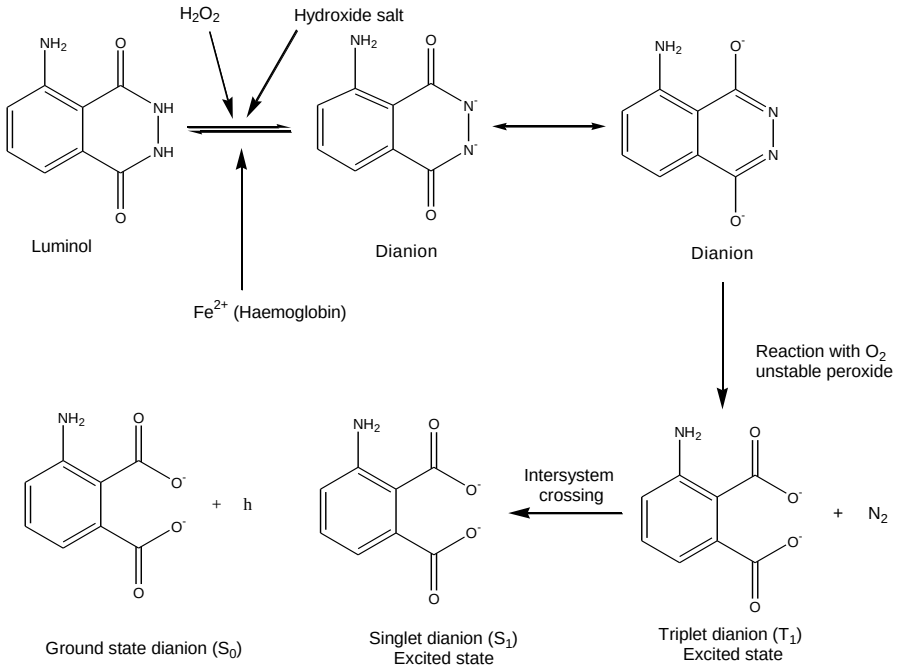


Figure 1.4 A schematic diagram of the chemical reaction of Luminol with blood. Adapted from BlueStar Forensic (2004).

Two other popular chemical presumptive tests with similar limits of detection (1:10000 blood dilution) are the Kastle-Meyer (KM) test and the Leucomalachite green (LMG) test (Virkler and Lednev 2009). These two tests are not as sensitive as Luminol which when diluted 1:1,000,000 times can successfully detect the presence of blood (Watkins and Brown 2004). Peroxidase in blood catalyses the reduction of hydrogen peroxide, liberating oxygen which in turn oxidises either LMG or KM solutions. A colour change from clear to either a blue/green colour (LMG) or pink colour (KM) change is seen when positive.

The KM test relies on phenolphthalein causing an alkaline solution to turn pink after being oxidised by peroxide in the presence of blood (Figure 1.5) but no false positives have been shown with other body fluids.

LMG also uses the catalytic properties of haemoglobin but in acidic conditions to produce a green colour. The heme group responsible for catalysing the reaction occurs widely in nature in cytochromes, plant peroxidases and some catalase enzymes. For this reason a positive result implies peroxidase activity and the presumed identification of blood. Oxidising agents such as copper salts and vegetable peroxidases may also yield false positives (Wecht and Rago 2006).

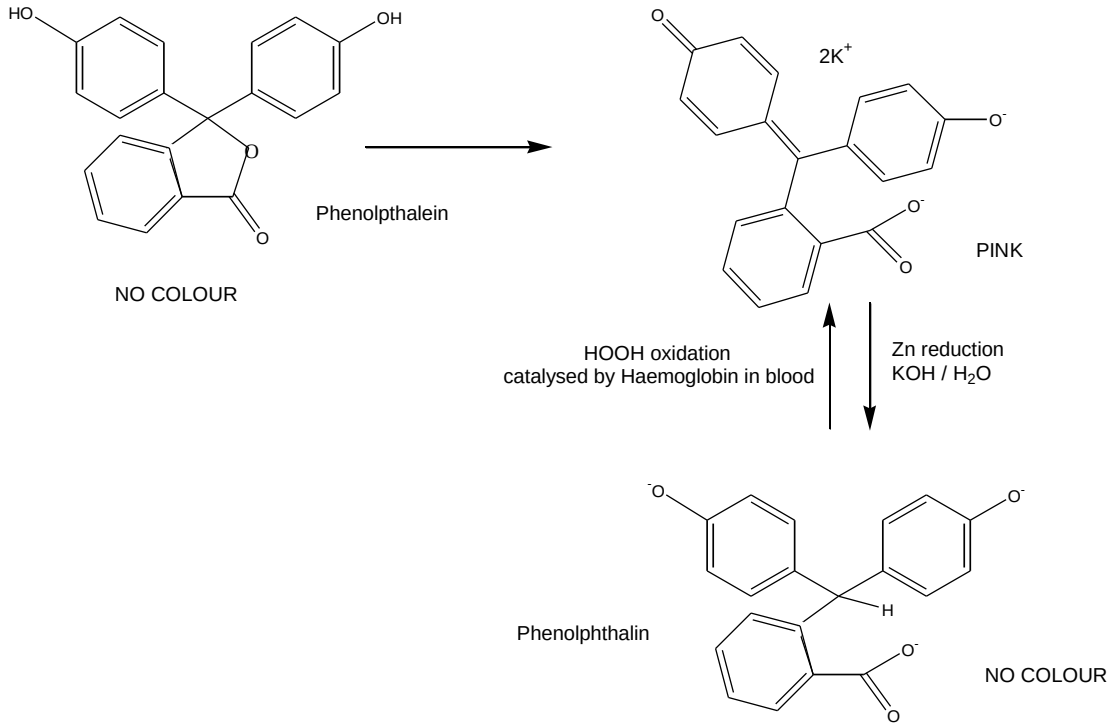


Figure 1.5 A schematic diagram of the Kastle Meyer chemical reaction in the presence of blood. Diagram adapted from Crosgray, W., 2004.

A blood stain can be confirmed by microscopic analysis with the identification of blood cells. A major disadvantage with the luminol, KM, LMG and microscopic examination tests is that none of them are species specific.

This problem can be overcome with immunodiffusion against antibodies specific to human haemoglobin (Otto and Somogyi 1974) and Enzyme-Linked Immunosorbant Assays (ELISA) (Kashyap, 1989). The most recently developed identification method uses an immuno-chromatographic strip. Independent Forensics of Illinois (IFI) produce the Rapid Stain Identification (RSID™). The lateral flow test strip uses two Human glycophorin A (GPA) antigens. Glycophorins are cell membrane proteins expressed in red blood cells (RBCs) and prevent cell aggregation. The detection limit of these strips is 1 µl of human blood with no cross reactivity seen with animal blood from the species tested. No cross reactivity was seen with human body fluids; vaginal fluid, semen, saliva and urine. Menstrual blood has not been tested with this kit (Schweers *et al*. 2008).

A major problem of interest to forensic scientists is to discriminate between menstrual blood and venous blood. This information could be critical in a sexual assault case where the female is undergoing menses. Although red in colour a more appropriate term to describe menstrual blood could be menstrual fluid as it is considered a suspension of blood and endometrium fragments within a mixture of

serum and cervico-vaginal fluid (Farage and Maibach 2006). The blood content of menstrual blood is dependent upon the degree of endometrial breakdown whereas the vaginal fluid portion is mainly water, electrolytes and proteins. As well as serum components and red and white blood cells (expected with venous blood), menstrual blood also contains waste tissue, endometrial proteases and vaginal fluid. Unlike venous blood, menstrual blood will not clot as it lacks clotting factors such as fibrinogen (Dockeray *et al*. 1987). mRNA (messenger Ribonucleic Acid) studies for the positive identification of menstrual blood have been shown with vaginal fluid primer sets but not circulatory blood primer sets (Juusola and Ballantyne 2005).

1.3.2 Saliva

There are only presumptive tests available for saliva. As with blood, a UV light can be used to locate saliva stains which appear blue/white in colour. The high water content of saliva makes it harder to detect than for example, a semen stain.

Most detection methods rely on detecting, α-amylase activity. There are two isoforms of α-amylase found within the human body. Both forms are encoded by genes on chromosome 1; salivary amylase (AMY1) and pancreatic amylase (AMY2) (James and Nordby 2003). However, salivary amylase is not found exclusively within saliva and pancreatic amylases are not found exclusively in the pancreas. Amylase found to be expressed within saliva, breast milk and sweat is encoded by AMY1 (locus 1). Amylase found expressed within the pancreas, vaginal fluid and semen is encoded by AMY2 (locus 2).

The Phadebas test is a popular test used routinely in forensic laboratories for detection of α-amylase activity. The Phadebas test consists of manufactured starch tablets which are dissolved in water and sprayed onto a piece of filter paper for the test. The starch is in the form of polymer chains, homogeneously interlinked to form spheres of known size, known as Bio-Degradable Starch Microspheres (DSMs). As starch is insoluble in water so are the DSMs. A water soluble blue dye is chemically attached to the microspheres and, as long as the dye is bound to the DSM, it remains insoluble in water. In the presence of saliva, α-amylase enzymatically degrades the DSMs. A positive reaction is indicated by the diffusion of liberated blue dye molecules (Figure 1.6).

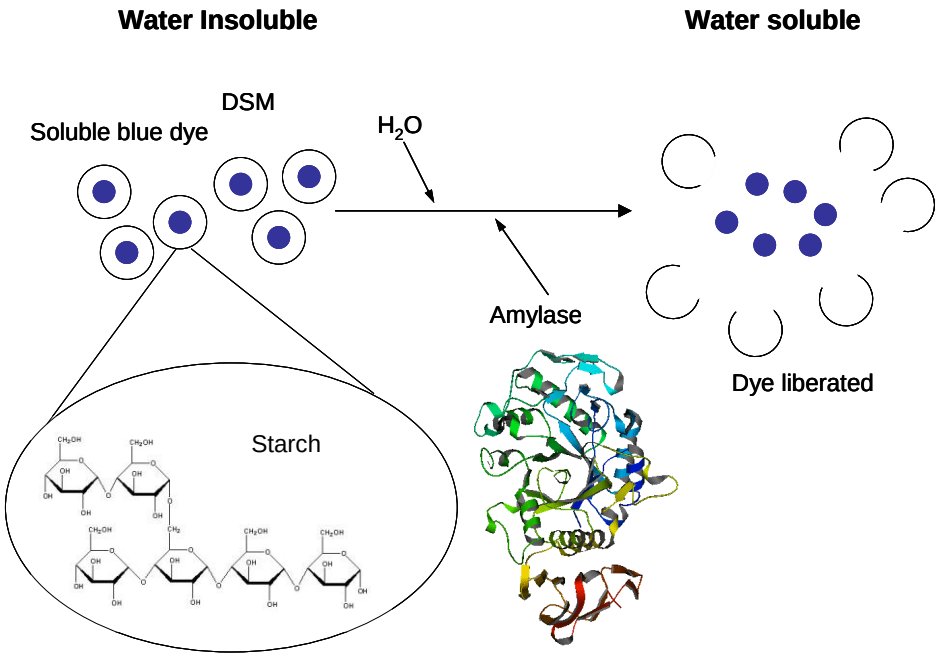


Figure 1.6 A schematic diagram showing the hydrolysis of starch during the Phadebas reaction.

This test, although specific for α-amylase, is not specific for saliva. As mentioned previously, other body fluids contain lower levels of α-amylase, and positive Phadebas results are shown by urine, blood and semen (Whitehead and Kipps 1975). The false positives shown with this test mean it is of little use for discriminating between saliva and semen.

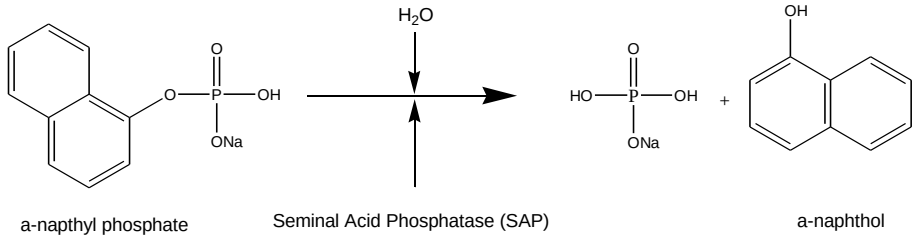
IFI have developed an immuno-chromatographic test strip for saliva using anti-human salivary (amyA) antibodies with a detection limit of 0.5 µl saliva. No cross reactivity was shown with any of the animal species saliva samples tested. No cross reactivity was shown with human body fluids; blood, semen, urine, vaginal secretions, or menstrual blood. Further discussion relating to this test can be found in Chapter 3.

1.3.3 Semen

Presumptive tests are available for semen. As for saliva, semen can be identified using an alternative light source (eg. the Wood's lamp). The Wood's lamp emits UV light (365nm) and was primarily used for identifying fungal or bacterial infections on the skin or scalp. When exposed to UV light at this wavelength semen has been seen to fluoresce on the surface of the skin but some ointments and creams can give false positives (Santucci *et al*. 1999).

The most commonly used biochemical test for the identification of semen is the Acid phosphatase (AP) test (Lundquist 1950). There are two steps in the reaction (Figure 1.7). In step one, the AP enzyme present within semen (Table 1.1 page 4) catalyses the hydrolysis reaction of α-naphthyl phosphate to sodium phosphate and α-naphthol (Kobilinsky *et al*. 2005). In the second step, the product α-naphthol then reacts with the chromagen, Brentamine Fast Blue (a diazonium salt) to produce an azo dye with a clear to purple colour change (James and Nordby 2003).

Stage 1 reaction



Stage 2 reaction

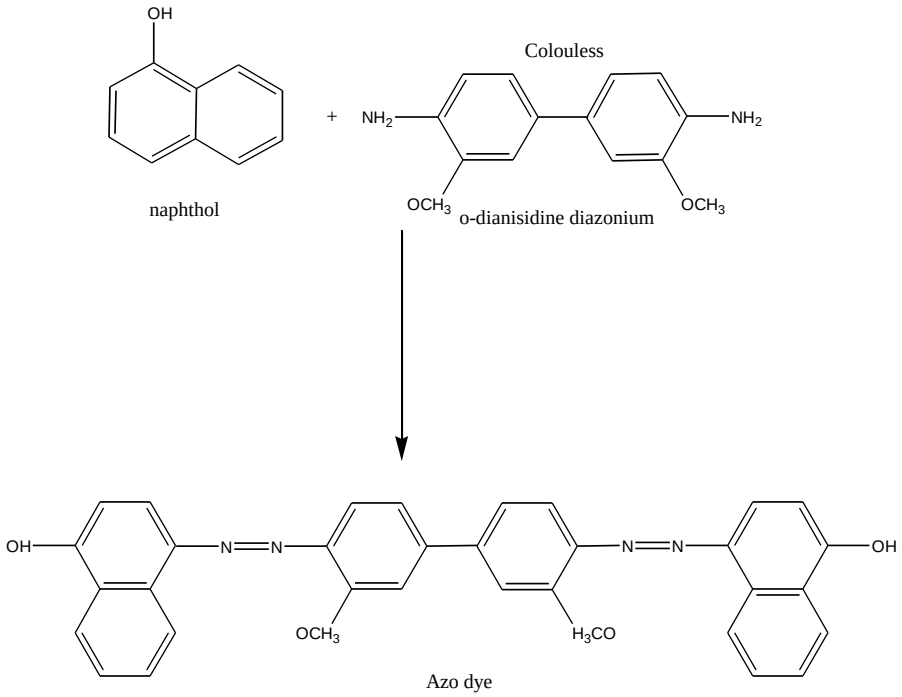


Figure 1.7 A schematic diagram showing the AP reaction in the presence of semen. Adapted from Shinohara and Ohkuma 1964

False positive reactions have been shown with plant matter such as cauliflower stems and as Table 1.1 on page 4 shows, AP has been found present within vaginal

fluid and female urine as well as semen (Graves *et al*. 1985). This could cause confusion if identifying samples for a suspected rape case were required, where identifying body fluid samples could imply whether sexual intercourse has occurred or not. It has been reported that the reaction with vaginal AP is much slower than that of seminal AP (Saferstein 2002), so timing could be used to determine the difference between samples giving a positive test result although this is not ideal and should not be used as a confirmatory test.

Isoelectric Focussing (IEF) (Toates 1979) and sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Stolorow *et al*. 1976) have been used to separate the two forms of AP. But these methods are not ideal for providing a quick identification of body fluid type prior to STR analysis. In addition to the presumptive tests mentioned it is common practice to confirm the presence of sperm cells within semen by light microscopy, staining the DNA within the sperm head to aid detection. Christmas tree stain, hematoxylin and eosin are commonly used. A problem could be encountered if there is a low sperm count (Oligozoospermia), an absence of sperm in the semen (Azoospermia) or if the donor had had a vasectomy. A useful identification method therefore should be more concerned with identifying a component of semen as opposed to a component of sperm cells.

Detection of prostate-specific antigen (PSA) by ELISA (Tsuda *et al*. 1984) has overcome this problem. It is present in seminal plasma and therefore is present in the semen of azoospermic males. However, PSA can be detected at low levels in other body fluids so a PSA test should have the detection threshold set above the low levels present in other body fluids so as not to cause false positive reactions.

IFI have developed an immuno-chromatographic test strip for semen using anti-human semenogelin antibodies detecting up to 1µl human semen. No cross reactivity was seen with animal semen from the species tested or with human body fluids; blood, saliva, urine, vaginal secretions or menstrual blood (Pang and Cheung 2007b).

1.3.4 Vaginal Fluid

In the past few years identification of vaginal epithelial cells and vaginal fluid have been intensively investigated as previous identification methods used have limitations.

Vaginal fluid composition varies between individuals, and as previously stated (Section 1.2.3) can be affected by hormone levels, and therefore changes through the

menstrual cycle. The Lugol's test detects glycogenated epithelial cells using a periodic acid-Schiff (PAS) reagent to stain the glycogen within the cytoplasm purple – the intensity of colour is proportional to the number of cells. Glycogenation is variable throughout the menstrual cycle and no glycogenated cells are visible in tissue from pre or postmenopausal women excluding two sectors of the female population. The test is not specific as glycogen-containing squamous epithelial cells have also been found in the male urethral mucosa (Alm and Colleen 1982). A large volume of sample is required for this test and would destroy any potential DNA evidence within the sample (James and Nordby 2003).

The oestrogen receptor has also been investigated as a potential marker for vaginal fluid identification using immunohistochemistry with monoclonal antibodies. Although positive results were shown for vaginal mucosa samples, false positives were also shown with male urethra samples (Hausmann *et al*. 1996). The false positives would limit interpretation of the data in any sexual assault case trying to distinguish between seminal and vaginal samples.

1.4 Limitations to Presumptive Tests

All of the identification tests described yield false positives. The tests are therefore indicative and only indicate the nature of the fluid. They also tend to be destructive towards the sample being identified.

One of the most important factors to consider when developing a method of identification for use within forensic science is that it is important to preserve as much of the sample as possible for further DNA analysis since the samples submitted for analysis are often limited. Another problem is that current methods for identification only identify one fluid at a time, so the analyst has to decide which test to use or to use each test in turn thus using more sample each time. An ideal test would use a number of different reactions in parallel.

Although microscopic examination can confirm the presence of blood and sperm cells, none of the routinely used immunological tests can definitely identify the presence of human saliva or vaginal secretions (Juusola and Ballantyne 2005). This is a serious problem in sexual assault cases where it is necessary to determine if a crime has taken place.

For example in an alleged rape, a defence lawyer could propose that a female DNA profile recovered from a penile swab originated from the victims epithelial cells present within her saliva during an oral sex act, but the prosecution might argue that the DNA present originated from the victim's vaginal fluid during sexual intercourse without consent. Without a test to discriminate between saliva and vaginal fluid samples, this case would rely on personal statements. Uncertainty could lead to a reduced charge being brought by a court for an oral sex offence rather than full intercourse. Another example to consider could be if a sexual assault occurred between two individuals living in the same house where a relative of a child for example, such as a stepfather, was suspected of sexually assaulting a child. The DNA profile of the stepfather could easily be recovered from samples taken from the child's underwear and bedding. Defence arguments for this case could suggest that the DNA profile originated from innocent transfer of DNA from the father's skin cells to the child's clothing and bedding from close contact in the house. If it could be proven that the source of the DNA profile was from a semen stain there would be strong evidence in support of a sexual assault taking place (Hanson *et al*. 2009).

1.5 New Technologies for the Identification of Body Fluids

This section looks into new technologies which are currently being developed for body fluid identification. Most of these ideas exploit the need for a universal confirmatory test capable of identifying a range of different body fluids working alongside current DNA techniques. A common theme is the analysis of immunological markers.

1.5.1 Immunohistochemistry

A technique used often in pathology to distinguish between fluid samples is immunohistochemistry. The method involves incubation with antibodies to antigens specific to the cell types of interest. Immunohistochemistry has recently been successfully applied in forensics, for example in the detection of the specific sweat antigen, G-81 (Sagawa *et al*. 2003) in the eccrine sweat gland with no staining visible in other tissues tested including the apocrine sweat gland. Immunohistochemistry also successfully identified deep central nervous system tissue (Miller *et al*. 2002) on fabric from a murder case. The technique has been reported to distinguish between epithelial cells from different origins such as buccal and vaginal cells (Paterson *et al*. 2006) by detecting differential antigen expression.

Epithelial cells can be distinguished by the positive antibody-antigen reaction for Cytokeratin (CK) intermediate filaments. In this 2006 study, vaginal fluid epithelial cells were exposed to antibodies specific to the oestrogen receptor and phosphodiesterase 5 (PDE5). These antibodies were unable to uniquely identify vaginal epithelial cells from the other cells tested in this study; the oestrogen receptor was not detected within vaginal epithelial cells whereas PDE5 was discovered in buccal cells and skin cells as well as vaginal epithelial cells. A similar study involving the oestrogen receptor gave a positive reaction with male urethral swabs (Hausmann *et al*. 1996). For this work to progress further it is necessary to characterise specific fluid antigens.

1.5.2 Raman Spectroscopy

Raman Spectroscopy is a non-destructive technique and has the potential to be used as a confirmatory test for body fluid identification. It is already being used in

forensic science to differentiate between types of material fibres (Jochem and Lehnert 2002), paints (Buzzini and Massonnet 2004) and inks (Claybourn and Ansell 2000).

Raman spectroscopy uses the scattering of low intensity laser light on a sample. The advantage of this method is that a sample amount in the pictogram range can be analysed, leaving sufficient sample for STR analysis. There is little interference from water making this technique ideal for analysis of body fluids.

Five body fluids have been tested: semen, vaginal fluid, saliva, sweat and blood. The resulting spectra from each body fluid were shown to be significantly different (Virkler and Lednev 2008). Canine and human semen samples were also distinguishable by Raman spectroscopy. The technique appears promising but needs further development. Further work should examine a larger number of samples as only two donors were used. The technique is claimed to be non destructive but it has not yet been demonstrated that a full DNA profile could be obtained from the body fluid sample after Raman spectroscopy. Samples would also have to be analysed in a laboratory rather than in situ at a crime scene.

1.5.3 PCR of Oral Streptococci

Oral Streptococci have been investigated for bite mark analysis, by measuring the persistence of bacteria on the skin after a bite mark to ascertain how long the bite mark has been present (Brown *et al*. 1984)**;** this is particularly useful in determining when a crime took place or in cases of child abuse. As well as isolating bacteria present on the skin it would be important to identify the criminal from the saliva left on the bite mark using DNA profiling.

With this in mind it has recently been suggested that the same techniques could be applied to using bacteria as a marker for saliva (Nakanishi *et al*. 2009) using PCR (polymerase chain reaction). This work looked at detecting and identifying the presence of two streptococci strains, *S.salivarius and S.mutans* both of which are known to be present within saliva. The detection rates given for *S.salivarius and S.mutans* were 100% and 90% respectively. There was no cross reactivity shown with other animal salivas tested or the body fluids tested (semen, urine, vaginal fluid) or skin surface. However when using crime scene productions such as cigarettes and fabric the detection success decreased to 80% for *S.salivarius* and 60% for *S.mutans*. It would seem that from these findings *S.salivarius* showed to be more reliable for repeated detection.

Following on from this work it has been postulated that bacteria from the vagina could be a marker for vaginal fluid identification. The vaginal microbiology was shown to be unchanged after the use of oral contraceptives and the use of tampons (Morris and Morris 1967) and of the 291 non pregnant women, aged between 18-45 studied the most predominant bacteria found to be present was *Lactobacilli.* The normal bacterial flora of women differs between individuals; terminal restriction length polymorphism (T-RFLP) has been used to compare 5 women over a two month period (Coolen *et al*. 2005).

Lactobacilli were shown to be the dominant vaginal bacteria (Antonio *et al*. 1999) occurring in 71% of the 302 women examined. Where *Lactobacilli* species were isolated (71% of the 302 women) the variation in species was high with a total of 9 species being isolated. There are limited tests available to differentiate between *Lactobacillus* species thus making it hard to use this as a potential vaginal fluid marker. *Lactobacillus acidophilus* is also found to be a cause of tooth decay so could cause confusion in fluid identity by being present in saliva.

There is a difference between the vaginal flora of women of different ethnic origins (Zhou *et al*. 2007). A sample population of 3012 healthy menstruating women between the ages 13-40 years were recruited and asked to classify themselves into one of four racial groups: white, black, hispanic or asian. Vaginal swabs taken from these individuals were analysed by T-RFLP analysis of the 16S rRNA (Ribosomal Ribonucleic acid) gene. Analysis of the 16S rRNA gene is used for phylogenic study as it is highly conserved between species and contains hypervariable regions specific to individual bacterial species, acting as a signature to each species (Weisburg *et al*. 1991). Internal regions of the 16S RNA genes were amplified in two separate reactions with fluorescently labelled primer sets (set one 8fm-926r and set two 49f-926r). Primer 8fm was labelled with VIC, 49f with NED and 926r labelled with 6-FAM. Restriction digests were performed with *Msp*I and *Hae*III, as these enzymes have been shown to provide greatest resolution for bacterial populations in vaginal samples. The restriction fragments were then analysed by capillary gel electrophoresis where the labelled ends of the amplicons were detected and the resulting electropherograms analysed by cluster analysis. A clone library was developed using full length 16S rRNA amplicons. Phylogenetically related clones with a 90% or above sequence homology were presumed to be members of the same genus and those with greater than 97% homology were assumed to be the same species. *L.salivarius, Actinobaculum sp, Anaerococcus sp, Mobiluncus mulieris, Mycoplasma sp, Peptococcus niger*, and

Peptostreptococcus sp were only found in Black women and not present in Caucasian women within the study again hampering the identification of a universal marker (Zhou *et al*. 2007)

1.5.4 mRNA

A common focus for body fluid identification has been Ribonucleic Acid (RNA) the intermediate molecule between DNA in the nucleus and encoded proteins within the cell (see Figure 1.8). DNA remains within the nucleus of the cell, limiting damage to the molecule whilst mRNA transports a copy of the genetic code out of the nucleus to the cytoplasm where proteins are formed at the ribosome from tRNA (transfer Ribonucleic Acid).

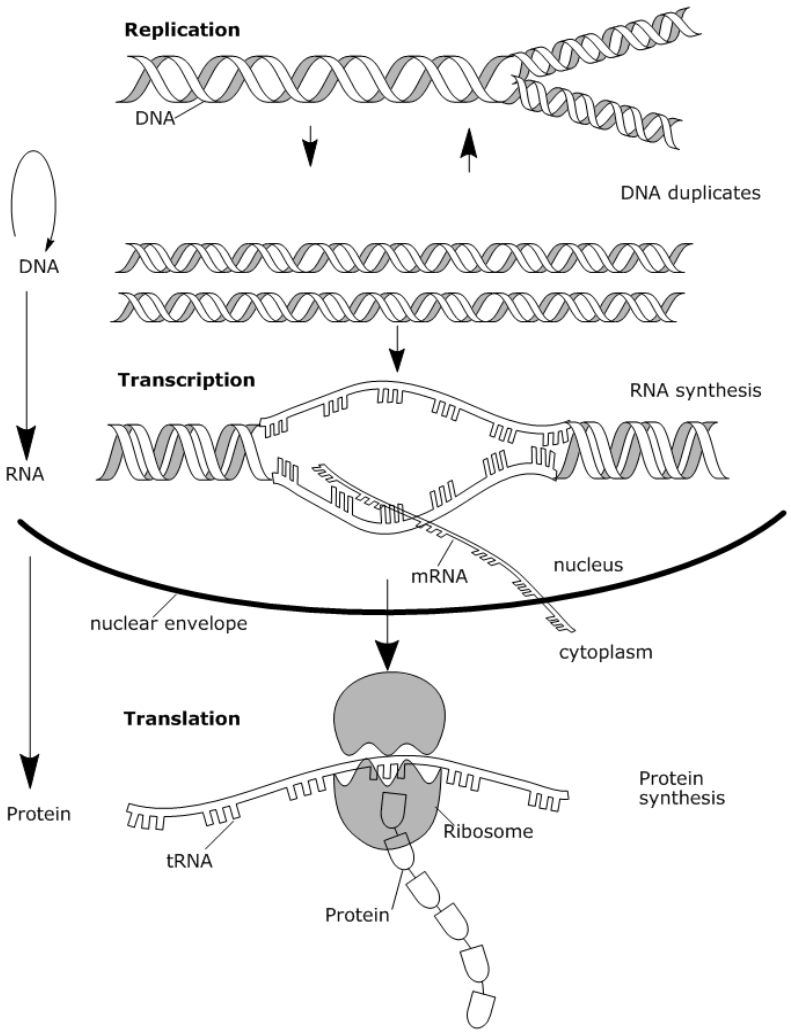


Figure 1.8 A schematic diagram of the Central Dogma showing the flow of information from DNA via RNA to protein. (adapted from Ussery, D.2000)

The genes expressed within the diverse range of tissues and fluids in the human body, are tissue specific. For example the genes expressed within the brain are different to those expressed in the blood giving them different structure and function within the body. Genes are responsible for encoding proteins, and both mRNA and proteins are also expressed in a tissue specific way.

Although gene expression is tissue specific it would be misleading to assume that changes in transcript levels of the mRNA indicate the same changes at a protein level, either in the amount expressed or the activity of the protein (Lilley *et al*. 2002). Commonly, cellular processes such as post-transcriptional splicing, translational regulation, and protein complex formation lead to mRNA and protein expression differences (Guo *et al*. 2008).

The levels of mRNA expression do not give any indications of post translational modifications shown by the proteins themselves which could lead to discovery of the protein's function within a cell type. Unlike the genome, which is constant and identical in every cell of a person's body, the proteome, the proteins actually expressed, are constantly changing in response to external environmental factors, thus different proteins are expressed in different cell types and at different times (Fey and Larsen 2001).

There are a number of groups working with mRNA expression to identify body fluid samples (Bauer and Patzelt 2002; Juusola and Ballantyne 2003; Alvarez *et al*. 2004; Fang *et al*. 2006; Nussbaumer *et al*. 2006; Zubakov *et al*. 2008; Haas *et al*. 2009). One of the major advantages of this approach is that it can be used in parallel with other current techniques by using a simple organic extraction method to co-extract both DNA and RNA. This ensures that a limited amount of sample is used in the analysis. Reverse transcription-polymerase chain reaction (RT-PCR) has been used to identify mRNA species that are cell type specific. Genes identified by each research group are summarised in Table 1.5. Ideally a method which is capable of identifying a number of body fluids in one application is preferred.

One way of achieving this would be to use a multiplex PCR, a technique where more than one pair of primers are used in one PCR reaction. This means that multiple regions of DNA or cDNA (copy Deoxyribonucleic Acid) are amplified in one reaction thus saving time and expense.

Table 1.5: Expressed genes identified as being body fluid specific

Body Fluid	*Gene ID*	*Gene name*	*Reference*
Blood	SPTB	β-spectrin	Juusola and Ballantyne 2005; Haas *et al*. 2009
	PBGD	Porphobilinogen deaminase	Juusola and Ballantyne 2005; Haas *et al*. 2009
	ALAS2	Erythroid δ-aminolevulinate synthase	Juusola and Ballantyne 2007
	HBA1	Haemoglobin alpha locus 1	Nussbaumer *et al*. 2006
	HBB	Haemoglobin beta	Haas *et al*. 2009
Saliva	STATH	Statherin	Juusola and Ballantyne 2005; Haas *et al*. 2009
	HTN3	Histatin 3	Juusola and Ballantyne 2005; Haas *et al*. 2009
	MUC4	Mucin 4	Nussbaumer *et al*. 2006
Semen	PRM1	Protamine 1	Juusola and Ballantyne 2005; Haas *et al*. 2009
	PRM2	Protamine 2	Juusola and Ballantyne 2005; Haas *et al*. 2009
	KLK3	Kallikrein 3	Nussbaumer *et al*. 2006;
	SEMG1	Semenogelin1	Fang *et al*. 2006
	SEMG2	Semenogelin2	Fang *et al*. 2006
	TGM4	Human prostate transglutaminase (type IV)	Fang *et al*. 2006
	MCSP	Human mitochondrial capsule selenoprotein	Fang *et al*. 2006
	PRB4	Proline-rich protein BstNI subfamily 4	Fang *et al*. 2006
Menstrual Blood	MMP-7	Matrix metalloproteinase 7	Juusola and Ballantyne 2005; Haas *et al*. 2009
	MMP-10	Matrix metalloproteinase 10	Juusola and Ballantyne 2007
	MMP-11	Matrix metalloproteinase 11	Bauer and Patzelt 2002; Haas,*et al*. 2009
Vaginal fluid	HBD-1	Human beta-defensin 1	Juusola and Ballantyne 2005; Haas *et al*. 2009
	MUC4	Mucin 4	Juusola and Ballantyne 2005; Haas *et al*. 2009
	ESR1	Oestrogen receptor 1	Fang *et al*. 2006

A multiplex assay comprising nine genes has been used to detect blood, saliva, semen, vaginal fluid and menstrual blood (Juusola and Ballantyne 2005). This assay was extended with the replacement of the blood gene porphobilinogen deaminase (PBGD) by erythroid δ-aminolevulinate synthase (ALAS2) and the addition of a menstrual blood gene, matrix metalloproteinase 10 (MMP 10) (Juusola and Ballantyne 2007) as well as a positive control primer set for the housekeeping gene Glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Successful endpoint PCR amplification was observed with the multiplex primer set reported by Juusola and Ballantyne (Haas *et al. 2009*).

Epigenetic events give rise to PTM (post translational modifications). Most proteins within the cell have some degree of PTM that determines the localisation or function of the protein and these modifications would not be detected by DNA or RNA based methods. It is also possible to determine the number of isoforms of a protein but this too cannot be determined from RNA based methods.

An additional problem of identifying body fluids/tissues by mRNA expression is raised by the methodology. Haemoglobin is a known inhibitor of Taq polymerase and blood samples submitted to forensic laboratories are routinely washed with ultrapure water to remove haemoglobin prior to DNA analysis. To remove haemoglobin completely is very expensive as it would involve complete protein removal compromising the amount of RNA/DNA available for analysis. Humus from soil is another well-known polymerase inhibitor and a frequent contaminant of forensic samples.

Using PCR as a potential method for body fluid identification could mean that inhibition problems are encountered in the discovery of primer sets for blood samples.

Because of the major problems of expense and poor reliability associated with performing RT-PCR, it was decided that proteomics could lead to more reliable, faster and cheaper methods for body fluid/tissue identification.

1.6. Proteomics

Proteomics is the large-scale analysis of proteins (Pandey and Mann 2000) or the proteome. The proteome or protein complement of a cell or tissue consists of all the proteins that are expressed within it. It is a representation of protein expression within a cell or tissue sample at a particular time and under a set of particular environmental conditions (Fey and Larsen 2001). Proteins can be separated in one dimension (1D), by molecular weight, although this is a simple technique many proteins will have the same molecular weight hence limiting the separating power of this method. Separation in two dimensions (2D), firstly by isoelectric point (p*I*) and then by molecular weight is a more powerful technique that has been in use since the 1970s (O'Farrell 1975) to separate proteins, each protein will reside as a single spot on a gel. The typical workflow employed in Proteomics is shown in Figure 1.9.

Initially proteins are isolated from their biological source. Separation of proteins by gel electrophoresis (GE) is performed either in one dimension (1DGE), separated by molecular weight or in two dimensions (2DGE), firstly, separated by p*I* and secondly by molecular weight. Protein spots are cut from the gel and trypsinised into peptides. Peptides are separated by liquid chromatography (LC) and sequenced by tandem mass spectroscopy (MS/MS). Once separation and sequence data has been obtained online databases can then be used for protein identification.

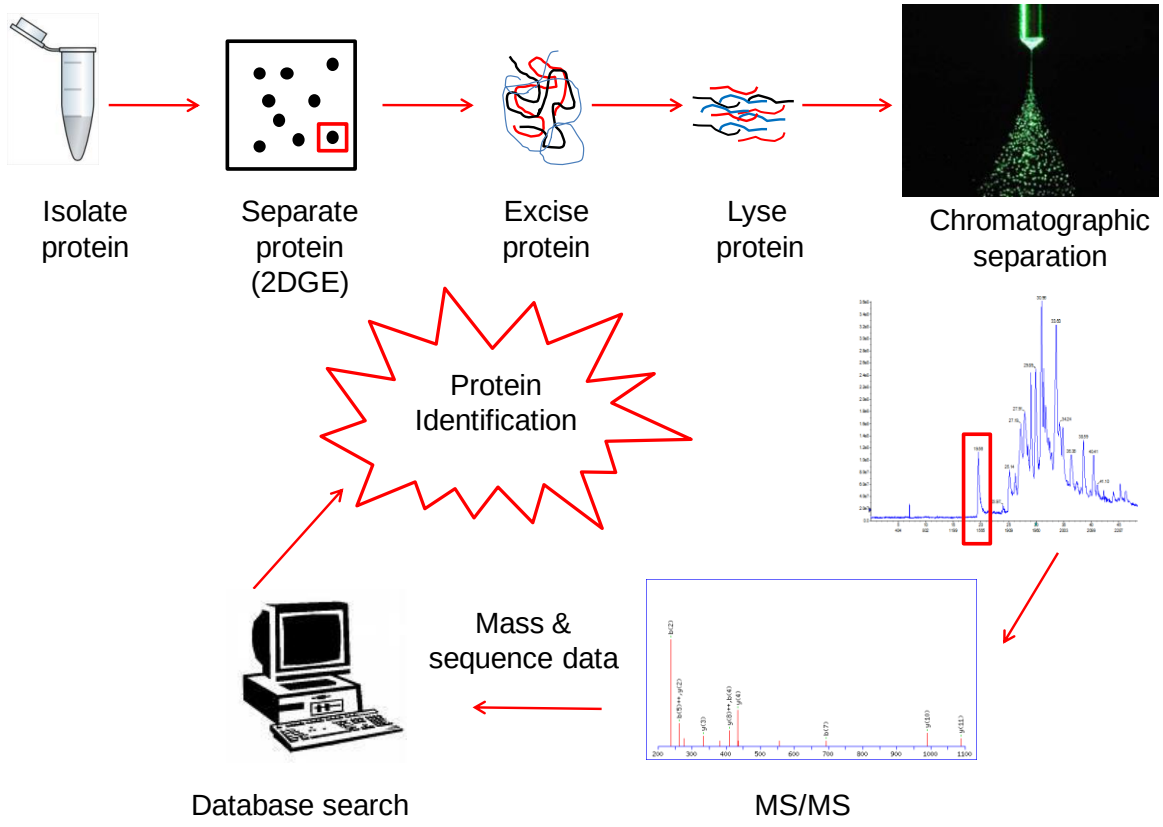


Figure 1.9: A schematic diagram representing each stage of a typical proteomics experiment using 2DGE and LC-MS/MS. Image adapted from Collins *et al*. 2006 and Precision combustion Inc. 2008.

The objective of proteomics is to identify and quantify proteins within a specific body fluid, cell type or tissue as well as looking at expression changes between two states of that sample type (Bernova-Giorgianni 2003) in order to gain a greater understanding of protein function within that sample or its role in a particular disease.

Proteomics has traditionally been used for a comparison of protein expression levels between two sample types i.e. diseased or non diseased tissue, or a sample pre and post treatment with a drug, to study protein-protein interactions (Pandey and Mann 2000).

A disadvantage of the technology is that it is not easily automated. Unlike mRNA technologies, 2DGE is reliant on the operator for sample preparation and gel set up, whereas post electrophoresis staining and spot analysis can be automated and robots are routinely used for these stages of analysis. Proteomics has benefited greatly from the genomic and bioinformatics eras, with the development of large databases that are accessible over the internet. These databases contain p*I,* molecular weight and protein sequence data to facilitate the identification of protein spots.

Some projects such as the Human Proteome Organisation (www.hupo.org) specifically focus on discovery of proteomes. So far their work has looked into characterisation of the human plasma proteome (Anderson *et al*. 2004) with ongoing research into the human liver and brain proteomes. It has been suggested that the study of body fluids would generate potential biomarkers for disease. Body fluids such as blood plasma contain secreted proteins. If a tissue expressed or secreted a protein characteristic of a particular disease it might be found with body fluids that are either in contact with that tissue or that pass through it.

So far there has not been any published work to date using proteomics as a method for forensic body fluid identification although data has been published using 2DGE with body fluids in other scientific areas.

For instance the sweat specific protein, G81 has been identified and excluded from other body fluids tested including saliva, semen and plasma (Sagawa *et al*. 2003) by 2DGE followed by Western blotting with G-81 antibody. Western blotting is a technique used to detect proteins separated by electrophoresis and then transferred to a nitrocellulose membrane. Proteins are identified using labeled antibodies. Interestingly, there was no cross reactivity with samples from other sweat producing mammals tested such as horses, donkeys and other primates.

1.6.1 Saliva

Some research groups have looked at the possibility of characterising the saliva proteome. Three hundred proteins were identified from one human donor's saliva (Hu *et al*. 2005). Whereas other research groups, identified 202 spots (Huang 2004) or 600 spots (Ghafouri *et al*. 2003). These differences could be accounted for by the length and pH range of the IPG (immobilised pH gradient) strips used for the 1st dimension separation along with the size of the gels the samples were run on in the second dimension. The longer the IPG strip the greater the separation of proteins and also the larger the gel the greater the separation. Some samples may contain more acid proteins in which case a strip with pH range 3-6 would give better separation than a strip with pH 5-8, which would be more suitable for a sample containing more basic proteins. For forensic purposes an appropriate pH range would need to be identified in order to resolve a maximal number of protein spots within the samples studied.

2D proteomics could be applied to the research problem addressed within this thesis. In this research project, it is important to investigate proteins that are expressed

population wide, therefore it would not be suitable to study samples provided by one person as reported by Hu *et a*l (2005).

Many factors affect the protein profile of saliva. Dietary influences and an individual's age have an impact on the composition and volume of saliva (Aps and Martens 2005), implying a wide variation between individuals. Each person may express a slightly different protein profile within the saliva proteome especially as we know that protein expression is in a state of dynamic flux – changing in response to external and internal stimuli. For this reason proteomics has been used to discover potential markers of disease. It would therefore not be suitable to base a body fluid identification tool upon one individual samples protein complement. A collection of samples would be required to be representative of the population in order to ensure that any proteins that are selected for use are characteristic of the human population and not unique to one or a group of individuals.

Two of the major proteins in saliva are amylase with a molecular weight of approximately 58 kDa and a p*I* of 6.47 (Hu *et al*. 2005) and IgA, with a molecular weight of approximately 51 kDa and a p*I* 0f 7.87 (Hu *et al*. 2005). Amylase is currently being used in forensic tests for the presumptive identification of saliva. To establish a confirmatory test it is necessary to identify proteins specific to saliva. Statherin and histatin have been studied as potential salivary marker proteins using mRNA techniques (Juusola and Ballantyne 2005).

1.6.2 Vaginal Fluid

The proteomic study of vaginal fluid has been limited so far, to analysing samples donated by pregnant women. Research has been focussed on searching for biomarkers within cervical-vaginal fluid (CVF) to monitor maternal and foetal health during pregnancy (Dasari *et al*. 2007).

The early proteomics of vaginal fluid looked at 1D SDS-PAGE. Four protein bands were observed at 79 kDa, 67 kDa, 58 kDa and 52 kDa which were thought to be transferrin, albumin, IgA and IgG respectively (Itoh and Manaka 1988). SDS-PAGE only separates proteins according to molecular weight so the observed bands could be comprised of more than one protein with the same molecular weight. Higher resolution methods have identified a greater number of proteins within the fluid (Klein *et al*. 2008, Di Quinzio *et al*. 2007 and Tang *et al*. 2007).

Shotgun proteomics using LC-MS/MS without an initial gel separation method has been used to identify 40 vaginal fluid proteins from women in late pregnancy (Klein *et al*. 2008). A major limitation of this study was that only seven pregnant donors were used so they were not representative of the general population. An initial 2DGE experiment was performed to show the distribution of vaginal fluid proteins but as vaginal fluid protein levels were low the gel images did not show much detail in the protein expression.

Recent studies using 2DGE have been investigated using samples from pregnant women (Di Quinzio *et al*. 2007). 400 protein spots were detected and of these 15 spots were identified as common to the 5 women donors. The number of donors used in this study did not give an accurate representation of the population. Another study reported on samples donated by 29 non-pregnant women (Tang *et al*. 2007). The total number of protein spots detected was not noted but 147 proteins were identified. No attempt was made in any of these studies to determine characteristic proteins that are not found in saliva

1.7 Mass Spectroscopy (MS) and Proteomics

Mass Spectroscopy (MS) is used to accurately identify protein spots by the determination of the ion masses. Traditionally Matrix-Assisted Laser Desorption/Ionisation- Time of Flight (MALDI-TOF) mass spectroscopy has been used for proteomic applications. Recent developments have seen tandem Mass spectroscopy using an electrospray ionisation (ESI) source with a triple quadrupole mass analyser being used increasingly for proteomic analysis. MS consists of an ion source (ESI or MALDI), a mass analyser and a detector (Aebersold and Mann 2003). The ion mass-to-charge ratio (*m/z)* is measured.

The power of MS alongside proteomics has developed in-line with the continual growth of gene and genome databases.

1.7.1 Matrix-Assisted Laser Desorption/Ionisation - Time of Flight (MALDI-TOF)

Until very recently MALDI-TOF was used to measure the mass of intact peptides. Peptide-mass fingerprinting, as it is more commonly known, identifies a protein by its specific peptide mass. Proteins are identified by matching a list of experimental peptide masses to a list of calculated masses within an online protein database (Aebersold and Mann 2003). The protein sample is crystallised along with the matrix onto a MALDI plate. MALDI ionisation is a soft form of ionisation, and does not penetrate the sample itself; most of the energy is absorbed by the matrix. Desorption occurs at the MALDI plate causing the matrix to become ionised (Figure 1.10). Proton transfer from the matrix to the sample causes ionisation of the sample. Due to the acidic crystalline nature of the matrix the ions produced have a positive charge.

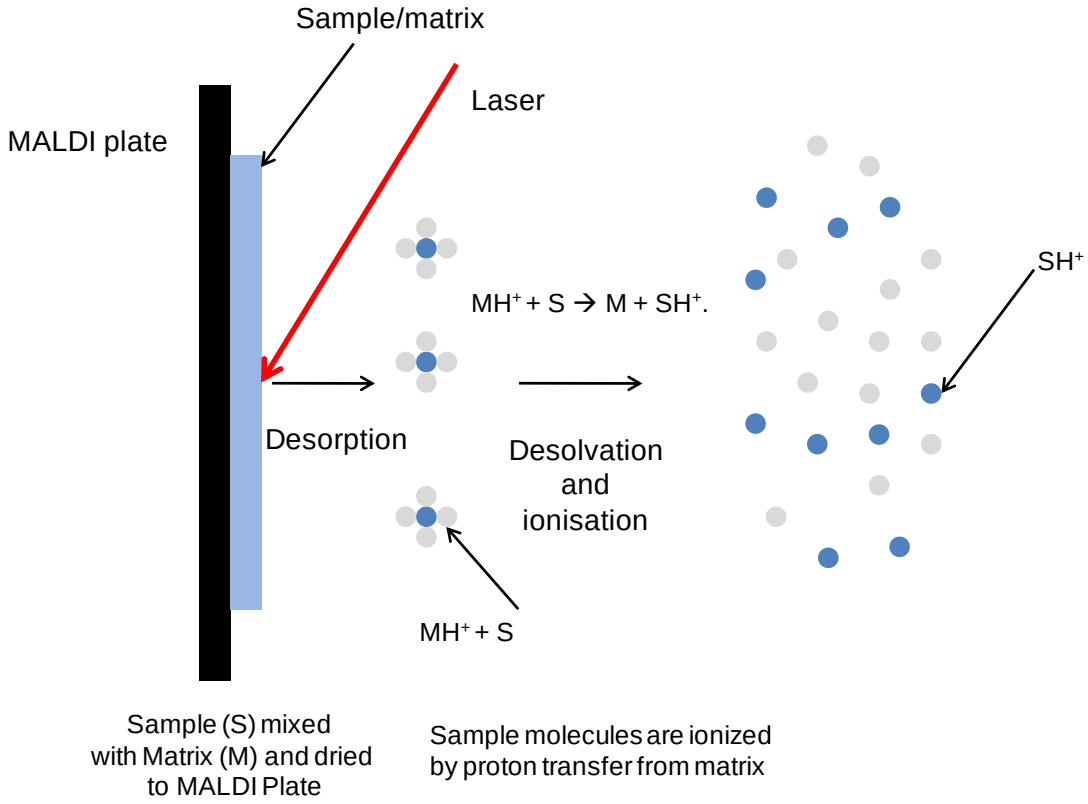


Figure 1.10: A schematic diagram representing matrix assisted laser desorption and ionisation adapted from Ashcroft, A.E. 2009.

The positive sample ions are accelerated through an electric field (Figure 1.11); the time the ion is trapped (takes to travel to the detector) is proportional to its *m/z* ratio. Ions with a large *m/z* (represented by red squares in Figure 1.11) will travel more slowly through the Time of Flight analyser than ions with a smaller *m/z* (represented by green triangles in Figure 1.11) (Smith and Busch 1999).

One drawback of this method for analysis is that it is difficult to automate because the sample must be mixed with the MALDI matrix. The result has been progress toward using a liquid chromatography approach coupled to MS/MS which can be easily automated for high throughput proteomic analyses.

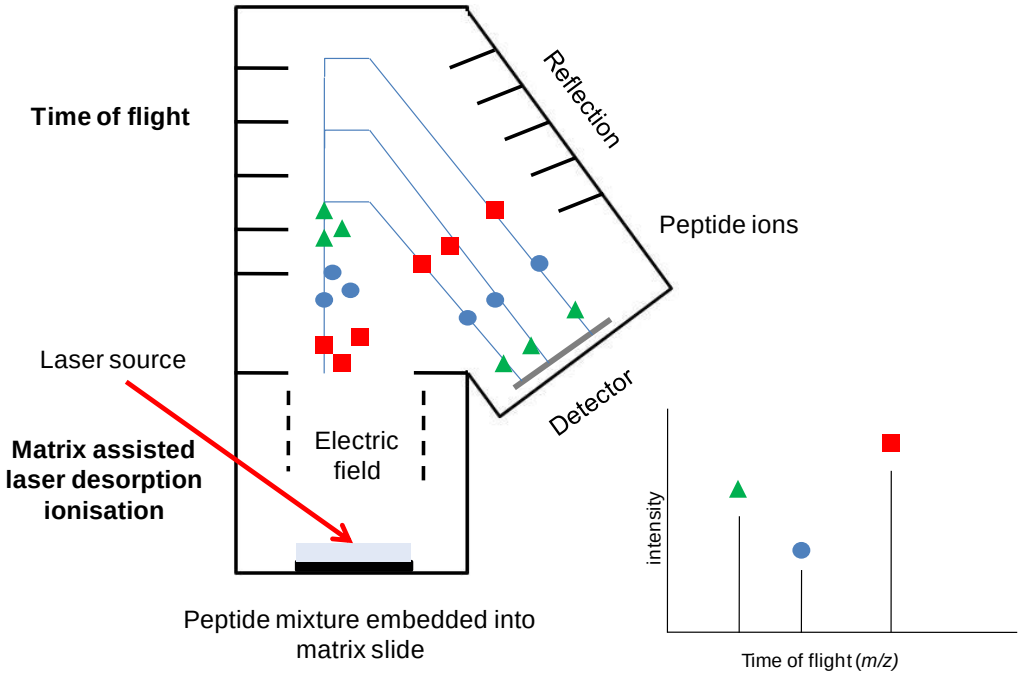


Figure 1.11: A schematic diagram to show how a MALDI-TOF mass spectrometry operates, and thus showing a representation of resultant mass spectra adapted from Nijmegen Proteomics Facility [online].

1.7.2 Liquid Chromatography Coupled to Tandem Mass Spectrometers LC-MS/MS

LC MS/MS gives better discrimination than MALDI-TOF for protein identification both ionisation pattern and peptide sequence are identified. It can be set up for automatic injection enabling 24 hour use. The method uses HPLC linked to a triple quadrupole mass analyser in combination with an electrospray/nanospray ion source (Figure 1.12). The protein of interest is initially cleaved with trypsin to produce a mixture of peptides which are easily ionised in the mass spectrometer. These peptides are then separated on HPLC and injected directly into the Mass spectrometer via a fine needle (electrospray). In the first quadrupole (Q1) a precursor ion consisting of a single peptide is selected based on its *m/z* and transferred to the second quadrupole (Q2) where it is subjected to collision induced dissociation (CID). Under low energy conditions the peptide ions fragment in predictable patterns (Delahunty and Yates 2005).

The fragmented peptides are analysed by the third quadrupole (Q3) to produce the product ion mass spectrum. The fragmentation conditions ensure cleavage of peptide bonds so the product ion spectrum is a representation of each amino acid in the peptide chain. The spectral peak pattern can be used to give information about the

peptide sequence in addition to obtaining the peptide masses as with MALDI-TOF analysis.

It is possible to ascertain theoretical spectra sequences within databases based on these predicted patterns. The spectra can then be searched with the fragmentation patterns produced by the mass spectrometer using computational algorithms. Protein identification is made using probability based matching between the experimental data and the theoretical data within the database. A score is given based on the significance of the match.

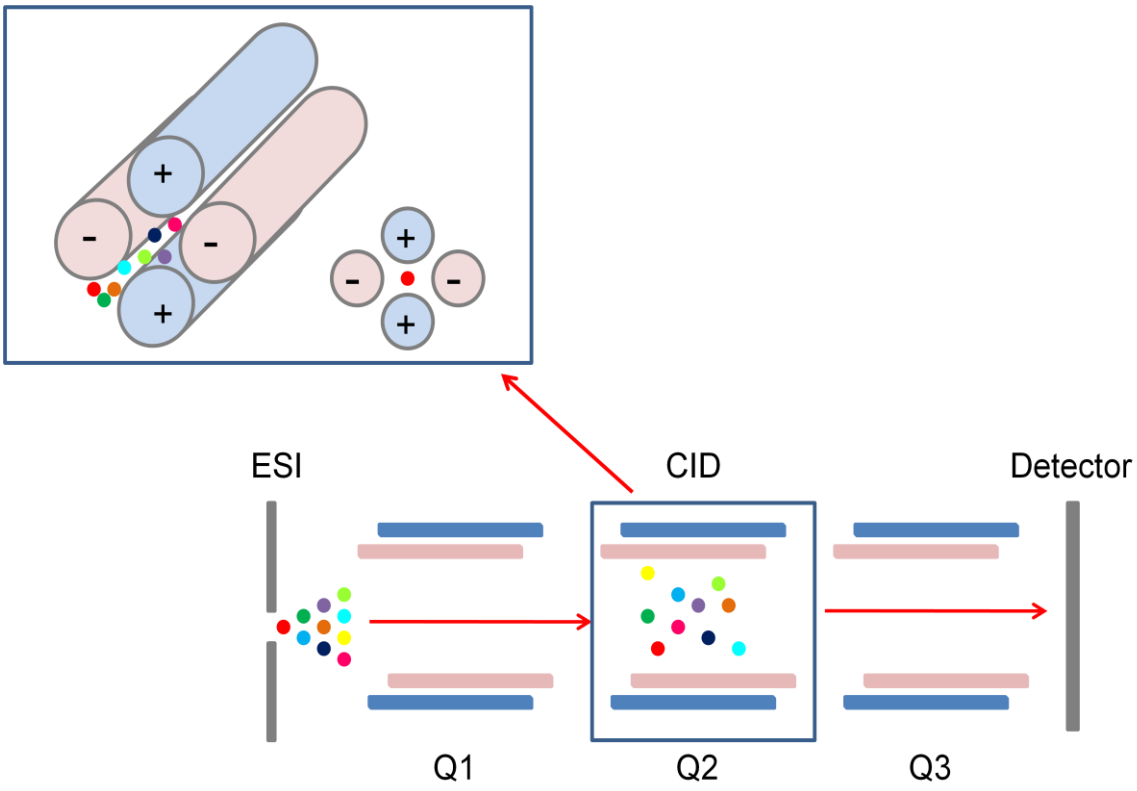


Figure 1.12: A schematic diagram to show how a LC-MS/MS operates adapted from Gates.P. 2009 [online].

1.8 Confirmatory Methods for Protein Identification

When putative protein identities have been obtained from Mass spectroscopy data it is necessary to confirm that the MASCOT database search correctly identified the protein of interest. Immunological techniques are commonly used as specific antibodies can confirm the presence of the protein and be used to determine quantitatively or qualitatively its expression in a given sample. The techniques most commonly used for this are ELISA (Enzyme Linked Immunosorbent Assay), dot blots and Western blots.

1.8.1 ELISA

ELISA is a technique used to quantitatively determine the presence of an antigen within a sample. It is usually performed in a 96 well microtitre plate alongside relative standards of known concentrations of the antigen of interest. The mechanism of the reaction (Figure 1.13) is based upon antibody-antigen interactions. An antibody specific to the protein of interest is immobilised onto the wells of the microtitre plate. When test samples are added to the microtitre plate, antigens in the sample will bind to the antibodies in the wells of the plate if the protein of interest is present within the sample. Unbound molecules are removed from the plate by washing and the plate is blocked to prevent further protein binding to reactive groups other than those of the antigen. A second antibody specific to another epitope of the sample antigen is then added to the plate. The second antibody is linked to an enzyme that catalyses a colour producing reaction, such as alkaline phosphatase or horseradish peroxidise. Both enzymes are capable of rapidly converting molecules of a colourless or non-fluorescent substrate into a coloured or fluorescent product. The amount of second antibody bound is directly proportional to the amount of antigen captured by the primary antibody. Hence by measuring the coloured or fluorescent signal with a spectrometer it is possible to determine quantitatively the amount of antigen in the sample. Controls lacking antigen are used so that cross-reactivity of antibody can be corrected for.

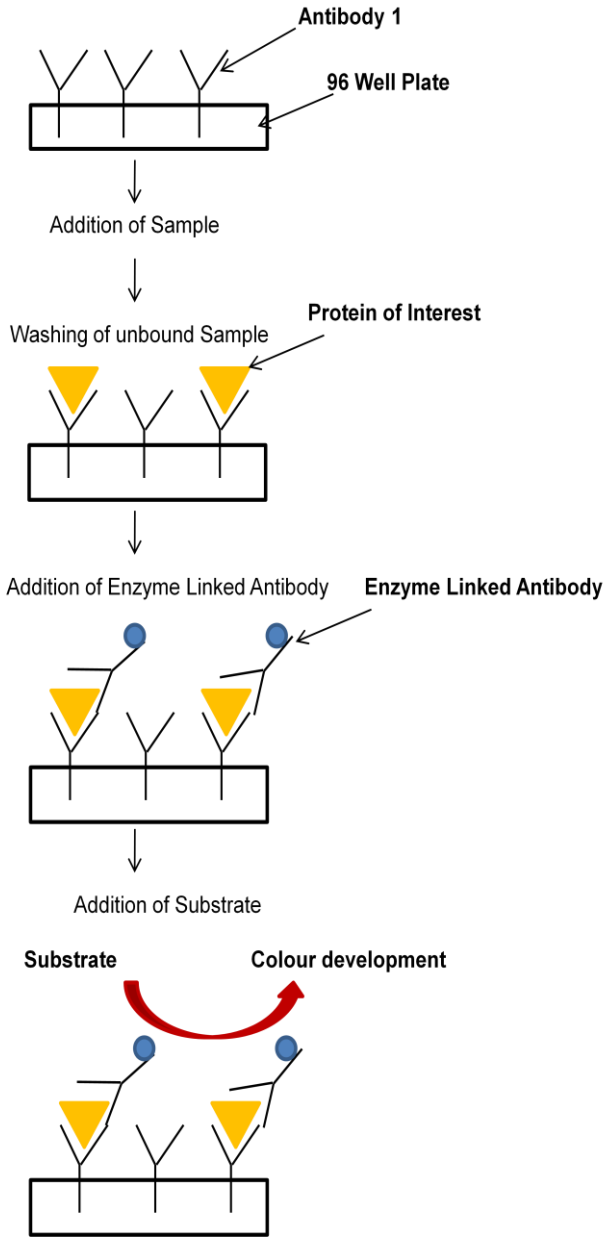


Figure 1.13: A schematic diagram to show the key steps of an ELISA reaction.

1.8.2 Dot Blot

Dot blots or slot blots (depending on the shape of the blot) can be used to identify DNA, RNA or proteins. The dot blot and the Western blot are based upon the same methodology of primary and secondary antibodies (Figure 1.14). The sample to be analysed is loaded directly to a membrane unlike a Southern, Northern or Western blot where the sample is separated by electrophoresis prior to blotting. Dot blots for protein identification are quicker and simpler than Westerns but do not identify individual proteins in a mixture. Without prior electrophoretic separation it is difficult to

ascertain whether the staining shown on the dot blot is due to cross reaction with other proteins in the mixture. After membrane blocking, the sample spots are incubated with antibodies specific to the antigen of interest (primary antibody), washed to remove any unbound antibody and then incubated with a antibody specific to the primary antibody (secondary antibody). The secondary antibody is enzyme linked, usually with alkaline phosphatase or horseradish peroxidise, Once again after incubation the membrane is washed to remove unbound antibody. Finally the membrane is incubated with a substrate that produces a coloured product, catalysed by the enzyme. The coloured product is directly proportional to the antigen of interest present in the sample.

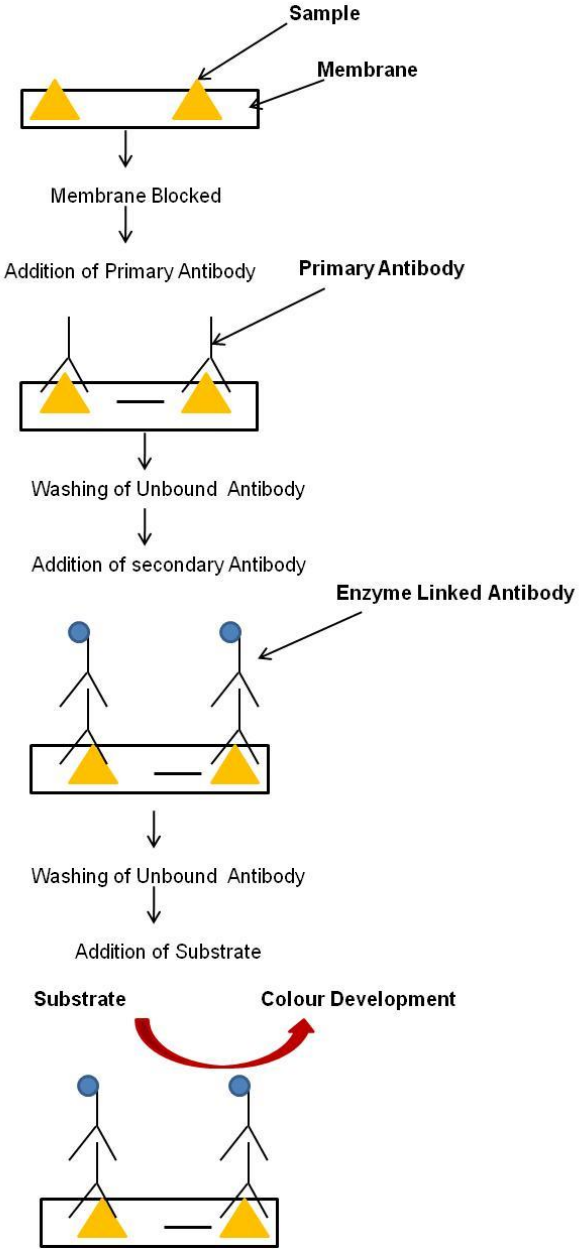


Figure 1.14: A schematic diagram to show the key steps of a Dot Blot or Western blot experiment.

1.8.3 Western Blot

A Western blot is a technique used after electrophoretic separation of proteins based on size to transfer them to a nitrocellulose or nylon membrane. The blot involves incubation of the membrane with primary antibody as with the dot blot, and then with a secondary antibody specific to the primary antibody. This secondary antibody is enzyme linked, common enzymes used are horseradish peroxidise and alkaline phosphatase. When the membrane is incubated with a substrate, a signal proportional to the amount of antigen present is produced. The enzyme of the secondary antibody catalyses a reaction, which could be result in an insoluble coloured product (colourmetric), or a product which emits light by excitation at a particular wavelength (fluorescence) or simply emits light as a result of the chemical reaction itself (chemiluminescence). Densitometry of the detected bands can be used for quantification of the antigen in the tested samples. A major advantage of Western blotting is that due to the separation step bands can be checked for the correct molecular weight.

1.9 Future Technological Advances for Body Fluid Identification.

If a protein is confirmed by MALDI-TOF or LC-MS/MS and found to be consistently and specifically present in a body fluid, it could be used in the unambiguous identification of body fluids. The next stage would be to exploit the antibody-antigen reaction by either creating a specific ELISA or to immobilise specific antibodies onto membranes and create a test strip which could be easily transported to a crime scene.

Lateral flow immunographic strips that immobilise specific antibodies into a cassette form have been developed for blood, saliva, semen and urine (see chapter 3) over the last two years. The advantage of the strips is that they can be taken directly to crime scenes or used within a laboratory setting. Results are obtained in a few minutes. There are however, problems with the current technology. The cassettes are only available for a few body fluids and the signal produced by the strips is only qualitative. Also, one test strip only identifies one antigen and hence one body fluid thus in order to definitively identify a fluid, more than one test kit may be required. If more than one type of kit is needed the costs are increased as well as the time taken to perform the tests. Ideally a platform capable of testing for a range of body fluids in one strip is needed.

Immunographic lateral flow test strips have been produced to test for a panel of six drugs using one sample of saliva (Medimpex United Inc.). This idea could be exploited for body fluids combining the individual test kits into one multi-body fluid test. The number of antibody lines that could be physically placed onto a lateral flow strip would limit the number of body fluids that could be identified in one hit. An alternative would be a microarray with antibodies to a range of proteins. Fluorescently labelled antibodies are deposited onto a glass surface via an attachment layer such as agarose. Due to the size of the protein arrays thousands of antibodies can be spotted onto one chip thus more than one target antibody per body fluid could be used, increasing the strength of any body fluid identifications made. The use of glass slides as a platform for antibodies has led to standard DNA microarray equipment (including detection scanners) being compatible with the protein arrays. As technology improves, protein arrays are becoming more commonly used in diagnostic applications. This more sophisticated approach would provide a quantitative as well as a qualitative approach

which could be advantageous if a mixture of body fluids was found at a scene. The method would also indicate the relative amounts of each body fluid.

1.10 Aims of Research

The current problems of human body fluid identification have been considered when addressing the aims of this study. The work presented in this thesis aims to combine knowledge of forensic science and biochemistry to develop a new technology capable of identifying a range of body fluids without some of the current disadvantages.

This study focuses on two body fluids, saliva and vaginal fluid, which are frequently encountered in sexual assault cases. Current body fluid identification methods are only capable of identifying saliva. There is no current method for vaginal fluid identification.

Published studies have used an mRNA approach to identify body fluids using RT-PCR. This thesis describes the development of a proteomics based technique to separate proteins within human body fluids. The proteomics approach enables the identification of changes at the protein level clearly by ascertaining whether or not a protein is expressed in one body fluid sample separated on a 2D gel compared with another sample. As this approach has not previously been used to distinguish body fluid components for identification optimisation of the methodology was necessary.

The aims of the thesis are therefore:

(i) To compare and asses three of the available methods of saliva identification currently used in Forensic science laboratories.

(ii) To develop and optimise an analytical technique for the separation and analysis of saliva and vaginal fluid proteins.

(iii) To identify protein biomarkers of saliva and vaginal fluid by 2DGE and LC-MS/MS.

(iv) To determine individual variation in biomarker levels

(v) To confirm protein identification by immunological methods.

1.10.1 Thesis Structure

The Thesis has an experimental methods section describing all the methods used in this study (Chapter 2). Following this Chapters 3-6 have a similar structure with a short introduction, results and discussion. Chapter 3 assesses the current techniques available for saliva identification. Chapter 4 looks at the proteomics methods used in this study and the steps that were taken to optimise those methods. The results of this study are split into two chapters, firstly looking at expression analysis of the saliva and vaginal fluid samples as well as identification of protein biomarkers from each fluid (Chapter 5) whilst the confirmation of biomarker specificity is addressed in Chapter 6. Finally a discussion of biomarker suitability is addressed in Chapter 7 as well as future work and a conclusion.

Chapter 2
Materials and Methods

Chapter 2 – Materials and Methods

All reagents were supplied by Fisher Scientific, UK unless otherwise stated

2.1 Reagents and Suppliers

12 cm square disposable weighing boats
2-D SDS-PAGE protein standard (Bio-Rad®)
30 ml plastic sample containers
7 cm focussing trays (Bio-Rad®)
7 cm reswelling trays (Bio-Rad®)
7 ml Bijoux tubes
Acetone HPLC grade
acetonitrile
30% acrylamide/bis (Sigma®)
Ammonium bicarbonate
Ammonium persulphate
Anti-goat IgG Antibody (Sigma®)
Anti-Mouse IgG Antibody (Sigma®)
Bio-Lyte 3-10 buffer (Bio-Rad®)
Bovine Immunoglobulin G (IgG) 1.45 mg/ml (Bio-Rad®)
Bovine Serum Albumin (BSA) 1.5 mg/ml (Bio-Rad®)
Bromophenol blue
CanAg SCC (Squamous cell carcinoma) EIA kit (Fujirebio™)
comprising:

- *Streptavidin Coated Microplate*
- *SCC Calibrators*
- *Biotin Anti-SCC*
- *Tracer, HRP Anti-SCC*
- *TMB HRP-Substrate*
- *Stop Solution*
- *Wash Buffer (25x)*

CHAPS
ChemiDoc™ EQ (Bio-Rad®)
Cotton swabs

Dithothreitol	
Eppendorf tubes	
Ethanol laboratory reagent grade	
Flamingo Fluorescent Gel stain	(BioRad®)
Formic acid	
Glacial acetic acid analytical reagent grade	
Glycerol	(ACROS Organic, Belgium)
Glycine	(ACROS Organics, Belgium)
Goat anti-human Cystatin SN Antibody	(R&D systems)
Goat anti-human Lipocalin -1 Antibody	(R&D systems)
Goat anti-human SerpinB1 Antibody	(Santa Cruz Biotechnology)
Gold trypsin	(Promega)
Human Serum Albumin protein	(abcam®)
Hybond-P membrane	(GE Healthcare, UK)
HYBRI-SLOT™	(GIBCO BRL)
Hyudroxycinnamic acid	
Instant dried skimmed milk	(Somerfield)
Iodoacetamide	(ACROS Organics, Belgium)
LABOPORT® N810 FT.18 vacuum pump	(KNF Neuberger, Germany)
MassPrep handling station	(MicroMass)
Methanol analytical grade	
2-mercaptoethanol	
Mineral oil	(Bio-Rad®)
Mini PROTEAN® tetra tank	(Bio-Rad®)
Mini gyro rocker Stuart SSM3	
MRX® II microplate reader	(Dynex Technologies, Worthing)
Mouse anti-human Cystatin SA Antibody	(R&D systems)
Paper wicks	(Bio-Rad®)
PharosFX™ molecular imager	(Bio-Rad®)
Phosphate buffered saline	(Oxoid)
Phosphoric acid	(ACROS Organics, Belgium)

PlusOne™ silver staining kit; comprising: (GE Healthcare, UK)

EDTA-Na_2.2H_2O

Formaldehyde

Gluteraldehyde

Silver nitrate solution

Sodium acetate

Sodium carbonate

Sodium thiosulphate

PowerPac® 200 (Bio-Rad®)

Protean® IEF cell (Bio-Rad®)

Purified Human IgG (R&D Systems)

Q-Trap triple quadrupole mass spectrometer (Applied Biosystems/MDS Sciex, Warrington, UK)

Recombinant Human Cystatin SA (R&D Systems)

Recombinant Human Cystatin SN (R&D Systems)

Recombinant Human Lipocalin-1 (R&D Systems)

Recombinant Human SerpinB1 (Abnova)

RC DC Protein Assay; comprising: (Bio-Rad®)

DC Reagent A: alkaline copper tartrate

DC Reagent B: dilute folin reagent

DC Reagent S: sodium dodecyl sulfate

RC reagent I (contains Universal Protein Precipitation Agent-I (UPPA-I))

RC reagent II (contains Universal Protein Precipitation Agent-II (UPPA-II))

Readystrip™ IPG strips 7 cm pH 3-10NL (Bio-Rad®)

Readystrip™ IPG strips 7 cm pH 3-6 (Bio-Rad®)

Readystrip™ IPG strips 7 cm pH 4-7 (Bio-Rad®)

Readystrip™ IPG strips 7 cm pH 5-8 (Bio-Rad®)

Rotary Evaporator (Rotovap)

SameSpots Prodigy™ software (Nonlinear Dynamics, Newcastle, UK)

SDS

SDS-PAGE Molecular Weight Standards, Broad Range (Bio-Rad®)

Sero-wel® Microtitre plate (Bibby Sterilin)

SIGMA*FAST*™ 3,3'-Diaminobenzidine tablets (Sigma®)

Sodium Chloride

Spin baskets

SYPRO® ruby protein gel stain	(Invitrogen™)
TCA	
TEMED	(National diagnostics)
Trifluoroacetic acid	
Tris base	
TWEEN-20	
UltiMate nanoLC system	(LC Packings, Camberley, Surrey)
Unstained precision plus protein™ standards	(Bio-Rad®)
Urea	(ACROS Organics, Belgium)
UV/white trans-illuminator	(Vilber Lourmat)
Vivapure®Anti-HSA kit; comprising:	(vivascience)
HSA-affinity resin (50% slurry)	
Binding Buffer	
Zinc-α-2-glycoprotein ELISA kit; comprising:	(Biovendor)
Antibody coated microtiter strips	
Conjugate Solution	
Dilution Buffer Concentrate (2x)	
Human Zinc-α-2-Glycoprotein standard	
Quality controls – High and Low	
Substrate Solution	
Stop Solution	
Wash Solution Concentrate (10x)	

2.2 Sample Collection

Saliva and vaginal fluid samples (25 of each) were donated by volunteers living in the Aberdeen area and attending the Golden Square sexual health clinic. Patients visiting the clinic elected whether to participate in the study after reading an information sheet provided by myself (Appendix I and II) or a clinic nurse. Informed consent was obtained from all donors prior to sample donation (Appendix III and IV). No further selection of donors was made, in order that samples were representative of the Aberdeen population. Donor's sex, age, time the sample was taken and the time since the donor last ate or drank were noted for all saliva samples. For vaginal fluid samples the donor's age, the stage of menstrual cycle, when the sample was taken and the form of contraceptive the donor was using were noted (shown in Table 2.1). Vaginal fluid samples were not taken from donors during menses in order to avoid potential contamination by menstrual blood. Semen, menstrual blood, blood and breast milk samples were obtained for comparison and optimisation experiments for these reasons only one donor was used in each case.

Table 2.1 A table to show the donor characteristics recorded for samples taken.

Sample type	***Donor characteristics***			
Saliva	Sex	Age	Time sample taken	Time since donor last ate or drank
Vaginal fluid		Age	Stage of menstrual cycle	Donor's form of contraceptive

The method of sample collection was dependent upon the fluid type. Saliva samples were collected in 7 ml plastic Bijoux tubes with approximately 3 ml saliva per donor. Vaginal fluid was collected onto cotton swabs (2 swabs per donor) from source, no samples were discounted for analysis by visual inspection. Additional samples of blood, menstrual blood, semen and breast milk were collected for comparative purposes, using one donor for each sample type and informed consent was obtained as before. Blood was collected by finger puncture and deposited onto a swab head and as with vaginal fluid, menstrual blood was also collected onto a swab head directly from

source. Semen ejaculate was collected by masturbation into a 30 ml plastic container and breast milk was expressed into a 30 ml plastic container. After collection samples were stored at -20°C until required.

2.3 Sample Preparation

2.3.1 Saliva

The preparation of the samples depended on whether they were to be used for optimising the methodology or for the comparative results stage of this research (see Figure 2.1). For optimisation each saliva sample was centrifuged at 12000 x g for 5 minutes at room temperature, to remove debris from the sample, and 300 µl of the saliva supernatant was then transferred to Eppendorf tubes and precipitation performed (see section 2.4). For comparative results each saliva sample was centrifuged as above and then pooled (see section 2.3.3) prior to precipitation.

2.3.2 Vaginal Fluid

The preparation of the vaginal fluid samples also differed depending on whether the samples were used for optimising the methodology or for the comparative results stage of this research (see Figure 2.1). For optimisation the swab head was transferred to an Eppendorf tube and the cotton part broken from the swab handle. An adapted phosphate buffered saline (PBS) wash was used (Martin *et al*. 2006). Onto the swab head 1 ml PBS was pipetted and vortexed briefly at room temperature to disrupt and detach the cells attached to the swab head. The swab was left to incubate on ice for 15 minutes. The tubes containing the swab heads were centrifuged at 12000 x g for 5 minutes at room temperature, with a spin basket in order to remove as much sample as possible from the swab. Diluted vaginal fluid supernatant (300 µl) was transferred to clean Eppendorf tubes and precipitation performed (see section 2.4). For comparative results the swab head was transferred to an Eppendorf tube and after the cotton part was broken from the swab handle the treatment was exactly as above but each sample extract was then pooled (see section 2.3.3) prior to precipitation (see section 2.4).

2.3.3 Sample Pools

For comparison of body fluids, individual sample pools were made from the saliva samples and another sample pool made from the vaginal fluid samples. Pools were made after samples had been centrifuged (see section 2.3.1 and 2.3.2). A main pool containing 50 µl of each sample supernatant was made as well as mini pools (see Table 2.2), based on donor data. For each sample 200 µl of supernatant was used, or

for pools comprising of only two samples, 300 µl of each sample supernatant was used. Each pool was made up in a 15 ml centrifuge tube. The pools were vortexed at room temperature and 300 µl aliquots were put in Eppendorf tubes and stored at -20°C until precipitation was performed (see section 2.4).

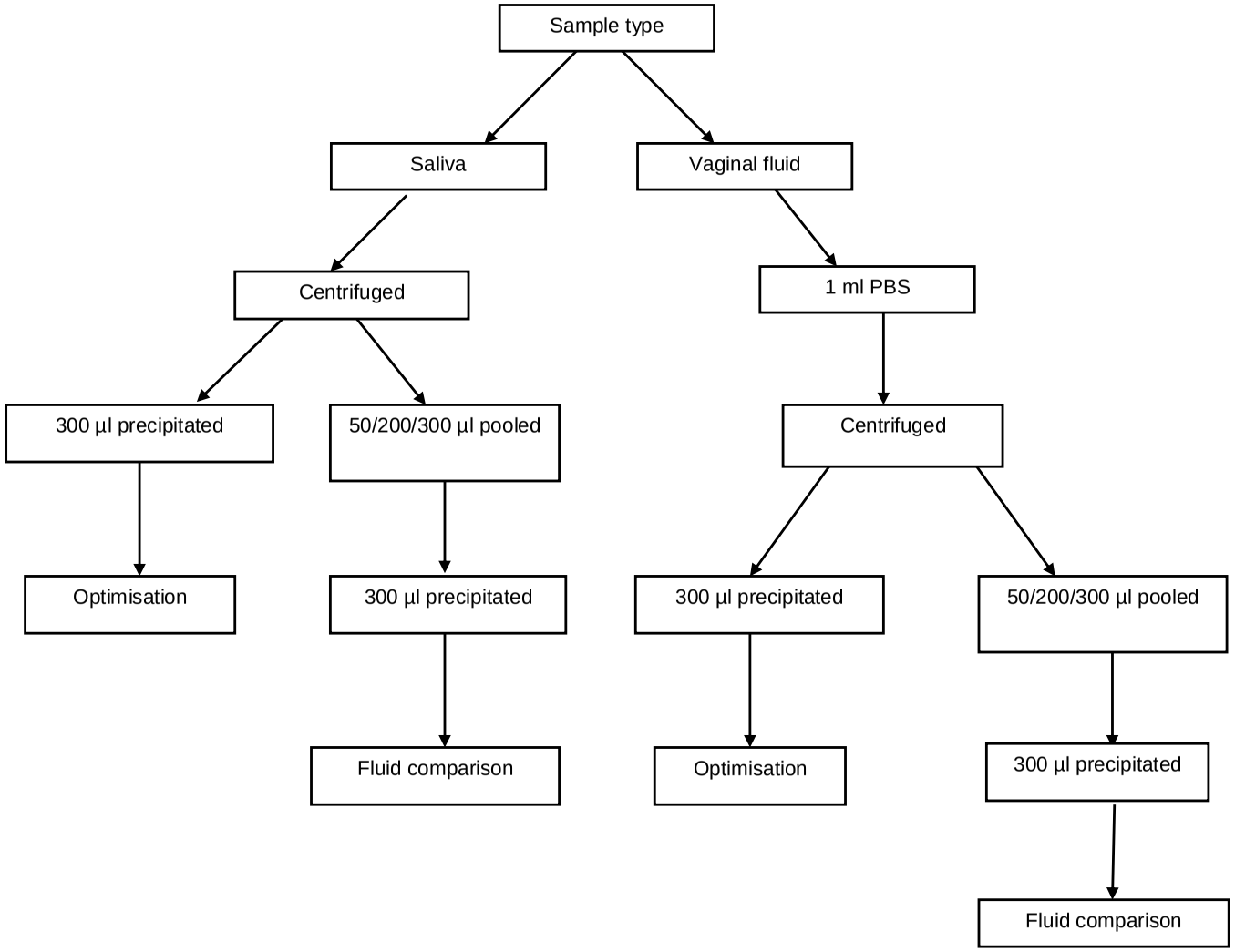


Figure 2.1 A flow diagram to show sample preparation of saliva and vaginal fluid samples for both the optimisation stage and comparative results stage of the study.

2.3.4 Semen

1 ml of each semen sample was centrifuged (12000 x g for 5 minutes at room temperature) to separate liquid semen from cellular components. Analysis of the liquid portion of semen (supernatant) only was performed as it is likely to always be present at a crime scene even if the male was azoospermic. The separated semen supernatant (300 µl) was transferred to Eppendorf tubes and precipitation performed (see 2.4).

2.3.5 Blood & Menstrual blood

Both the blood and menstrual blood samples were treated in the same manner. After ethanol swabbing blood was deposited onto a cotton swab head by pricking the donor's finger. Approximately a 5 mm diameter spot on the swab tip was collected. Menstrual blood was collected onto swab heads directly from the donor during early menstruation when it was most easily collectable. The swab heads were cut from the wooden swab handles and transferred to Eppendorf tubes. 500 µl PBS was added to the swab head using a pipette and the tubes vortexed briefly at room temperature. The tubes were pulse centrifuged at room temperature, with swab heads in spin baskets in order to remove as much sample as possible from the cotton swab head. The samples were then passed through an albumin depletion column (see 2.3.5.1) in order to remove the major protein from the samples.

2.3.5.1 Albumin Depletion using Vivapure®Anti-HSA Kit (vivascience).

2.3.5.1 (i) Vivapure®Anti-HSA/IgG Kit Protocol

The Anti-HSA affinity resin (50% slurry) was resuspended according to manufacturer's recommendations with distilled water. 440 µl affinity resin was added to each spin column followed by 20 µl of either blood or menstrual blood supernatant. Each spin column was then incubated on a rotary shaker for 15 minutes at room temperature before centrifugation at 400 x g for 2 minutes at room temperature. The resulting eluate was collected in a separate tube. Binding buffer (200 µl) was added to the same spin column and incubated on a rotary shaker for 2 minutes at room temperature. The spin column was once again centrifuged as above and the eluate collected once more and precipitation performed (see section 2.4).

2.3.6 Breast Milk

Untreated breast milk (300 µl) was transferred directly to Eppendorf tubes prior to precipitation (see section 2.4).

A summary of preparation steps for each body fluid samples is represented in Table 2.2. The method of sampling for each body fluid had some bearing on the preparation steps involved prior to precipitation.

Table 2.2: A summary of body fluid sample preparation.

	Semen	***Blood/Menstrual Blood***	***Breast milk***	***Saliva***	***Vaginal fluid***
Sample Preparation Steps	Separate cells from cellular fluid Precipitation	Deposited onto Swab head Add 500µl PBS Albumin removal Precipitation	Precipitation	See Figure 2.1	

2.4 Protein Precipitation Methods

Body fluid samples were kept on ice throughout the precipitation procedure.

2.4.1 TCA (Trichloroacetic Acid) – Acetone

The precipitation method was adapted from Chen *et al.* (2002). Ice cold 10% TCA and 0.10% DTT solution (900 µl) was added to the Eppendorf tubes containing 300 µl sample and kept on ice. The tubes were vortexed briefly and incubated on ice overnight.

The next morning the tubes were centrifuged at 12000 x g for 10 minutes at room temperature. The tubes were then washed with ice cold acetone twice and the acetone decanted off. The protein pellets were left to air dry for ten minutes. The sample pellets were resuspended by pipette action using 183 µl sample reswelling buffer (9M urea, 4% CHAPS, 0.50% Bio-Lyte 3-10 buffer, 0.001% Bromophenol Blue) and 17 µl 15% (w/v) DTT per tube.

2.4.2 Ethanol

An adapted version of the ethanol precipitation performed by Rajan *et. al.* (1999) was performed using Ice cold ethanol (900 µl) added to the Eppendorf tubes containing 300 µl sample and kept on ice. The tubes were vortexed briefly and incubated on ice overnight.

The tubes were then centrifuged at 12000 x g for 20 minutes at room temperature and the ethanol supernatant removed. The protein pellets were treated as in 2.4.1 above.

2.4.3 Acetone

Acetone precipitation was adapted from Hardt *et al.* (2005a). 900 µl ice cold acetone was added to the Eppendorf tubes containing 300 µl sample and kept on ice. The tubes were vortexed briefly and incubated on ice overnight.

The tubes were then centrifuged at 12000 x g for 20 minutes at room temperature and the acetone supernatant removed. The protein pellets were treated as in 2.4.2 above.

2.5 Protein Measurement by RC DC Protein Assay (Bio-Rad®)

2.5.1 Assay Protocol

The Bio-Rad® *RC DC* (reducing agent compatible detergent compatible) Protein assay is a colorimetric assay for determining protein quantity. The assay is a microplate version (Bio-Rad®) of the Lowry assay (Lowry *et al*. 1951) further modified to be compatible with reducing agents and detergents. The protein standard used for the determination of protein concentration varied with the major protein component of the body fluid being tested, e.g. bovine immunoglobulin G (IgG) for vaginal fluid, saliva, semen and breast milk and bovine serum albumin (BSA) for blood and menstrual blood. Protein standards were prepared in sample buffer (9M urea, 4% CHAPS, 0.50% Bio-Lyte 3-10 buffer, 0.001% Bromophenol Blue) ranging from 0.18 mg/ml to 1.45 mg/ml for IgG and 0.20 mg/ml to 1.50 mg/ml for BSA. Five protein dilutions and a zero protein concentration were used for each standard curve.

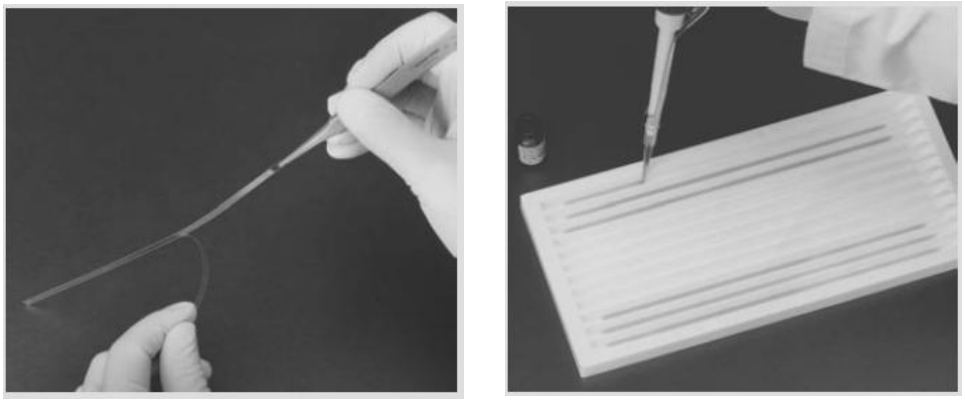
The protocol supplied with the assay was followed; 25 µl of standard or sample was pipetted into an Eppendorf tube and 125µl *RC* Reagent I (contains Universal Protein Precipitation Agent (UPPA) -I) was added. The tubes were incubated at room temperature for 1 minute then 125 µl *RC* Reagent II (contains UPPA-II) was added to each tube and centrifuged at 12000g for 5 minutes at room temperature. The supernatant was decanted and the pellets resuspended in 25 µl Reagent A (5 µl of *DC* Reagent S (sodium dodecyl sulfate) added to 250 µl of *DC* reagent A (alkaline copper tartrate). Tubes were left at room temperature for 5 minutes, and the contents transferred to a microtitre plate with 200 µl Reagent B (dilute folin reagent) being added to each well and mixed by pipette action. The microtitre plate was incubated at room temperature for 15 minutes.

The microtitre plate was run on a MRX® II microplate reader (Dynex Technologies, Worthing) at 650 nm. The absorbance was read and the protein concentration of the samples determined from the standard curve produced.

2.6 Two-Dimensional SDS-PAGE

2.6.1 IPG Strip Rehydration

The Protean® isoelectric focusing cell (Bio-Rad®) was used to separate protein samples in the first dimension using immobilised pH gradient (IPG) 7 cm Readystrips ™ (Bio-Rad®). The IPG strips are in dehydrated form when purchased and need to be rehydrated with sample overnight prior to focussing. The protein load depended on the sample type and was run alongside a 2-D SDS-PAGE protein standard (Bio-Rad®); 4 µl of standard was mixed with the sample and sample reswelling buffer (9M urea, 4% CHAPS, 0.50% Bio-Lyte 3-10 buffer, 0.001% Bromophenol Blue) to a final volume of 125 µl. The 125 µl buffer/protein mixture was pipetted into the middle of an unused well of a 7 cm plastic 12 well IPG reswelling tray (Bio-Rad®). The plastic protective strip was removed from the IPG strip using forceps (see Figure 2.2) and the IPG strip was gently bent into a U shape, gel side down, into the well of the reswelling tray on top of the buffer/protein mixture.



a) **b)**

Figure 2.2 Photographs showing a) the plastic backing being removed from a dehydrated IPG strip prior to sample rehydration and b) sample loading into a focussing tray (ReadyStrip IPG strip instruction manual).

The strip was left for an hour to allow the liquid to distribute throughout the whole strip and then 1.50 ml of mineral oil (Bio-Rad®) was added on top of the strip and it was left to rehydrate overnight.

2.6.2 Isoelectric Focussing

Paper wicks (Bio-Rad®) were placed over each electrode of the 7 cm focussing tray (Bio-Rad®). Water (10 µl) was added to each wick to dampen it and thus maintain a good contact with the electrode. The IPG strip was removed from the reswelling tray and gently blotted onto tissue paper in order to remove any excess liquid. The strip was placed gel side down into the focussing tray with the positive end of the strip orientated towards the positive end of the focussing tray (see Figure 2.3).

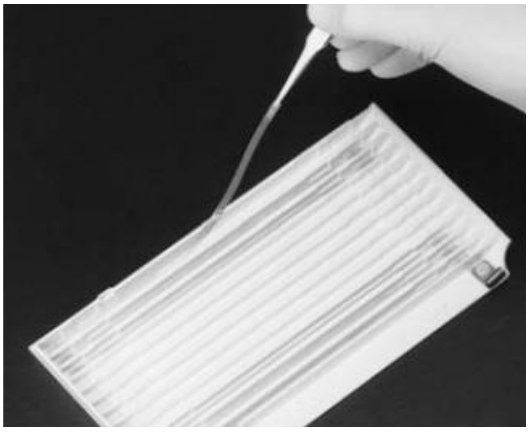


Figure 2.3: A Photograph to show an IPG strip being lowered into the focussing tray prior to being positioned into the IEF cell (ReadyStrip IPG strip instruction manual).
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The strip was covered with 1.50 ml of mineral oil. The lid was placed on top of the tray and the tray was positioned onto the peltier platform of the Protean® IEF cell (see Figure 2.4) ensuring that there was sufficient contact with the electrodes. Isoelectric focussing was performed overnight using a programme with the following settings:

Rapid ramping to 200 V for 15 minutes
Rapid ramping to a maximum of 4000 V for 2 hours
Rapid ramping to a maximum of 4000 V for 20000 Vhr
Held at 500 V for a minimum of 30 minutes.

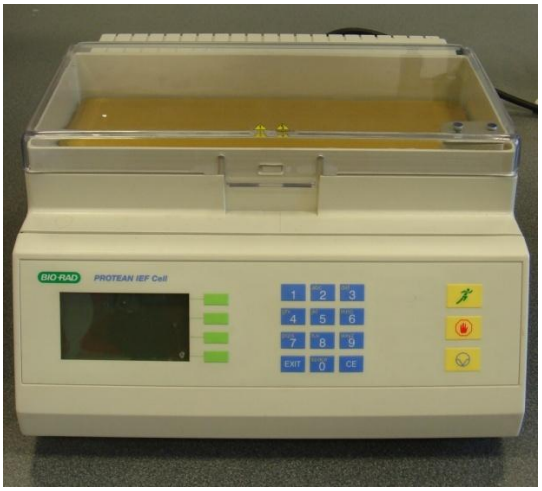

Figure 2.4: A Photograph to show the Protean® IEF cell (BioRad®) (photograph taken in house).

The IPG strips were either kept at 500 V or the focussing tray was removed from the platform, wrapped in cling film and stored at -80°C depending on whether the second dimension stage was to be performed immediately following focussing or up to a week later.

2.6.3 Separation in the Second Dimension – SDS PAGE

Table 2.3: A table to show the composition of 2D – SDS PAGE gels

Solution	***15% acrylamide gel***	***12% acrylamide gel***	***10% acrylamide gel***
30% acrylamide/bis	5 ml	4 ml	3.30 ml
MilliQ water	2.30 ml	3.30 ml	4 ml
1.5 M tris-HCL pH 8.8	2.50 ml	2.50 ml	2.50 ml
10% (w/v) SDS	0.10 ml	0.10 ml	0.10 ml
10% (w/v) Ammonium persulphate (APS)	100 µl	100 µl	100 µl
TEMED	4 µl	4 µl	4 µl

The second dimension was performed using the mini-PROTEAN® tetra cell (Bio-Rad®). The casting stand was set up with 1 mm glass plates according to the Bio-Rad® instruction manual (see Figure 2.5) and the appropriate gel mix made (see Table

2.3) in a conical flask. The APS (ammonium Persulphate) and TEMED (Tetramethylethylenediamine) were added finally before the gel was poured.

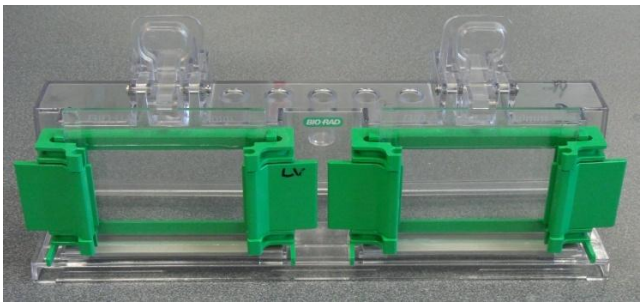

Figure 2.5: A Photograph to show the SDS-PAGE gel (7 cm wide) set up (photograph taken in house)

The gel mixture was swirled gently to initiate polymerisation. The gel was poured between the two glass plates of the casting stand using a disposable Pasteur pipette, to approximately 0.50 cm from the top of the shortest glass plate. Water was gently placed on the top of the gel using a pipette, to create a straight line when the gel sets, in order for the IPG strip to sit evenly on top of the gel. The gel was left to polymerise for 45 minutes. This time changed depending upon the temperature in the laboratory at the time. If the focussing tray had been stored at -80°C for up to a week, the focussing tray was removed from the freezer for a maximum of 15 minutes to allow the IPG strips to defrost sufficiently to be removed with ease from the tray. If the focussing tray was still positioned on the focussing platform, the program was paused and the IPG strips were removed from the focussing tray. Once removed from the focussing tray the IPG strips were blotted with tissue paper in order to remove excess liquid. Each IPG strip was placed gel side up in a fresh well of a reswelling tray specifically used for the equilibrium stage of the procedure. Firstly 2 ml of DTT (Dithiothreitol) equilibrium buffer (50 mg DTT in 2.5 ml equilibrium base buffer stock (6 M Urea, 2% SDS, 50 mM Tris-HCl pH 8.8, 20% glycerol)) was added to each strip and the lid placed onto the tray. The tray was placed onto an orbital shaker at low speed for 10 minutes. DTT is incorporated into the first step for the reduction of the disulphide bonds and complete unfolding of proteins (Gorg *et al*. 2000). The DTT buffer was decanted from each well of the reswelling tray and 2 ml of Iodoacetamide buffer (62.50 mg Iodoacetamide in 2.50 ml equilibrium base buffer stock (6 M Urea, 2% SDS, 50 mM Tris-HCl pH 8.8, 20% glycerol)) was added to each strip. Iodoacetamide is added to the second buffer stage to remove excess DTT which would cause streaking in the 2D image (Gorg *et al*. 2000). Once more the lid was returned to the tray and the tray

placed onto the orbital shaker at low speed again for 10 minutes. After 10 minutes the Iodacetamide buffer was decanted from the reswelling tray and the strips were left in 1 x Laemmli running buffer (Laemmli 1970) diluted from 10 x Laemmli running buffer stock (0.25 M Tris base, 1.92 M Glycine,1% (w/v) SDS) until the second dimension gels were ready to run.

Once the gels had set, the water was poured off and any residue absorbed by tissue paper. The glass plates were removed from the casting stand and positioned at an angle allowing for the IPG strip to be pushed down with the plastic coating facing the front of the glass plates and the + end of the strip to the left hand side of the gel. The strip was pushed into place using a small spatula until it made contact with the gel. Unstained precision plus protein™ standards (Bio-Rad®) were run alongside the gel, to give a representation of molecular weight in the second dimension. A paper wick (Bio-Rad®) was cut in half using scissors and 5 µl of precision plus protein™ standard was added to it and stored at -20°C until required. One of these standards was lowered onto the right hand edge of each gel. Overlay agarose (Bio-Rad) was melted using a hot water bath and 1 ml was poured on top of the IPG strip to keep it in place and to monitor the electrophoresis run. Once the agarose had set the glass plates were assembled into the mini-PROTEAN® tetra electrophoresis cell (see Figure 2.6). The cell was filled with 1L of 1x running buffer and connected to a PowerPac™ 300 (Bio-Rad®) and gels were run at 200 V for 40-45 minutes.

a) **b)**

Figure 2.6: A Photograph to show a) the casket assembly and b) the electrophoresis set up for two 7 cm wide gels (photograph taken in house).

2.7 1D SDS- PAGE

Table 2.4: A table to show the composition of 1D – SDS PAGE gels

Solution	***4% Stacking gel***	**10% Separating gel**
30% acrylamide/bis	0.51 ml	3.30 ml
MilliQ water	2.04 ml	4 ml
1.50 M tris-HCl pH 8.8	-	2.50 ml
0.50 M tris-HCl pH 6.8	0.375 ml	-
10% (w/v) SDS	30 µl	0.10ml
10% (w/v) (APS)	30 µl	100 µl
TEMED	3 µl	4 µl
Total	3 ml	10ml

The SDS-PAGE separating gel setup was performed in the manner described for the second dimension (section 2.6.3) using the mini-PROTEAN® tetra cell (Bio-Rad®). The separating gel mixture was made using the volumes described above (Table 2.4) and poured between the glass plates to approximately 1.5 cm from the top of the shortest glass plate. Water was gently overlaid as before to create a straight edge to the gel and the gel left to set. After approximately 40 minutes, when the separating gel had set, the water was poured off and a stacking gel was mixed (Table 2.3), poured on top of the separating gel and a 10 well comb was placed in between the glass plates. Once the gel had set, the plates were removed from the casting stand and placed into the gel tank. The tank was filled with 1 x Laemmli running buffer diluted from 10 x Laemmli running buffer stock (0.25 M Tris base, 1.92 M Glycine,1% (w/v) SDS) and the combs removed from the glass plates. Each sample was prepared by transferring the desired load into an Eppendorf tube and adding 3 µl of 3 x dissociation buffer (0.50 M Tris-HCL pH 6.8, 25% (w/v) SDS, 21% (v/v) 2-mercaptoethanol, 27% glycerol, 0.01% Bromophenol blue) to it in a fume hood. An unstained standard was prepared also using 5 µl stock SDS-PAGE Standards (Bio-Rad®) and adding 3 µl of 3 x dissociation buffer (0.50 M Tris-HCL pH 6.8, 25% (w/v) SDS, 21% (v/v) 2-mercaptoethanol, 27% glycerol, 0.01% Bromophenol blue) to it in a fume hood. The sample tubes were placed into a heating block at 95°C for 5 minutes. Samples were pulse centrifuged and then the whole volume loaded into the gel wells using a pipette. The mini PROTEAN® tetra tank was plugged into a PowerPac® 300 (Bio-Rad®) and gels were run at 200 V for 40-45 minutes.

2.8 Post Electrophoretic Staining

Once electrophoresis had been completed the gels encased in glass plates were removed from the gel tank and separated using a small plastic separator. The gels were placed into individual 12 cm square disposable plastic weighing boats for staining.

2.8.1 Staining with Colloidal Coomassie Blue G-250

All steps were performed with gentle agitation on a 3D orbital shaker. Firstly the gels were placed in 30 ml fixing solution (50% (v/v) ethanol, 25% (v/v) phosphoric acid) in a disposable weighing boat and left overnight. The gel was transferred to a clean weighing boat and washed in distilled water three times each for 30 minutes. The gel was then transferred to another weighing boat and put in equilibrium buffer (17% (w/v) ammonium sulphate, 34% (v/v) methanol, 2% (v/v) Phosphoric acid) for one hour. A small spatula (approx 10 mg) of Coomassie Brilliant Blue (CBB) G-250 powder was added to the equilibrium buffer avoiding direct contact with the gel and left to stain for up to three days.

The gel was rinsed with distilled water to remove any unbound CBB stain until the background staining was minimal.

2.8.2 Detection of Proteins by Silver Staining (using PlusOne^{TM} Silver Staining Kit GE Healthcare, UK)

The staining protocol was performed according to the manufacturer's recommendations and all steps were performed with gentle agitation on a 3D orbital shaker.

Initially the gel was placed in fixing solution (40% (v/v) Ethanol, 10% (v/v) glacial acetic acid) overnight and the following morning the gel was transferred to sensitizing solution (30% (v/v) Ethanol, 0.50%(v/v) Gluteraldehyde, 4% (v/v) Sodium thiosulphate, 6.8% (w/v) sodium acetate) for 30 minutes. The gel was then washed in distilled water (5 minutes x 3) and then in silver solution (10% (v/v) silver nitrate solution, 0.04% (v/v) formaldehyde) for twenty minutes. After this the gel was washed in distilled water twice for 1 minute each time.

The gel was placed in developing solution (2.50% (w/v) sodium carbonate, 0.02% (w/v) formaldehyde) for 2½ minutes and then transferred to stop solution (1.50%

(w/v) EDTA-$Na_2.2H_2O$) for 10 minutes to prevent over developing of the gel. The gel was finally washed in distilled water three times for 5 minutes each time.

2.8.3 Detection of Proteins using SYPRO® Ruby (Invitrogen™) Fluorescent Gel Stain.

The staining protocol was performed according to the manufacturer's recommendations and all steps were performed with gentle agitation on a 3D orbital shaker.

Initially the gel was placed in 30 ml fixing solution (40% (v/v) methanol, 10% (v/v) glacial acetic acid) for one hour. The gel was then transferred to a clean weighing boat and 30ml SYPRO® Ruby (Invitrogen™) protein gel stain added to it, and covered in aluminium foil as SYPRO® Ruby (Invitrogen™) is a photosensitive stain.

The following morning the gel was transferred to a clean weighing boat and 30 ml wash solution (10% (v/v) methanol analytical grade, 7% (v/v) glacial acetic acid) was added for 1 hour.

2.8.4 Detection of Proteins using Flamingo™ Fluorescent Gel Stain (Bio-Rad®).

The staining protocol was performed according to the manufacturer's reccommendations. All steps were performed with gentle agitation on a 3D orbital shaker. Firstly the gel was fixed in 30 ml of fix solution (40% (v/v) methanol, 10% (v/v) glacial acetic acid) for 2 hours. The gel was then transferred to 30 ml of 1 x working staining solution (stock solution diluted 1:10 with MilliQ water) for 3 hours and the gel covered with aluminium foil to protect the photosensitive stain from light.

2.9 Image Analysis of Protein Gels.

Initially images were obtained using a ChemiDoc™ EQ (Bio-Rad®) Gel documentary system using either the epi white setting with a white conversion plate for CBB and PlusOne™ Silver stained gels, or trans UV setting for fluorescently stained gels – SYPRO® and Flamingo™. The digital images produced using this method were 8 bit resolution which provided images with insufficient grey tones for software analysis. An alternative imaging system was required. A PharosFX™ molecular imager (Bio-Rad®) was used to obtain images with 16 bit resolution providing a greater number of grey tones per pixel more suitable for analysis using SameSpots Prodigy™ software (Nonlinear Dynamics, Newcastle, UK). Images were analysed to determine acceptable expression fold differences between identical spots on duplicate gels of the same sample. These were then used to identify expression changes between sub groups of samples and determine spots suitable for identification. The SameSpots Prodigy software guides the user through each stage of 2D gel analysis, by firstly ensuring images are of sufficient quality for analysis and matching the gel images to a master gel image through 21 matched expression vectors (identical spot matches) between each gel and the master gel image. These manually assigned vectors are used to align each gel positioning each gel relative to one another and hence allowing comparison between them to be performed. Once aligned, gel images are sorted for spot analysis and spots of interest are selected and a report formed containing expression data for each spot of interest on each gel analysed.

2.10 Spot Excision

Proteins fixed in two-dimensional gels were located with a UV/white trans-illuminator (Vilber Lourmat). Protein spots were excised from the two dimensional gel using a pasteur pipette. The tip of the pipette was used to cover the spot and by pushing down on the gel the spot was cut from the gel. Each gel plug was then transferred to an Eppendorf tube. To ensure sufficient protein was present within each tube a duplicate spot was cut for each sample. The spot samples were stored in 100 µl distilled water at 4°C until further analysis.

2.11 LC-MS/ MS

Gel plugs were trypsinised using the MassPrep (see Figure 2.7) automated handling station (MicroMass). The gel plugs were destained (50% acetonitrile, 0.05M ammonium bicarbonate), reduced (1.54 mg/ml dithiothreitol) and alkylated (10.20 mg/ml iodoacetamide). Each stage took 30 minutes. The protein plugs were trypsin digested for 5 hours (Promega Gold trypsin in 0.1 M ammonium bicarbonate). Tryptic peptides were extracted from the gel plugs (40% acetonitrile, 0.10% formic acid) for 1 hour. All operations were performed at 37°C and the peptides were evaporated to dryness using a Rotovap.

Figure 2.7: A Photograph to show the Mass prep automated liquid handling system (Rowett Institute of Nutrition and Health)

The samples were prepared using 20 µl 0.10% formic acid, sonicated for 30 minutes and transferred to sample vials for injection into the HPLC. An UltiMate nano LC system (LC Packings, Camberley, Surrey) was used with a C18 PepMap 100 nanocolumn (see Figure 2.8), 15 cm x 75 µm id, 3 µm, 100 Å (LC Packings). The system was operated with a column flow rate of 0.30 µl / min, a Famos autosampler set to an injection volume of 10 µl and a switchos microcolumn switching device set at a flow rate 0.03 ml / min using 0.10% formic acid. Two HPLC grade solvents were used in a gradient system (see Table 2.5); solvent A: 2% ACN (acetonitrile) and 0.10% formic acid and solvent B: 80% ACN and 0.08% formic acid. The gradient started at 5% B 95% A, moving to 50% of B and A over 30 minutes ramping to 80% B 20% A. The system was equilibrated at 95% A for 9 minutes prior to injection of the next sample.

The samples were held in the trap column for 7 minutes before entering the Mass spectrometer.

Figure 2.8: A Photograph to show the UltiMate nanoLC system and Q trap Quadrupole MS (photograph taken in house).

Table 2.5: The HPLC solvent ramping sequence.

Time (min)	Flow Rate (µL/min)	% Solvent A	% Solvent B
0.00	0.30	95	5
30.00	0.30	50	50
32.00	0.30	20	80
42.00	0.30	20	80
51.00	0.30	95	5
60.00	0.30	95	5

The Mass Spectrometry was performed using a Q-Trap (Applied Biosystems/MDS Sciex, Warrington, UK) triple quadrupole mass spectrometer fitted with a nanospray ion source, where Quadrupole 3 (Q3) operated as a linear Ion trap. The nanospray needle was set at 2800V. Oxygen free nitrogen (OFN) was used as the curtain gas and the collision gas. The mass range in Quadrupole 1 (Q1) was set to *m/z* 400-1200 with a scan rate of 4000 amu/s. The criteria for selection of ions for the fragmentation (Quadrupole 2 (Q2)) were ions of 10^5 cps (counts per second) or above. The trap fill time (Quadrupole 3 (Q3)) was 250ms and the scan rate was 1000 amu/s (Atomic Mass Unit/second).

2.12 Database Searching

Obtaining protein identification is not a simple process of data entry into a database resulting in an identification being produced. The experimental data produced by an LC-MS/MS experiment consists of the mass of the intact peptide, the peptide fragments and the retention time. Data output is in the form of a TIC (total ion count) spectra which displays a molecular ion scan of the retention times (in the LC capillary) of the molecular ions within the peptide mixture (Figure 2.9). This spectra alone does not provide any information regarding the protein identification.

The TIC spectra were highlighted between 10 min and 55 min (which is where most of the peptides are detected and shown in the spectra). Following LC separation CID fragmentation of the molecular ion into daughter ions allows structural determination to be ascertained from the mass intervals of the daughter ions. BioAnalyst™ deconvolution software transforms the TIC spectra obtained from LC-MS/MS into an electronic format compatible with the MASCOT database to identify the protein of interest. The area outlined in red would be highlighted by the analyst and the data transferred to the MASCOT MS/MS Ion search database (Matrix science).

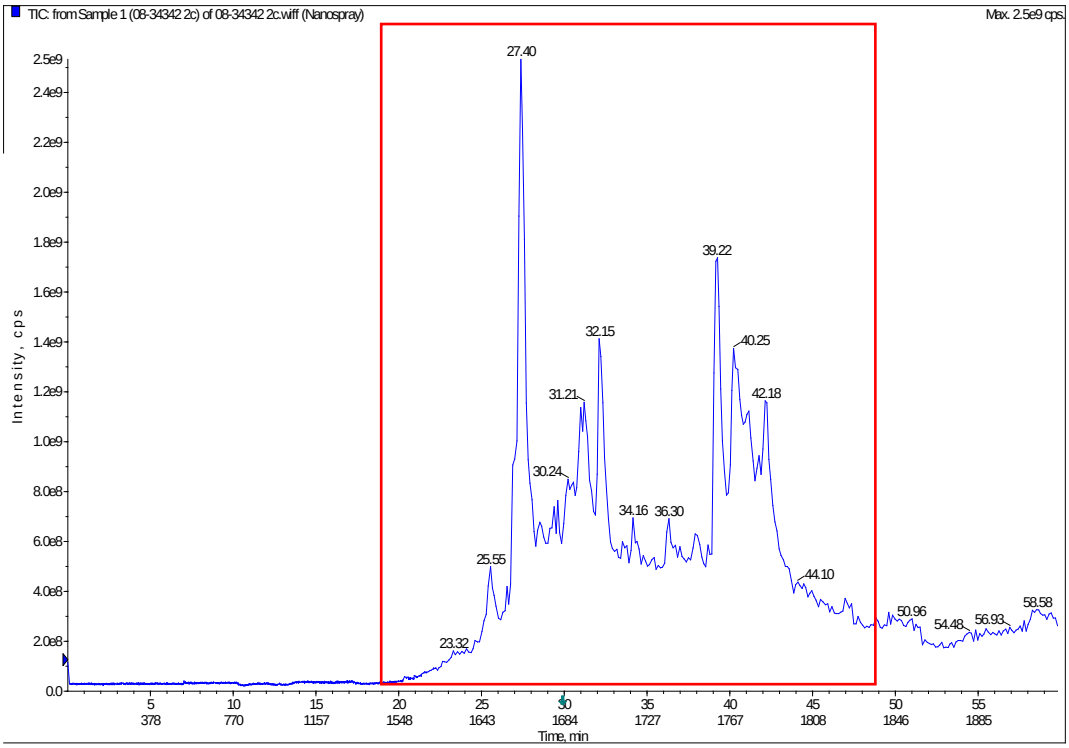

Figure 2.9: Total Ion Count spectra of sample control 1C.

The following search parameters were used: the MSDB database, species homo sapien; allowance of zero or one missed cleavage; peptide mass tolerance at ± 1.50 Da; digestion enzyme trypsin; carbamidomethyl modification of cysteine; partial modification methione oxidation and charged state of MH^+ (see Figure 2.10).

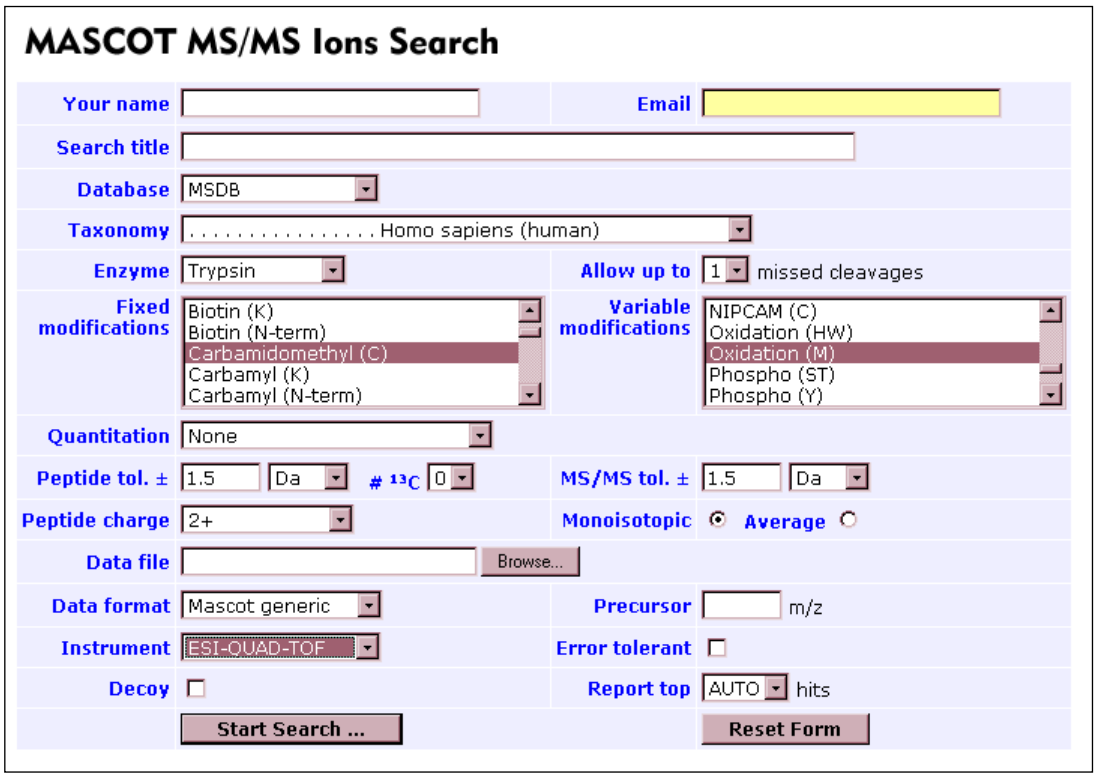

Figure 2.10: An example of the MASCOT MS/MS Ions search screen with desired search parameters set.

2.13 ELISA

Saliva, breast milk, urine and semen were dispensed into 500 µl aliquots. The second vaginal fluid swab taken from each donor was used for the ELISA tests. Swabs were removed from their holders and the swab heads removed from their wooden sticks using scissors and placed into Eppendorf tubes. 300 µl PBS was added to each swab head and were vortexed for approximately 1 minute. The swab head was placed into a spin basket and pulse centrifuged to ensure the entire sample was recovered from the swab. Blood swabs were treated as in section 2.3.5 without removal of albumin.

2.13.1 CanAg SCC EIA ELISA kit (FUJIREBIO™)

The manufacturer's protocol was followed as below. Assay solutions were made prior to commencing the ELISA protocol. Each SCC Calibrator (Cal A-E) was reconstituted with 0.75 ml water, mixed gently by inversion and left to stand for 15 minutes. Wash solution was diluted from the 25x stock solution to a 1x working solution. Antibody solution was prepared by adding the vial of Tracer, HRP Anti-SCC to the bottle of Biotin Anti-SCC.

Each strip of the 96 well plate was washed once with wash solution. The SCC calibrators (A-E) and samples (diluted 1:50) were pipetted (25 µl) into the wells of the 96 well ELISA plate according to the template (Figure 2.11). Antibody solution (100 µl) was added to each well and the plate was incubated for 1 hour at room temperature on a microplate shaker. Each well was washed six times using 200 µl wash solution. TMB HRP-substrate was added to each well (100 µl) and the plate covered in aluminium foil and incubated for 30 minutes at room temperature on a microplate shaker. Stop solution (100 µl), was added to each well and the plate shaken briefly on a microplate shaker to mix. The absorbance was read using a MRX® II microplate reader (Dynex Technologies, Worthing) at 405nm within 15 minutes of the stop solution addition. The results of this ELISA were expressed as bar graphs.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Cal A 0µg/L	Cal E 46µg/L	Vag 4	Vag 8	Vag 12	Vag 16	Vag 20	Vag 24	Menstrual Blood			
B	Cal A 0µg/L	Cal E 46µg/L	Vag 4	Vag 8	Vag 12	Vag 16	Vag 20	Vag 24	Menstrual Blood			
C	Cal B 1.1µg/L	Vag 1	Vag 5	Vag 9	Vag 13	Vag 17	Vag 21	Vag 25	Breast Milk			
D	Cal B 1.1µg/L	Vag 1	Vag 5	Vag 9	Vag 13	Vag 17	Vag 21	Vag 25	Breast Milk			
E	Cal C 5.1µg/L	Vag 2	Vag 6	Vag 10	Vag 14	Vag 18	Vag 22	Blood	Saliva			
F	Cal C 5.1µg/L	Vag 2	Vag 6	Vag 10	Vag 14	Vag 18	Vag 22	Blood	Saliva			
G	Cal D 24µg/L	Vag 3	Vag 7	Vag 11	Vag 15	Vag 19	Vag 23	Semen	Urine			
H	Cal D 24µg/L	Vag 3	Vag 7	Vag 11	Vag 15	Vag 19	Vag 23	Semen	Urine			

Figure 2.11: A diagram to show the CanAg SCC EIA ELISA plate layout. Vaginal fluid samples labelled Vag1-25.

2.13.2 Human Zinc-α-2-Glycoprotein (ZA2G) ELISA Kit (BioVendor).

The manufacturer's protocol recommends a 1:5000 dilution of serum samples with kit dilution buffer, but the test samples were diluted 1:2 as it was thought that the antigen would be expressed in lower amounts in the samples being studied compared with the human serum samples that the kit was designed for.

Dilution buffer was diluted 2 fold to produce a 1 x working buffer. Human Zinc-α-2-Glycoprotein standard was reconstituted by adding 1.4 ml of 1 x dilution buffer to the vial making a stock solution of 100 ng/ml. Both the High and Low quality controls were also reconstituted using 1x dilution buffer (1 ml to each vial). Wash solution was diluted ten-fold in distilled water to produce a 1x working solution.

Standards were prepared by diluting Human Zinc-α-2-Glycoprotein master standard stock with dilution buffer (see Table 2.6).

Table 2.6 A table to show the composition of ZA2G standards.

Concentration	Volume of standard	Standard diluent
100 ng/ml	Stock	-
50 ng/ml	300 µl of stock	300 µl
25 ng/ml	300 µl of 50 ng/ml	300 µl
12 ng/ml	300 µl of 25 ng/ml	325 µl
6 ng/ml	300 µl of 12 ng/ml	300 µl
3 ng/ml	300 µl of 6 ng/ml	300 µl

Diluted standards (Std), quality controls (QC), blank (dilution buffer) and samples (1:2 dilution) were pipetted (100 µl) into the wells of the 96 well ELISA plate according to the template (Figure 2.12).

The plate was incubated for 1 hour at room temperature on a microplate shaker. The plate wells were washed 3 times (200 µl) with wash solution. After the final wash the plate was inverted onto paper towel and tapped to remove the remaining wash solution trapped in the wells. Conjugate solution was added to each well (100 µl) and the plate incubated for 1 hour at room temperature on a microplate shaker. The plate wells were washed 3 times (200 µl) with wash solution. After the final wash the plate was inverted onto paper towel and tapped to remove the remaining wash solution trapped in the wells. Substrate solution was added to each well (100 µl), the plate covered with aluminium foil and incubated for 10 minutes at room temperature without

shaking. Colour development was stopped by adding stop solution (100 µl) to each well. The absorbance was read using a MRX® II microplate reader (Dynex Technologies, Worthing) at 450nm within 5 minutes of the stop solution being added to the wells. Results expressed as bar graphs.

	1	2	3	4	5	6	7	8	9	10	11	12
A	std 100	std 100	QC Low	QC Low	Sal 8	Sal 8	Sal 16	Sal 16	Sal 24	Sal 24		
B	std 50	std 50	Sal 1	Sal 1	Sal 9	Sal 9	Sal 17	Sal 17	Sal 25	Sal 25		
C	std 25	std 25	Sal 2	Sal 2	Sal 10	Sal 10	Sal 18	Sal 18	Menstrual blood	Menstrual blood		
D	std 12	std 12	Sal 3	Sal 3	Sal 11	Sal 11	Sal 19	Sal 19	Breast Milk	Breast Milk		
E	std 6	std 6	Sal 4	Sal 4	Sal 12	Sal 12	Sal 20	Sal 20	Urine	Urine		
F	std 3	std 3	Sal 5	Sal 5	Sal 13	Sal 13	Sal 21	Sal 21	Blood	Blood		
G	blank	blank	Sal 6	Sal 6	Sal 14	Sal 14	Sal 22	Sal 22	Semen	Semen		
H	QC High	QC High	Sal 7	Sal 7	Sal 15	Sal 15	Sal 23	Sal 23	Vaginal fluid	Vaginal fluid		

Figure 2.12: A diagram to show the ZA2G ELISA plate layout. Saliva samples labelled as Sal 1-25.

2.14 Dot Blot

Dot blots were performed according to an adapted version of a dot blot protocol reccommended by R&D Systems (R&D Systems 2009. *Dot Blot Protocol*. [online]).

Initially Antibody sensitivity was tested using recombinant proteins to act as positive controls for the primary antibody reactions. Commercial recombinant proteins were sourced from R&D Systems (Cystatin SA, Cystatin SN and Lipocalin-1) and Abnova (Serpin B1). The method below was used throughout.

A piece of Hybond-P (GE Healthcare) PVDF (Polyvinylidene Fluoride) membrane was cut to an appropriate size for the dot blot apparatus and the number of samples being blotted. 5 µl of loading buffer (0.12M Tris-HCl, pH 6.8, 6% (w/v) SDS, 12% (v/v) Glycerol, 6mM DTT, 0.03% (w/v) Bromophenol blue) was added to each sample and they were incubated at 95°C for 5 minutes. The membrane was washed in methanol in a large plastic weighing boat and then washed in TBST (50 mM Tris base, 0.5 M NaCl, 0.05% TWEEN-20, pH 7.4). The membrane was placed between the plastic blocks of the blotting manifold (GIBCO) and the screws tightened to keep the membrane in place. The Laboport vacuum pump was switched on (KNF Neuberger, Germany) and the samples loaded to the slot wells. The vacuum was kept on until the samples had fully penetrated the membrane; this took approximately 30 minutes. The vacuum was switched off and the membrane removed from the manifold and placed in block solution (8% dry skimmed milk in TBST) for 1 hour at room temperature. The membrane was incubated with primary antibody (diluted in block solution) for 1 hour at room temperature. Working antibody concentrations are listed in Table 2.7. The membrane was washed 3 times (10 minutes TBST, room temperature) then incubated with secondary antibody (diluted in TBST) for 1 hour at room temperature. Working antibody concentrations are listed in Table 2.7. The resultant blots appeared more like a line in shape rather than a dot due to the shape of the apertures of the blot equipment used.

Table 2.7: Working Primary and Secondary antibody concentrations.

Antibody name	***Concentration***	***Dilution***	***Working concentration***
Anti-human Cystatin SN	0.10 mg/ml	1:1000	0.10 µg/ml
Anti-human Cystatin SA	500 µg/ml	1:500	1.00 µg/ml
Anti-human Lipocalin-1	0.10 mg/ml	1:1000	0.10 µg/ml
Anti-human SerpinB1	200 µg/ml	1:200	1.00 µg/ml
Anti-Goat IgG	7.90 mg/ml	1:8000	1.00 µg/ml
Anti-Mouse IgG	0.80 mg/ml	1:4000	0.20 µg/ml

The membrane was once again washed in TBST 3 times (10 minutes each time). Two SIGMA*FAST*™ *DAB* with cobalt chloride tablets (SIGMA) two urea hydrogen peroxide tablets were removed from the freezer and allowed to reach room temperature. 10 ml of MilliQ water was added to a 15ml centrifuge tube and the tablets added and vortexed until they had dissolved. The membrane was transferred to a clean plastic weighing boat and the DAB solution was poured over the top. Colour development took approximately 5-10 minutes. The membrane was washed in MilliQ water to stop colour development progressing once the desired intensity had been reached.

The reaction mechanism for DAB colour development is shown below in Figure 2.13. The Cobalt Chloride enhances the blue black colour produced in the DAB reaction.

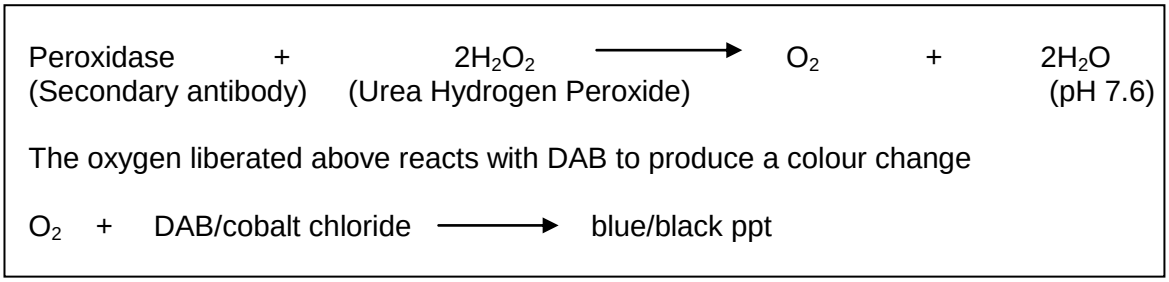

Figure 2.13 A schematic diagram showing the reaction of DAB with peroxidase labelled antibodies

In the technical information provided from the antibody suppliers Anti-human Cystatin SA antibody (R&D systems) stated 100% cross reactivity with human Cystatin SN. Anti-human Lipocalin-1 antibody (R&D systems) stated 5% cross reactivity with

Lipocalin-2. Anti-human Cystatin SN antibody (R&D systems) stated 30% cross reactivity with Cystatin SA, 15% cross reactivity with Cystatin S and 5% cross reactivity with Cystatin C. Anti-human SERPINB1 (Santa Cruz) antibody did not state any information on cross reactivity. No information was provided regarding the specificity of the secondary antibodies.

Chapter 3
Identification of saliva: A comparison of three detection methods

Chapter 3 - Identification of saliva: A comparison of three detection methods.

3.1 Introduction

Historically saliva stain identification has been based on the detection of the most abundant and resilient enzyme to be found within saliva, amylase (Kirk 1963). Amylase is an enzyme that exists in two forms, α-amylase is found in animals whereas β-amylase is found in bacteria and plants. In humans there are two isoforms of the α-amylase enzyme produced in saliva glands and in the pancreas and secreted into the digestive system, as a consequence of this small amounts diffuse into blood and are removed in sweat and urine (Barni *et al*. 2006) thus being detected in low levels in a number of other body fluids. Amylase has been shown to be present in perspiration, tears, breast milk, vaginal secretions, urine and seminal fluid (Merritt *et al*. 1973). From these findings it is important when considering using amylase as a detection enzyme for saliva, especially in sexual assault cases, to ensure that the kit is capable of distinguishing between the two isoforms of α-amylase. Failing that, positive results could be given for vaginal secretions and seminal fluid thus causing confusion in case circumstances.

This chapter looks to compare two currently marketed identification methods (RSID-Saliva from Independent forensics, a chromatographic membrane strip test, and SALIgAE® tube test) with the more traditional Phadebas® (Magle Life Sciences) paper method currently employed in forensic laboratories throughout the UK. The work described in this chapter investigates test sensitivity with human saliva and cross reactivity with different mammalian species and other human body fluids. In order to determine whether the tests are compatible with routine forensic DNA extraction methods, saliva detection was tested with a range of alternative extraction buffers to those provided with the kits.

3.1.1 Phadebas® Paper Test

Willott first reported the use of the Phadebas test in 1974, testing for amylase activity within a number of biological stains. The method used involved cutting out the stain, mixing with water and dissolving Phadebas tablets in the sample mixture and measuring the colorimetric change of the sample. This method confirmed the presence of amylase at low levels in other fluids as well as saliva. This method was further adapted into a touch test by dissolving Phadebas tablets in water and spraying the resultant mixture onto filter paper. This non destructive method is the method used more commonly today as the sample itself is not destroyed in any way and a DNA profile can be extracted from the exhibit after the test. In the Phadebas test amylase activity is tested with a blue dye crossed-linked to starch microspheres. These microspheres are immobilised onto filter paper and in the presence of amylase, the starch is hydrolysed, releasing the water soluble blue dye which stains the paper.

3.1.2 RSID-Saliva Test

The RSID-Saliva kit is a recently marketed kit which claims to use antibodies specific to human salivary α-amylase for detection. The manufacturers claim that it is the most sensitive test for human saliva with no animal saliva cross reactivity (Independent Forensics 2009). The test uses two mouse monoclonal antibodies specific to human salivary α-amylase, and detects the presence of salivary amylase rather than the activity of the enzyme. It does this in a lateral flow strip format.

One of the antibodies is conjugated to a colloidal gold dye to generate a signal and is deposited underneath the sample window. A capture antibody is at the test line of the strip. Anti-mouse IgG antibody is used as a positive control on each strip. The sample is loaded at position S (Figure 3.1); the sample moves through the membrane by capillary action and when it comes into contact with antibodies at the test line (T) an antigen-antibody complex is formed and a red line becomes visible if the sample contains amylase. At the control line (C) a similar reaction occurs between anti-mouse IgG and the mouse antibodies flowing past the test line, ensuring the sample fluid was transported through the strip effectively (Figure 3.1).

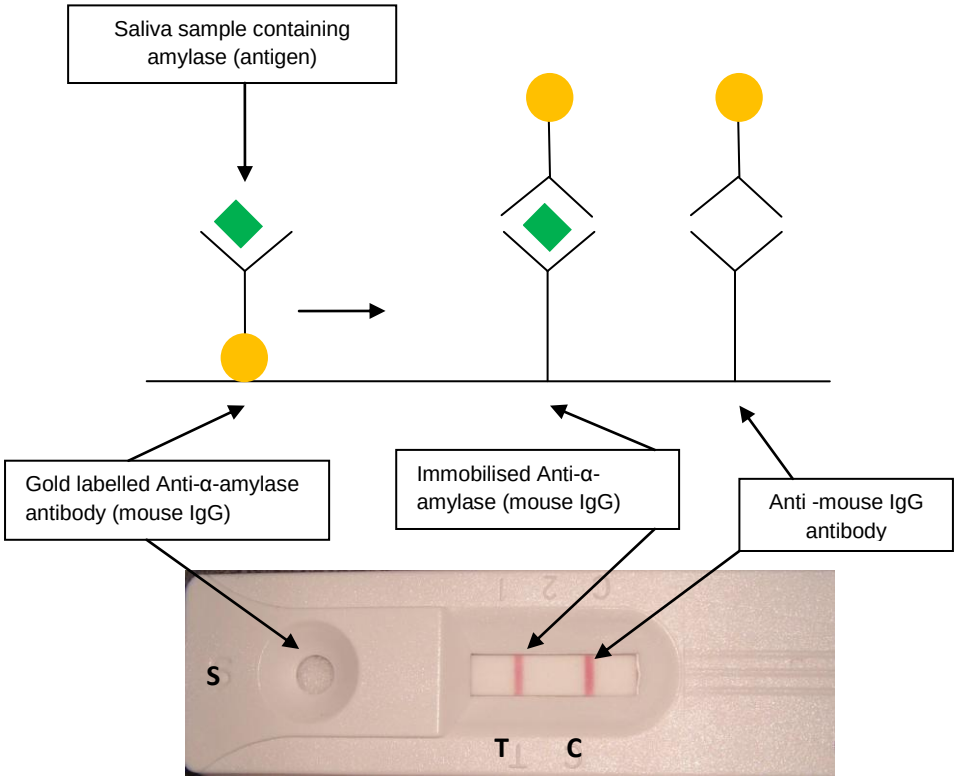

Figure 3.1: A schematic diagram to show the positive antibody-antigen reaction which occurs when a saliva sample has been added to an RSID immunochromatographic test strip (image adapted from Wide University 2001) alongside a test strip displaying both a positive control band (T) and a band representing a positive result for saliva (C). Sample added at site S.

3.1.3 SALIgAE Test

The SALIgAE® test is based upon the presence of salivary amylase within a sample and consists of a transparent solution in a small glass vial. In the presence of a saliva sample a colour change from colourless to yellow is visible. The manufacturers have not released any information regarding the mode of action for this test yet.

3.3 Methods

3.3.1 Samples

3.3.1.1 Body Fluids

Human saliva, urine, blood, menstrual blood vaginal fluid, semen, sweat and breast milk samples were obtained with informed consent (Appendix V) from volunteer donors. Samples of vaginal fluid and menstrual blood were collected onto cotton swabs. For ease of comparison, the other samples were also applied to cotton swabs by dipping the swab head into the fluid sample. All samples were air dried at room temperature for 15 minutes, then stored at -20°C until required.

3.3.1.2 Saliva from Animals

Three saliva samples were taken from each of cow, domestic cat and dog by collection onto swabs. The swabs were dried for 15 min at room temperature and stored at -20^{o}C for use in this study.

3.3.1.3 Commercial Amylases

Two commercial α-amylases were tested for cross reactivity in all the tests. α-amylase from porcine pancreas (A3176, sigma) and α-amylase from *Bacillus licheniformis* (A3403, sigma) were diluted with deionised water to 6.25 units/µl and 26.10 units/µl respectively before use.

3.3.2 RSID-Saliva

Dried saliva on cotton swabs was the source material for analysis. One quarter of each cotton swab was cut off and placed into a labelled Eppendorf tube. PBS (1 x 250 µl) was added to each tube and left to extract for two hours at room temperature. After a brief vortex, 20 µl sample was mixed with 80 µl of TBS in a clean labelled Eppendorf tube and the contents deposited into the sample window of the kit (marked S in Figure 3.2). Almost immediately a pink control line appeared. The reaction state was recorded after 10 minutes in accordance with manufacturer's instructions, this allowed the background of the strip to fade. A positive test result was shown by red/pink lines appearing in the T and C regions of the cassette. A negative result was shown by a line appearing in the C region of the cassette only (Figure 3.2).

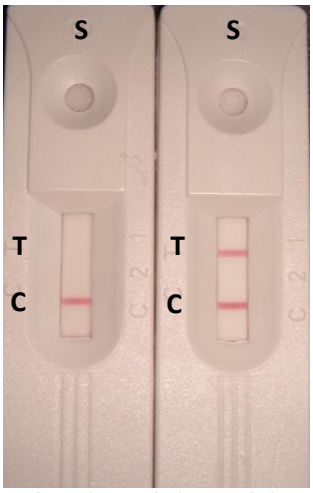

Figure 3.2: RSID strip tests showing from left to right negative and positive test results respectively (photo taken in house).

3.3.3 SALIgAE®

The manufacturer's instructions were followed with a minor variation. The recommended volume of extraction buffer for the swab was 50 µl. In this work, the volume was increased to 500 µl, as when 50 µl was added to the swab for extraction it was completely absorbed by the swab making recovery of the liquid very difficult.

In accordance with the instructions, half a swab was used for extraction in 500 µl distilled water at room temperature for 30 minutes. The test vials were equilibrated to room temperature before saliva samples were added. Sample (8 µl) was added to the test vial and the reaction timed for 10 minutes. A clear to yellow colour change was

shown if the test was positive and no colour change shown in the negative reactions (Figure 3.3)

Figure 3.3: SALIgAE vial tests showing from left to right, positive and negative test results respectively (photo taken in house)

3.3.4 Phadebas Test

Phadebas paper was produced according to the manufacturer's guidelines. A positive reaction was observed when saliva was spat directly onto a piece of Phadebas paper, resulting in a blue colour being formed characteristic of the presence of amylase (Figure 3.4).

Body fluid stains on white cotton fabric were tested. One Phadebas tablet was dissolved in 10 ml deionised water and transferred to filter paper using a fine aerosol spray and left to dry for approximately 15 minutes until touch dry. The paper was then placed sprayed side down onto the fabric, the back of the paper lightly sprayed with water and covered with a piece of plastic , A weight was applied to ensure a good contact was made and the reaction left for approximately 40 minutes or when the blue dye could be seen through the back of the paper.

Figure 3.4: A piece of phadebas paper showing a positive reaction for the presence of saliva (photo taken in house).

3.3.5 Determination of Test Sensitivity

In order to determine the limits of each test five saliva dilutions were made to a final volume of 50 µl (Tables 3.1, 3.2 and 3.3) with the extraction buffer of each kit. Water was used to dilute samples used in the Phadebas test.

The methods used for this were described in sections 3.3.2 and 3.3.3 except that 50 µl was loaded to each swab head and for RSID the whole swab head was extracted in 1 ml of 1x PBS for 2 hours, whereas for the SALIgAE test the method was not changed. For the Phadebas test, saliva dilutions were prepared in Eppendorf tubes to a final volume of 50 µl and then the whole volume of saliva was deposited onto cotton fabric

Table 3.1: Serial dilution of saliva used for RSID test sensitivity.

Sample identification	*Dilution of saliva loaded to swab*	*Final saliva Dilution*	*Equivalent saliva in final test volume RSID (µl)*
A	undiluted	1:1,000	1.00
B	1:1	1:2,000	0.50
C	1:4	1:5,000	0.20
D	1:9	1:10,000	0.10
E	1:49	1:50,000	0.02
F	1:100	1:100,000	0.01

N.B Equivalent saliva in final test volume stated to compare with RSID validation study published by Independent Forensics (Old *et al.* 2009).

Table 3.2: Serial dilution of saliva used for SALIgAE test sensitivity.

Sample identification	*Dilution of saliva loaded to swab*	*Final saliva Dilution SALIgAE*
A	undiluted	1:500
B	1:1	1:1,000
C	1:4	1:2,500
D	1:9	1:5,000
E	1:49	1:25,000
F	1:100	1:50,000

Table 3.3: Serial dilution of saliva used for Phadebas test sensitivity.

Sample identification	*Dilution of saliva loaded onto fabric for Phadebas*
A	undiluted
B	1:1
C	1:4
D	1:9
E	1:49
F	1:100

3.3.6 Determination of Specificity

3.3.6.1 Body Fluids

All body fluids listed were used to determine the specificity of all three tests by extracting the samples as described in section 3.3.2 and 3.3.3. For the Phadebas test the samples were either deposited directly onto cotton fabric or the swab head moistened with distilled water and rubbed against the fabric (depending upon the sample type) and left to air dry before the test protocol was performed.

3.3.6.2. Species Specificity

All animal body fluids listed were used to determine the specificity of each test, again the swab heads were extracted according to the RSID and SALIgAE protocols (sections 3.3.2. and 3.3.3) and for the phadebas test the swabs were moistened using distilled water and rubbed onto cotton fabric and left to dry before the test was performed

3.3.6.3. Commercial Specificity

In order to maintain the same conditions across each test and between samples the number of units of amylase loaded onto each swab for each test was kept the same. Swabs were extracted as described for the RSID and SALIgAE tests (section 3.3.2 and 3.3.3.) and deposited onto cotton fabric for the Phadebas test.

3.3.7 Compatibility of Tests with Alternative Extraction Buffers.

Saliva was collected into a bijoux tube and swabs were dipped into the saliva. Each swab head was cut from the stick and transferred to Eppendorf tubes labelled with the extraction buffer to be used in each case. Extraction buffers used were Lysis buffer, TE (Tris-EDTA) buffer, PBS buffer, water and the kit extraction buffer for RSID. Both tests were run as before (section 3.3.2 and 3.3.3); 1 ml of test extraction buffer was added to the corresponding labelled tubes for that test and vortexed briefly. The samples were left to extract for 1.5 hours and the appropriate amount of sample tested.

3.4. Results and Discussion

In this chapter the results and discussion will be combined together due to the integral part this initial study plays in the method development of the work presented in this thesis.

3.4.1 Sensitivity

A summary of the results for kit sensitivity when tested with human saliva is shown in Table 3.4. Human saliva was diluted to determine the sensitivity of each test. The RSID-Saliva test performed best and could detect a 1:50,000 dilution of neat saliva applied to the test cassette. The SALIgAE could detect saliva at a 1:2500 dilution.

RSID-Saliva instructions state that the reaction is not linear, which agreed with findings from this experiment. A standard curve was drawn using densitometry readings for the test strip band intensities using the GelDoc in association with QuantityOne software (Bio-Rad) (Figure 3.5). On the graph drawn the intensity of bands seemed to level off with higher volumes of saliva being tested. No positive results were obtained for the Phadebas test used with the same saliva dilutions; this could be due to insufficient saliva being deposited onto the fabric.

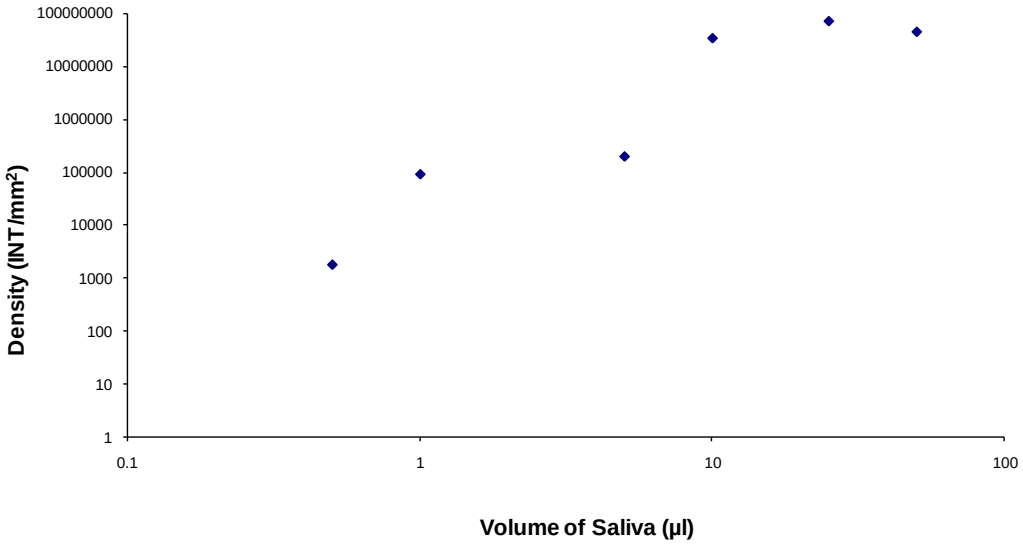

Figure 3.5: A calibration curve to show the density of the test band of the RSID kits used in the saliva sensitivity test.

Table 3.4: The sensitivity of three different saliva detection methods.

Sample identification	*Dilution of saliva*	*SALIgAE-Saliva test*	*RSID-Saliva test*	*Dilution of saliva loaded to fabric for Phadebas*	*Phadebas-Saliva test*
A	1:500	+++	+++	undiluted	-
B	1:1000	+++	+++	1:1	-
C	1:2500	+++	++	1:4	-
D	1:5000	-	+	1:9	-
E	1:25000	-	+	1:49	-
F	1:50000	-	-	1:100	-

+++ strong signal, ++ medium signal, + barely detectable signal, – no signal.

These detection limits fall within reported values. Independent Forensics (manufacturers of the RSID – Saliva test) claim that the kit can detect as little as 1 µl of saliva (Old *et al.* 2009). However a higher detection limit was seen by (Pang and Cheung 2008) of 1:10000 saliva dilution which would be the equivalent of 0.1 µl saliva.

Abacus SALIgAE (Miller and Hodges 2005) showed a positive result for a 1:1000 saliva dilution but not 1:10000. In this work SALIgAE gave a positive result with a 1:2500 dilution of saliva. The findings from this experiment are consistent with work carried out by Lim. Si-Keun *et al*. (2008).

In contrast, the results using Phadebas paper did not confirm reported values. Previous work has shown Phadebas to successfully detect saliva at 1:100 (Pang and Cheung 2008) and 1:200 (Myers and Adkins 2008) using the Phadebas tube test. Using Phadebas paper the lowest detection dilution has been reported as 1:5 (Lim. Si-Keun *et al*. 2008). In this work saliva was dropped directly onto the Phadebas paper. The volumes used in this experiment did not detect saliva even when undiluted. The low detection limit for Phadebas detection is reported as 2000U/L (Magle Life Sciences). Human saliva has an average amylase concentration of 263000-376000 U/L (Whitehead and Kipps 1975). From this it was expected that at least the undiluted saliva would have shown a positive result with the Phadebas paper. Therefore this experiment proves that the antibody identification methods detecting the actual presence of the enzyme are more sensitive than the Phadebas method detecting the activity of the enzyme or that the amylase tested was not active.

3.4.2. Body Fluids

Previous validation experiments have tested a limited range of body fluids. The manufacturer of RSID-Saliva states that the test is specific for human salivary amylase as there was no cross reactivity observed with blood, semen, urine, vaginal secretions, or menstrual blood (Old *et al.* 2009). This claim was investigated and sweat and breast milk were also tested (Table 3.5).

The results from this experiment showed a weak positive reaction produced from the sweat and breast milk samples. The reaction time was set at 10 minutes as recommended by the manufacturer, outside this time-frame a weak signal was produced for the semen test cassette. It should be noted that this result was clearly not as strong a reaction as the saliva sample. Previous work has shown positive reactions with fecal swabs, breast milk and urine (Pang and Cheung 2008) but the authors gave no indication as to the strength of the signal produced from these fluids. There was no other cross reactivity with any of the other body fluids tested. It was also noted that the timing of this test was a very important factor in interpreting results.

It seems likely that the SALIgAE saliva test is based upon the presence of salivary amylase. Miller and Hodges (2005) reached a similar conclusion. In an independent validation of this test there was no cross reactivity with human semen, vaginal secretions, sweat or blood. In addition to these body fluids menstrual blood, breast milk and urine were also tested using the SALIgAE kit (Table 3.5). In this experiment, positive test results were shown with breast milk and urine as well as saliva. These experimental findings confirm those of Pang and Cheung (2008). The recommended test time is 10 minutes. Positive results have been shown for nasal secretions within this time-frame (Lim. Si-Keun *et al*. 2008). Other positive results have been detected after longer incubations with sweat, urine, vaginal fluid and semen emphasising the importance of time when identifying potential saliva samples. Unlike the RSID false positives the colour changes for SALIgAE were seen to be as strong as those for saliva.

The Phadebas test is specific for amylase but not necessarily specific for saliva, as amylase is found within other body fluids at lower levels than in saliva. A positive result was seen with the breast milk sample as well as the saliva sample when saliva was spat upon the Phadebas paper. The presence of salivary amylase in body fluids other than saliva can be justified. Salivary amylase was shown to be detected in all volunteers of a study looking at whether salivary amylase is destroyed during digestion

(Fried *et al*. 1987). Salivary amylase has also been detected in breast milk (Lindberg and Skude 1982) and in semen samples (Auvdel 1986).

Table 3.5: Testing three different saliva detection methods with various body fluids.

Body fluid	*SALIgAE-Saliva test*	*RSID- Saliva test*	*Phadebas – Saliva test*
Blood	-	-	-
Breast milk	++	+	++
Menstrual blood	-	-	-
Saliva	+++	+++	+++
Semen	-	-	-
Sweat	-	+	-
Urine	+++	-	-
Vaginal fluid	-	-	-

+++ strong signal, ++ medium signal, + barely detectable signal, – no signal.

3.4.3 Species Specificity

No cross-reactivity was found in any of the tests with cat, cow or dog saliva (Table 3.6). The manufacturer of RSID-Saliva state that their test is specific for human salivary amylase and that in their validation study there was no cross reactivity observed with other animal saliva samples. The SALIgAE test had also previously given negative results with animal saliva samples. This result shown here, confirmed the trend also (Table 3.6). Others have reported similar results with cat and dog saliva (Pang and Cheung 2008). The same authors however reported positive test results for RSID with rat saliva only and positive results for pig, rat, mouse, rabbit, guinea pig and hamster were seen for both the SALIgAE and Phadebas tests; further samples were not tested in this experiment due to lack of accessibility of samples.

Table 3.6: Testing three different saliva detection methods with animal saliva.

Sample	*SALIgAE-Saliva test*	*RSID- Saliva test*	*Phadebas – Saliva test*
Cat	-	-	-
Cow	-	-	-
Dog	-	-	-

+++ strong signal, ++ medium signal, + barely detectable signal, – no signal.

3.4.4 Commercial α-amylases

Due to difficulties obtaining a human pancreatic amylase sample a commercial porcine pancreatic α-amylase sample was tested along with a commercial bacterial α-amylase. The RSID test gave negative results for each of the two samples (Table 3.7). The SALIgAE test gave a negative result for the bacterial α-amylase but a positive result for the porcine pancreatic α-amylase. Positive results were observed with both samples when the Phadebas paper was used.

Table 3.7: Testing three different saliva detection methods with commercial α-amylases.

Sample	*SALIgAE-Saliva test*	*RSID -Saliva test*	*Phadebas –Saliva test*
Porcine pancreatic α-amylase	+++	-	+++
Bacillus α-amylase	-	-	++

+++ strong signal, ++ medium signal, + barely detectable signal, – no signal.

These results are in agreement with previous work (Pang and Cheung 2008) showing the same trends as seen in this experiment for the same samples and tests.

3.4.5 Compatibility of Tests with Alternative Extraction Buffers.

It is important when using a saliva detection kit to know if the test is compatible with techniques used currently within forensic laboratories. A major difference lies in the different buffers used for extracting forensic samples. Therefore it was of interest to determine whether the kits tested could be used in different buffers such as those commonly encountered for DNA extraction. A compatible buffer would enable a potential forensic submission to be utilised for both body fluid identification and DNA profiling without having to extract two separate samples. For the RSID test the test buffer was used along with PBS, Water, Lysis buffer and TE buffers for the comparison. For the SALIgAE test, as water is used for the test extraction buffer, PBS, Lysis buffer and TE buffer were used for the comparison. From the results produced from this experiment (Table 3.8) it can be seen that the type of extraction buffer used does not seem to affect either set of test results.

Table 3.8 : Testing RSID – Saliva and SALgAE kits with different extraction buffers

Buffer	*SALIgAE-Saliva test*	*RSID - Saliva test*
PBS	+++	+++
Water	+++	+++
Lysis buffer	+++	++
TE buffer	+++	+++

+++ strong signal, ++ medium signal, + barely detectable signal, – no signal.

3.5 Chapter Summary

This chapter has compared current methods for saliva identification with different methodologies. RSID proved to be the most sensitive followed by SALIgAE and lastly Phadebas was the least sensitive method of those tested. The Phadebas test took longest whereas the other two tests were relatively quick. RSID - Saliva was most specific and showed the least cross reactivity with other body fluids. Positive results were seen for breast milk with both the Phadebas and the SALIgAE tests. Urine also showed a positive result with the SALIgAE. None of the animal saliva samples gave positive test results with any of the kits tested. Porcine pancreatic amylase gave a positive reaction with both the SALIgAE and the Phadebas tests. The Phadebas test gave a positive reaction with the bacterial amylase also. No cross reactivity was seen with the RSID kits with the commercial amylases. Concluding from these findings the RSID saliva test is the most accurate method currently on the market for saliva identification. It should also be noted that due to time constraints the findings were only tested once.

Chapter 4
Optimisation of Proteomic Conditions for the Analysis of Body Fluids

Chapter 4. Optimisation of Proteomic Conditions for the Analysis of Body Fluids

4.1 Introduction

2DGE is a method that is capable of delivering high resolution images with the ability to simultaneously separate hundreds to thousands of proteins (Herbert *et al*. 2001). It has been a powerful tool for both the analysis and the detection of proteins since the 1970's (O'Farrell 1975). The methods used between laboratories tend to differ greatly as there is not a set of standard running conditions for 2DGE which makes comparison of results among laboratories a challenge. Definitive comparison can only be made once a protein has been successfully identified by MS (mass spectroscopy). In order to obtain the best results from 2DGE, sample treatment and efficient protein extraction are important considerations (Herbert *et al*. 2001). Due to the complexity of a sample proteome it cannot be completely represented by a single extraction method and separation step. 2DGE captures a static image of the proteome at a particular point in time and under certain conditions.

This chapter describes various steps taken to optimise the stages associated with proteomic methodology as depicted in Figure 4.1, firstly giving an introduction to the techniques used (section 4.1) and then describing (section 4.2) and discussing (section 4.3) the results obtained during each stage of the proteomic process.

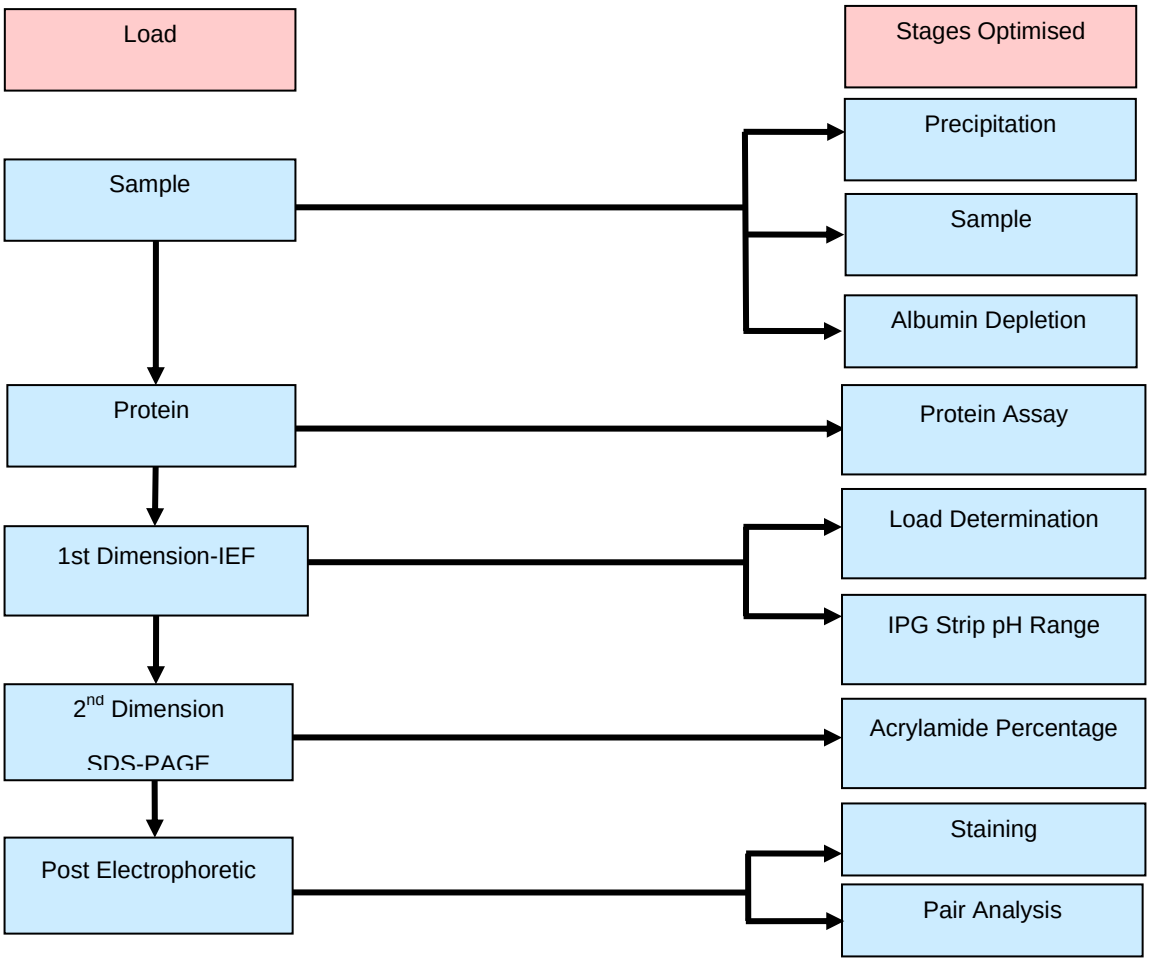

Figure 4.1: Flow chart showing each stage of the methodology optimised to study the proteomics of body fluids.

4.1.1 Sample Preparation

Sample preparation is an important step prior to 2DGE. Ideally samples should have a high protein concentration and be free of components which could interfere with electrophoresis such as salts, nucleic acids and lipids (Jiang *et al.* 2004). Sample preparation methods depend on the individual sample to be analysed and what portion of that sample is of interest. In the work described in this chapter no prior extraction method is employed as the proteins are precipitated directly from the fluid source.

There are several methods that can be applied to ensure the removal of interference components and these include dialysis, desalting and precipitating of the protein. Unlike desalting or dialysis to remove mineral salts, protein precipitation acts as a concentrating step; this is particularly useful when a dilute source of protein is being used as for example, when studying body fluids.

4.1.1.1 Precipitation Methods used in the Isolation of Proteins

Body fluids and tissues contain many substances which if left in the sample would interfere with the electrophoresis process causing insufficient focussing of protein spots, streaking or smearing on the gel, all of which would make interpretation and consequent identification of protein spots extremely difficult.

Protein precipitation is a method used to concentrate protein within a given sample and to separate the protein from the unwanted components of the sample. Once proteins have been precipitated from the body fluid sample they are mixed with an IEF compatible sample buffer to ensure that the sample itself does not interfere with the focussing step of electrophoresis.

Saliva samples can be easily collected in liquid form within a tube, but a cotton swab is used in vaginal fluid collection. In order to recover proteins from the swab, the swab head must be immersed into a suitable liquid. In forensic laboratories it is common to wash both vaginal cells and spermatozoa from swab heads using water followed by agitation. This technique is used both for visualisation on microscope slides and also for extraction of DNA for profiling. It is known that a hypotonic solution such as water, will give low cell recovery, due to cells bursting open during the washing stage. For this reason isotonic PBS is used as an alternative to water (Martin *et al*. 2006) as a wash solution. It is important when precipitating protein from a sample that as much protein as possible is recovered without the removal of portions of the proteome.

4.1.1.2 Depletion of Abundant Proteins

Proteomic studies have shown that body fluid samples can contain up to 1,000 proteins (Shen *et al*. 2004). Each individual protein may have a number of associated isoforms or post translational modifications that are separated on a 2D gel. The dynamic range of protein expression remains a challenge when trying to identify potential biomarkers, in this instance for fluid type. It is important to determine whether target proteins are highly expressed and therefore clearly shown on a protein gel, or expressed at lower levels which could mean that they are masked by the presence of more abundant proteins. High abundance proteins such as immunoglobulins in saliva or albumin in blood can be removed from samples enhancing the resolution of proteins in lower abundance. This can be performed using affinity columns to bind the high abundant proteins. An example of this is depicted in Figure 4.2 where a serum sample

is shown on a 2D gel before and after treatment with an albumin affinity column showing the removal of a large albumin spot revealing smaller spots otherwise hidden.

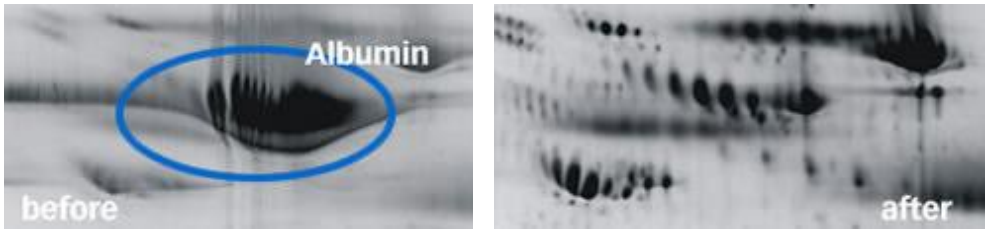

Figure 4.2: A 2D gel image showing a human serum sample before and after treatment with Vivapure® Anti HSA/IgG kit removing Albumin. *(source: Sartoriuns Stedim Biotech).*

4.1.2 Determination of Protein Concentration

It is important to determine the concentration of protein in the sample after precipitating protein from solution and prior to performing electrophoresis (1D or 2D). This enables accurate determination of the protein load whether loading to an IPG strip or depositing into a well of a SDS-PAGE gel. It is important that the method chosen is quick, sensitive and reproducible. The most common methods to determine protein concentration are the Bradford assay and the Lowry assay. The Lowry assay uses Folin phenol reagent in a two step reaction a) with copper in alkali, and b) reduction of phosphomolybdic-phoshotungstic reagent by the copper treated protein (Lowry *et al*. 1951) when in the presence of protein in a sample. Disadvantages of this method include a variability in colour development with different protein mixtures and the colour development being shown not to be completely proportional to protein concentration (Lowry *et al*. 1951). Interference has also been seen with Tris, EDTA, and magnesium and potassium ions (Bradford 1976). The Bradford assay involves CBB G-250 dye binding to protein which causes a shift in absorbance from 465nm to 595nm. The test is rapid and reproducible with colour stability for up to 1 hour. Drawbacks to this test show that detergents such as SDS can interfere with the test results.

Both these methods have disadvantages with interference and sensitivity. A new *RCDC* protein assay has been developed by Bio-Rad based on the Lowry assay. This protein assay is particularly useful as the IEF sample buffer recommended by Bio-Rad contains DTT CHAPS and UREA, and for 1DGE compatible with β-mercaptoethanol. The assay is linear between 0.2 mg/ml and 1.5 mg/ml, it is quick and simple to use and can be adapted for a microplate version rather than the cuvette form (Bio-Rad *RCDC* protein assay guide) and for this reason was used in this study.

4.1.3 First Dimension Optimisation

The post genomic era has seen the proteomics field rapidly advance in two-dimensional technology. One of the first new technologies introduced was IPG strips for the first dimension (Bjellquist *et al.*1982) as an alternative to rod gels using carrier ampholytes. The pH gradients of rod gels were considerably variable (Ong and Pandey 2001) thus making reproducibility difficult.

The IPG strips require reswelling with sample and sample buffer to rehydrate the gel attached to the strip prior to isoelectric focussing. In comparison to the previously used rod gels they are quick and easy to prepare. They have demonstrated improved reproducibility both within the same research group and inter-laboratory, higher loading capacity and increased resolution (Gorg *et al*. 1988).

After the IPG strips have been focussed they must be equilibrated prior to 2DGE. It is important that all proteins are loaded to the second dimension gel with SDS for improved transfer from first to second dimension. Proteins bind more strongly to the IPG strips than to rod gels, used previously. Strips are therefore washed twice with equilibrium buffer; firstly with the addition of DTT to reduce disulphide bonds and enhance protein unfolding, and secondly with iodoacetamide to remove excess DTT and to alkylate proteins. This second process prevents reoxidation of sulphydral groups during the second dimension (Gorg *et al*. 2000).

4.1.3.1 IPG Strip Load Determination

Protein dynamic range poses a problem with 2DGE. It is important when obtaining a 2D image that as much of the proteome is represented as possible. This plays an important role when ascertaining the appropriate sample load for an optimal 2D gel image. The less abundant proteins should be stained so that they are visible without over-staining the more abundant proteins. For example, in a body fluid such as blood, serum albumin is present at a concentration of approximately 40 mg/ml whereas cytokines are expressed at a picogram level showing a dynamic range variation of 10^9 (Rabilloud 2002). If too much protein is loaded on an IPG strip the gel image produced will be impaired. Horizontal streaking can be caused by protein aggregation and precipitation at a particular protein's isoelectric point, preventing complete separation in the first dimension. The load capacity of narrow-range IPG strips is substantially higher than that of broad-range IPG strips enabling the visualisation and identification of proteins that would not be seen on the broad range strips.

Protein load is dependent upon the type of staining used for gel visualisation. For example, there could be intense staining of high abundance proteins if the protein load is too high or the proteins would hardly be visible if not enough protein was loaded to the strip.

The amount of protein that can be loaded onto an IPG strip depends upon the strip length. The recommended range for protein loading onto a Readystrip™ 7cm IPG strip (Bio-Rad®) is between 5-100 µg of protein dependent upon the chosen staining method and the sample type (Bio-Rad® Readystrip™ IPG strip instruction manual). For Silver/SYPRO® Ruby (Invitrogen™) staining the recommended amount of protein to load is 5-20 µg, and for CBB it is 50-100 µg. The maximum load for a 7cm strip is 500 µg (Bio-Rad® Readystrip™ IPG strip instruction manual).

Different sample types show variability in the amount of protein that can be loaded to IPG strips. Lopez *et al*. (2000) used 50 µg protein standards run on mini gels with silver and SYPRO® Ruby stains, which is over twice the maximum recommended load stated by the manufacturer. Previous work looking at saliva proteins used 300 µg protein with 13 cm IPG strips and stained with CBB (Huang 2004).

4.1.3.2 IPG Strip Range Determination.

Each protein has a pH represented by a position on the IPG strip where it has a charge of zero; this is known as the p*I* of the protein. Proteins are separated in the first dimension based upon their p*I*. IPG strips can cover a wide range of pHs such as a broad range strip with a pH of 3-10 or can be limited to a much smaller range such as a zoom strip with a pH of 3.9-5.1. Initially when dealing with a new sample type it is common practice to use a broad range strip such as pH3-10 (Ong and Pandey 2001) to give an overview of the proteins present within the sample and to determine whether the sample contains mainly basic or acidic proteins and then focus in on that specific area of interest. IPG strips can also be used as an alternative to separating more complex samples into acidic and basic fractions prior to analysis using ion exchange chromatography. Overlapping narrow range IPG strips in the first dimension of a two-dimensional gel can be used to show protein separation across a range of pHs thus avoiding aggregation of multiple proteins at a single spot (Gorg *et al*. 2000).

When separating in 2D using a broad range pH range strip, only a small proportion of the whole proteome is shown. This is due to insufficient spatial resolution and the difficulty of seeing low copy number proteins in the presence of abundant

proteins (Gorg *et al*. 2000). Resolution and the separation power are lower using broad range strips compared to the narrow range strips but the representation of the sample as a whole is greater. By using a narrow range strip the proteins shown to be one spot on a broad range strip could in fact be a number of smaller spots that had not resolved completely. Thus a strip with a smaller pH range could display a larger number of protein spots with better resolution than a strip with a wider pH range.

4.1.4 Second Dimension Optimisation

4.1.4.1 The Determination of Gel Acrylamide Percentage

When separating proteins in the second dimension it is important to consider the range of molecular weight proteins to be analysed within the sample. The ease at which proteins move through the gel matrix is determined by the pore size of the gel and the size and shape of the protein. Pore size is inversely proportional to the amount of acrylamide added to the gel mixture. Gels with a higher acrylamide percentage have a smaller pore size and thus have a greater ability to separate smaller molecules. Gels with a lower acrylamide percentage have larger pore sizes, separating larger molecules more easily. Although the separation of larger molecular weight proteins would be seen with a lower acrylamide percentage gel, smaller proteins could move too quickly through the gel matrix and be lost off the end of the gel. For these reasons the proteins within the sample should be used as a guide to determine the gel percentage suitable for use.

4.1.4.2 Protein Detection using Post Electrophoretic Stains

When looking at a staining method for use with two-dimensional electrophoresis it is important to consider the sensitivity of the stain, the reproducibility of the staining, and its compatibility with downstream techniques associated with protein characterisation. For expression analysis a stain must provide a linear measure of protein abundance (Smales *et al*. 2003) in order to determine accurately the expression changes in protein spots between gel images.

In the past, the most commonly used staining methods for 2D gels have been CBB and silver staining. These stains have not been ideal due to poor sensitivity (CBB) and poor compatibility (silver) with MS applications downstream (Lopez *et al*. 2000). Within the last few years the introduction of fluorescent dyes such as SYPRO® Ruby

(Invitrogen™) and Flamingo™ protein stain (Bio-Rad®) have added further possibilities in staining techniques.

4.1.4.2 (i) Protein Detection using Colloidal CBB G-250

CBB has been a popular method for protein staining since the early 1960s (Williams 2001) as it is easy to use, relatively cheap and is compatible with downstream techniques (it does not alter the protein configuration in any way).

CBB staining has low sensitivity and limited detection of proteins, so an adapted colloidal CBB method has been developed (Neuhoff *et al*. 1988) using progressive staining until reaching an endpoint. Equilibrium is set up between colloidal particles and free dye in solution. The low concentration of free dye penetrates the gel matrix, staining the proteins. The colloidal particle is excluded from the gel preventing background staining, overcoming detection limitations by increasing the stain's sensitivity. By using CBB as a colloidal stain the sensitivity of the method is increased from detection of 30-100 ng of protein down to 8-10 ng of protein (Patton 2002). The dye has been seen to have a linear dynamic range over a 10-30 fold range (Patton 2002) although a disadvantage of this method is the increase in staining time (up to three days).

4.1.4.2 (ii) Protein Detection using Silver Staining (PlusOne™ Silver Staining Kit, Amersham Biosciences)

Silver staining is based on the origins of photography development and was first introduced for protein staining post electrophoresis in 1970s (Kerenyi and Gallyas 1972). Although silver staining has been the stain of choice for its sensitivity, it is destructive for MS applications, so the benefits of resolving 1,000 protein spots is offset by the inability to identify them. Additionally silver acts as a strong oxidising agent that can lead to chemical modification or destruction of proteins. Furthermore the presence of sensitising agents such as gluteraldehyde can cross-link to the proteins causing covalent modifications and preventing efficient digestion by trypsin prior to MS analysis (Shevchenko *et al*. 1996; Scheler *et al*. 1998). To resolve these problems an adapted silver staining method has been used by Shevchenko and colleagues (1996) permitting further proteomic analysis using mass spectroscopy by the omission of gluteraldehyde. Silver staining is a multistep procedure with four main steps common to

most protocols; (i) fixation, (ii) sensitisation, (iii) silver impregnation and (iv) image development. Unlike CBB, it is not an end point stain. The staining intensity of spots can vary from gel to gel making reproducibility increasingly difficult to achieve. This may also cause problems if using software to analyse silver stained gels when determining whether spot intensity variation is due to an expression change or simply a difference in the staining between gels.

Silver staining is achieved by saturating the gels with silver ions, washing the molecules that are less tightly bound from the gel matrix, and then reducing the bound metal ions to form metallic silver. Silver staining does not act by specific binding to the protein, rather it interacts with lysine residues (Lopez *et al*. 2000). The sensitivity of PlusOne™ Silver staining kit (Amersham biosciences) is 0.20-0.60 ng protein (PlusOne™ Silver staining kit Protein instructions) whereas the linear dynamic range is limited (10-fold) (Patton 2002). This has made it difficult to determine subtle differences in protein quantities (Lopez *et al*. 2000).

4.1.4.2 (iii) Protein Detection using SYPRO® Ruby Fluorescent Stain (Invitrogen™).

SYPRO® Ruby (Invitrogen™) is a relatively new stain introduced to proteomics that contains Ruthenium which is chelated into a fluorophore (White *et al*. 2004). This stain does not require the use of any protein modifying agents and is therefore compatible with downstream spectrometric techniques. It binds non covalently to basic amino acids; lysine, arginine and histidine residues (Lopez *et al*. 2000) via an electrostatic interaction similar to CBB (Miller *et al*. 2006). SYPRO® Ruby (Invitrogen™) staining has a sensitivity that is similar to silver. It is described as an endpoint stain therefore over-staining cannot cause visibility problems when viewing the gels. It is also is a simple method with only three staining steps in comparison with silver staining which has many more steps involved. Protein detection between 4-8 ng is possible with this staining method giving an alternative in sensitivity to silver staining and a quicker, easier staining method to use. The linear dynamic range of the dye extends over three orders of magnitude; greater than that of both silver and colloidal CBB stains (Patton 2002).

4.1.4.2 (iv) Detection of Proteins using Flamingo™ Fluorescent Stain (Bio-Rad®)

Flamingo™ fluorescent gel stain (Bio-Rad®) is reported to be a more sensitive alternative to SYPRO® Ruby gel stain (Invitrogen™). The dye in this stain only fluoresces when bound to denatured protein (Bio-Rad Literature Bulletin 5346). It has the advantage of a short and simple protocol which can be completed within five hours and has only two steps (lower than any other stain used in this experiment). Like SYPRO® Ruby (Invitrogen™), Flamingo™ (BioRad®) stain is compatible with downstream techniques such as mass spectrometry and enzymatic digestion. It has been reportedly shown to display sensitivity of 0.50-2 ng using a UV transilluminator and is therefore the most sensitive of all the stains tested.

4.1.5 Optimisation of Image Analysis – Gel Repeat Expression Comparison.

Image capture is critical for the successful analysis of 2D gels. Image dynamic range is described as the range of grey tones within a digital image whereas 'bit depth' refers to the number of bits used to define each pixel and hence the number of grey tones generated. The larger the bit depth, the larger the range of grey tones per pixel. For example, an 8 bit image file produced by a Charged Coupled Device (CCD) camera (ChemiDoc™ EQ) has 256 (2^8) shades of grey per pixel. This compares to 16 bit image files with 65536 (2^{16}) shades of grey per pixel obtained when the laser scanner (PharosFX™ molecular imager) was used for image capture for the same 2D gels. A higher number of shades of grey per pixel (16 bit images) enables the software to differentiate between low abundance spots and the background of the gel thus increasing the number of spots detected and analysed (Miller *et al*. 2006).

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4.2 Results

All SDS-PAGE gels run in duplicate in parallel throughout this study and the gel images displayed in this results section are representative of the two duplicates unless otherwise stated.

4.2.1 Sample Preparation

4.2.1.1 Precipitation Methods used in the Isolation of Saliva Proteins.

Three of the most widely used precipitation methods were studied; TCA-acetone, ethanol, and acetone precipitations. The most effective precipitation method was selected and applied for 2D gel electrophoresis experiments conducted in this research.

An independent experiment was performed three times using 300µl of one donor's saliva in duplicate to compare the three precipitation methods (see sections 2.3 and 2.4). Figure 4.3 shows the average results for each saliva precipitation. The total protein precipitated was measured using the *RCDC*™ protein assay (Bio-Rad®)(see section 2.5).

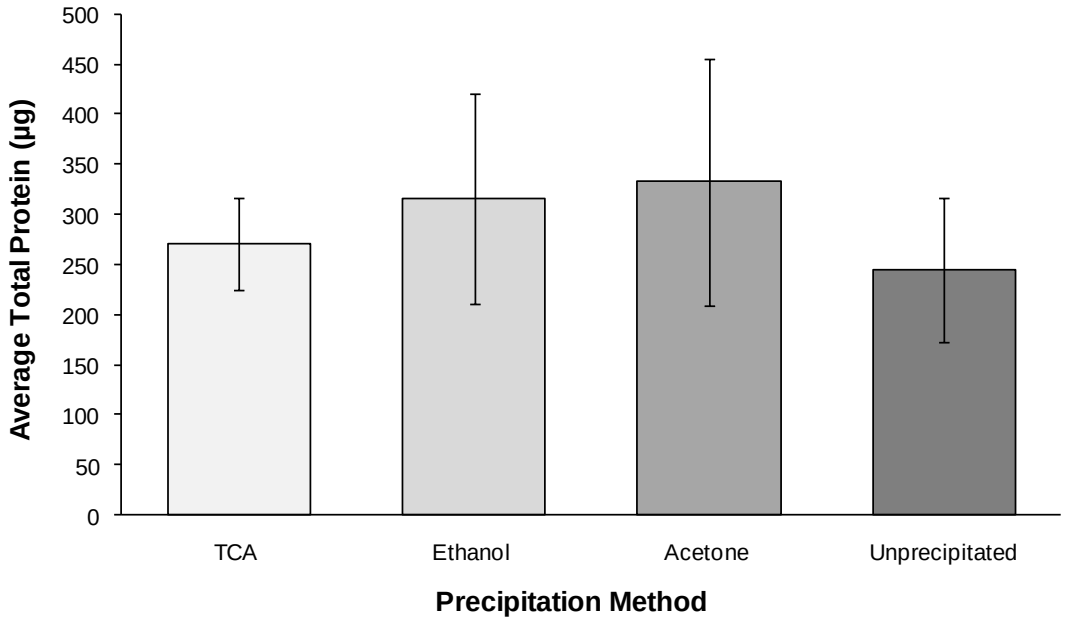


Figure 4.3: Comparison of TCA-acetone (TCA), Ethanol and Acetone precipitation methods with unprecipitated saliva protein. Showing total saliva protein recovered by each method. Average total protein of three replicate experiments was plotted ±1SD from the three averages, each average based on two duplicates.

The results for saliva precipitation (Figure 4.3) show that using acetone to precipitate protein from a 300 µl saliva sample afforded the highest average total protein (332 µg). Both Ethanol and Acetone gave the greatest recovery, and ability to concentrate protein from the original saliva sample with little difference between them. TCA-acetone produced the lowest total protein (270 µg) among all three methods tested. Although absolute differences in means were found, variation on the three replicates shows overlapping results.

From these results the method that yielded the highest level of total protein and the lowest total protein were compared to determine whether differences could be seen in a 2D gel image depending upon the precipitation method used (see Figure 4.4). Acetone and TCA-acetone precipitated saliva samples were run in the first dimension using 3-10NL Readystrip™ IPG strips (see section 2.6) and in the second dimension (see section 2.6) using 12% polyacrylamide gels, stained with SYPRO® Ruby gel stain (see section 2.8.3).

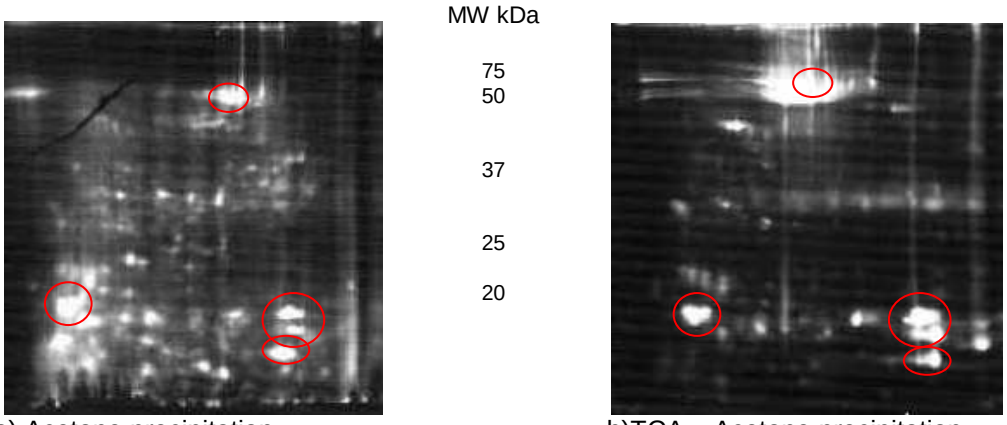


Figure 4.4: Saliva protein (50 µg) precipitated with a) acetone and b) TCA- Acetone using 3-10NL Readystrip™ IPG strips and 12% polyacrylamide gels, stained with SYPRO® Ruby fluorescent gel stain.

Distinct differences can be seen in the gel images produced by each precipitation method. Although the main protein groups seem to be present within each image (those highlighted in red), a greater number of spots are visible in a) when compared with b). The spots in the TCA-acetone sample image are less resolved than those in the acetone sample. It is therefore very important to keep the precipitation method constant throughout a study when comparisons of gel images are involved.

4.2.1.2 Protein Extraction from Cotton Swab Heads

An experiment was performed to determine the total protein recovered from a swab head when immersed in either water or PBS (see section 2.3.2), followed by an acetone precipitation (see section 2.4.3). The total protein within each sample was measured using an *RCDC*™ protein assay (BioRad®)(see section 2.5). This experiment was repeated three times and the results are presented in Figure 4.5.

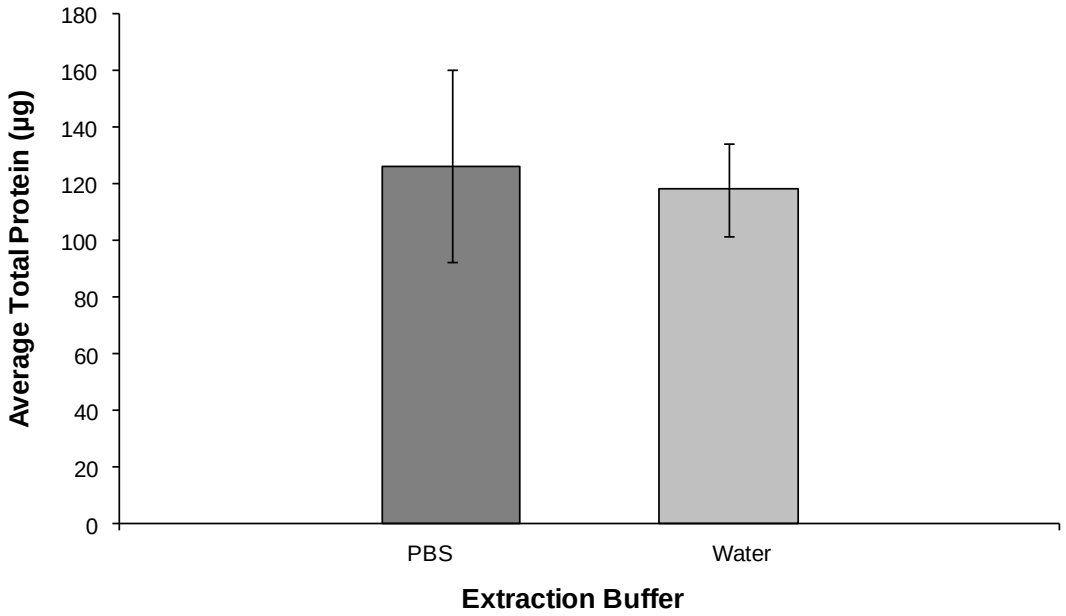


Figure 4.5: A comparison of protein extraction from a single swab head using PBS or water. Average total protein plotted as a result of three swab heads tested independently with duplicate readings from each swab head ±1SD.

The comparison of PBS and water to elute protein from a vaginal swab head showed little difference between the different wash buffers used. The total protein recovered using PBS was slightly higher (126 µg) than the total protein recovered using water (118 µg). Due to compatibility with different DNA extraction methods used in forensic laboratories either method could be used prior to precipitation. For this study PBS was chosen to avoid potential osmotic imbalances within the cells.

4.2.1.3 Precipitation Methods used in the Isolation of Vaginal Fluid Proteins

The same three precipitation methods were tested once again using one donor's samples but by looking at vaginal fluid instead of saliva. The average results from this experiment are displayed in Figure 4.6.

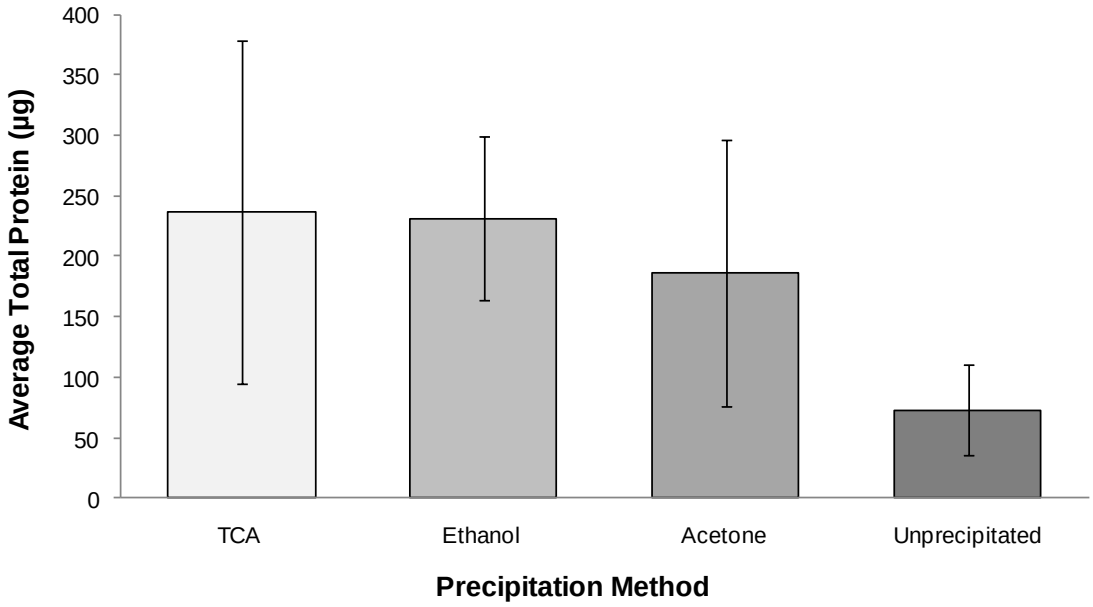


Figure 4.6: Comparison of TCA-acetone (TCA), Ethanol and Acetone precipitation methods showing total vaginal fluid protein recovered by each method. Average total protein plotted ±1SD (n=3).

The total protein measurement of unprecipitated vaginal fluid was low (73 µg) in comparison with unprecipitated saliva (244 µg). This was likely in part due to a dilution of vaginal fluid samples in 1 ml PBS prior to precipitation, in order to recover protein from the swab head. These results demonstrate different findings to those of the saliva experiment. TCA-acetone and ethanol recovered a higher average total protein (237 µg and 231 µg respectively) than acetone precipitation (186 µg) but the high standard deviation shown for the TCA-acetone method means a high level of variability in the average total protein recovered using this method. In this study it is important to consider a method that is shown to yield a consistently high concentration of protein with a simple method as more steps give a greater chance for loss of protein. To enable comparison between samples, the acetone precipitation method was chosen and applied for each body fluid used in this study.

4.2.1.4 Depletion of Abundant Proteins

In order to efficiently remove abundant proteins from samples, columns containing antibodies raised against the abundant human proteins IgG and HSA were used. The samples to be analysed were passed through the column and the high abundance proteins removed by binding to the antibodies. In this research, all blood samples were treated with the Vivapure® anti HSA/IgG columns (Sartorius) prior to being applied to 2D gels. The samples were also run on 1D gels to show the eluted fractions from the columns. Blood and menstrual blood sample swabs from one donor were used to avoid person to person differences having an effect on the data produced. Both samples were run through anti HSA/IgG columns (Vivapure®) and the eluted fractions collected in separate tubes (see section 2.3.5). The samples were quantified using *RCDC* protein assay (Bio-Rad®). 1D gels were poured (see section 2.7) and run in duplicate for each sample type. An aliquot of sample that had not been treated with the column was run alongside each column eluate and a protein molecular weight standard (see Figure 4.7). Each well was loaded with 10 µg protein sample and after electrophoresis, the gels were stained with colloidal CBB stain (see section 2.8.1).

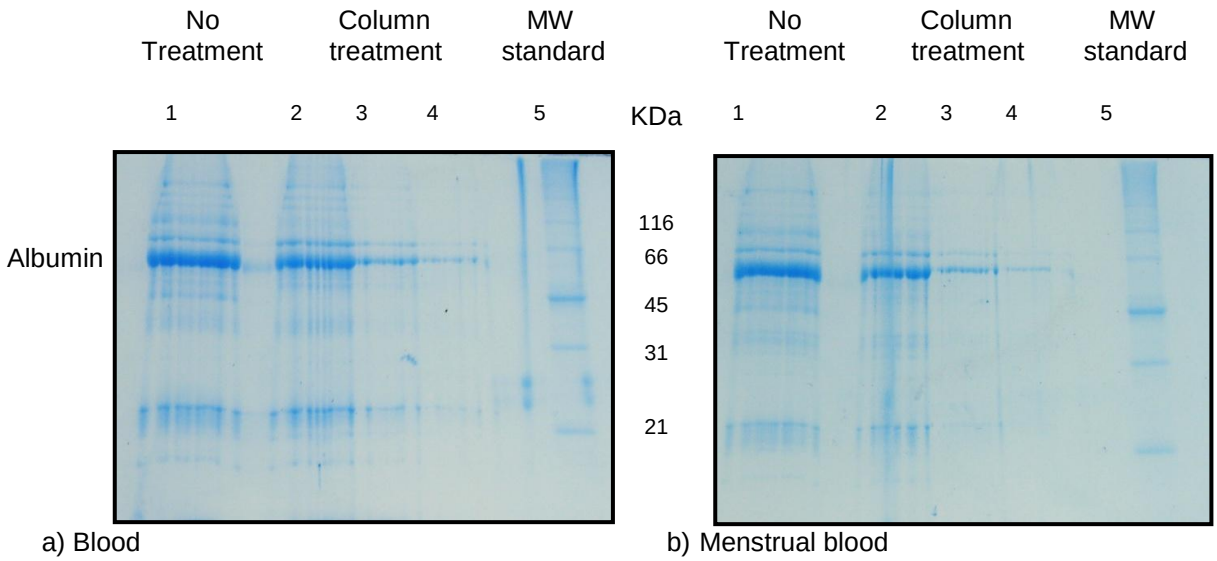
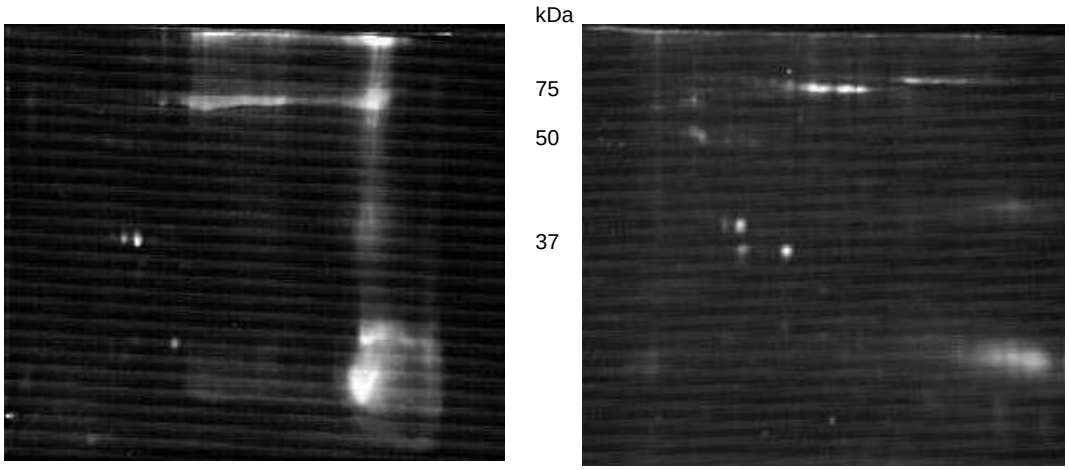


Figure 4.7: Two 1D gel images of a) blood and b) menstrual blood samples. Lane 1 – before treatment, lanes 2, 3 and 4 show successive eluted fractions after treatment with an anti HSA/IgG column (Vivapure) and lane 5 contains the SDS-PAGE protein standard (*Bio-Rad*).

The successive column elutes displayed a reduction in the albumin band visible at approx 66 kDa and also showed a reduction in the total protein visible on the 1D gel.

Lane 1 (loaded with sample not passed through the column) did not show distortion in the albumin band as would be expected with blood samples. From this it was possible to infer that the level of albumin present within both the blood swab and the menstrual blood swab was much lower than from clinical blood samples. In respect of the blood swab this could be explained by the small volume of blood being deposited onto the swab. Although the menstrual blood sample was taken when menses was heaviest, the proportion of blood present in the sample could be lower than expected due to being a mixture of blood, endometrium and vaginal fluid. Sample dilution with PBS during extraction from the swab led to a lower proportion of blood within the sample and thus less entering the column. The general use of anti HSA/IgG columns is for whole blood samples collected direct from the vein of a donor which would tend to have a higher concentration of albumin present than forensic samples deposited onto a swab head or exposed to air for a period of time.

2D gels were used to assess the composition of both blood samples in more detail (see Figure 4.8).

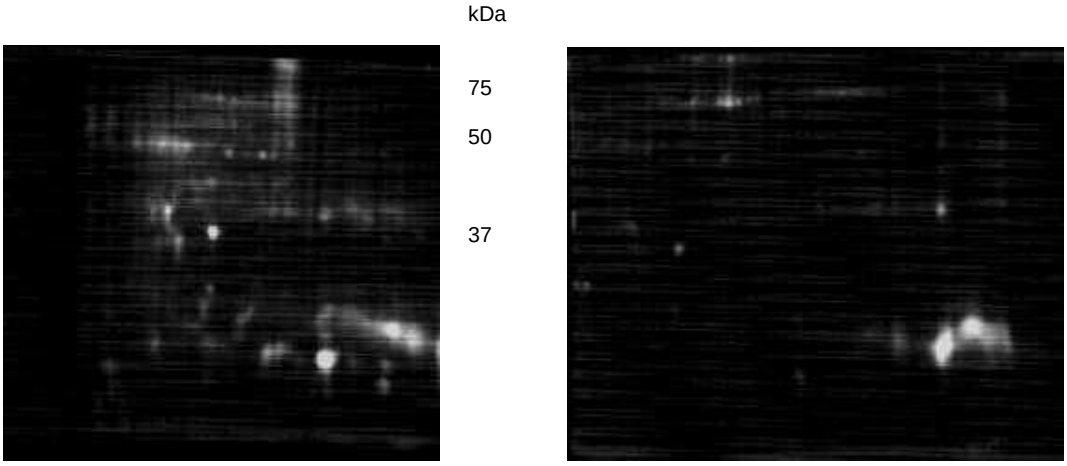


a) Blood without treatment b) Blood passed through column

Figure 4.8: 2D gel images of blood protein (acetone precipitation, 50 µg protein load, pH 5-8 Readystrip™ IPG strips, 12% polyacrylamide gels, SYPRO® Ruby stained (Invitrogen™)). Panel a) sample not passed through the column and panel b) sample having been treated with the anti HSA/IgG column (vivascience®).

The molecular weight standards run on these gels have been removed from the images (Figures 4.8 and 4.9) for aesthetic purposes. There is little difference between the 2D images produced by blood protein (Figure 4.8) but a higher load of protein may provide a better comparison. From these images it is clear that the sample that had not been passed through the column does not show albumin masking spots at 66 kDa

where expected, and hence a column may not be necessary for this application. Menstrual blood samples were also compared by 2DGE (Figure 4.9).



a) Menstrual blood without treatment b) Menstrual blood passed through column

Figure 4.9: 2D gel images of menstrual blood protein (acetone precipitation, 50 µg protein load, pH 5-8 Readystrip™ IPG strips, 12% polyacrylamide gels, SYPRO® Ruby stained (Invitrogen™). Panel a) sample not passed through the column and panel b) sample having been treated with the anti HSA/IgG column (vivascience®).

The results from the menstrual blood sample show more protein spots are visible in both gel images when compared with the images for the blood sample. There does not appear to be a strong band visible at 66 kDa that would be indicative of albumin being in high abundance within menstrual blood and these results are consistent with those from the blood sample. Panel b) shows the major effect of the anti HSA/IgG column is to eliminate a variety of some protein spots which could be characteristic of menstrual blood. Published reports of using columns has shown that as well as albumin and immunoglobulin, other proteins may be lost in the process (Joo *et al*. 2003). For this reason it was decided not to use the columns in any future experiments.

4.2.2 Determination of Protein Concentration – A Comparison of Two Protein Assays

Two protein assays were compared in order to determine whether IPG strip reswelling buffer had an effect on the accuracy of the standard curve. Hence the Bradford protein assay (Bradford 1976) and the *RCDC* protein assay (Bio-Rad®) were compared in one experiment. The *RCDC* assay is known to be compatible with a number of different buffers, reducing agents and detergents that may be present in a sample as a result of the extraction methods. Both assays were tested using IgG

protein standards in the range of 0.10 mg/ml – 1mg/ml. The standards were diluted using the reswelling buffer (as the samples would be) in order to ascertain whether or not the buffer had an effect on the quality of the standard curve produced. Thus inaccurate protein concentrations for samples associated with that particular assay would be avoided if this was the case. Figure 4.10 shows the standard curve produced using the Bradford assay and in comparison Figure 4.11 shows the standard curve produced using the *RCDC* protein assay. From these standard curves, the R^2 value for the Bradford assay was 0.75 and for the RCDC assay was 0.97. Therefore the reswelling buffer appears to affect the standard curve accuracy in the Bradford assay but not in the *RCDC* assay. The closer the R^2 value is to 1, the more linear the relationship between the optical density of the sample and its concentration for the range of standards used in the experiment, indicating that more reliable protein concentrations can be determined from the curve. On the basis of these results, the *RCDC* assay was selected for this work.

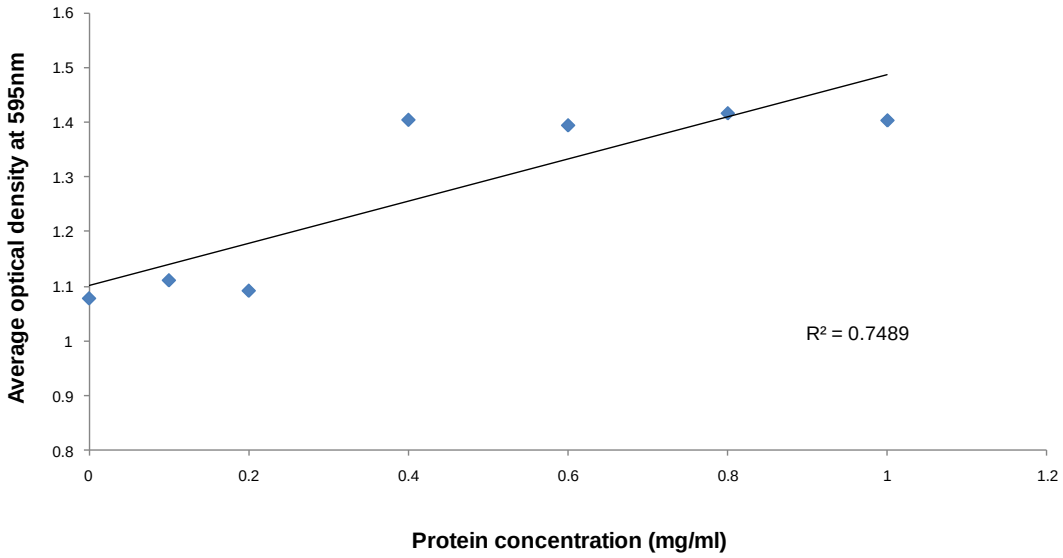


Figure 4.10: A Bradford assay using IgG standards with reswelling buffer, values taken as an average of two optical density readings.

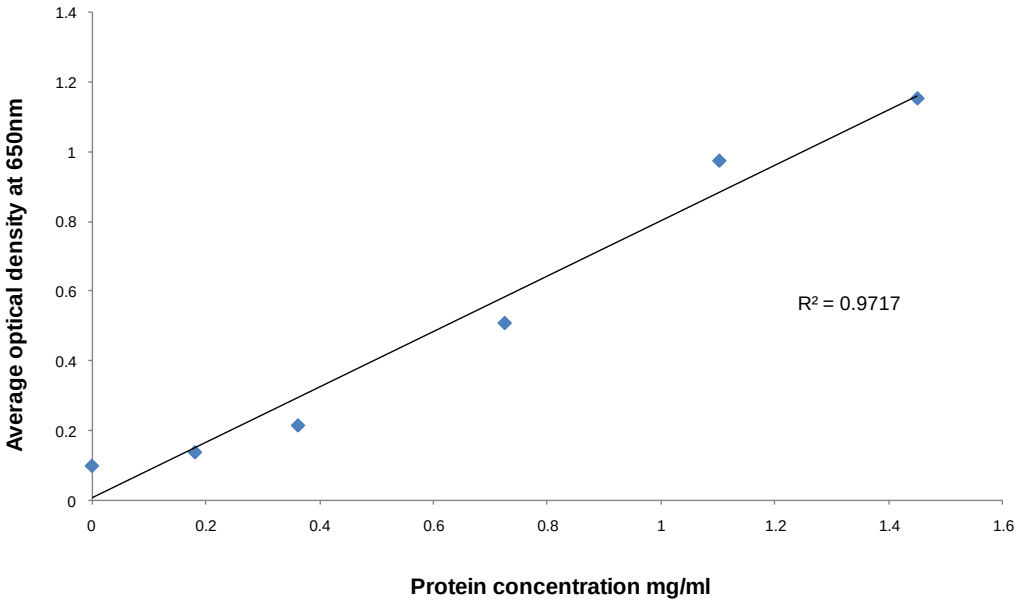
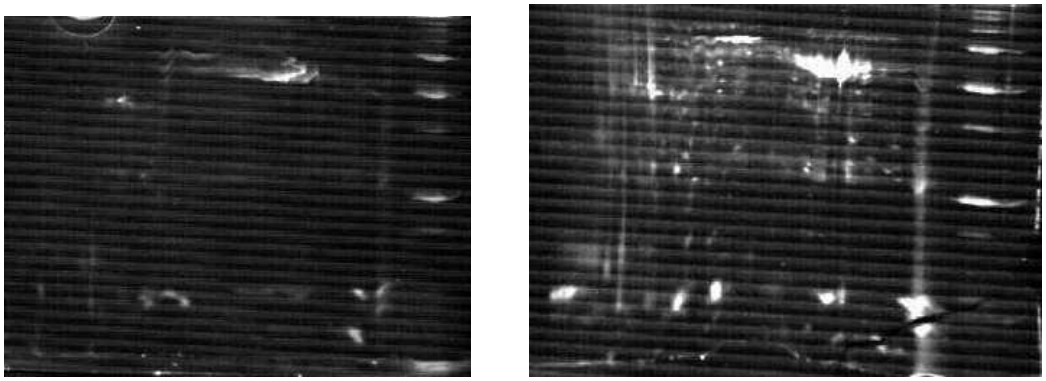


Figure 4.11: A *RCDC* protein assay using IgG standards with reswelling buffer, values taken as an average of two optical density readings.

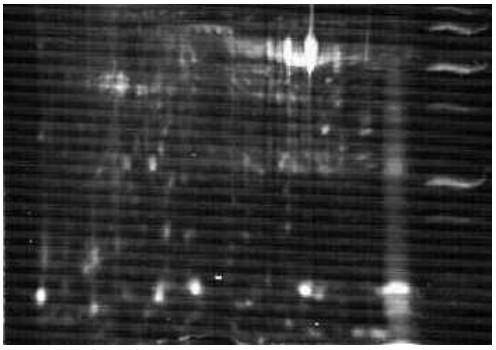
4.2.3 First Dimension Optimisation

4.2.3.1 A Comparison of IPG Strip Load Variation using Saliva Protein

An experiment was conducted to determine the optimal protein load for each body fluid (Figure 4.12). Initially, for saliva and vaginal fluid, three load quantities were chosen; 25 µg, 50 µg and 75 µg. This was limited in later experiments to a narrower range based upon the findings of this experiment. Gels were run in duplicate using 7cm pH 5-8 IPG and in the second dimension run on 12% polyacrylamide gels (see section 2.6) and stained with SYPRO® Ruby (Invitrogen™) gel stain (see section 2.8.3).



a) 25 µg load b) 50 µg load



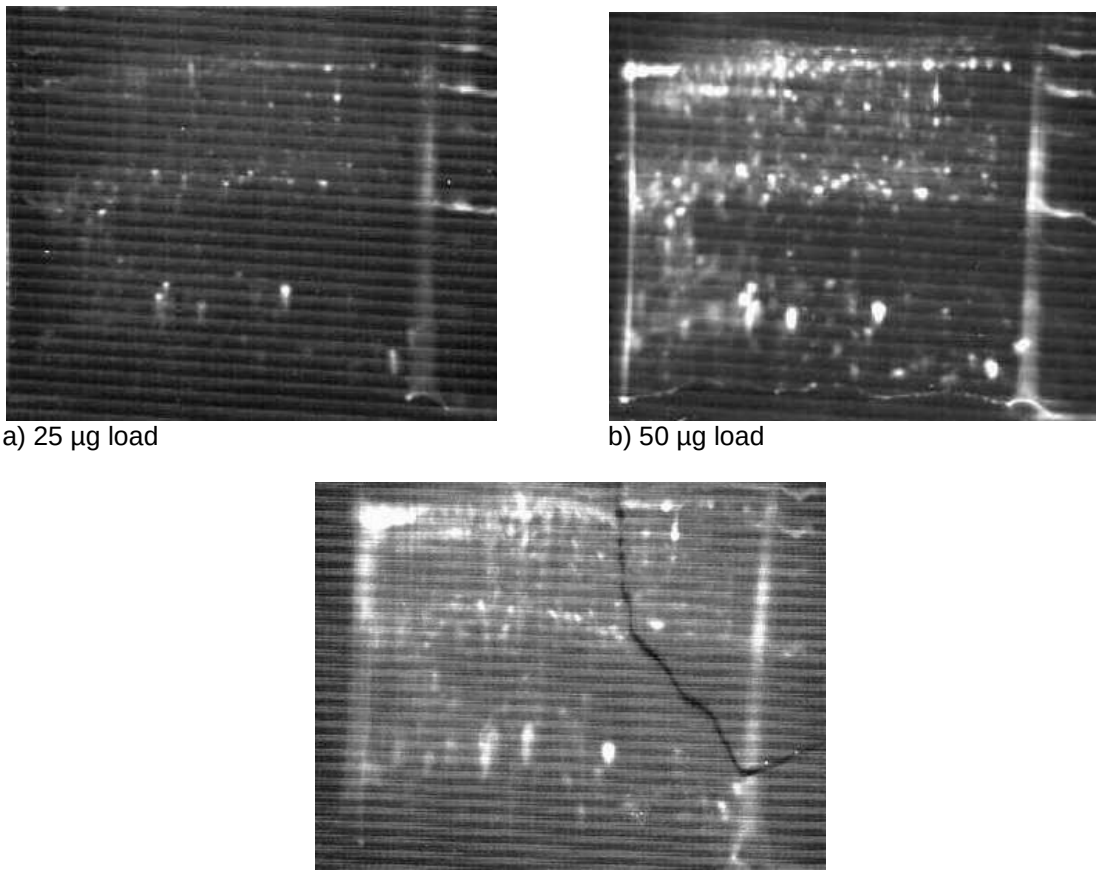
c) 75µg load

Figure 4.12: 2D gel images of a) 25 µg, b) 50 µg and c) 75 µg saliva protein (acetone precipitation, pH 5-8 Readystrip™ IPG strips, 12% polyacrylamide gels and SYPRO® Ruby (Invitrogen™) stain.

Results from this experiment (see Figure 4.12) showed that the lowest load (25 µg) of saliva protein displays only four major protein groups. The results also demonstrated that medium load (50 µg) generally displayed more detail within the gel. A greater number of smaller protein spots were also visible as well as emphasising the major protein groups. The highest load (75 µg) showed the greatest detail overall but the major protein spots seemed over stained resulting in low resolution and the more abundant protein spots were distorted on the gel. From these findings a 50 µg protein load was determined to be suitable for subsequent experiments using saliva samples.

4.2.3.2 Determination of IPG Strip Loading for Vaginal Fluid Protein

A similar experiment to that described in section 4.2.3.1 was performed to determine the optimal protein load from vaginal fluid. Gels were run in duplicate using 7cm pH 5-8 IPG and in the second dimension on 12% polyacrylamide gels (see section 2.6). Both were stained with SYPRO® Ruby (Invitrogen™) gel stain (see section 2.8.3).



a) 25 µg load b) 50 µg load

c) 75 µg load

Figure 4.13: 2D gel images of a) 25 µg, b) 50 µg and c) 75 µg vaginal fluid protein (acetone precipitation, pH 5-8 Readystrip™ IPG strips, 12% polyacrylamide gels and SYPRO® Ruby (Invitrogen™) stain).

Figure 4.13 shows the images produced with different vaginal fluid protein loads. A similar trend to that noted in respect of saliva protein is demonstrated and there are fewer protein spots visible with the lowest load (25 µg). A larger number of spots were visible with the medium load (50 µg), with good separation and resolution. However the gel with the highest load (75 µg) showed distortion and masking of some of the areas by abundant proteins, which appeared overloaded (shown by very bright staining in the top left corner of the gel). On the basis of these results a 50 µg load was chosen as a suitable load for vaginal fluid.

4.2.3.3 IPG Strip Range Determination.

Experiments were performed to identify the optimal strip pH range in order to achieve the best protein separation. Four strip ranges were chosen; pH 3-10, pH 3-6, pH 4-7, and pH 5-8. Each strip was run in the second dimension using 12%

polyacrylamide gels. For each body fluid type the protein load remained constant; 50 µg of protein was loaded onto each strip so that accurate comparisons could be performed. Alongside the samples, 2D SDS PAGE protein standards were run (Figure 4.14) in order to determine the p*I* and molecular weight of proteins within the gel. Table 4.1 describes the p*I* of the protein standards represented in Figure 4.14 (numbers shown below the spot of interest). A molecular weight standard was also run on the right hand side of each gel. This also enabled the confirmation of protein identification from MASCOT search data to be ascertained based on the cut spots position within the 2D gel.

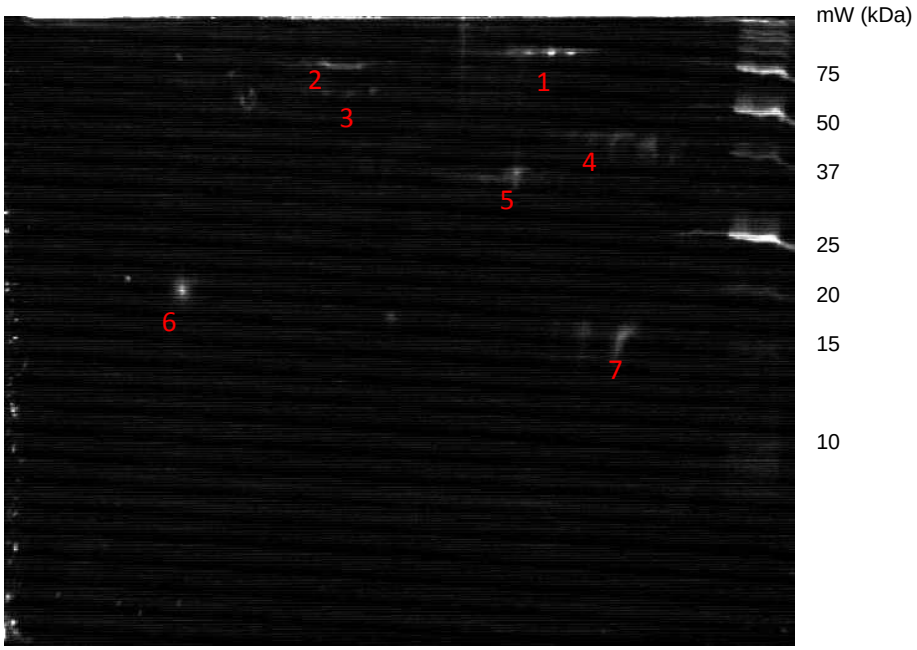


Figure 4.14: A 2D gel image to show 2D SDS-PAGE standards (Bio-Rad) run using pH 3-10 strips run on a 12% gel stained with SYPRO Ruby gel stain (Bio-Rad).

The proteins displayed in Figure 4.14 have their name, p*I* and molecular weight listed in table 4.1.

Table 4.1: 2D SDS-PAGE standard protein data (ref: Bio-Rad 2D SDS-PAGE standards instruction manual).

Protein	***Molecular weight***	***pI***
1. Hen egg white conalbumin 1	76,000	6.0, 6.3, 6.6
2. Bovine serum albumin	66,200	5.4, 5.6
3. Bovine muscle actin	43,000	5.0, 5.1
4. Rabbit muscle GAPDH	36,000	8.3, 8.5
5. Bovine carbonic anhydrase	31,000	5.9, 6.0
6. Soybean trypsin inhibitor	21,500	4.5
7. Equine myoglobin	17,500	7.0

N.B Standards with more than one isoform separate showing more than one spot on a 2D gel and hence have more than one p*I* value.

4.2.3.4 A Comparison of IPG Strips pH Range using Saliva Protein

It is evident from the separation shown by each strip (Figures 4.15 and 4.16) that although the broad range strip (pH 3-10) gives a good overview of the proteins expressed within the saliva sample, the resolution and the number of spots visible increased when narrower pH range strips were used. The gel image of strip pH 4-7 displayed blurred (unfocussed) spots towards the acidic end of the strip range but showed an increase in spot numbers at the neutral end. The pH 3-6 strip gave better separation than the broad range strip for the same pH range and the proportion of spots visible was greatly increased. The pH 5-8 strip displayed the greatest number of spots with the highest resolution of the strips tested thus making it the most suitable strip for further analysis. Choosing only one pH range would limit any biomarkers identified to this range hence dismissing any potential biomarkers outside this range. Ideally the whole range would be covered however the benefit this could provide could be limited by the extra gels run and the time taken to do so.

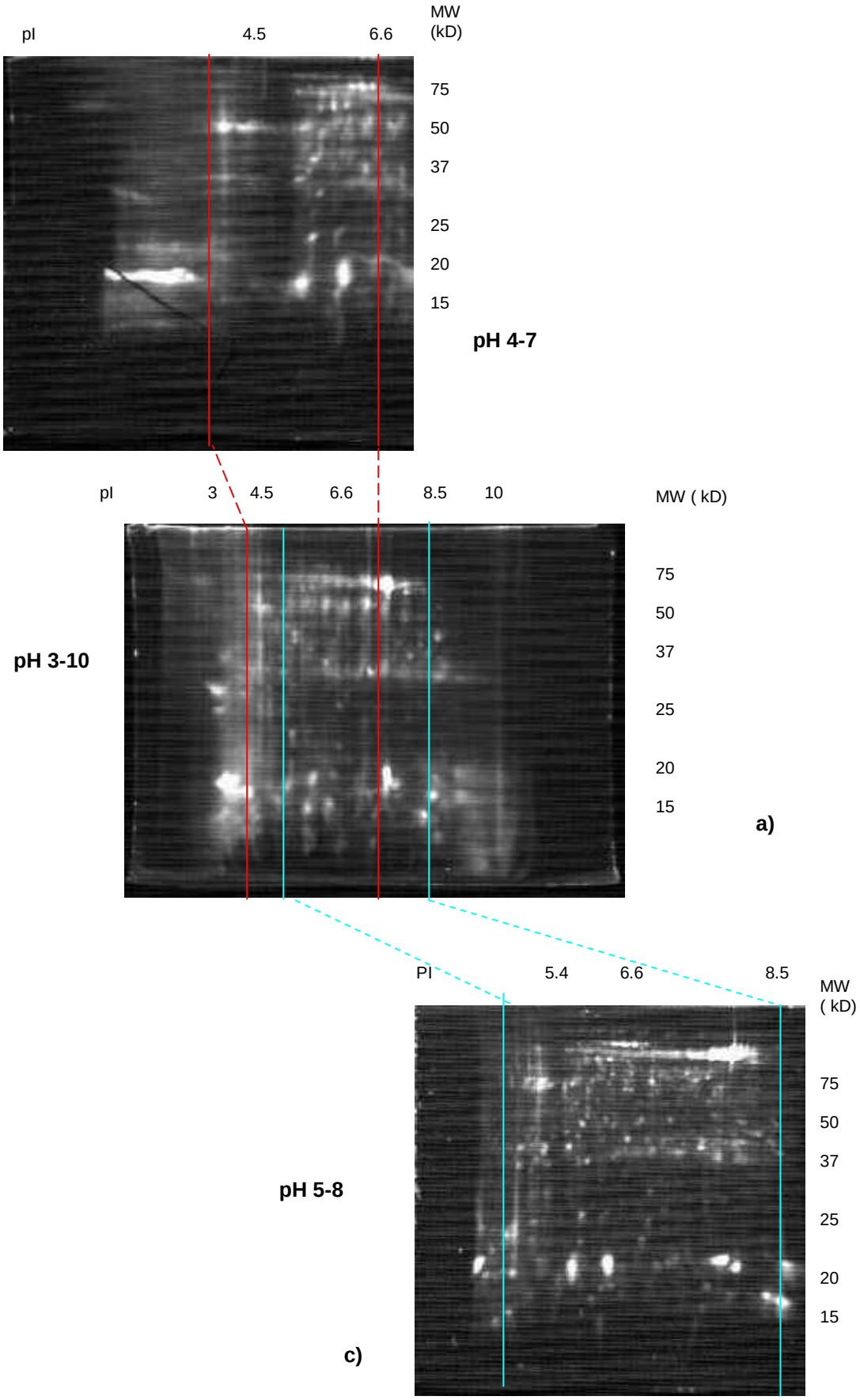


Figure 4.15: Saliva protein 2D gel images (12% polyacrylamide) IPG strips of range a) pH 4-7, b) pH 3-10 and c) pH 5-8. Stained with SYPRO® Ruby (Invitrogen™).

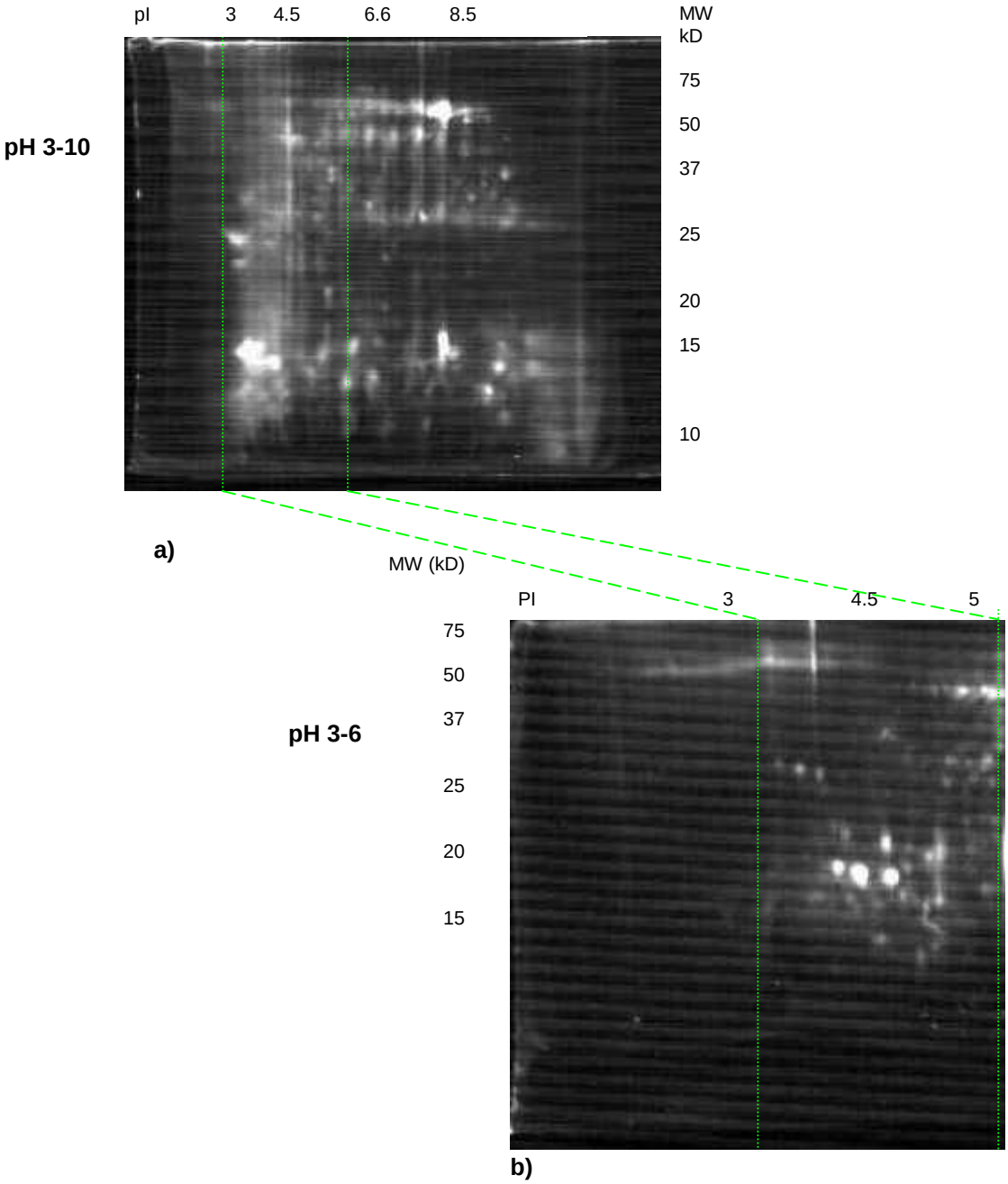


Figure 4.16: Saliva protein 2D gel images (12% polyacrylamide) IPG strips of range a) pH 3-10, and b) pH 3-6, stained with SYPRO® Ruby (Invitrogen™).

A further increase in resolution and separation of protein spots could be made by extending the separating distances using strips of different lengths. This was not possible for this study as there were no tanks available for running longer length gels. Total protein extracted from body fluid samples was shown to contain proteins with isoelectric points ranging from pH3 - pH12. When these samples were loaded to IPG strips with a narrow pH range (such as a pH 5-8 strip - Figure 4.17) many proteins were

found to lie outside the range of the strip. As a result, many of the proteins migrate towards the ends of the IPG strip and precipitate in concentrated lines.

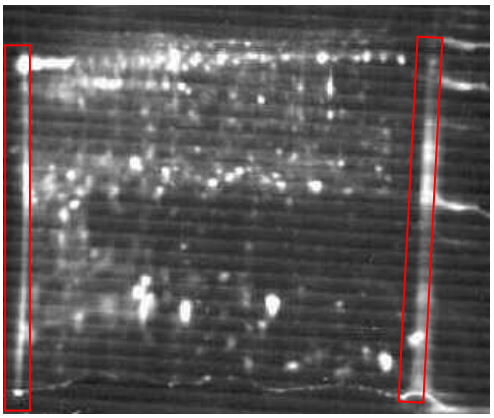


Figure 4.17: A 2D gel image of 50µg saliva protein (acetone precipitation, pH 5-8 Readystrip™ IPG strips, 12% polyacrylamide gels, and SYPRO® Ruby (Invitrogen™) stain.

4.2.3.5 A Comparison of IPG Strips pH Range using Vaginal Fluid Protein.

A similar trend to that described in section 4.2.3.4 was seen when the experiment was repeated using vaginal fluid (Figures 4.18 and 4.19). The broad range strip pH 3-10 showed an overview of protein expression for the sample showing that the majority of protein expression was in the middle of the gel. Once again, separation was improved when narrower range strips were used. The gel images demonstrate that pH strip 5-8 gave the greatest number of resolved spots and whereas the pH 3-6 strip displayed the least spots for this sample. IPG strip 4-7 showed a reasonable number of clearly focussed spots but not as many as the 5-8 strip. Therefore, in order to make accurate comparisons between samples pH 5-8 strips were used in this project.

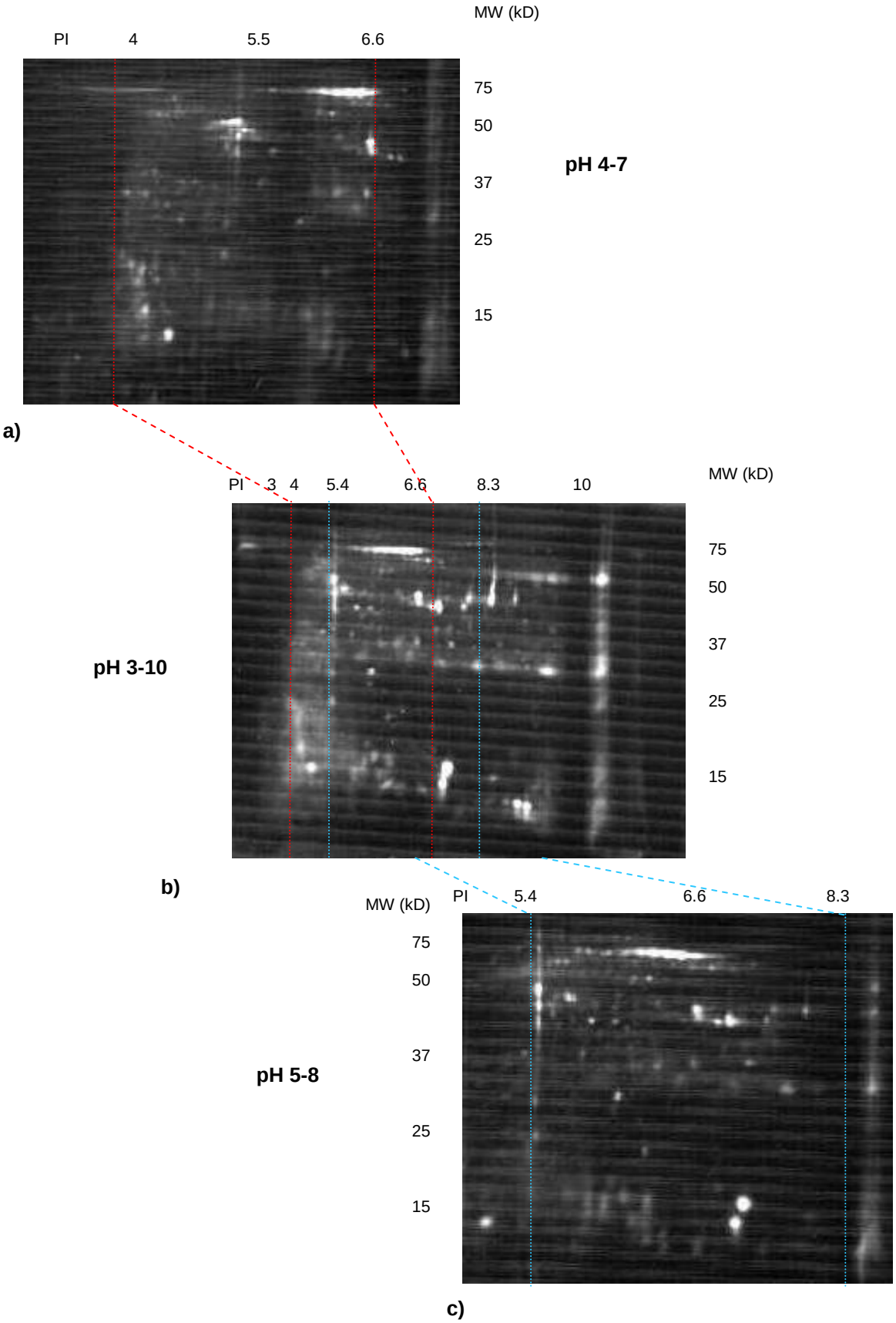


Figure 4.18: Vaginal fluid protein 2D gel images (12% polyacrylamide) IPG strips of range a) pH 4-7, b) pH 3-10 and c) pH 5-8, stained with SYPRO® Ruby (Invitrogen™).

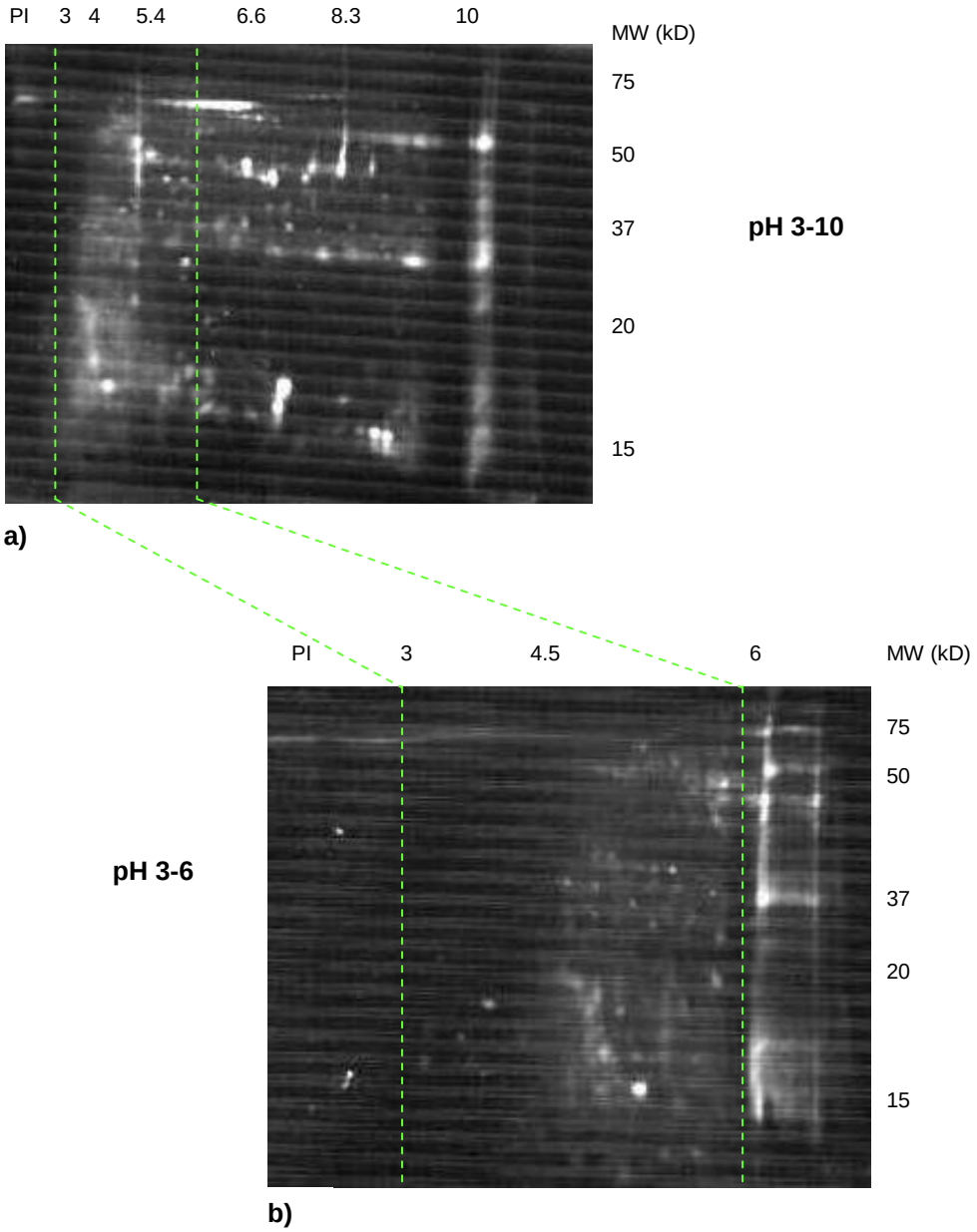


Figure 4.19: Vaginal fluid protein 2D gel images (12% polyacrylamide) IPG strips of range a) pH 3-10 and b) pH 3-6, stained with SYPRO® Ruby (Invitrogen™).

4.2.4 Second Dimension Optimisation

4.2.4.1 A Comparison of Gel Acrylamide Percentage using Saliva Protein

In this experiment three different acrylamide percentage gels were run with 50 µg saliva protein loaded using pH 3-10NL (non linear) IPG readystrips.

Each set of gels was run in duplicate and all gels were stained with SYPRO® Ruby gel stain (Invitrogen™). Figure 4.20 showed that when using a 15% acrylamide gel the high abundance proteins did not move through the gel far enough thus leaving a large space at the bottom of the gel. The 12% gel showed that all the proteins were well distributed throughout the gel providing a better separation of the high abundance proteins. Finally, the 10% gel showed that the low molecular weight proteins at the bottom right of the gel have run off the end. From these results the 12% gel was selected for use throughout this research as this was considered to give the best protein separation.

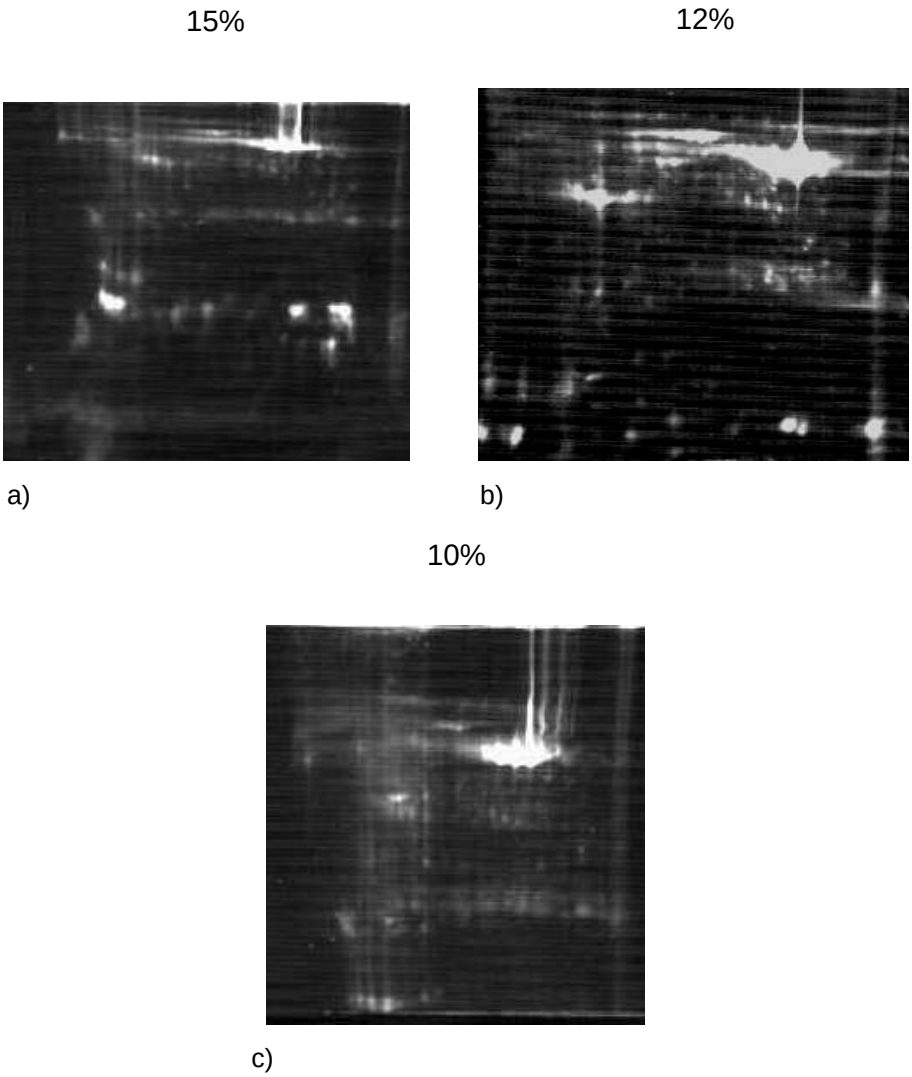


Figure 4.20: 2D gel images showing the separation of saliva protein using IPG strips of range pH 3-10 with a) 15% acrylamide, b) 12% acrylamide and c) 10% acrylamide gels stained with SYPRO® Ruby (Invitrogen™).

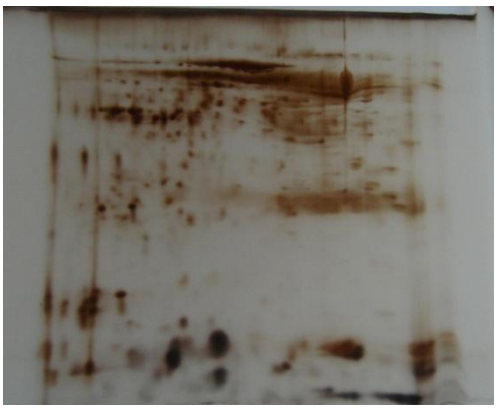
4.2.4.2 Protein Detection using Post Electrophoretic Stains

In order to determine the most effective staining method for use within this study, a comparison study of four protein stains was undertaken. Two of the older commonly used stains; Colloidal CBB and silver staining (PlusOne silver stain kit) were compared with two newer fluorescent stains; SYPRO Ruby and Flamingo. Three independent experiments were performed in order to examine the sensitivity, linearity and reproducibility of each stain.

To determine the sensitivity of each stain by visual comparison, 50 µg of saliva protein was loaded onto four 7cm IPG strips with pH range of 5-8. The second dimension was run using 12% polyacrylamide gels and then each gel was stained

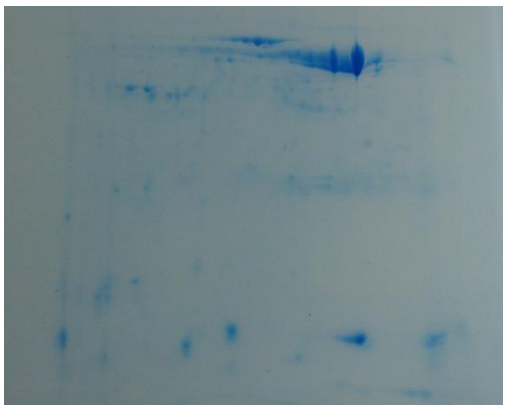
using a different method. The experiment was run in duplicate (see Figure 4.21). The gel images showed that PlusOne™ silver staining (Amersham Biosciences) and SYPRO® Ruby (Invitrogen™) were the most sensitive methods of staining displaying the greatest number of resolved protein spots (panels a) and c) respectively). Colloidal CBB G-250 (panel b) showed that the most abundant proteins were clearly visible. However, Flamingo™ (BioRad®) showed very poor staining with some of the abundant proteins visible (panel d) although not as clear as could be seen with Colloidal CBB G-250 stain which was the most economical staining technique used.

PlusOne Silver Stain



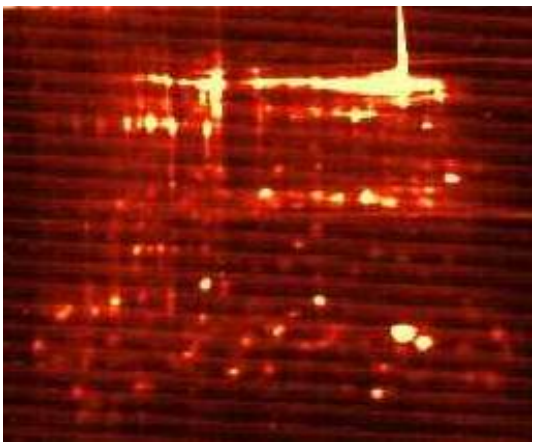
a)

Colloidal CBB



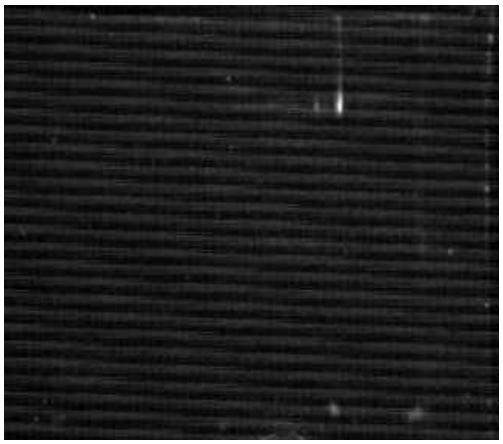
b)

Sypro Ruby



c)

Flamingo



d)

Figure 4.21: 2D gel images of saliva protein using pH 5-8 7cm IPG strip stained with a) PlusOne™ silver stain kit (Amersham Biosciences) b) Colloidal CBB G-250 c) SYPRO® Ruby (Invitrogen™) and d) Flamingo™ gel stain(BioRad®).

As well as determining sensitivity by eye, looking at the number of protein spots present on the 2D gels (Figure 4.21) an experiment was also performed measuring the

sensitivity and linearity quantitatively. Four 1D gels were loaded with broad range Precision Plus Protein™ standards in serial dilution. The serial dilution was based upon the amount of protein present within the 50 kDa protein band of the broad range standard. The instruction manual stated this as 750 ng in the total 1 ml provided. The range of protein tested wthin the 50 kDa band was 20 ng – 0.50 ng. The experiment would enable the limits of the dye to be detected and would thus facilitate comparisons with published data. The linearity of each stain was determined by measuring the density of each 50kDa band and plotting this against the protein load within that band.

Finally, reproducibility was analysed by running 1D gels loaded with a serial dilution of saliva protein. The densitometry of the highest molecular weight protein bands (approx 66 kDa) was determined using the ChemiDoc (BioRad) for densitometry analysis once again (Figure 4.22). The U1- U7 displayed on Figure 4.22 is the area used to calculate density with the ChemiDoc. Each area is the same size and measures the density within the rectangle drawn by the Quantity One software. This was repeated three times in order to determine whether the results obtained each time were reproducible.

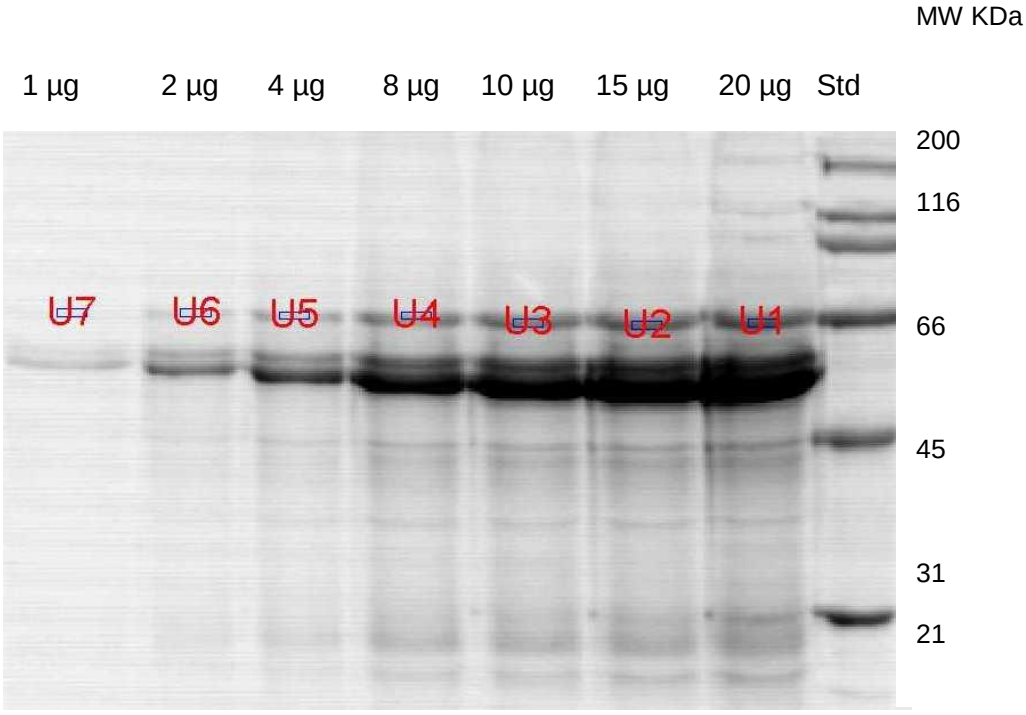


Figure 4.22: A 1D gel image of saliva protein in a serial dilution stained with SYPRO ruby. Density measurements recorded of U1-U7 (n=3).

4.2.4.2 (i) Protein Detection using Colloidal CBB G-250

Colloidal CBB G-250 staining (Figure 4.23) demonstrated that the lowest 50 kDa band visible was 0.75 ng protein. Clear bands were detectable from 20 ng to 3.25 ng although beyond this, the level of detection was faint. Staining with Colloidal CBB was shown to be linear (Figure 4.24) with an R^2 value of 0.96. It seemed likely that characteristic marker proteins for body fluids may not necessarily be the most abundant proteins in the sample. Thus due to the low detection limits of Colloidal CBB it would not be the most suitable stain for this project as it may not be sensitive enough to stain low abundance proteins. Reproducibility of Colloidal CBB staining was demonstrated (Figure 4.25). The serial dilution of saliva proteins follows a negative trend as expected (the less protein loaded to a well, the lower the density/mm^2 in that band). There is some variation in the density values between the repeats which is surprising when using an endpoint stain as the staining would be expected to reach a maximum and maintain this level. Colloidal CBB G-250 staining method demonstrated the least variability among the four methods, thus confirming the rationale for its historical use in the Bradford protein assay for protein quantification.

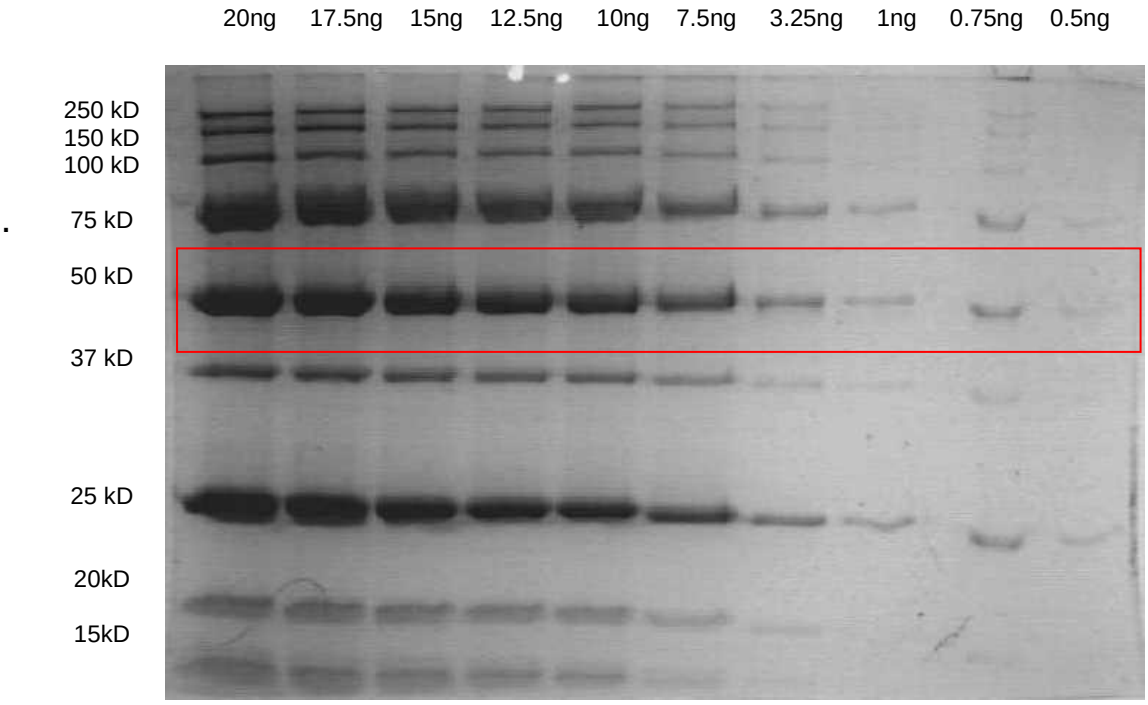


Figure 4.23: A 1D gel showing Precision Plus Protein™standards (BioRad®) in serial dilution stained with Colloidal CBB G-250 staining. The values used to determine the sensitivity of the stain within the 50 kDa band are highlighted by the red box.

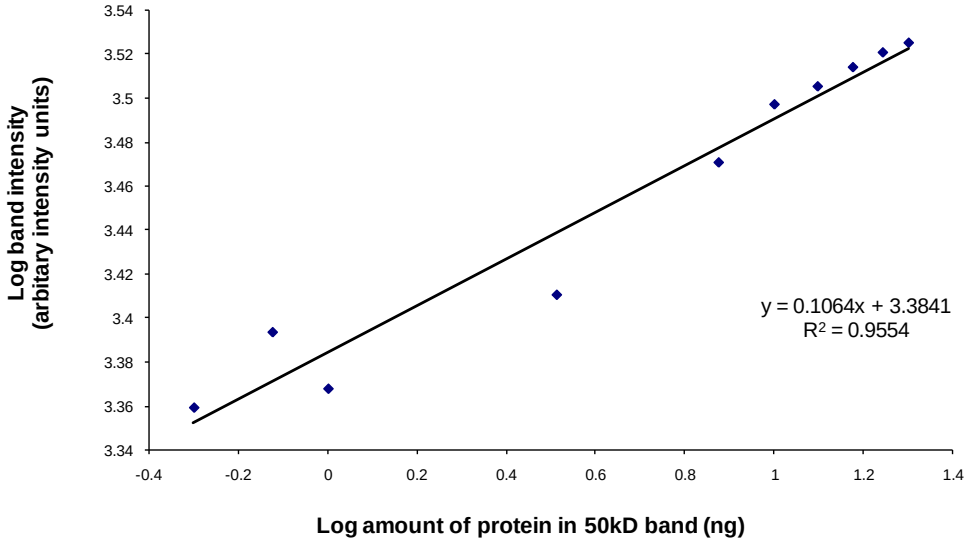


Figure 4.24: The linearity of colloidal CBB G-250 staining by measurement the protein in the 50kD band.

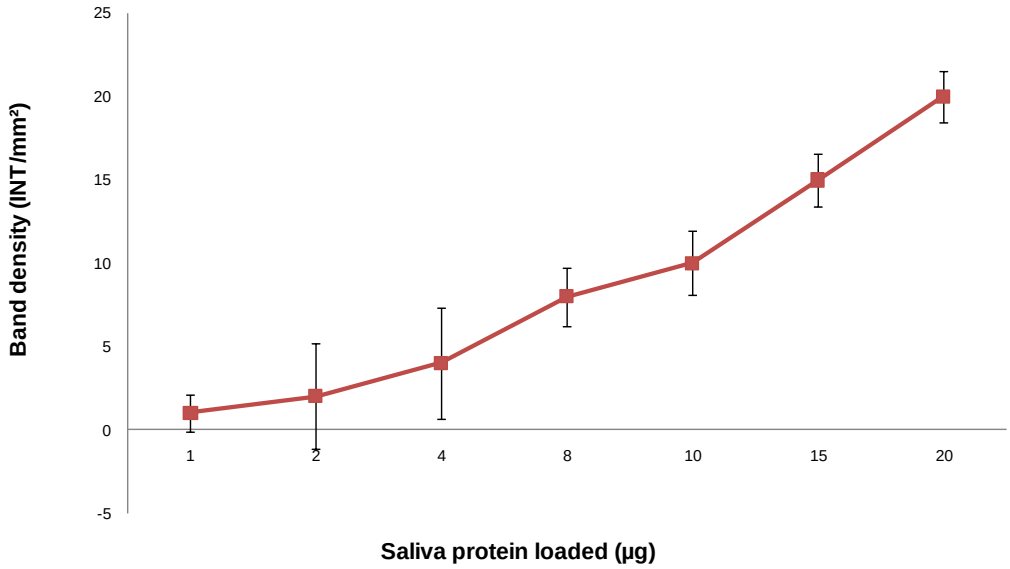


Figure 4.25: The average density of saliva protein bands for reproducibility of colloidal CBB G-250 staining (n=3).

4.2.4.2 (ii) Protein Detection using Silver Staining (PlusOne™ Silver Staining Kit, Amersham Biosciences)

One concern when using the silver staining method is that silver acts as a strong oxidising agent which can lead to chemical modification or destruction of the protein. The use of sensitizing agents such as gluteraldehyde can also result in covalent modifications to proteins and resulting in low sequence coverage (Shevchenko, Wilm *et al*. 1996; Scheler, Lamer *et al*. 1998)

An adapted silver staining method has been used (Shevchenko, Wilm *et al*. 1996) permitting further proteomic analysis using mass spectroscopy by the omission of gluteraldehyde which can cause covalent modifications of proteins.

Silver staining is achieved by saturating the gels with silver ions, washing the molecules that are less tightly bound from the gel matrix, and then reducing the bound metal ions to form metallic silver. Silver staining does not act by specific binding to the protein. Silver nitrate stain interacts with lysine residues while SYPRO® Ruby stain detects lysine, arginine and histadine residues (Chou1990). The sensitivity of PlusOne™ Silver staining kit (Amersham biosciences) is 0.2-0.6 ng protein (PlusOne™ Silver staining kit Protein instructions). PlusOne™ silver staining showed a visible 50 kD band at 0.5 ng protein (Figure 4.26) this is consistent with the published data given above.

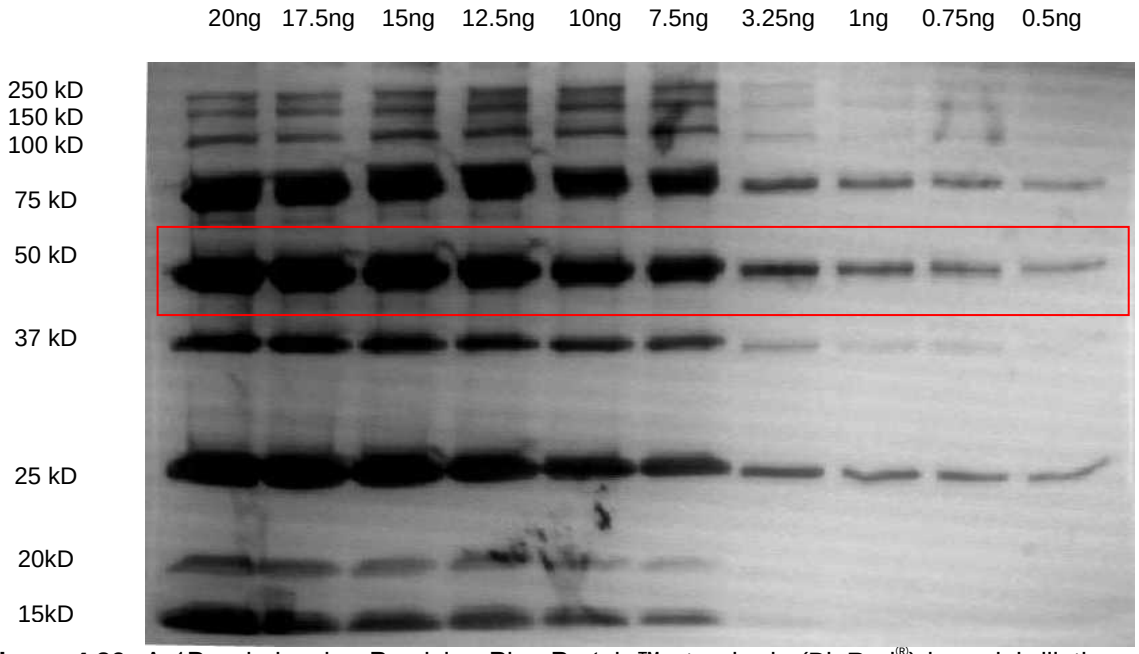


Figure 4.26: A 1D gel showing Precision Plus Protein™ standards (BioRad®) in serial dilution

stained with PlusOne™ silver staining kit (Amersham Biosciences). The values used to determine the sensitivity of the stain within the 50 kDa band are highlighted by the red box.

Silver staining also showed linearity in the range tested (Figure 4.27) this is unusual as silver staining is not known to be linear. Silver staining has a detection range of 1-10 ng of protein (Rabilloud, Brodard *et al*. 1992).

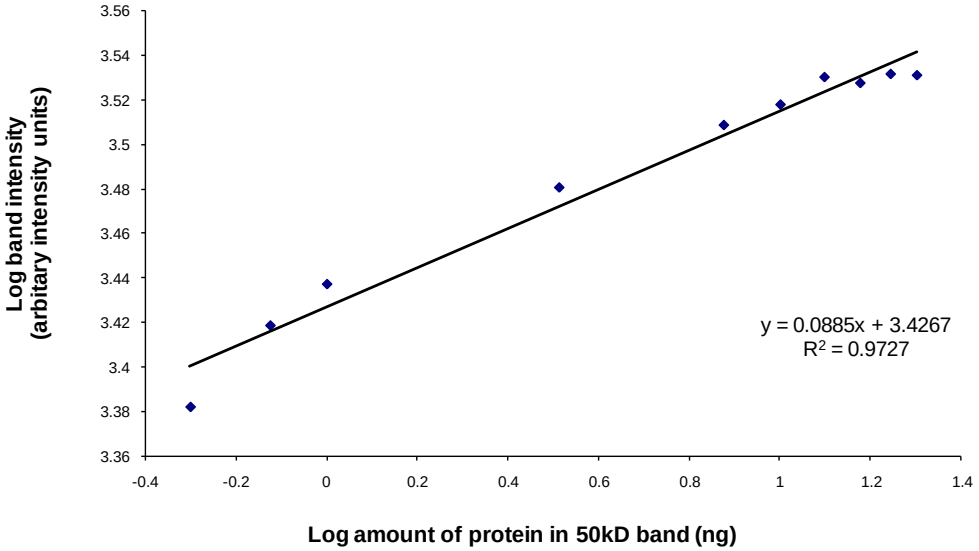


Figure 4.27: The linearity of PlusOne™ silver staining (Amersham Biosciences).

Unlike Coomassie and SYPRO® Ruby stains silver stain is not an endpoint stain, it needs to be stopped at an arbitrary point this can limit the reproducibility of the staining method unless an automated staining method is used. Silver staining is a multi step procedure that is quite time consuming and can display run to run variability in staining. The multiple staining steps produced a problem when trying to test the reproducibility of the staining method. In order to test this accurately each step was timed using a stopwatch in order to maintain the same conditions each time the staining was performed. A way to overcome this would be to use an automated staining vessel which would maintain the same staining times each time it was used. The results for this showed that in order to obtain suitable staining of the gel the density of staining was similar for many of the dilutions (Figure 4.28) which did not show discrimination in the amount of protein loaded to each well. Although a decrease in density/mm^2 was shown for the serial dilution it was minimal.

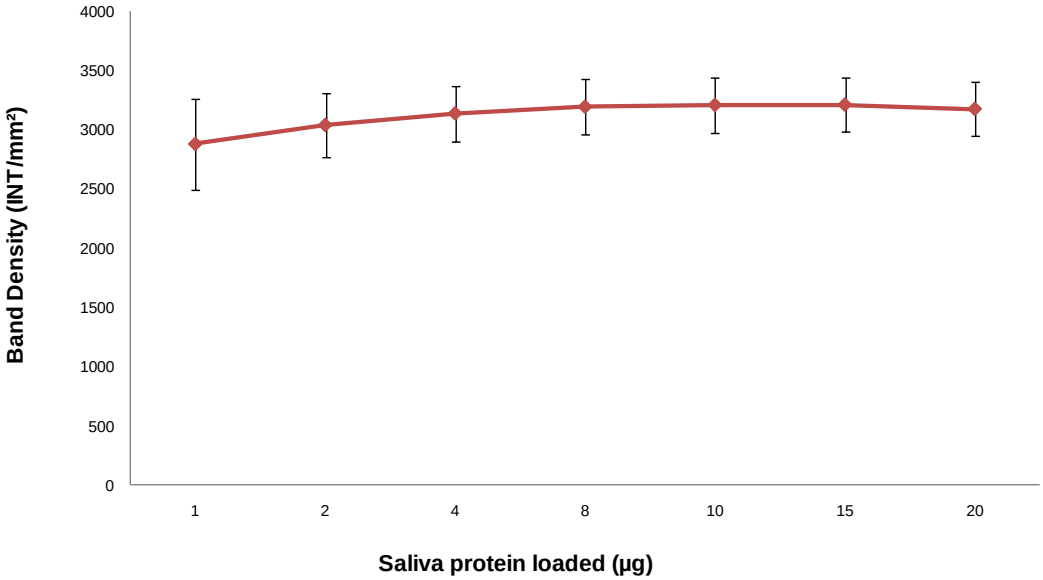


Figure 4.28: The average density of saliva protein bands for repeatability of PlusOne™ silver staining (Amersham Biosciences) (n=3).

Negative staining was shown in the silver stained images (Figure 4.29), this can occur where high abundant proteins are over stained, this could be a limitation with this method in order to achieve a suitable staining intensity for some of the smaller proteins within a gel image the high abundant protein may become negatively stained.

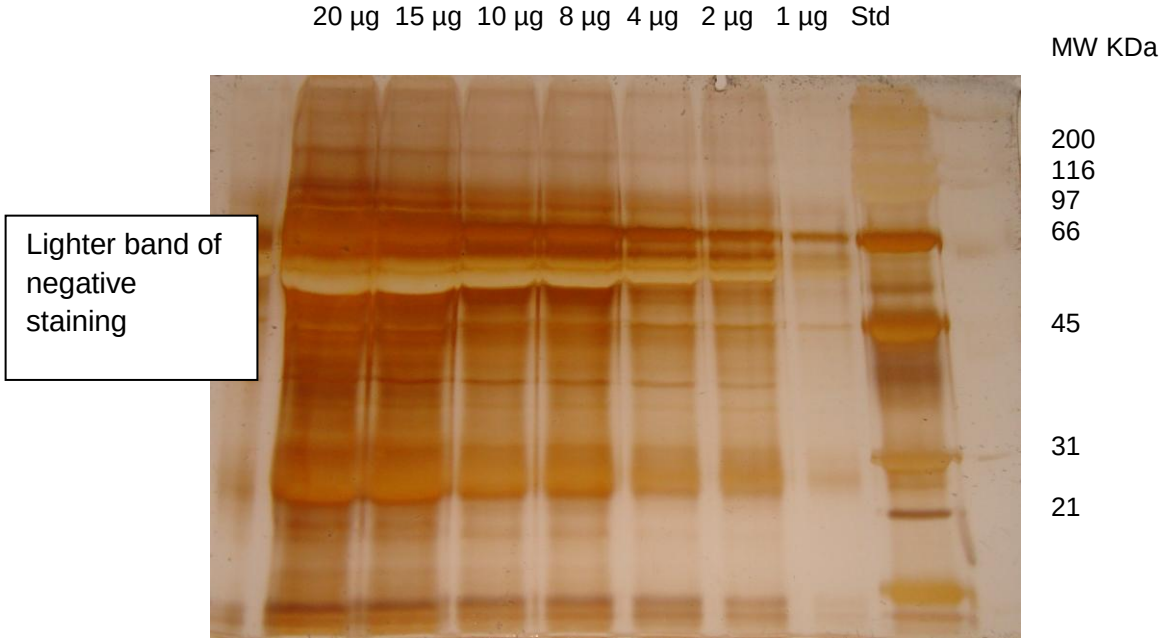


Figure 4.29: A 1D gel showing Saliva protein in serial dilution stained with PlusOne™ silver staining kit (Amersham Biosciences) showing an example of negative silver staining highlighted at approximately 55 kDa.

4.2.4.2 (iii) Protein Detection using SYPRO® Ruby Fluorescent Stain (Invitrogen™)

Decreasing density/mm^2 was demonstrated (Figure 4.30) as for the other endpoint stains tested, although unlike colloidal CBB G-250, SYPRO® Ruby showed an increase in variability between the repeats. SYPRO® Ruby (Invitrogen™) stained a 50 kD band at 0.50 ng (Figure 4.30) similar to that seen with PlusOne™ silver stain. This result was expected as SYPRO® Ruby is reported to have sensitivity similar to silver staining. SYPRO® Ruby (Invitrogen™) was as linear (Figure 4.31) as colloidal CBB G-250 and silver staining over the range tested, with little increase in signal seen at higher (20 ng) protein amounts.

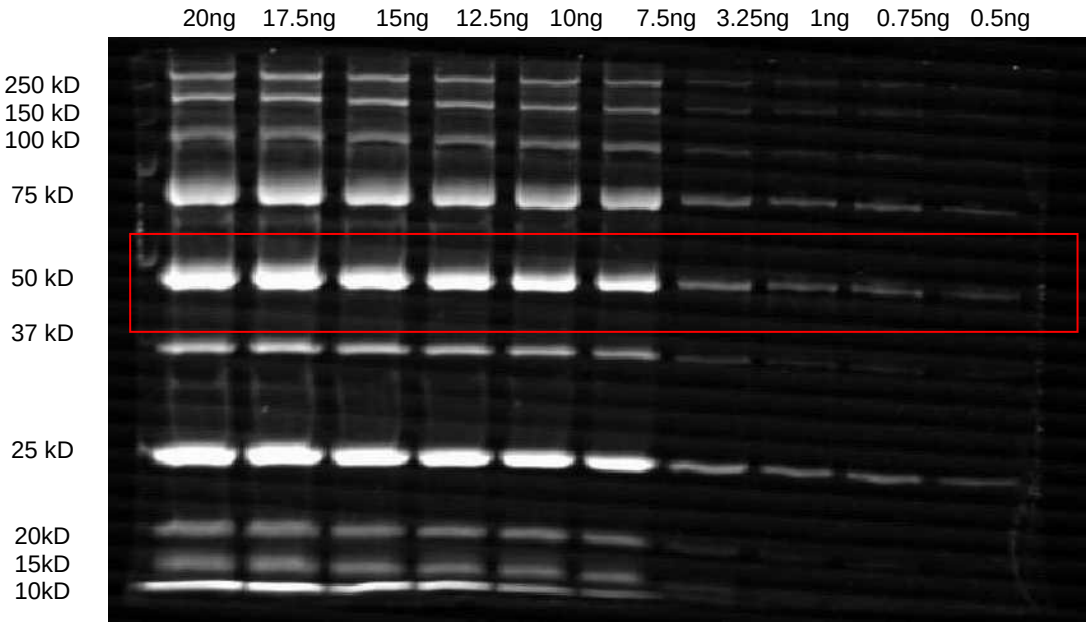


Figure 4.30: A 1D gel showing Precision Plus Protein™ standards (BioRad®) in serial dilution stained SYPRO® Ruby gel stain (Invitrogen™). The values used to determine the sensitivity of the stain within the 50 kD band are highlighted by the red box.

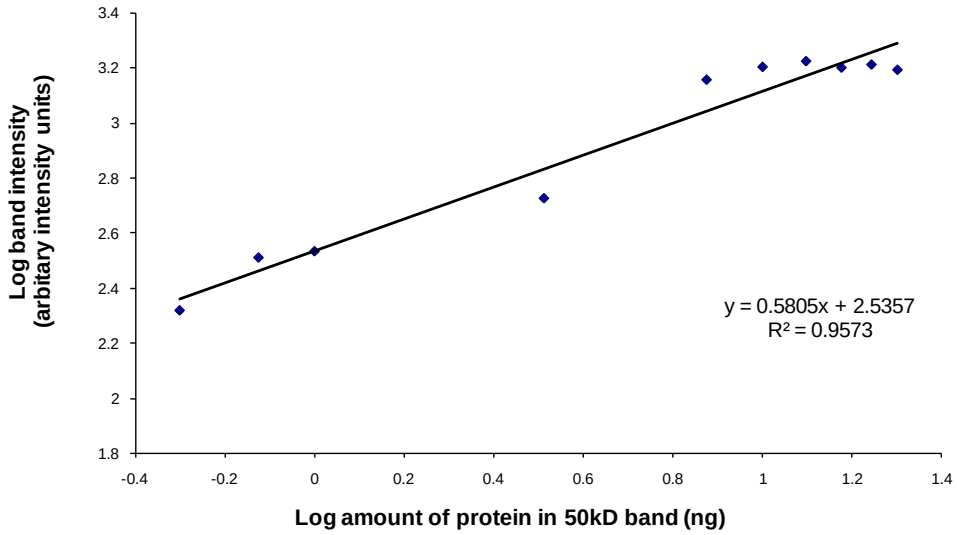


Figure 4.31: The linearity of SYPRO® Ruby gel stain (Invitrogen™).

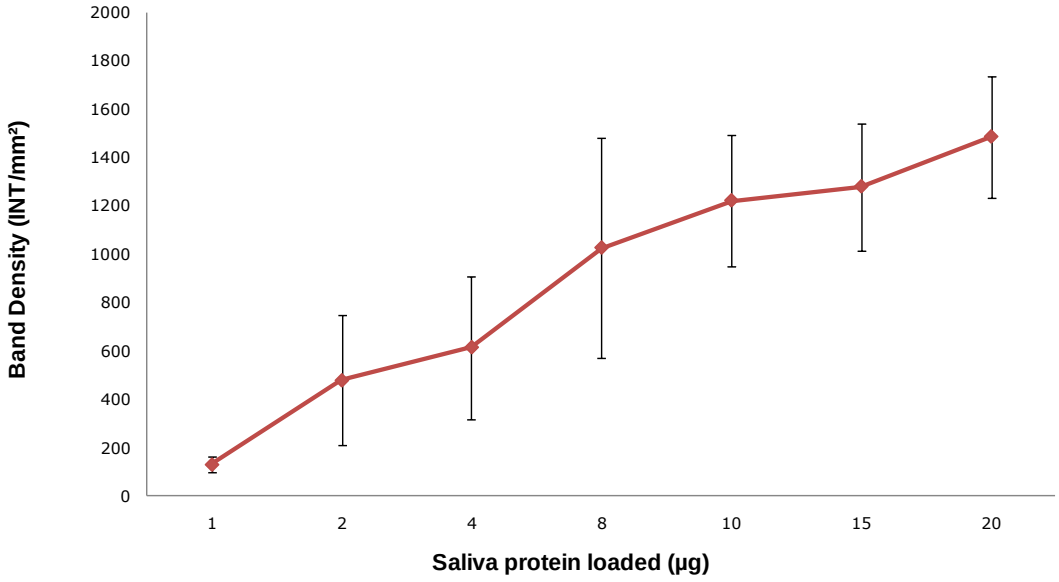


Figure 4.32: The average density of saliva protein bands for repeatability of SYPRO® Ruby staining (n=3).

4.2.4.2 (iv) Detection of Proteins using Flamingo™ Fluorescent Stain (Bio-Rad®)

Flamingo stain is less sensitive than SYPRO® Ruby (Invitrogen™), detecting a 50 kDa band at 7.5 ng as compared to 0.5 ng (Figure 4.33). The linearity of flamingo™ (BioRad®) (Figure 4.34) was less than that of all the other stains tested, especially at the higher protein concentrations. Reproducibility studies demonstrated (Figure 4.35)

that the staining method was unable to detect the two smallest serial dilutions of saliva unlike the other stains. Therefore the results were more variable than the other methods used. The first serial dilution displayed a greater density/mm^2 than that of the previous well with a greater protein load. The results from this comparison showed that as well as having a low sensitivity Flamingo staining was also variable between repeated gels.

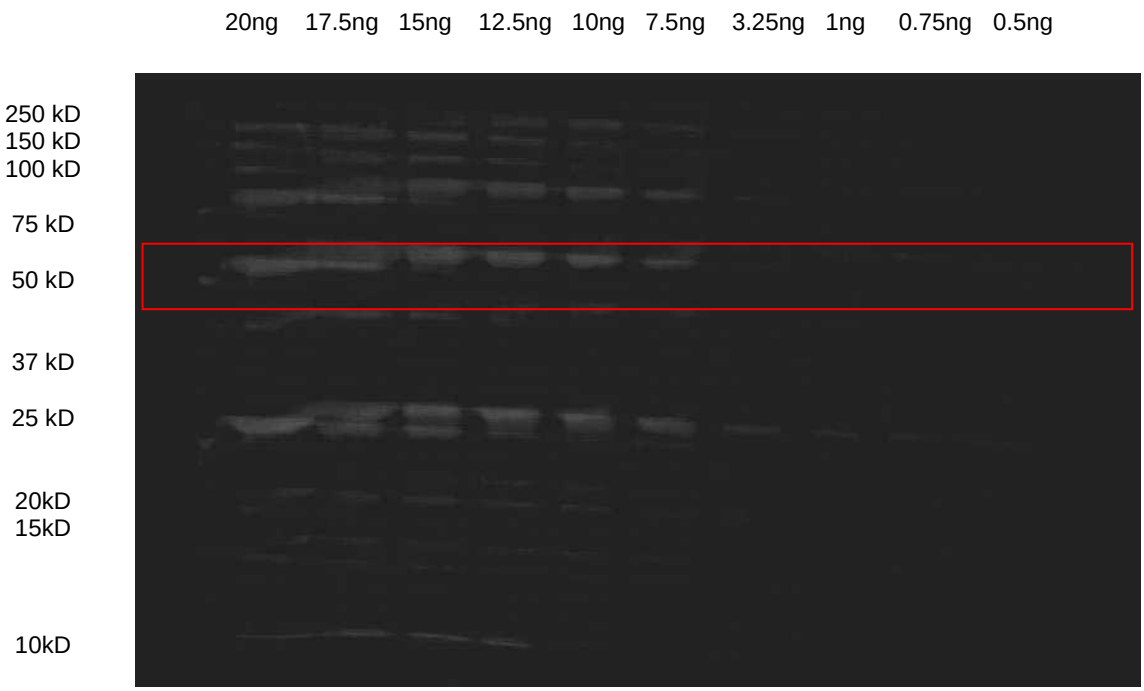


Figure 4.33: A 1D gel showing Precision Plus Protein™ standards (BioRad®) in serial dilution stained Flamingo™ fluorescent gel stain (BioRad®). The values used to determine the sensitivity of the stain within the 50 kDa band are highlighted by the red box.

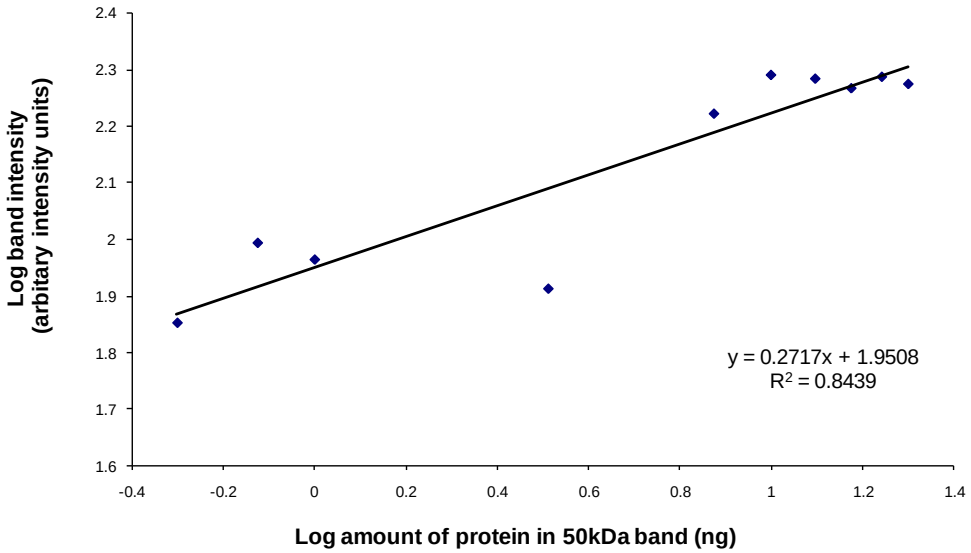


Figure 4.34: The linearity of Flamingo™ fluorescent gel stain (BioRad®).

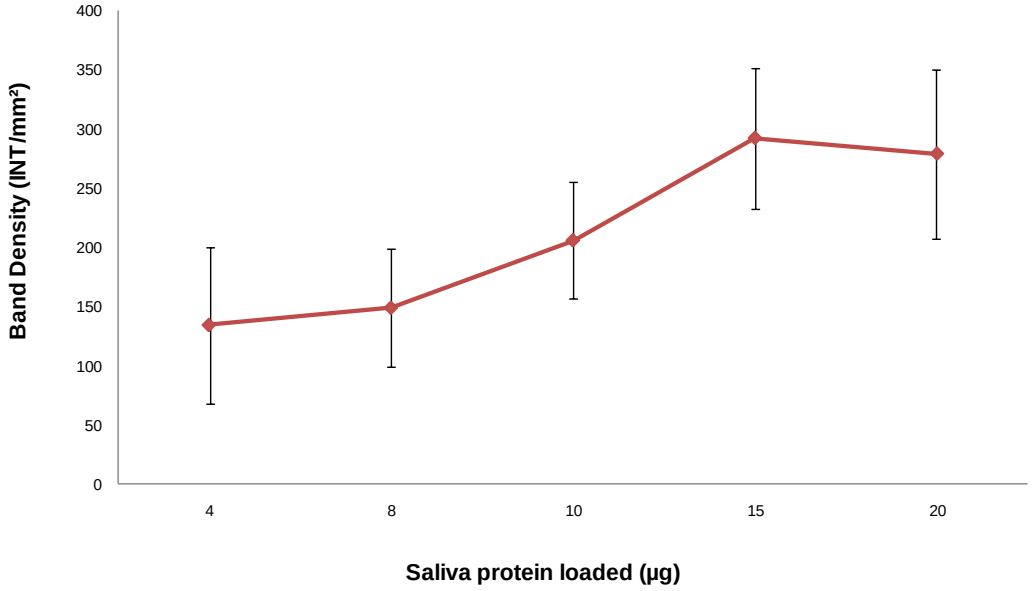


Figure 4.35: The average density of saliva protein bands for repeatability of Flamingo staining (n=3).

A summary of the staining methods compared (Table 4.1) is shown below.

Table 4.2: Summary of the four staining methods compared showing limits of Sensitivity, Linearity and Reproducibility of each method.

Stain	***Colloidal CBB***	***PlusOne Silver Stain***	***SYPRO Ruby***	***Flamingo***
Sensitivity	0.75 ng	0.5 ng	0.5 ng	7.5 ng
Linearity (R^2)	0.9554	0.9727	0.9573	0.8469
Reproducibility	***	*	**	*

4.2.5 Optimisation of Image Analysis – Gel Repeat Expression Comparison

Images were grouped according to sample pools using the Prodigy same spots software. For example, the gels from vaginal pools 4 and 4a were placed in one group to allow direct comparison between them and the gels from saliva pools 4 and 4a were placed in a separate group to allow comparisons to be made between them.

A pair of gel repeats were chosen for each sample type based on their similarity and lack of any gel distortion. Gels PV4 and PV4a were selected for vaginal fluid sample comparison (Figure 4.36) and gels PS12a and PS12b (Figure 4.37) were selected for saliva sample comparison. These pairs were selected as they showed the least visual variation between them. The highest fold difference shown between matching spots for the vaginal fluid gels was 1.6 (see Figure 4.38) and for saliva gels 1.9 (see Figure 4.39).

PV 4 PV 4a

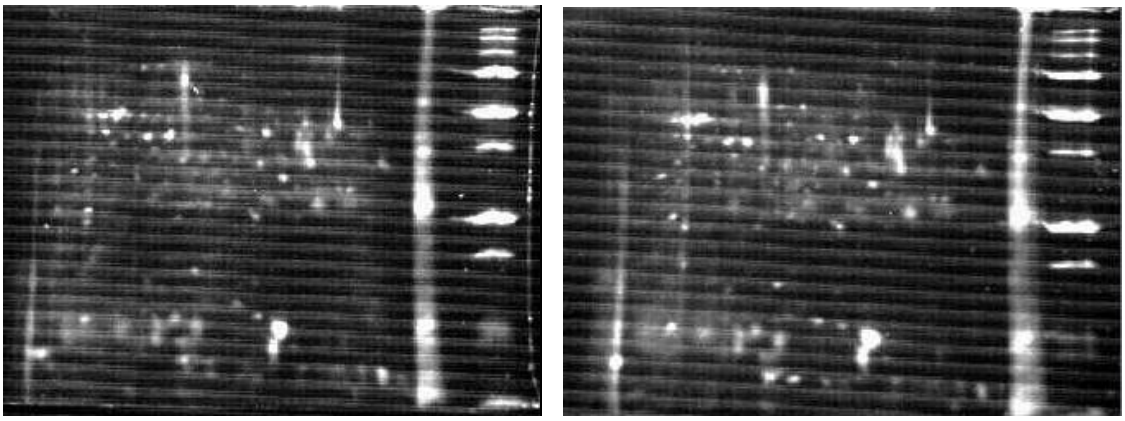


Figure 4.36: Duplicate 2D gel images (vaginal pool 4 and 4a respectively) used for comparison of spot expression fold differences.

PS 12 PS 12a

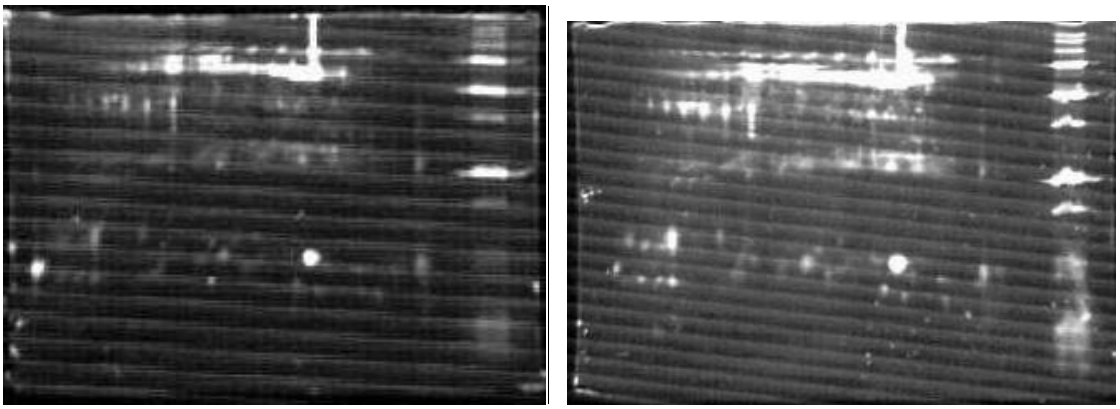
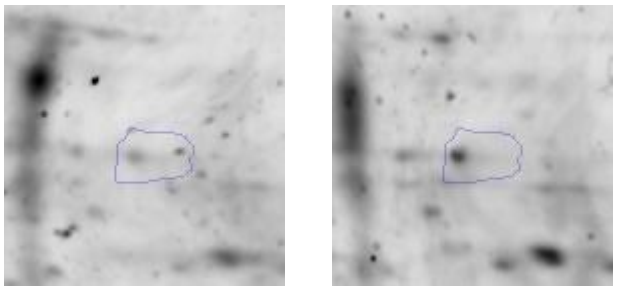


Figure 4.37: Duplicate gel images (saliva pool 12 and 12a respectively) used for comparison of spot expression fold differences.

From the values displayed in Figures 4.38 and 4.39 it was determined that the lower limits for spot selection would be 2.5 for vaginal samples and 3 for saliva samples. This would allow for expression changes to be deemed acceptable between factor sub groups such as age and time of cycle.

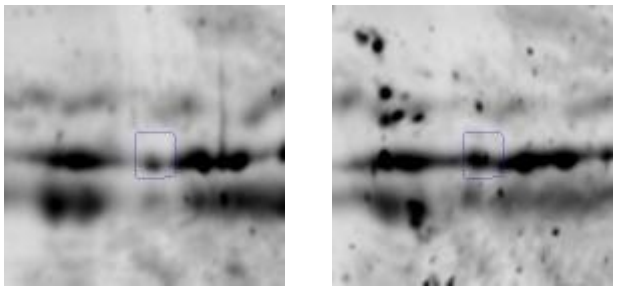
PV4 PV4a



a) b)

Figure 4.38: Images to show pair analysis of gels a) PV4 and b) PV4a showing the highest fold difference of 1.6 between matching spots on duplicated gels.

PS12 PS12a

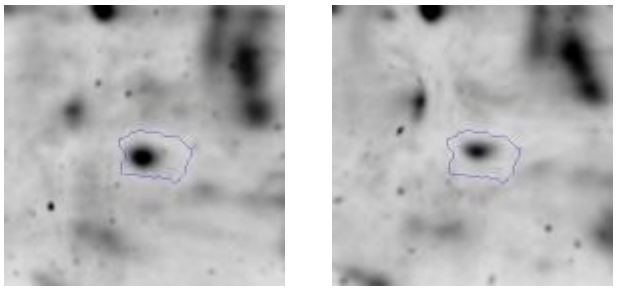


a) b)

Figure 4.39: Images to show pair analysis of gels a) PS12 and b) PS12a showing the highest fold difference of 1.9 between matching spots on duplicated gels.

The lowest fold differences demonstrated for vaginal fluid gels was 1.2 (Figure 4.40) and for saliva was 1.0 (Figure 4.41). Spots with a fold difference of 1 show the same intensity in the gel images. Spots with a fold difference between these values and the lower limit set above were used for comparison of spots between samples when identifying potential markers for uniform expression across all sample gels. “Speckling” can be observed on gel images (Figure 4.39) as the SYPRO Ruby stain is susceptible to crystallising on the gel during the staining process. It is important to recognise this speckle by eye when analysing gel images so as not to confuse these with true protein spots that appear with a less sharp outline.

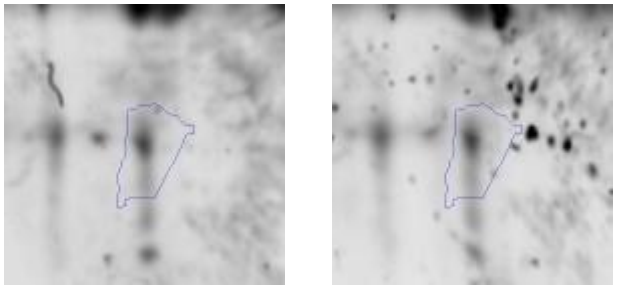
PV4 PV4a



a) b)

Figure 4.40: Images to show pair analysis of gels a) PV4 and b) PV4a showing the lowest fold difference of 1.2 between matching spots on duplicated gels.

PS12 PS12a



a) b)

Figure 4.41: Images to show pair analysis of gels a) PS12 and b) PS12a showing the lowest fold difference of 1.0 between matching spots on duplicated gels.

4.3 Discussion

4.3.1 Optimisation of Body Fluid Proteomics

It is important when comparing samples by 2DGE that the sample preparation and running conditions are kept constant to allow accurate comparisons to be made between gels showing relative protein expression changes (Ong and Pandey 2001). It is also important to remember that 2DGE is known for run to run variability and inter laboratory variability. Therefore gel images cannot be compared easily without suitable software capable of compensating for slight gel to gel differences.

Unlike the genome which is identical in every somatic cell of the body the proteome is in a state of dynamic flux (Fey and Larsen 2001). A 2D gel is a representation of the sample, saliva or vaginal fluid at a particular moment in time under a particular set of environmental conditions (Fey and Larsen 2001; Ong and Pandey 2001). As with all other proteins, salivary proteins are transcribed from mRNA and translated into proteins with post translational modifications occurring prior to secretion into the oral cavity. Further salivary protein modifications occur in the oral cavity where both host and bacterial enzymes cleave proteins secreted from the salivary glands, thus the proteins detected in saliva have undergone significant changes from the initial mRNA transcripts in the salivary glands (Helmerhorst and Oppenheim 2007). It is for these reasons that proteomics was the chosen method over mRNA analysis as it is more representative of the biology of human body fluids (Fey and Larsen 2001) for this study.

4.3.1.1 Sample Preparation Prior to Proteomic Analysis

Sample preparation is an important step in obtaining reliable results in any proteomics study. Protein samples commonly contain substances capable of interfering with downstream proteomic methods. Sample preparation should look to remove salts and detergents and any other factors that could interfere with 2DGE (Jiang, He *et al*. 2004).

4.3.1.1(i) Precipitation

Precipitation of protein followed by pellet uptake in IEF sample buffer is the most common method used to concentrate and purify proteins (Jiang, He *et al*. 2004)

precipitation is particularly useful for body fluid proteomics analysis as it minimises protein depletion in the sample (Martins de Souza, Oliveira *et al*. 2008). For saliva and vaginal fluid the precipitation method affording the highest total protein differed, ie. Acetone and TCA-acetone respectively (Figure 4.3 and 4.6). When the two methods were compared, however, and a 2D gel was run, clear distinctions were seen between the two protein profiles produced by the same saliva sample (Figure 4.4). Acetone precipitation produced a more resolved image with a greater number of spots visible on the gel and for this reason, was the precipitation method chosen. TCA-acetone precipitation has been used by others for both saliva and vaginal fluid proteomics studies (Giusti, Baldini *et al*. 2007; Tang, De Seta *et al*. 2007) whereas acetone has only been used for saliva (Hardt, Thomas *et al*. 2005). Ethanol precipitation has been applied to precipitate proteins from vaginal fluid (Rajan, Cao *et al*. 1999) and so was therefore used for comparison. When the precipitation is performed the proteins within the sample are concentrated and resuspended in a smaller volume of IEF sample buffer, thus protein assay values for the average total protein in precipitated samples would be expected to be higher compared with non precipitated samples, this was the case with vaginal fluid samples (Figure 4.5 and 4.6).

4.3.1.1 (ii) Protein Extraction

Unlike saliva, where proteins are readily obtained in solution, a problem was encountered with vaginal fluid collected by swab. A suitable buffer was required to wash the vaginal fluid from the swab without substantial protein loss in the process. A comparison of PBS and water (Figure 4.5) showed that more protein was afforded with PBS than with water. Both samples were assayed prior to precipitation hence the average total protein in the sample was lower than the values obtained in the precipitated samples (Figure 4.6) due to a dilute sample being assayed. A buffer compatible with downstream techniques such as in 2DGE used in this study and DNA typing in a forensic laboratory would also be advantageous. No adverse effect was observed with PBS when vaginal cells was washed from swabs for DNA profiling or Haematoxylin and Eosin staining (Martin, Pirie *et al*. 2006).

4.3.1.1 (iii) Depletion of Abundant Proteins

Biomarker discovery in blood samples is often hampered by the high proportion of albumin and IgG present within the sample (Echan, Tang *et al*. 2005). Although in this study blood and menstrual blood samples were not studied in depth for biomarker discovery, sample optimisation was carried out with the depletion of abundant proteins using Vivapure® anti HSA/IgG columns (Sartorius). 1DGE showed a pronounced reduction of protein (Figure 4.7) in both blood and menstrual blood samples. When comparisons were made with 2DGE significant protein loss was shown in the gels where the blood (Figure 4.8) and menstrual blood (Figure 4.9) had been passed through an anti HSA/IgG column. Albumin has been previously shown to interact with low molecular weight proteins, acting as a carrier by binding to cytokines and hormones resulting in their removal from the samples (Veenstra, Conrads *et al*. 2005). More defined protein spots were observed in the gels with samples that had not been passed through the anti HSA/IgG column. Therefore it was decided that for further work with either blood or menstrual blood, the anti HSA/IgG column would not be used. Menstrual blood is composed of a mixture of blood and cervical and vaginal secretions (Fraser, McCarron *et al*. 1985). It was thought that the level of blood protein present was much lower than in traditional plasma or serum samples. The volume of blood that was deposited onto a swab head by finger prick was small and hence could account for the low levels of protein in the sample.

4.3.1.1.4 Protein Assay Comparison

Traditional assays used for protein measurement are the Bradford (Bradford 1976) and the Lowry assays (Lowry, Rosebrough *et al*. 1951). After protein precipitation, the pellets were reconstituted in the IEF sample buffer containing urea, CHAPS and DTT (compatible with the *RC DC* assay). Comparison of the *RC DC* protein assay with an adapted microplate version of the Bradford assay showed that the IEF sample buffer reduced the linearity of the standard curve given by IgG. The standard curve produced with the Bradford assay gave an R^2 value of 0.7489 (Figure 4.10) in comparison with the standard curve from the *RC DC* assay giving an R^2 value of 0.9717 (Figure 4.11). The linearity of the Bradford assay was clearly compromised in the presence of IEF sample buffer hence the RC DC assay was used for protein quantification when IEF sample buffer was used. Other researchers have shown a preference for the RC DC protein assay (Giusti, Baldini *et al*. 2007).

4.3.2 First Dimension Optimisation

4.3.2.1 IPG Strip Load

The dynamic range of serum proteins is greater than 10 orders of magnitude which poses a problem. The six most abundant proteins constitute approximately 85% of the total protein content (Martins de Souza, Oliveira *et al*. 2008), when resolving both high and low abundance proteins. Increasing the protein load may also increase the intensity of the low abundance proteins but in turn this could cause overloading of the IPG strip causing poor resolution of proteins on the gel (Ong and Pandey 2001). For both saliva (Figure 4.12) and vaginal fluid (Figure 4.13) 50 µg was determined to be the optimal protein load when tested with SYPRO Ruby as the detection stain. At this protein load high abundant proteins were clearly resolved and the smaller low abundant protein spots were visible on the gel without distortion of the high abundant proteins or any lost of details from the disappearance of less abundant proteins.

4.3.2.2 IPG Strip Range

It is common practice when analysing a new sample type to use a broad range IPG strip in order to determine the range of protein p*I*s (Ong and Pandey 2001). One limitation of the broad range strips is that some of the spots that appear to be a single defined spot can be a mixture of two or more proteins migrating together (Ong and Pandey 2001). This was seen when a pH 3-6 IPG was used with saliva, where what appeared to be a large single protein in the pH 3-10 gel (Figure 4.16) was separated into three large proteins in the pH 3-6 gel. The use of narrow range IPG strips gives greater resolution and more separation on the gel. A disadvantage of using more than one narrow range strip to create a wider picture of protein expression within the sample means running a larger number of gels and more labour. The number of spots from both saliva and vaginal fluid gels was increased with narrower range IPG strips pH 4-7 and pH 5-8 (Figure 4.15 and 4.18 respectively). For both saliva and vaginal fluid the gel run with IPG strip of pH 3-6 gave fewer protein spots in that range than with other gels. Those that were expressed had increased resolution and separation (Figure 4.16 and 4.19 respectively).

4.3.3 Second Dimension Gel Optimisation

4.3.3.1 Gel Percentage

The percentage of acrylamide used in the second dimension can determine the distribution of proteins on the gel. In this work 10%, 12% and 15% acrylamide were studied (Figure 4.20). Of these percentages, 12% acrylamide showed the most even protein distribution while ensuring lower molecular weight proteins were not lost off at the end of the gel while the higher molecular weight proteins had moved a sufficient amount through the gel avoiding aggregation at the top of the gel. Giusti *et al.* (2007) used this acrylamide gel percentage to successfully separate whole saliva and these results were confirmed in this study.

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4.3.3.2 Staining

A comparison of several protein gel stains showed that silver staining and SYPRO were the most sensitive with the greatest number of visible gel spots. High abundant proteins were observed on the gel stained with SYPRO whereas hardly any spots were visible with Flamingo staining. A summary of the staining methods compared (Table 4.2) showed comparisons each method more simply. Quantitative analysis for the limit of detection of each stain showed that with Colloidal CBB G-250 staining (Figure 4.23) showed that the lowest visible 50 kD band was at 0.75ng protein. This value was much lower than the 8-10 ng limit reported by Neuhoff *et al*. (1998). This could be due to a greater sensitivity of the silver staining kit used in this study. A similar experiment conducted by Kang *et al.* (2002) showed that the limit of detection with colloidal CBB staining was 1ng protein/band which is consistent with that seen in this experiment. Staining with Colloidal CBB was shown to be linear (Figure 4.24) with an R^2 value of 0.9554. This is consistent with Colloidal CBB being used to quantify proteins in the Bradford assay (Kang. D, Y *et al*. 2002). Linearity of staining has been reported up to 3 orders of magnitude (Patton 2002). In this study less than two orders of magnitude were studied.

PlusOne™ silver staining gave a visible 50 kD band at 0.5 ng protein (Figure 4.26). Silver staining also showed linearity in the range 0.5 ng-20 ng protein tested (Figure 4.27). This was unexpected as silver staining is not known to give linear calibration curve (White, Pickford *et al*. 2004). Silver staining had previously shown to

have a detection range of 1-10 ng of protein (Rabilloud, Brodard *et al*. 1992) which is higher than that seen in this study.

Negative staining, (Figure 4.29) has been a common problem with silver staining(Miller, Crawford *et al*. 2006). In order to achieve a suitable staining intensity for some of the smaller proteins within a 2D gel image the high abundant proteins may become negatively stained. Unlike colloidal CBB G-250 stain, silver stain is not an endpoint stain, since it needs to be stopped at an arbitrary point. As a result this can limit the reproducibility of the staining method unless an automated staining method is used. The negative staining seen with this method when using 1D gels shows that the stain is more suitable for 2D gels where proteins are distributed throughout the gel rather than being confined to bands in the 1D gels. If a large area of high abundant protein was present in a 2D gel, for example amylase in saliva or albumin in blood negative staining may be experienced.

Silver staining is a multi step procedure and is relatively time consuming and can result in staining variability between runs. When testing the reproducibility of the staining method, problems were encountered with the multiple staining steps. In order to test this accurately each step was timed using a stopwatch to ensure the same conditions each time the staining was performed. This could be overcome by using an automated staining vessel which would maintain the same staining times each time it was used.

SYPRO® Ruby (Invitrogen™) displayed a 50 kD band at 0.5 ng (Figure 4.30) thus showing a similar sensitivity to silver staining. This was lower than the previously reported detection limit for SYPRO Ruby of 1ng (SYPRO Ruby data sheet, SIGMA). Although being an endpoint stain like colloidal CBB G-250, SYPRO® Ruby showed an increase in variability between the repeats as shown by larger standard deviations (Figure 4.32). SYPRO® Ruby (Invitrogen™) showed a similar linearity to both colloidal CBB G-250 and silver staining (Figure 4.31) over the range tested, although a linear response has been seen over three orders of magnitude (Berggren, Schulenberg *et al*. 2002). The intensity of Flamingo staining was much lower than that of SYPRO® Ruby (Invitrogen™). A 50 kD band was detected at 7.5 ng (Figure 4.33). The linearity of flamingo™ (BioRad®) (Figure 4.34) is less than that of the other stains tested. Flamingo™ (BioRad®), like SYPRO® Ruby (Invitrogen™), is an endpoint stain although in this study it has the disadvantage of having a lower sensitivity.

The primary fluorescence excitation maximum of Flamingo fluorescent gel stain is at 512 nm. There is a minor excitation maximum at 271 nm (Flamingo stain user guide).

The stain was stated to be compatible with a UV transilluminator with a 300 nm bulb but in our hands the images obtained using this method were poor. A more sophisticated imager may be necessary for this stain such as a laser scanner rather than a CCD camera. Sensitivity has been stated at 0.5 ng of protein using a UV transilluminator with a wavelength of 300nm and a sensitivity of 0.25ng using a laser scanner with a wavelength of 532nm. The filter used in the ChemiDoc was 302nm inferring that the fluorescence exhibited by the Flamingo stain should have been in the sensitivity of that stated by the manufacturer. Increasing the length of time the gel spent in fix could have increased the sensitivity, as residual SDS on the gel may hamper imaging at 300nm, this was not performed but could be considered if a more economic alternative to SYPRO was considered. Reproducibility studies demonstrated (Figure 4.35) that the staining method was unable to detect the two smallest serial dilutions (1 µg and 2 µg) unlike the other stains described above. Therefore the results with Flamingo staining were more variable than the other methods used. The well containing 15 µg saliva protein displayed a greater density/mm^2 than that of the previous well with 20 µg saliva protein but the standard deviations show variability between repeats hence this could be an anomaly in the results and not a significant decrease in the protein level in this band. The results from this comparison showed that as well as having a low sensitivity, Flamingo staining was also variable between repeated gels.

Variability in the staining method chosen by laboratories still defines how many protein spots are identified within a 2D gel. Although the introduction of fluorescent stains has been seen to increase the sensitivity of the proteomics system, some laboratories still prefer to stain with colloidal CBB. This has been the case for the identification of potential spots likely to yield successful spectroscopy results (personal communication).

4.3.3.3 2DGE Image Analysis

Gels were scanned to generate 16 bit images for analysis. Bit depth is a term given to describe the number of bits defining each pixel of a digital image (Miller, Crawford *et al*. 2006). This determines how many levels of grey that can be generated. The greater the bit depth, the greater the range of grey tones represented by a pixel. The image analysis software is able to distinguish between these different levels of grey. Thus the more levels of grey represented in an image the better the ability of the

analysis software to differentiate low abundance spots from background and hence the greater the accuracy of the software analysis (Miller, Crawford *et al*. 2006).

Ideally all replicate gel images of a sample would contain the same number of spots with the same staining intensity (individual spot volumes), with each spot migrating to exactly the same gel position. However this is not the case with 2DGE and there will always be process variability factors due to different conditions in a 2D gel run. This variation can arise at any stage during the 2D process and as a result of this in order to identify any significant protein expression changes accurately, the error in the system must be identified (Smales, Birch *et al*. 2003). Matching spot pairs could be examined between the samples, thus showing the highest and lowest fold difference (expression change) seen by matching spots between gel repeats of the same pool.

The highest fold difference shown between matching spots for the vaginal fluid gels was 1.6 (see Figure 4.38) and for saliva gels 1.9 (see Figure 4.39). The lowest fold differences exhibited by vaginal fluid gels was 1.2 (Figure 4.40) and by saliva was 1.0 (Figure 4.41).

These two values were used to indicate the level of fold difference seen between gel repeats and hence determine whether a fold difference could be considered as a true change due to biological factors or to gel to gel run differences between the duplicates.

These figures were used to determine inclusion and exclusion criteria when selecting potential spots for further analysis. For example there should be a limit for fold difference to determine whether a spot should be selected for further analysis. Traditionally when looking to identify expression differences between samples this limit should be above the highest fold difference seen between duplicate gels used for the pair analysis. In matching spots within duplicate gels an expected fold difference would be of 1, although this is rarely seen due to inter-gel variability throughout the 2D process. In this project if the fold difference of the same spot was shown to be a similar level across different sample pools within the same sample type, it could be determined that the protein spot may be of interest as a marker for that fluid type as expression seemed constant in each of the pools tested.

4.4 Chapter Summary

The work described in this chapter has determined the optimum conditions for 2DGE of body fluids under investigation.

In future work the samples will be used either directly from source or extracted into PBS before protein concentration measurement by the RCDC protein assay. After acetone precipitation electrophoresis will be run using 7cm pH 5-8 strips in the first dimension and 12% polyacrylamide gels in the second dimension. Post electrophoretic staining will be performed using SYPRO Ruby fluorescent gel stain (Invitrogen). Image analysis will be performed by Prodigy samespots software.

Chapter 5
Protein Expression Analysis of Saliva and Vaginal Samples

CHAPTER 5 – Protein Expression Analysis of Saliva and Vaginal Samples

5.1 Introduction

The aim of work described in this chapter was to identify proteins with expression specific to one fluid type, i.e. only expressed within either saliva or vaginal fluid.

5.1.1 Factors Effecting Protein Expression in Saliva

Proteomic analysis of saliva can be complicated by gland specific effects of circadian rhythm (Hardt *et al*. 2005). Daily oscillations in the secretion of saliva can be affected by physical factors such as exposure to light and changing time zones and biological factors including age, sex, emotions and stress. For these reasons gender, age and collection time were selected as factors which may show proteomic variability.

5.1.1.1The Effect of Gender on Protein Expression in Saliva

Previous workers have reported that unstimulated whole saliva flow rate is greater in males than in females (Percival *et al*. 1994). To date proteomic studies investigating whether gender has an effect on changes in protein expression have been limited. It has been shown that there are no differences between men's and women's α-amylase levels (Nater *et al*. 2007). A mouse study stated that mRNA expression is higher for many genes in salivary glands of males than females (Treister *et al*. 2005a).

5.1.1.2 The Effect of Age on Protein Expression in Saliva

Previous studies have focussed on saliva flow rate rather than protein expression changes with age. Un-stimulated whole saliva flow rate has been shown to decrease with age (Percival *et al*. 1994; Yeh *et al*. 1998). Age has no effect on the level of α-amylase within saliva (Nater *et al*. 2007), but the concentration of mucins in saliva decreases with age (Vissink *et al*. 1996). IgA concentrations increase with age; this is thought to occur in response to alterations in the oral microbiota (Ambatipudi *et al*. 2009) comparing two saliva pools from donors aged 20-30 and 55-65 years old.

5.1.1.3 The Effect of Sample Donation Time on Protein Expression in Saliva

Salivary flow rate and saliva salt concentrations have been shown to vary over a 24 hour period (Dawes 1974). Day time variations in saliva protein expression have been studied with particular interest being paid to α-amylase. The autonomic nervous system controls both the flow (parasympathetic stimulation) and composition of saliva (sympathetic stimulation). Salivary α-amylase is regulated by the sympathetic nervous system and measurement of salivary α-amylase levels are proposed to reflect changes in the nervous system especially under stress conditions (van Stegeren *et al*. 2008). Levels of α-amylase change throughout the day as shown by Ferguson *et al*. (1973) where low levels were seen in the morning and high levels in the afternoon (Ferguson *et al*. 1973).

5.1.1.4 The Effect of Food Consumption on Protein Expression in Saliva

Previous research has inferred that a carbohydrate rich diet can increase the level of α-amylase within saliva (Squires 1953). During eating the proportion of parotid saliva increases, participating in the bolus formation and digestion by α-amylase (Neyraud *et al*. 2009). It is thought that α-amylase levels increase after food consumption whereas eating or drinking was found to have no effect on α-amylase expression (Nater *et al*. 2007). The literature on this subject remains limited.

5.1.2 Factors Affecting Protein Expression in Vaginal Fluid

5.1.2.1 The Effect of Age on Protein Expression in Vaginal Fluid

For the age range studied changes in the vagina are mostly linked to the reproductive cycle and its termination (Farage and Maibach 2006). Menopause is defined by the World Health Organization as the permanent cessation of menstruation due to the loss of ovarian follicular activity. The term perimenopause is used to describe the period ending 12 months after the final menstrual period (Burger 1996). There is a decrease in vaginal secretions after this time. There does not appear to be substantial literature relating to proteomic changes in vaginal fluid at the time of writing.

5.1.2.2 The Effect of Contraception on Protein Expression in Vaginal Fluid

The protein lactoferrin within vaginal secretions differs according to oral contraceptive use. In women not taking oral contraceptives the level of lactoferrin is at its lowest in the luteal phase and at its highest in the follicular phase immediately after menses. In comparison women taking oral contraceptives have lower levels of lactoferrin which remains unchanged throughout the cycle (Wilson 2005).

5.1.2.3 The effect of the Menstrual Cycle on Protein Expression in Vaginal Fluid

The vaginal mucosa is sensitive to ovarian hormone cycling (Farage and Maibach 2006). Oestrogen causes the thickness and glycogen content of the epithelium to peak at mid cycle during the ovulatory phase. The most abundant immunoglobulin in vaginal fluid is IgG (Wilson 2005). The level of this protein has been seen to peak before ovulation and decline in the luteal phase (Usala *et al*. 1989). Other anti-microbial proteins in the vaginal fluid have also shown fluctuations during the menstrual cycle. Human *β*-defensin (HBD) 5 has been shown to increase throughout the menstrual cycle reaching a maximum during the luteal phase (Wilson 2005).

5.2 Methodology

5.2.1 Sample Selection

Samples (25 for each saliva and vaginal fluid) were obtained from volunteer donors as described in Section 2.2. The samples were categorised according to predefined variables. Saliva samples were taken;

a) at different times of the day

b) at different times after food or drink

c) from males and females

d) from people of different ages.

Vaginal fluid samples were taken from women;

a) of different ages,

b) at different stages of the menstrual cycle (not menses)

c) using different forms of contraception.

The information used for characterisation was obtained from the consent form filled in by each donor (Appendices III & IV). Each variable had 25 samples associated with it spread across the sample pools (Figure 5.1 and 5.2) for that variable.

Table 5.1: Composition of pools for saliva samples.

Saliva pool identification number	***Saliva pool description***	***Total number***	***Sample composition of pool****
PS1	All saliva samples pooled	25	1,2,3,4,5,6,7,8,9,10,11,12,13,14,15,16,17,18,19, 20, 21,22,23,24,25
PS2	Sex: - Male samples	8	2,9,12,13,14,15,20,21
PS3	Sex: - Female samples	17	1,3,4,5,6,7,8,10,11,16,17,18,19,22,23,24,25
PS4	Age < 23	14	2,3,4,5,7,8,11,18,19,21,22,23,24,25
PS5	Age 24 - 27	7	9,10,13,14,15,17,20
PS6	Age 28 - 31	4	1,6,12,16
PS7	Samples donated in the morning between 9am - 12pm	6	20,21,22,23,24,25
PS8	Samples donated at lunchtime between 12pm - 2pm	10	1,2,3,6,7,8,9,10,11,19
PS9	Samples donated in the afternoon between 2pm - 5pm	7	4,5,13,14,16,17,18
PS10	Samples donated in the evening after 6pm	2	12,15
PS11	Time since eating < 15 minutes	7	1,7,9,10,15,16,18
PS12	Time since eating < 60 minutes	13	2,3,4,5,6,14,17,19,20,21,22,24,25
PS13	Time since eating > 60 minutes	5	8,11,12,13,23

*Each pool was composed of samples relevant to that pool type for examples all male samples in the male pool. For anonymity reasons samples were allocated a number.

Table 5.2: Composition of pools for vaginal fluid samples.

Vaginal fluid pool identification number	***Vaginal fluid pool description***	***total***	***Sample composition of pool****
PV1	All samples pooled	25	1,2,3,4,5,6,7,8,9,10,11,12,13,14,15,16,17,18,19,20, 21,22,23,24,25
PV2	Age – teens	3	2,6,19
PV3	Age – 20s	9	1,5,7,9,15,17,20,23,24
PV4	Age – 30s	9	3,8,10,12,13,14,16,21,25
PV5	Age – 40s	2	4,11
PV6	Age – 50s	2	18,22
PV7	Combined pill	7	1,3,5,8,15,16,17
PV8	Pill	5	2,7,21,22,23
PV9	None	6	4,10,11,13,18,25
PV10	Implanon implant	2	6,12
PV11	Depo-Provera injection	2	9,24
PV12	Condoms	3	14,19,20
PV13	Not menstruating	4	9,18,20,24
PV14	Cycle stage - Follicular	8	1,2,4,6,8,17,21,25
PV15	Cycle stage - Ovulatory	5	5,7,12,16,23
PV16	Cycle stage - Luteal	8	3,10,11,13,14,15,19,22

*Each pool was composed of samples relevant to that pool type for examples all samples from donors in their 30s in the age 30s pool. For anonymity reasons samples were allocated a number.

5.2.2 2DGE

All pooled samples were analysed by 2DGE. Each gel was run in duplicate in parallel using pH 5-8 IPG strips (section 2.6.2, 2.6.3) and stained with SYPRO Ruby (section 2.8.3). The gels were scanned using a PharosFX™ molecular imager and analysed with SameSpots Prodigy™ software (section 2.9). The gel running conditions were identical for each fluid type in order that gel images were comparable.

In order to identify potential biomarkers for each fluid type it was important to ascertain whether a particular protein was consistently present or absent within each of the pools. If protein expression remained constant across all pools of a fluid the protein was considered a potential biomarker for that fluid. If expression of a protein was affected by different biological variables it would be unreliable as a marker and was therefore rejected. 268 spots were observed in the saliva samples (see appendix VI) and 193 spots were observed in the vaginal fluid samples (see appendix VI)

Each protein spot was expressed in terms of its individual volume in the gel. Protein spots on each gel were measured using normalised spot volumes. Spots were normalised as a percentage of the total volume of all spots present in a gel. This was performed automatically by the SameSpots analysis software ensuring any run differences between gels did not have an effect on the expression data of proteins between gels.

Spots chosen for LC-MS/MS analysis were those observed to be uniquely and consistently expressed in each fluid type. Spots were cut from the polyacrylamide gels and then submitted to the ROWETT Research Institute for LC-MS/MS analysis. Proteins were identified from their ion fragmentation pattern and peptide mass fingerprint data through a MASCOT search (see sections 2.11 and 2.12).

Positive protein identifications were made from MASCOT output data by considering the following criteria;

(i) the probability MOWSE (Molecular Weight Search) score calculated by the MASCOT software should be >40 indicating identity or extensive homology ($p<0.05$);

(ii) the expected and observed molecular weights and p*I*s of protein hits should match the protein spots from the gel

(iii) the protein hit should be consistent with the source tissue. When these criteria were met, the protein determined, was considered a **good** hit. On the other hand if only one of the criteria was satisfied, the protein hit was described as **poor**.

5.3 Results - LC-MS/MS Analysis of Proteins

5.3.1 Using IgG Standards as an Internal Control for LC-MS/MS Output

An internal control standard IgG protein (Bio-Rad) was run on a 12% 2D gel using pH 3-10 IPG strips (see section 2.6.2. and 2.6.3). Two spots (1C and 2C) were cut from that gel and submitted for blind analysis as positive controls (see Figure 5.1). Two spots with different degrees of staining intensity were chosen to determine whether this factor may affect excised protein spot identification.

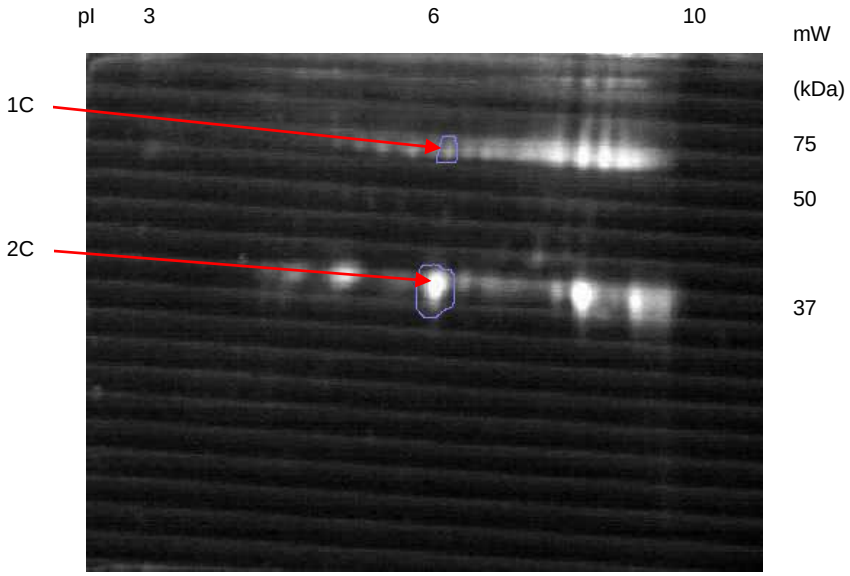


Figure 5.1: A 2DGE image showing IgG protein standard (Bio-Rad) run with pH 3-10 strips on a 12% polyacrylamide gel. Labels 1C and 2C represent the control spots submitted for LC-MS/MS analysis.

Blind LC-MS/MS analysis on the two control spots gave different results; control 2C gave a positive hit for Immunoglobulin λ chain variable region with a MOWSE score of 45 and 2 peptide hits. Interestingly another protein hit (MOWSE score 35) gave a positive identification of HSV-I stimulating-related protein with 1 protein hit was also seen. These two hits are shown as small bars in Figure 5.2, the taller the bar the more peptide hits associated with the MOWSE score. However control 1C did not identify IgG as the matched protein after MASCOT search. The distribution of hits was skewed with the majority having a MOWSE score under 40 (see Figure 5.3) and hence did not meet the predefined criteria (section 5.2.2).

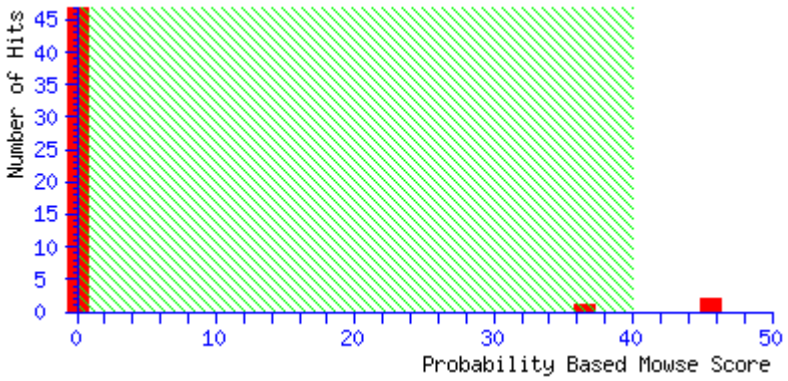


Figure 5.2: Distribution of protein hits and their associated MOWSE scores for blind control sample 2C.

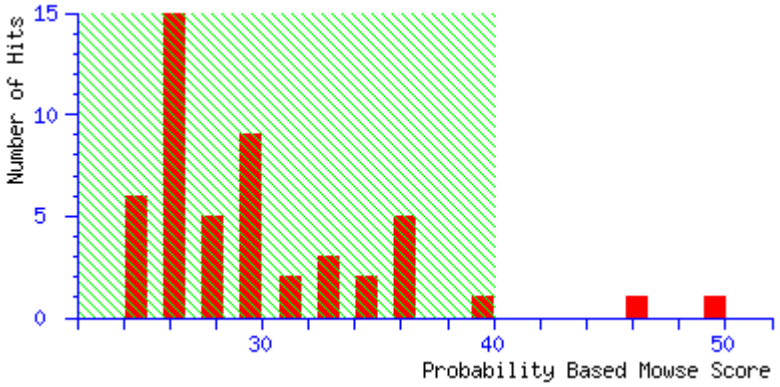


Figure 5.3: Distribution of protein hits and their associated MOWSE scores for blind control sample 1C.

The details of the MASCOT hits with MOWSE scores > 40 are summarised in table 5.3. The identification hits for sample 1C are poor, i.e. Putative anti-CNG α 1 cation channel translation product and centrosomal protein 2. Although the MOWSE score is high, the theoretical p*I* / mass differs from the observed p*I* / mass. For sample 2C a good identification hit for Immunoglobulin λ chain variable region was seen. The MOWSE score was >40 and the theoretical and observed p*I* / mass match. The blind samples confirmed the validity of the hit criteria on page 158.

Table 5.3: A summary of MASCOT output data for the two IgG control spots submitted for analysis.

Sample ID	*MASCOT protein hit name*	*MOWSE score*	*Theoretical pI / Mass*	*Observed pI / Mass*	*Peptides matched*
1C	Putative anti-CNG α 1 cation channel translation product Bottom of Form	50	11.39 / 9081	6 / 78000	2
	Centrosomal protein 2	47	5.21 /19393	6 / 78000	3
2C	Immunoglobulin λ chain variable region (Fragment)	45	6.19 / 35000	6 / 38000	2

5.3.2 Protein Expression Analysis of Saliva Samples

Spots were selected for excision if they were consistently expressed within all saliva sample pools (see Table 5.4 and Figure 5.4). The fold difference threshold of 1.00 (see section 4.2.5) was adhered to for comparison of samples for this experiment. Table 5.4 shows the fold difference determined by comparison of the highest normalised volume pool against the pool of lowest normalised volume for each individual spot. Spots were automatically ranked according to fold change by the Prodigy same spots software and thus allocated the spot numbers listed in Table 5.4. Nineteen spots were excised for saliva with the fold differences between spots ranging from 1.60 to 8.20.

Table 5.4: Saliva spot identification number and expression fold difference for spots analysed by LC-MS/MS.

Spot Identification Number	Fold Difference
6	8.20
34	7.80
13	7.40
66	5.80
27	5.60
33	4.60
83	3.80
47	3.80
41	3.40
46	3.20
81	2.80
79	2.80
76	2.70
70	2.50
43	2.30
89	2.20
72	1.90
59	1.90
85	1.60

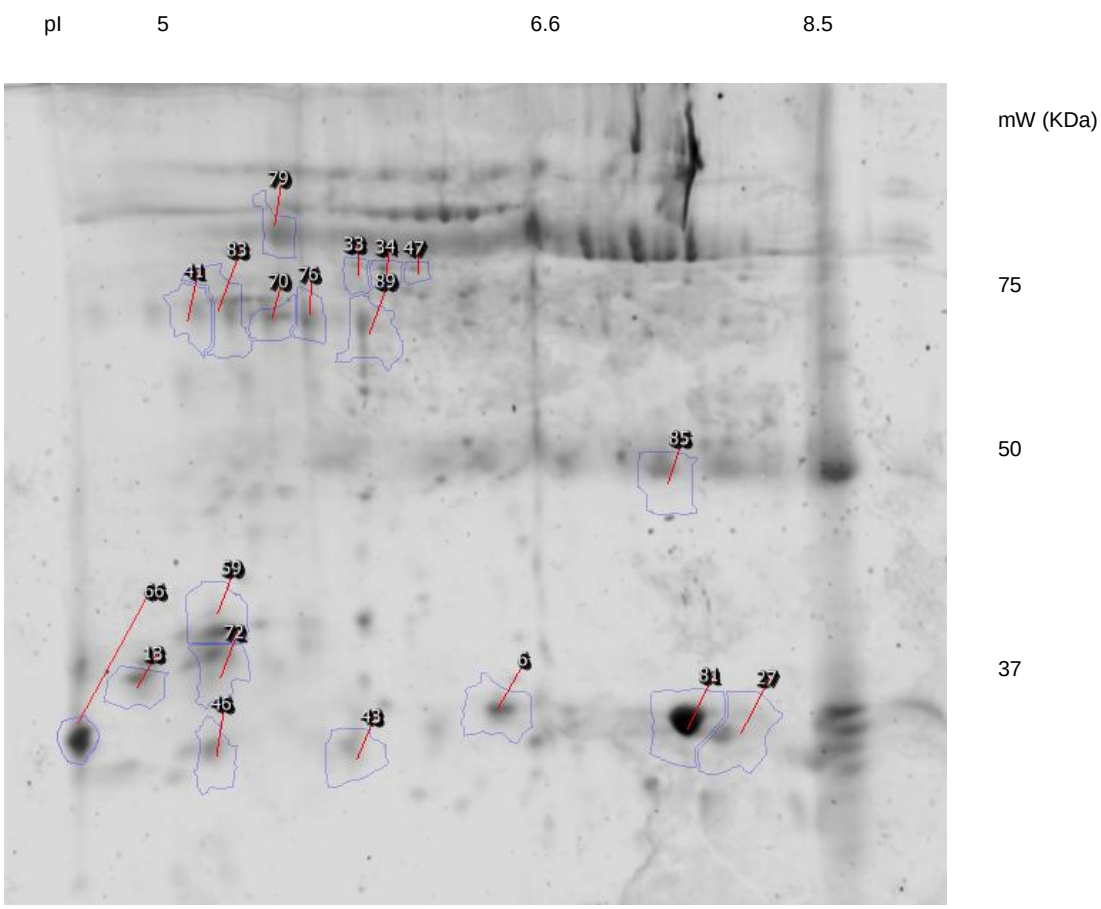


Figure 5.4: A 2D gel image of the master comparison gel (pool 1) to show spot identification numbers and gel position of saliva spots chosen for analysis.

A summary of MS/MS ion MASCOT search data is described in Table 5.5, showing **good** protein identification hits for spots excised from saliva sample gels. Nine protein spots were successfully identified using LC-MS/MS data using a MASCOT search.

Table 5.5: Good protein identifications for saliva sample spots from a MASCOT search of LC-MS/MS data.

Spot number	MASCOT Mass	Theoretical Mass / pI	Observed Mass / pI	SwissProt accession number	Name	MOWSE score	Peptides matched
41	42108	41661.65 / 5.31	40000 / 5.40	ACTG_HUMAN	Actin-γ-1 - human	120	4
59	18078	17918.44 / 4.93	23000 / 5.60	LC1L1_HUMAN	Lipocalin-1 precursor (Von Ebner gland protein)	76	1
66	16719	16444.69 / 4.85	17000 / 5.20	CYTT_HUMAN	Cystatin SA precursor	125	2
70	31767	33872.21 / 5.57	39000 / 5.50	ZA2G_HUMAN	Zinc-α-2-glycoprotein precursor	116	3
76	31767	33872.21 / 5.57	39000 / 6.00	ZA2G_HUMAN	Zinc-α-2-glycoprotein	144	3
79	55203	54154.06 /6.21	60000 / 5.50	IGHA1_HUMAN	Ig α-1 chain C region	144	2
81	16579	16361.62 / 6.82	20000 / 7.80	CYTN_HUMAN	Cystatin SN precursor	57	1
83	42108	41792.84 / 5.31	40000 5.60	ACTG_HUMAN	Actin-γ-1 - human	166	4
89	31767	33872.21 / 5.57	40000 / 6.00	ZA2G_HUMAN	Zinc-α-2-glycoprotein, chain A	43	1

Table 5.6: Poor protein identifications for saliva sample spots from a MASCOT search of LC-MS/MS data.

Spot number	MASCOT Mass	Theoretical Mass / pI	Observed Mass / pI	SwissProt accession number	Name	MOWSE score	Peptides matched
6	16154	15935.74 / 5.70	20000 / 6.60	SODC_HUMAN	superoxide dismutase	40	1
13	25865	25766.52 / 11.58	22000 / 5.40	Q7M4M7	tumor-associated antigen DF3 - human	35	1
27	15922	21891.92 / 5.66	17000 / 8.50	PRDX2_HUMAN	Peroxiredoxin 2	35	1
33	207706	205127.21 / 5.85	52000 / 6.00	PLXB2_HUMAN	Plexin-B2 precursor (MM1)	30	1
43	139786	138448.32 / 5.78	17000 / 6.00	CBPC1_HUMAN	ATPVGTP binding protein 1	50	2
46	120722	119143.02 / 6.32	17000 5.60	Q5XWD3_HUMAN	Leucine-rich repeat protein LRIG1	47	1
47	139786	138448.32 / 5.78	52000 / 6.20	CBPC1_HUMAN	ATPVGTP binding protein 1	50	2
72	130062	129003.96 / 5.49	22000 5.40	Q6U8A4_HUMAN	Ubiquitin-specific protease 7 isoform	53	2
85	11773	11608.86 / 5.58	28000 / 7.80	IGKC_HUMAN	Ig light chain C region	37	1

Total ion count spectra for each spot can be found in Appendix VIII. The MOWSE scores for these proteins ranged from 43 – 166 with the number of matching peptides for ranging from 1 – 4. As well as the above scores, the theoretical data for molecular mass and p*I* also appeared to be approximately the same as the protein spot gel position. The error in these two values ranged between 500 and 6000 kDa for molecular weight with 0.07 and 0.80 for p*I.* Hit distribution graphs for the saliva spots identified can be seen in Appendix VII.

Salivary proteins identified were; Lipocalin-1, Zinc- α-2 glycoprotein (ZA2G), two Cystatin proteins; Cystatin SA and Cystatin SN, Actin and immunoglobin.

It has been hypothesised that Lipocalin-1 or Von Ebners gland (VEGh) protein has a role in bitter taste perception (Holzfeind *et al*. 1996). The tertiary structure of the protein contains a hydrophobic pocket providing a binding site for small lipophilic molecules. In the mouth (a hydrophilic environment) this function allows the transportation of lipophilic bitter compounds to the taste buds (Holzfeind *et al*. 1996; van't Hof *et al*. 1997).

ZA2G is a multifunctional protein which is expressed within a number of body fluids (Hassan *et al*. 2008), and is thought to be related to urinary glucocorticoids (Airoldi *et al* 2009).

Cystatins are extracellular secreted proteins found in relatively high concentrations in body fluids (Turk *et al*. 2008). They function as competitive, reversible inhibitors of cysteine proteases responsible for protein metabolism or to protect tissues from attack by bacteria or viruses (Isemura *et al*. 1991). Cystatins were segregated into three types by Barrett *et al* (1986) based upon their molecular structure. Salivary Cystatins fall into the type 2 Cystatin superfamily which are localised mostly within body fluids (Isemura *et al*. 1987). Type 2 cystatins have two disulfide bonds and a molecular mass of approximately 14 kDa (Hiltke *et al*. 1999). Table 5.5 showed that the theoretical mass and the observed mass (Figure 5.4) were higher than the weight stated by (Hiltke *et al*. 1999). Cystatin SN (spot 81) and Cystatin SA (spot 66) each had theoretical masses of approximately 16 kDa and observed masses of 17 kDa and 20 kDa respectively.

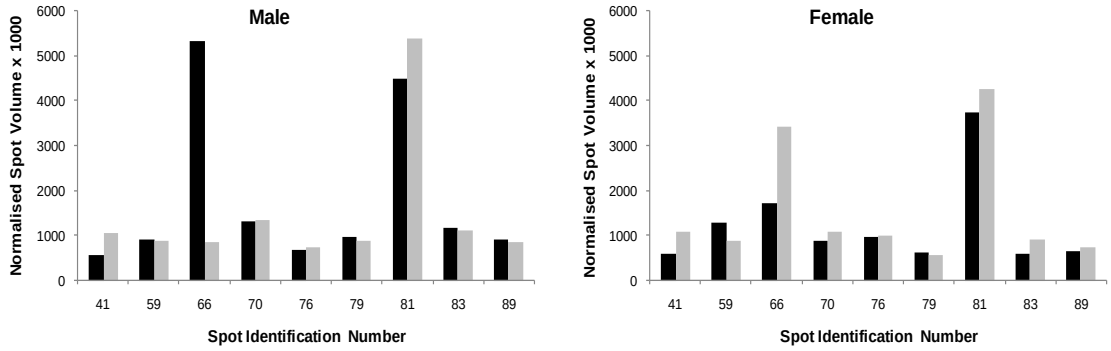
The final two proteins, actin and immunoglobulin-α (IgA) were not investigated further as they are almost ubiquitous. Actin is a globular protein which forms filaments for a support role within the cell cytoskeleton (Hennessey *et al*. 1993) and therefore localisation is not confined to saliva alone.

IgA is the predominant immunoglobulin in saliva of normal individuals (Norhagen *et al*. 1989). Secreted into the oral cavity from the salivary gland epithelial cells and plays a role in defence of the mucosal surface against infection. Localisation of immunoglobulin alpha is also not expressed solely in saliva but is found within all body secretions.

LC-MS/MS data also provided nine protein identifications rated poor (see Table 5.6). MOWSE scores for these proteins ranged from 30-50 and the number of matching peptides to make these identifications was either 1 or 2. Insufficient data was available to successfully identify spot 34 by LC-MS/MS. The poor protein identifications (Table 5.6) were rejected based either upon their MOWSE score or a comparison between theoretical and actual protein data. They were not used in further analysis.

Expression bar graphs of the nine proteins positively identified by LC-MS/MS were drawn for each saliva sample pool. The graphs compare protein expression by measurement of normalised spot volume for each pool. Each Gel duplicate (duplicate gels were run at the same time) is shown as a separate bar on the graph. Comparisons were made between each pool subset which are; sex (Figure 5.5), age (Figure 5.6), time of sample collection (Figure 5.7) and time since eating (Figure 5.8).

5.3.2.1 Protein Expression Analysis of Saliva Samples – Gender Comparison



a) b)

Figure 5.5: Bar graphs illustrating protein expression measured using normalised spot volume between a) male and b) female pools, with two gel repeats. Gel repeat 1 represented by black bars and gel repeat 2 represented by grey bars.

Protein expression between the gel duplicates for both male (represented in **panel a)** and female samples (represented in **panel b)** was shown to be low for the majority of spots (<1400) with consistent expression between gel repeats represented by a similarity in bar height between gel duplicates (Figure 5.5). Normalised volumes ranged between 573 (spot 41 – actin-γ) and 1362 (spot 70 – zinc-α-2 glycoprotein) for male samples and 570 (spot 79 immunoglobulin α) and 1280 (spot 59 – Lipocalin-1) for female sample pools. However, protein spot 66 (Cystatin SA) and 81 (Cystatin SN) were high in both male (Figure 5.5 a) and female (Figure 5.5 b) duplicates. For gel one, expression of protein spot 66 (Cystatin SA) in male samples (Figure 5.5 a) was 5337 whereas for gel duplicate two, the expression of Cystatin SA was greatly reduced to 855. The differences in spot intensity can be seen in figures 5.6 a and b. A smaller difference was seen in the female samples (Figure 5.5 b). Duplicate one showed a normalised volume of 1717 whereas duplicate two showed almost double that of 3424. This expression difference is displayed in figures 5.6 c and d.

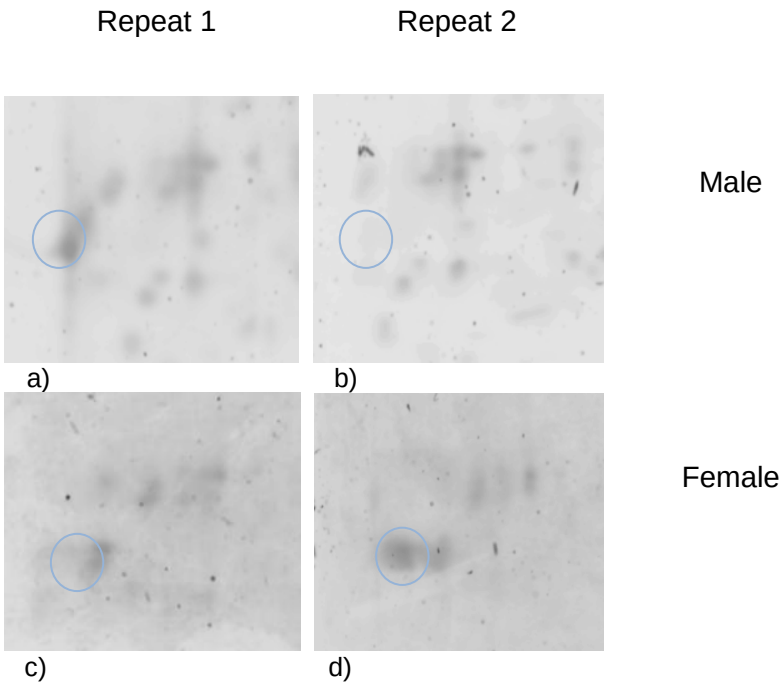


Figure 5.6: Spot 66 expression represented on 2D gel images. Male saliva samples repeat 1 and 2 (a and b respectively) and Female saliva samples repeat 1 and 2 (c and d respectively).

Expression of protein from spot 81 (Cystatin SN) for repeat one of the male samples (Figure 5.5 a) was 4506 and for repeat two was 5396, almost 3 times the normalised volume noted for some of the other spots listed. For the female samples (Figure 5.5 b), the expression was shown to be lower; 3761 and 4270 for duplicates one and two respectively. A similar trend to spot 81 (Cystatin SN) was seen in protein spots 70 (Zinc-α-2 glycoprotein), 79 (immunoglobulin α) and 83 (actin-λ): expression was slightly higher in the male gel duplicates (Figure 5.5 a) than in the female gel duplicates (Figure 5.5b). Expression of protein spot 41(actin-λ) was consistent between the sex pools, with normalised volumes of 573 and 602 (duplicate one male and female respectively) and 1075 and 1083 (duplicate two male and female respectively). Spots that showed a higher normalised volume in the female samples (Figure 5.5 b) compared to the male samples (Figure 5.5 a) were 59 (Lipocalin-1) and 76 (Zinc-α-2 glycoprotein). Differences in the spot volume for these spots are shown in Figures 5.7 and 5.8 spot 59, Lipocalin-1 and spot 76, Zinc- α-2 glycoprotein respectively. Male pool duplicates shown in panels a and b and female duplicates in panels c and d.

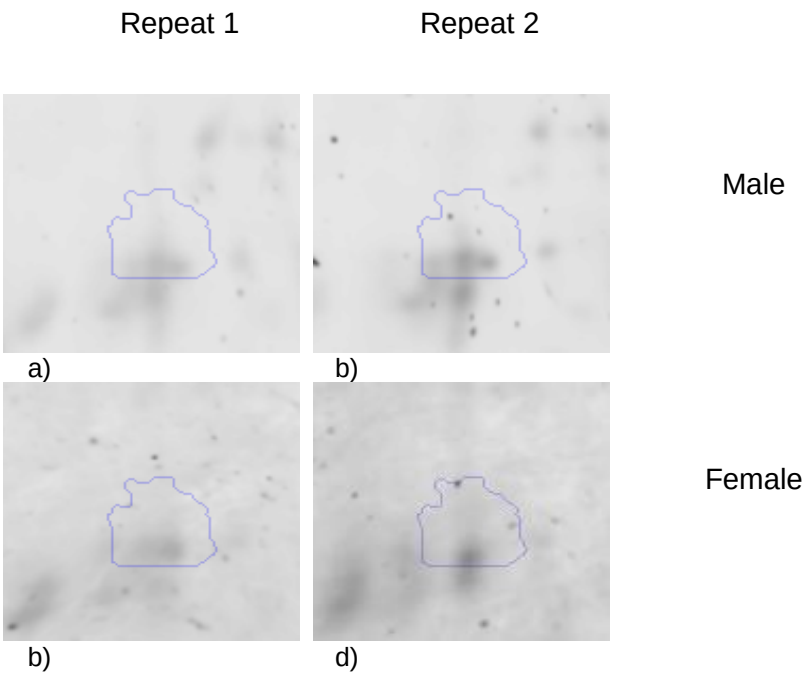


Figure 5.7: Spot 59 expression represented in 2D gel images. Male saliva samples repeat 1 and 2 (a and b respectively) and Female saliva samples repeat 1 and 2 (c and d respectively).

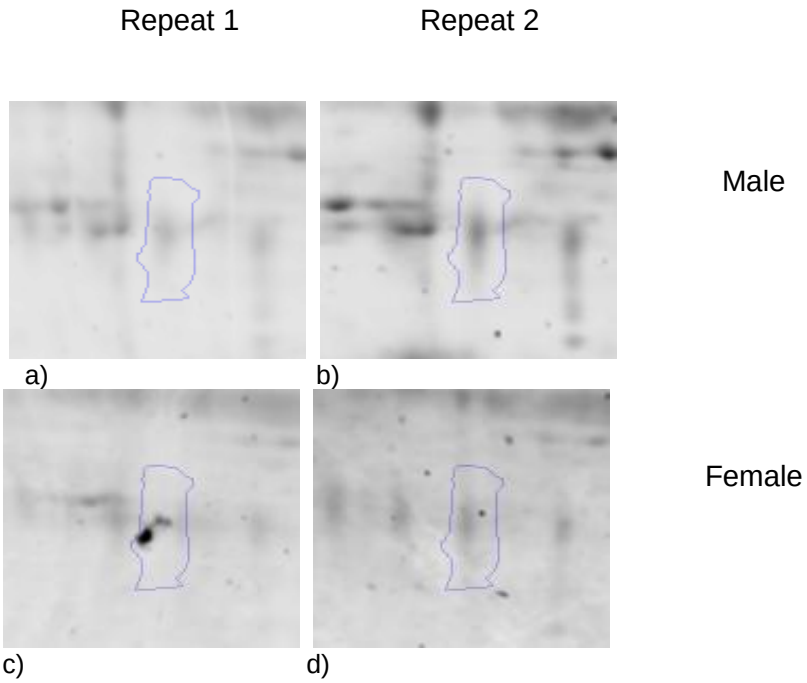
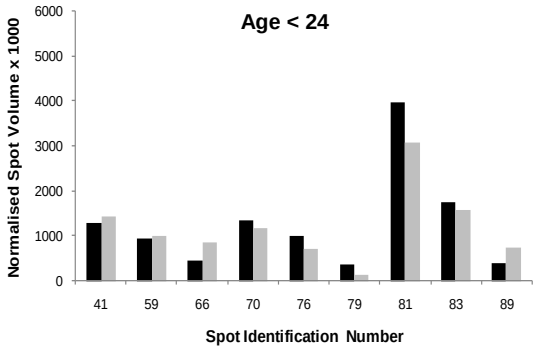


Figure 5.8: Spot 76 expression represented in 2D gel images. Male saliva samples repeat 1 and 2 (a and b respectively) and Female saliva samples repeat 1 and 2 (c and d respectively).

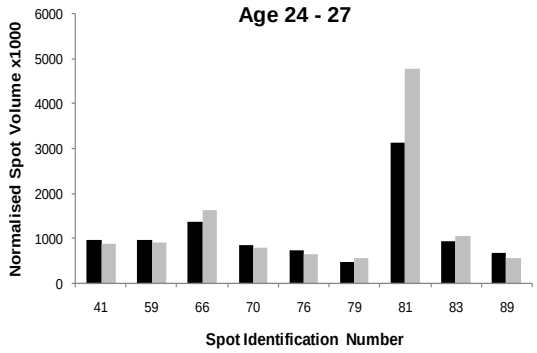
When assessing normalised spot volume it is important to consider the 2D gel as a 3D entity whereas these images only represent a 2D image of the spot.

5.3.2.2 Protein Expression Analysis of Saliva Samples – Age Comparison

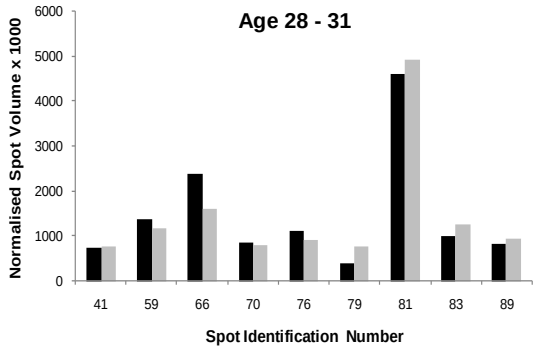
Samples were pooled according to age (Figure 5.9). Samples donated by individuals under the age of 24 years are represented in **panel a,** samples donated by individuals aged between 24 and 27 years in **panel b,** and samples donated by individuals aged between 28 and 32 in **panel c**. When the expression graphs for the saliva samples pooled into different age groups (Figure 5.9) were compared it was noted that the expression of spot 81 (Cystatin SN) was again the highest across all age groups. The expression of Cystatin SN increased with age ranging from 3078 (24 - 27 duplicate 1) to 4938 (28 - 31 duplicate 2), double the expression of the rest of the proteins across all pools. One exception was spot 66 (Cystatin SA) that showed greatest expression in the highest age pool (Figure 5.9 c) with 2397 (duplicate 1) and 1609 (duplicate 2). Proteins 41 (actin-γ) and 70 (Zinc-α-2 glycoprotein) decreased with age. Protein spot 79 (immunoglobulin α) showed the lowest expression across all the three pools. There was no similarity in the expression between the three Zinc-α-2 glycoprotein spots (70, 76 and 89). However, the two spots identified as actin-γ (spots 41 and 83) showed a decrease in expression from above 1000 to below 1000 in the two highest age ranges (Figure 5.9 b and c) compared with the under 24 age group (Figure 5.9a).



a)



b)



c)

Figure 5.9: Protein expression measured using normalised spot volume between donors aged a) < 24 years, b) 24 - 27 and c) 28 - 31. Gel repeat 1 represented by black bars and gel repeat 2 represented by grey bars.

5.3.2.3 Protein Expression Analysis of Saliva Samples – Sampling Time Comparison

Samples were pooled depending upon the time of donation. Protein expression from the earliest (9am and 12 noon) are represented in **panel a,** with the samples taken over lunchtime between 12 noon and 2pm in **panel b**. Samples donated in the afternoon between 2pm and 5pm in **panel c** and finally, samples donated in the evening after 5pm in **panel d.** Once again, expression of Spot 81 (Cystatin SN) was higher than the other spots represented in three out of the four graphs shown in Figure 5.10 (a, b and d). However, expression of spot 83 (actin-γ) was doubled in the samples taken between 2pm and 5pm (Figure 5.10 c) at approximately 2000 in comparison with the other expression values of approximately 1000. Spots 66 and spot 81 (Cystatin SA and Cystatin SN respectively) seemed to follow a similar expression trend to spot 59 (Lipocalin-1) with a decrease in expression as the time of sampling progressed throughout the day until the final evening sampling where expression was highest (Figure 5.10 d). Expression of Cystatin SN was particularly high in the evening (Figure 5.10 d) reaching 5574 and 3869 (duplicate 1 and 2 respectively). Expression of spots 41 and 83 (actin γ) followed a similar trend with expression increasing as the time of day progressed but, finally decreasing in the evening (Figure 5.10 d).

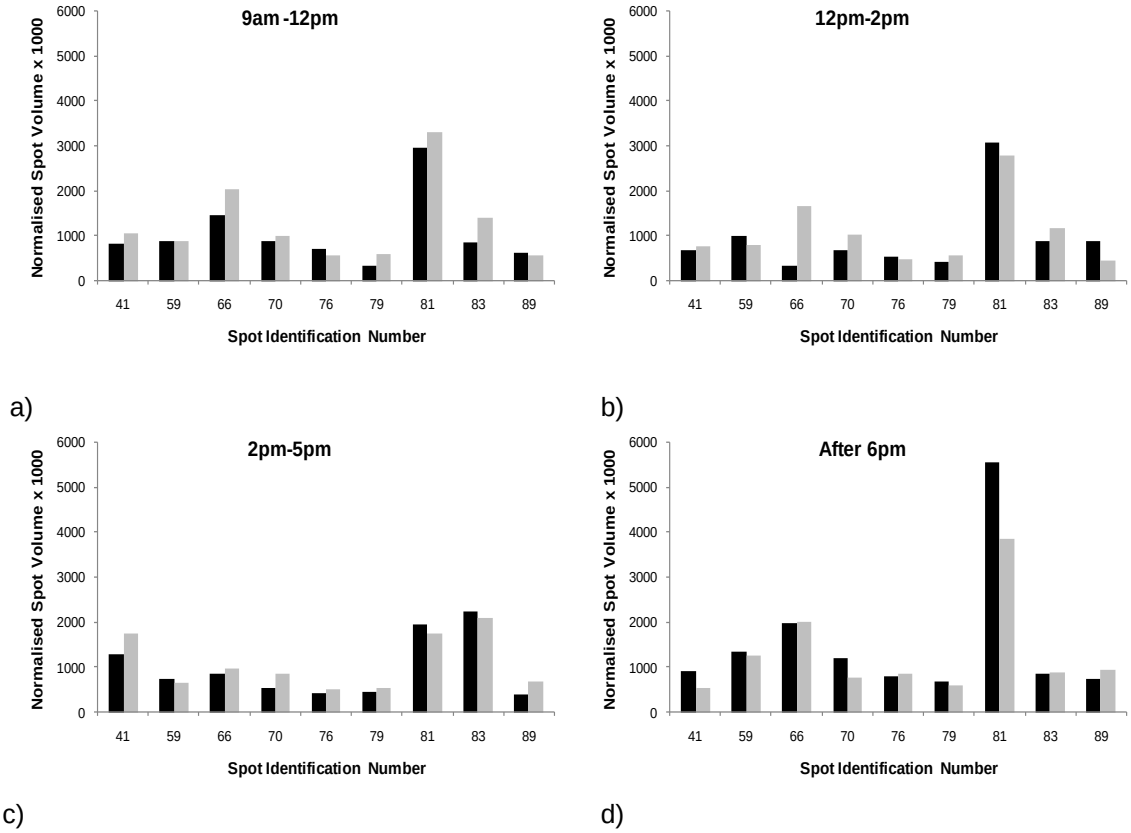
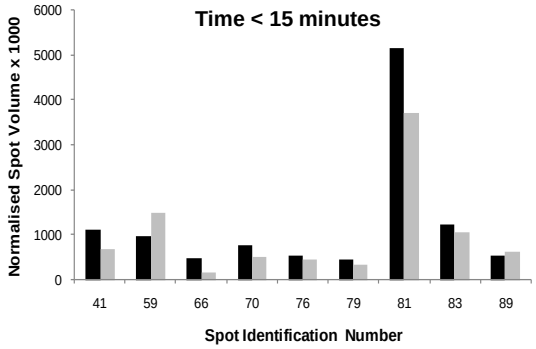


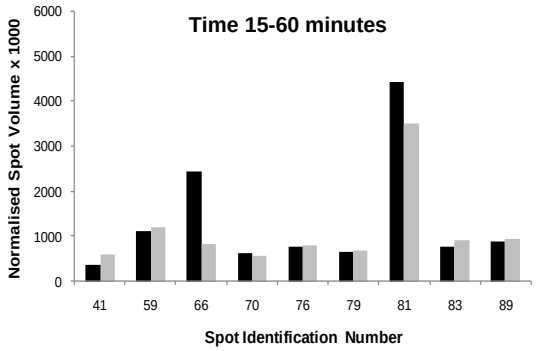
Figure 5.10: Protein expression measured using normalised spot volume between samples taken between a) 9am-12pm, b) 12pm and 2pm, c) 2pm and 5pm and d) after 5pm. Gel repeat 1 represented by black bars and gel repeat 2 represented by grey bars.

5.3.2.4 Protein Expression Analysis of Saliva Samples After Food Consumption

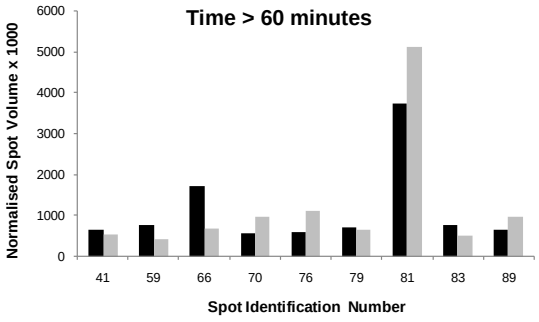
Samples were pooled according to the time since the donor previously had consumed food or drink. **Panel a** represents those samples where the donor had last consumed food or drink up to 15 minutes prior to sample donation**, panel b** between 15 minutes and 1 hour and **panel c** over 1 hour. Spot 81 (Cystatin SN) expression was again seen to be higher than the expression of other spots represented in Figure 5.11 and expression is shown at a similar level in each graph. Expression of spots 41 and 83 (actin-γ) as well as 59 (Lipocalin-1) was inversely related to the length of time since food or drink consumption, decreasing as the time since eating increased. Expression of spots 70, 76 and 89 (zinc-α-2 glycoprotein) increased with length of time since eating or drinking (over a range of approximately 200). Cystatin SA expression was the highest where time since eating ranged between 15 minutes to an hour (Figure 5.11 b).



a)



b)



c)

Figure 5.11: Protein expression measured using normalised spot volume between samples where the time since eating or drinking was a) <15 minutes, b) 15minutes <1 hour, and c) > 1 hour. Gel repeat 1 represented by black bars and gel repeat 2 represented by grey bars.

5.3.3 Protein Expression Analysis of Vaginal Fluid Samples

Spot selection for vaginal fluid samples was processed in the same way as described in section 5.2 for saliva spot selection. Consistent expression was checked across all pools and the protein spot was selected for spot picking. The sixteen spots chosen for LC/MS-MS analysis are summarised in Table 5.7 and their gel position shown in figure 5.9. The fold differences between spots ranged from 2 to 19.30. The fold difference threshold of 1.20 (see section 4.2.5) was adhered to for comparison of samples for this experiment. Table 5.7 shows the fold difference determined by comparison of the highest normalised volume pool against the pool of lowest normalised volume for each individual spot. Spots were automatically ranked according to fold change by the Prodigy same spots software and thus allocated the spot numbers listed in Table 5.7.

Table 5.7: A table to show vaginal fluid spot identification number and fold difference in expression for each spot cut.

Spot Identification number	Fold difference
6	19.30
19	15.00
17	7.50
39	6.20
14	5.80
8	5.50
71	5.30
53	4.70
59	4.40
74	4.20
64	3.60
73	3.50
69	3.10
85	2.90
81	2.30
86	2.00

All MOWSE scores for good protein hits (Table 5.8) were greater than 41 thus making them statistically significant. The lowest MOWSE score given for protein spots identified from the vaginal fluid samples was seen to be 56 for spot 17, identified as Serpin B3 (Squamous cell carcinoma antigen (SCCA)1) and the highest was 184 for spot 59 (leukocyte elastase inhibitor). Protein isoforms were represented by a line of protein spots with the same molecular weight and differing p*I*. This was seen with spots 17, 14 and 19. Spots 17 and 19 were identified as Serpin B3. Spot 14 was unsuccessfully identified possibly to due to the small size of the spot. Another example of this was seen with spots 85 and 86, identified as immunoglobulin κ light chain fragment.

IgG is the predominant class of antibody present within vaginal secretions, whose main function is to prevent microbial adhesion and invasion of the epithelial surface and neutralise toxins or other harmful substances. Levels of IgG have been shown to be higher before ovulation than in the luteal phase afterwards. Two spots lying next to each other on the 2D gel image (Figure 5.12) were identified as IgG. This trend was found to be true for spot 86 with the highest expression shown in the sample pool representing the follicular phase of the menstrual cycle. Spot 85, also identified as IgG showed consistent expression at each phase of the menstrual cycle.

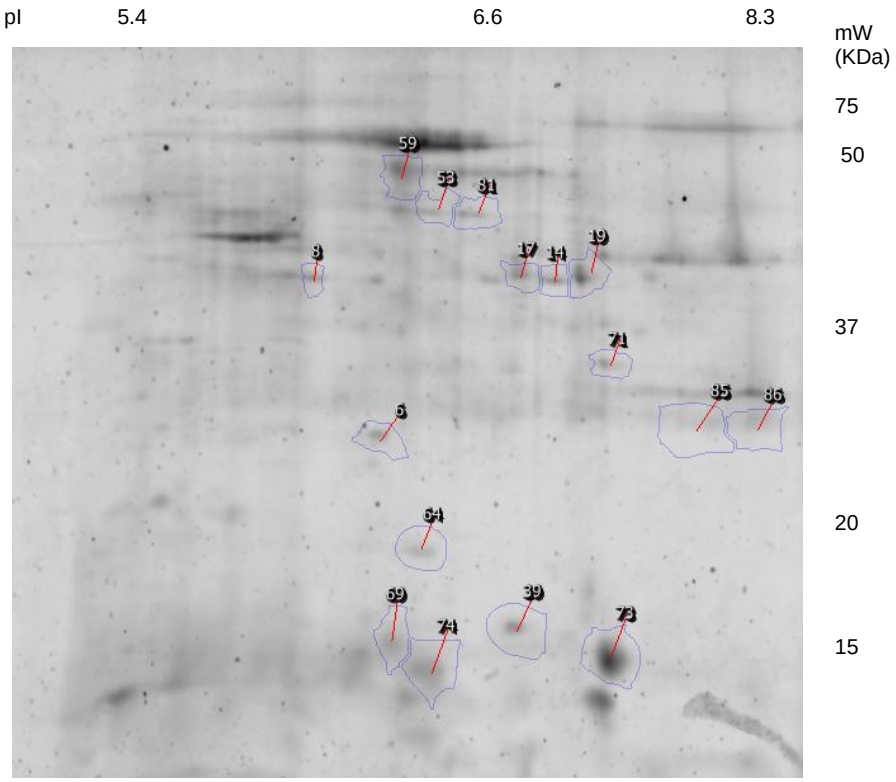


Figure 5.12: A 2D gel image to show positions and identification numbers of vaginal fluid spots chosen for spot cutting.

Nine protein spots were successfully identified using LC-MS/MS data in a MASCOT search. The total ion count spectra can be found in Appendix VIII. The MOWSE scores for these proteins ranged from 56-184, with the number of peptides matching for a positive identification ranging from 2-5 (Table 5.8).

Vaginal fluid protein identifications were; SERPINB3, Leukocyte elastase inhibitor, superoxide dismutase, fatty acid binding protein, calgranulin B and immunoglobulin.

SERPINB3 or SCCA and leukocyte elastase inhibitor (SERPINB1) are serine protease inhibitors of the ov-serpin clade family (Vidalino *et al*. 2009).

Leukocyte elastase inhibitor participates in phagocytosis degrading bacterial components. Elevated levels of SCCA are characteristic of advanced serological cell carcinomas and can be used to monitor the response of a patient to therapy (Cataltepe *et al.* 2000).

Superoxide dismutases are enzymes expressed in seminal plasma and the vaginal epithelium that act to protect spermatozoa from oxidative stress within the vaginal tract (Zini *et al*. 2002).

Fatty acid binding proteins bind lipophilic molecules and have a role in maintaining cellular lipid balance and regulation of lipid pathways (Storch and Thumser 2000).

Calgranulin B (Calprotectin) is a zinc and calcium binding protein with a protective role as its name suggests, produced by squamous mucosal epithelial cells. It inhibits the growth of *E.coli, Staph. aureus* (Sohnle *et al*. 1991) and *Can. Albicans* (Sohnle *et al*. 2000) by preventing growth on mucosal cells *(*Sohnle *et al*. 1991) by competitive binding of zinc required for microbial growth. Calgranulin B has been identified within a number of forensically relevant body fluids including; blood plasma (Dale 1990), saliva (Cuida *et al.* 1995) and urine (Holt *et al.* 1983).

Both IgA and IgG have been shown to be present within the vaginal mucosa but IgG is the predominant antibody present. Secretions have shown to vary during the menstrual cycle with levels being dependent upon hormonal regulation. Half the IgG present are produced by the cells of the genital tract mucosa whereas the remaining half are derived from the circulation. High levels of IgG have been identified within semen and urine (Mestecky 2006) for this reason the protein posed no interest for further testing.

LC-MS/MS data also showed three poor protein identifications with MOWSE scores ranging from 26-47 and the number of matching peptides from 1-3 (Table 5.9). Hit distribution graphs for each spot can be found in Appendix IV. Because of insufficient data from LC-MS/MS, identification of spots 53, 71 and 81 using a MASCOT search was not possible. Therefore further analysis was not carried out on those spots. Database search results produced low MOWSE scores (Table 5.9) and mismatches between theoretical and observed data for mass and p*I* (Table 5.9). Spot 86 was identified with a high MOWSE score of 152 but the molecular weight data obtained from SwissProt only provides values for the whole protein. The fragment molecular weight was not identified so this spot not included in the table. Spot 85 was identified as expected from the spot position (Figure 5.12) but a similar mismatch in data was also seen.

Expression graphs were drawn for the nine proteins positively identified by LC-MS/MS. A comparison of normalised spot volume was made for sample pools (Figures 5.13 - 5.15).

Due to a gel effect running through the middle of the gel, this had hampered the analysis of spot expression data for the second gel image. Therefore vaginal pool

2 data is only based on results from one gel and hence only one data series is represented within the graph for this pool (Figure 5.13a).

Table 5.8: Good protein identifications for vaginal fluid sample spots from a MASCOT search of the LC-MS/MS data.

Spot number	MASCOT Mass	Theoretical Mass / pI	Observed Mass / pI	SwissProt accession number	Name	MOWSE score	Peptides matched
17	44534	44564.60 / 6.35	42000 /6.60	SPB3_HUMAN	Serpin B3/Squamous cell carcinoma antigen 1 /Serine (Or cysteine) proteinase inhibitor (ovalbumin)	56	2
19	44534	44564.6 / 6.35	42000 / 6.80	SPB3_HUMAN	Serpin B3/Squamous cell carcinoma antigen 1 /Serine (Or cysteine) proteinase inhibitor(ovalbumin)	70	2
59	42829	42741.81 / 5.90	48000 / 6.00	ILEU_HUMAN	Leukocyte elastase inhibitor	184	5
64	16154	15935.74 / 5.70	19000 / 6.20	SODC_HUMAN	Superoxide dismutase	89	2
69	15366	15164.43 / 6.60	15000 / 6.00	FABPE_HUMAN	Epidermal Fatty acid-binding protein	156	3
73	15295	15164.43 / 6.60	15000 / 7.00	FABPE_HUMAN	Epidermal Fatty acid binding protein	145	4
74	12770	13241.99 / 5.71	14000 / 6.30	S10A9_HUMAN	Calgranulin B	117	4
85	29067	* 8.80	30000 /8.10	A2NYV5_HUMAN	Immunoglobulin kappa light chain VLJ region (fragment)	169	3
86	26503	* 8.80	30000 /8.30	KV125_HUMAN	Immunoglobulin kappa light chain VLJ region (fragment)	152	3

*Theoretical Molecular Weight not stated due to protein identification of fragment only

Table 5.9: Poor protein identifications for vaginal fluid sample spots from a MASCOT search of the LC-MS/MS data.

Spot number	MASCOT Mass	Theoretical Mass / pI	Observed Mass / pI	SwissProt accession number	Name	MOWSE score	Peptides matched
6	57867	57561.07 / 5.84	30000 / 6.00	API5_HUMAN	Apoptosis inhibitor 5	43	2
8	38673	37353.44 / 5.16	40000 / 5.80	Q96CS8_HUMAN	Hypothetical protein C11or f49	47	2
39	162285	160970.22 / 7.10	16000 / 6.80	NOS1_HUMAN	Nitric oxide synthase	26	1

5.3.3.1 Protein Expression Analysis of Vaginal Fluid Samples – Age Comparison

Spot expression throughout the six age groups sampled is shown in Figure 5.13. Both spots 73 and 69 were identified as fatty acid binding protein isoforms, they demonstrated different levels of expression across all the age groups examined. Spot 73 showed the highest expression in all age groups except in the 50s age group where the expression was similar to one of spot 74 duplicates (calgranulin B). Spots 85 and 86 (immunoglobulin κ) started with high expression in the teen's age group with the level dropping throughout the 20s and 30s. The highest level was found in the 40s group and eventually a decrease was observed in the 50s age group. Spot 86 (immunoglobulin κ) had a lower normalised volume than spot 85 (immunoglobulin κ) for each age group. Spots 17 and 19 (SerpinB3) also showed a similar expression pattern with low expression throughout the teens and 20s increasing to a maximum in the 30s age group. A decrease was noted in the 40s and 50s age groups. Spot 19 (SerpinB3) demonstrated a slightly greater expression than that of spot 17 (SerpinB3). Expression of spots 59 (leucocyte elastase inhibitor) and 64 (superoxide dismutase) peaked in the 20s age group and decreased again in the 50s age group.

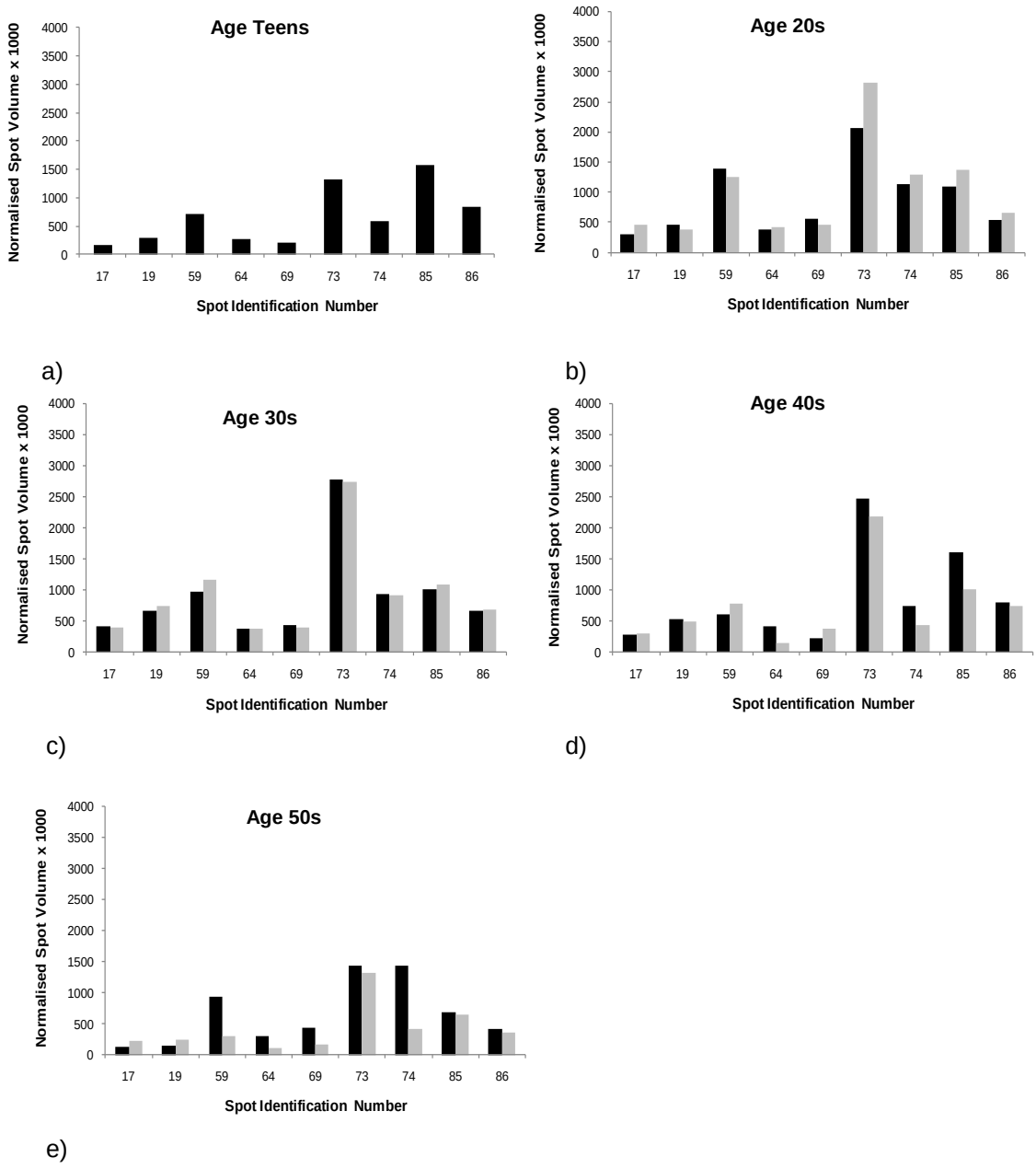


Figure 5.13: Bar charts to show each sample pool for donor's age; a) teens, b) 20s, c) 30s, d) 40s and e) 50s showing normalised spot volume to measure protein expression for each spot cut. Gel repeat 1 represented by black bars and gel repeat 2 represented by grey bars.

5.3.3.2 Protein Expression Analysis of Vaginal Fluid Samples– Contraception Comparison

Samples were pooled according to the method of contraception that the donors were using (Figure 5.14). Hormonal and non-hormonal methods of contraception were compared. In an examination of the groups according to hormonal methods; the combined pill is represented in **panel a**, the pill represented in **panel b**, the implanon implant and Depo-provera injection, represented in **panels c and d** respectively. Non hormonal methods of contraception (e.g. women using the condom) represented in **panel e** and **panel f** includes women not using any contraceptives.

Once again the expression of spot 73 (fatty acid binding protein) was consistently high in all groups except the pill. The highest levels were found for the combined pill and the contraceptive implant – implanon. In contrast spot 69 (fatty acid binding protein) showed a lower level of expression in all graphs, except for the combined pill (Figure 5.14 a) with a spot volume value under 500. Spots 85 and 86 (Immunoglobulin κ) showed a consistent level of expression across each pool. Spot 86 expression was lower (ranging from 384-670) in each pool than spot 85 (ranging from 695 -1364). Spots 17 and 19 (SerpinB3) displayed a slightly higher expression in the pools where no contraceptive method was being used and where condoms are used. The lowest expression was seen in the pool where donors were using the Depo-provera injection (Figure 5.14 c). Spot 59 (Leucocyte elastase inhibitor) showed reduced expression where no contraceptive was being used (Figure 5.14 f) whereas the highest levels were seen with both the combined pill (Figure 5.14 a) and pill (Figure 5.14 b) pools. Spot 64 (Superoxide dismutase) expression was low (less than 500) for each pool with the lowest expression of all the protein spots described so far.

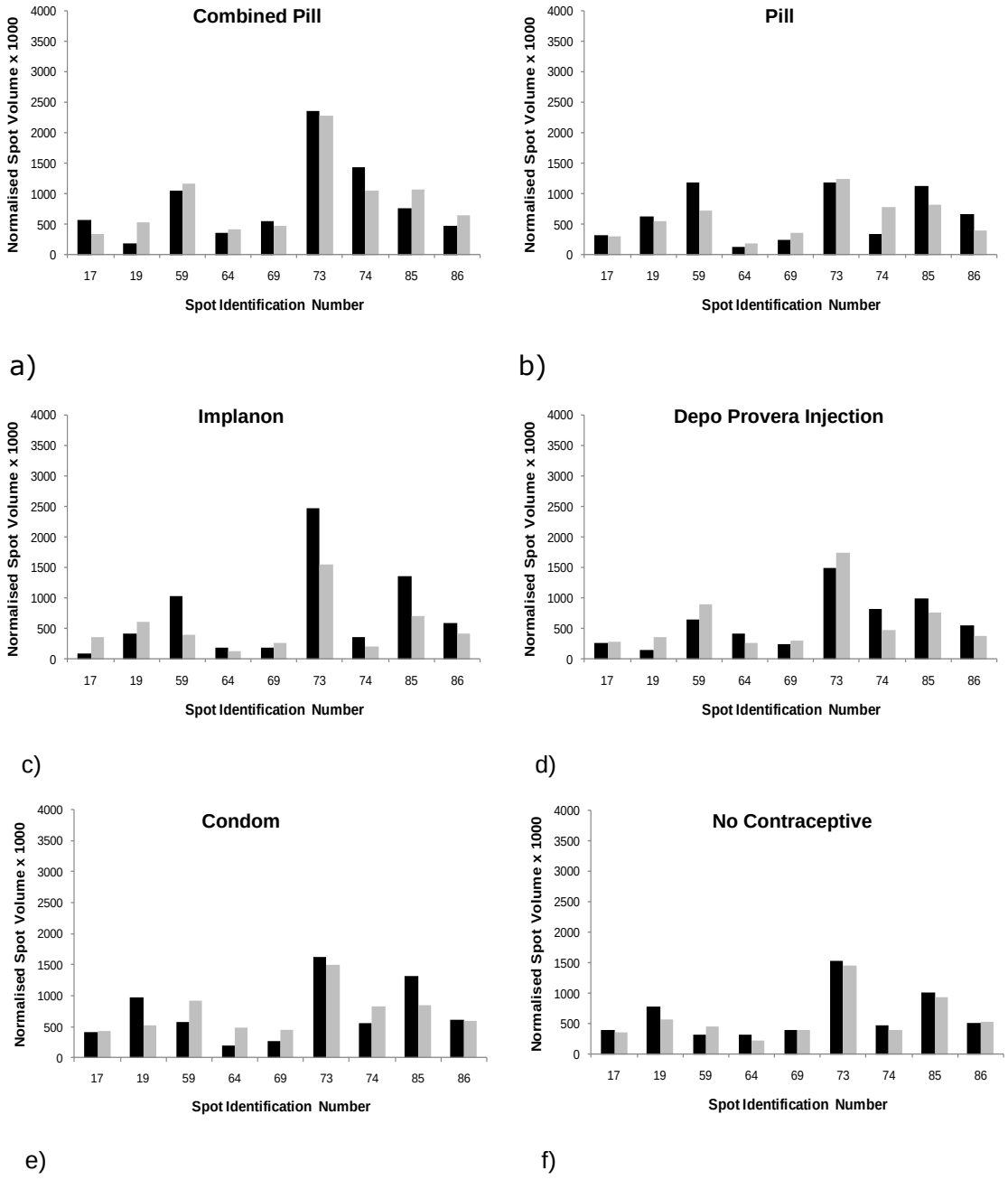


Figure 5.14: Bar charts to show each sample pool for donor's method of contraception; a) combined pill, b) pill, c) Implanon, d) Depo-Provera injection e) condom and f) none showing normalised spot volume to measure protein expression for each spot cut. Gel repeat 1 represented by black bars and gel repeat 2 represented by grey bars.

5.3.3.3 Vaginal Fluid Samples – Menstrual Cycle Comparison

The expression of each protein throughout the menstrual cycle is shown in Figure 5.15. Samples were pooled by the day of the cycle upon which they were sampled. Samples from women not menstruating were pooled together and the results represented in **panel a**. Samples taken in the follicular stage (day 8-12) are shown in **panel b**, those in the ovulatory stage (day 13- 15) are shown in **panel c**, and those in the luteal stage (day 16-28) are shown in **panel d**.

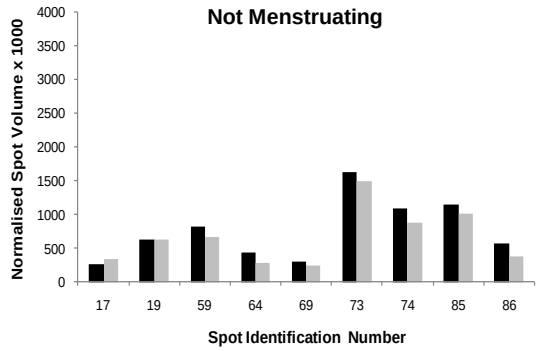
Spot 73 (Fatty acid binding protein) exhibited the highest expression of all the spots identified in each stage of the menstrual cycle, with expression increasing from the follicular phase (Figure 5.15 b), peaking in the ovulatory stage (Figure 5.15 c) and remaining high in the luteal stage (Figure 5.15 d) of the menstrual cycle. The other spot identified for fatty acid binding protein (spot 69) also followed a similar pattern but with expression much lower than spot 73. Similarly, its lowest level was in the follicular phase (Figure 5.15 b) increased to a maximum at ovulation (Figure 5.15 c) and then decreased again in the luteal phase (Figure 5.15 d).

Spots 86 (immunoglobulin κ), 69 (fatty acid binding protein), and 17 (SerpinB1) showed expression levels lower than the follicular stage (Figure 5.15 b) in the pools from the women not menstruating (Figure 5.15 a) All the other spots showed a level of expression slightly higher than the levels seen in follicular phase (Figure 5.15 d). Spots 85 and 86 (Immunoglobulin κ) showed consistent expression levels across each pool with spot 85 being approximately twice as high in expression as spot 86 (as demonstrated in Figures 5.13 and 5.14).

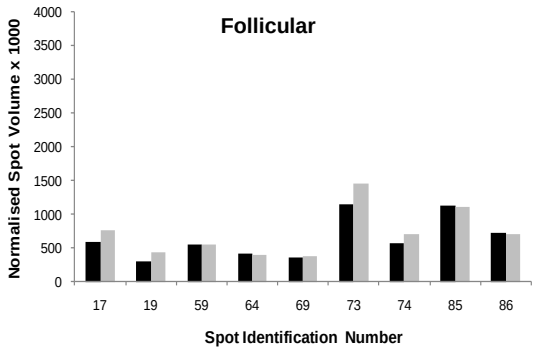
SerpinB3 (spots 17 and 19) had similar expression patterns to one another. Expression increased from the follicular phase (Figure 5.15 b), peaking at the ovulatory phase (Figure 5.15 c) and decreased at the luteal phase (Figure 5.15 d). The only difference as mentioned above was that Spot 17 showed the lowest level of expression in the pool from non menstruating women (Figure 5.15 a), whereas for spot 19 this pool showed a higher expression than shown in the follicular pool (Figure 5.15 b). Spot 74 (Calgranulin B) and spot 59 (Leucocyte elastase inhibitor) showed an increase in expression from the follicular phase (Figure 5.15 b) peaking at the ovulatory phase (Figure 5.15 c) and decreasing slightly in the luteal phase (Figure 5.15 d).

The level of expression in the non-menstruating pool (Figure 5.15 a) was slightly higher than the level seen in the follicular phase (Figure 5.15 b) for these two spots. Expression of spot 64 (superoxide dismutase) decreased throughout the

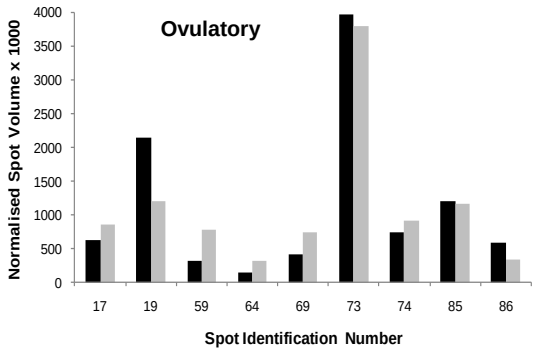
menstrual cycle with the highest expression seen in the samples from the women not menstruating (Figure 5.15 a).



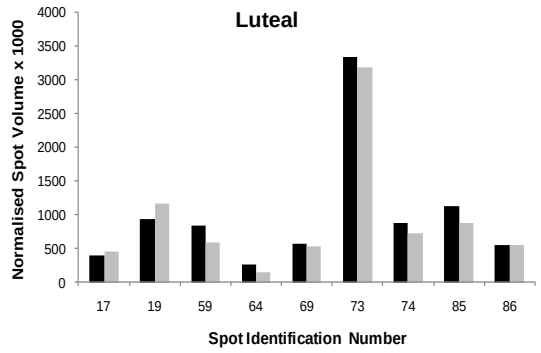
a)



b)



c)



d)

Figure 5.15: Bar charts to show each sample pool to show the stage of menstrual cycle when each sample was donated; a) not menstruating, b) Follicular (day 8-12) , c) Ovulatory (day 13-15), and d) Luteal (day 16-28), showing normalised spot volume to measure protein expression for each spot cut. Gel repeat 1 represented by black bars and gel repeat 2 represented by grey bars.

5.4 Discussion

5.4.1 Protein Expression Analysis

A limitation of 2DGE is the inability to resolve all the proteins present in the body fluid. It is estimated that the protein complement of a cell could contain at least 10,000-30,000 different proteins with between 2000-10,000 visible on a silver stained gel and not all of these would occur at sufficient levels for MS analysis (Martins de Souza, Oliveira *et al*. 2008). The number of protein spots identified in this study was considerably less than the figures stated above. A total of 268 and 193 spots were identified in the saliva and vaginal samples respectively. In cervical-vaginal fluid 400 spots were detected in one recent study with 157 protein identifications made (Di Quinzio, Oliva *et al*. 2007) whereas in another publication, the number of spots was considerably lower with only 147 identified (Tang, De Seta *et al*. 2007). The 2DGE running conditions in this study differed from those used in recent studies mentioned above. For example longer IPG strips (11cm and 13 cm respectively) were used for separation with different pH ranges (pH 3-11 and pH 4-7 respectively). More spots were detected when SYPRO Ruby stain was used (Di Quinzio, Oliva *et al*. 2007) rather than CBB (Tang, De Seta *et al*. 2007). It would seem that the number of spots resolved can be limited by size of the gels and the pH range of the IPG strip used as well as the post electrophoretic staining method employed. Tang *et al* (2007) used larger sized gels and more than one pH range IPG strip per sample resulting in an increased the number of proteins resolved. However the disadvantage is that the image analysis would require more time with the increase in gel numbers and the comparison of more than one pH range.

The number of samples within the sample population used for proteomics studies of saliva and vaginal fluid has seen great variability. In this study, 25 saliva and vaginal fluid samples were considered a suitable representation for a pilot experimental study within the geographic location used. To comply with ethical approval the number of samples obtained should be relevant to the study itself without collecting and sampling from donors unnecessarily. A broad range of samples should be collected if an unbiased view of the population is to be represented. Of course there are limitations of this, time and labour of processing a large number of samples would be inappropriate and not productive to the results for the study. For saliva proteomic studies the number of samples used has been seen to range from using a single donor (Hu, Xie *et al*. 2005) to using 12 donors (Giusti, Baldini *et al*. 2007). For vaginal fluid

proteomic studies donor samples have been taken from between 6 (Zegels, Van Raemdonck *et al*. 2009) and 29 (Tang, De Seta *et al*. 2007) women. From comparison of the number of samples used in this study with previous work, the population used in this study (n=25) was of similar size.

5.4.1.1 Using IgG Standards as an Internal Control for LC-MS/MS Output.

A blind control protein was tested to act as a quality control for the protein identifications produced. The identification hits with significant MOWSE scores obtained for blind control sample 1C can be discounted firstly, as the protein loaded to the 2D gel was a known control sample of pure IgG. Secondly the theoretical p*I* and molecular mass for the protein hits identified in Table 4.4 showed a mis-match with the position that the protein spot was removed from the gel. Spot 1C was removed from the 2D gel at approximately 75 kDa and p*I* of approximately 6.2 in the middle of the gel. The theoretical p*I* for Putative anti-CNG protein was given as 11.39, which was out of the range of IPG strip (pH 3-10) used in the 1st dimension of this gel hence a protein with this p*I* would not be visible within the gel range. The molecular weight for this protein was also seen to be much lower than that of the spot taken for analysis. The second significant hit for Centrosomal protein 2 had a lower p*I* than that of the spot picked and the molecular weight once again was much lower than that of the location the spot was selected from on the gel. In addition to the mis-matched theoretical molecular mass and p*I* data of the 2D gel image, the distribution of protein hits and their MOWSE score shown in Figure 5.3 showed that the majority of hits for control sample 1C are below the significance threshold of 40, with the hits that were significant not yielding a true identification. Control spot 2C on the other hand showed one successful immunoglobulin identification hit with a MOWSE score of 45, and the rest with very low MOWSE scores and hence were not considered for analytical purposes. A real match, which is a non-random event, gives a score which is beyond the green shaded area (Figure 5.2 and 5.3). This successful hit also yielded matching theoretical molecular mass and p*I* data with the location the spot was picked from the 2D gel image. The two spots chosen were different in size and their stain intensity. The relative expression differences between these spots may account for the differences in the identifications between them.

Advances in the stain sensitivity, such as the fluorescent stain SYPRO Ruby being used in this study has meant that some spots chosen may be too small to be

successfully identified by the LC-MS/MS system. This is why some laboratories still use Colloidal CBB (personal communication). Insufficient protein in the spot could lead to a failure to obtain a statistically significant positive identification from the MASCOT search. It is believed to be the case with control spot 1C, as the spot chosen was considerably smaller than spot 2C. This meant that the level of material in the spot selected for analysis was insufficient for a positive identification using the system employed. This problem was overcome by submitting two identical spots per sample submitted for analysis from the two repeated gels providing more protein material per sample in the spots selected.

5.4.2 Protein Expression Analysis of Saliva Samples

All the protein hits described as “good” identifications had MOWSE scores greater than 41 while the highest MOWSE score observed was 120. All these protein identifications were considered statistically significant based upon their MOWSE score.

MOWSE score is not directly dependent on protein quantity as spot 81 was shown to be the most intense spot on the gel (Figure 5.4) but its MOWSE score was shown to be the second lowest of all the spots identified. Some of the MOWSE scores for the poor protein identifications were high, for example spots 43, 46, 47 and 72 all have MOWSE scores over 45. A mismatch was seen between the theoretical mass and p*I* compared with the actual mass and p*I* shown by the position the spots were cut from the gels. Some of the smaller protein spots, such as spot 33 and 47 did not provide a positive identification. Their small size or an insufficient amount of protein present within the spot to be able to provide an accurate protein identity may explain this. Other spots with poor identifications were spots larger in size but the intensity of staining shown on these spots was quite faint (spots 6, 13, 27, 43, 46, 72 and 85). A mismatch can be seen for the theoretical molecular mass and p*I* and the observed mass and p*I* estimated from the 2D gel image for these spots. The sensitivity of SYPRO Ruby may limit the proteomic identification obtained by LC-MS/MS, since although the spot can be seen on the gel there may not be enough material within the spot itself to accurately determine an identity.

A major limitation of the MASCOT search database is that accuracy depends on the quality of the data held within it. As the proteomics field develops the number of protein entries held on the database will increase and thus so will the accuracy of the search hits performed. A downside to this is that a search is likely to yield slightly different results if repeated at a later date due to the changes in the database.

The nine good salivary protein identifications determined by the MASCOT search have all been positively identified previously in saliva either by 2DGE followed by MALDI-TOF (Ghafouri, Tagesson *et al*. 2003; Huang 2004; Hu, Loo *et al*. 2007) or by LC-MS/MS (Hu, Loo *et al*. 2007).

Direct comparisons cannot be made between the gel images produced in this study with those produced in the previously published literature above due to differences in experimental conditions, as discussed earlier (sections 7.1.2.2 and 7.1.3.2). Small gels were cast in this study using 7 cm IPG strips with a pH range of 5-8. The size of the gels was limited due to the equipment available. Longer IPG strips, 11cm IPG strips (Hu, Loo *et al*. 2007) and 13 cm IPG strips (Huang 2004) have been used previously to provide greater spot separation in the first dimension. The pH ranges of the strips used also differed from those in this study. Strips with a pH range of 3-10 (Huang 2004; Hu, Loo *et al*. 2007) and strips with a pH range of 4-7 (Ghafouri, Tagesson *et al*. 2003) have been used with saliva. As was shown in Chapter 3 section 3.2.3.2 (i) the pH strip range alters the 2D pattern shown on the gel and direct comparisons are made difficult.

Saliva protein expression is under neurological control, with protein output being dependant upon the stimulus. When attempting to discover potential biomarkers of saliva it is important to consider the dynamic environment of the oral cavity where there is a continuous supply of newly synthesised proteins and their removal by swallowing (Helmerhorst and Oppenheim 2007). It is also important to be aware of variables which could affect protein expression between individuals; a forensic biomarker would need to be expressed population wide and at a consistent level throughout the day, not having expression affected by external factors.

5.4.2.1 The Effect of Gender on Saliva Protein Expression

Spot expression was higher in the male sample pool than in the female pools (Figure 5.5), for proteins Cystatin SN, and IgA. Although ZA2G (spots 70 and 89) showed higher expression in the male sample pool the other ZA2G spot (76) was higher in the female pool. Lipocalin-1 was the only other spot to show higher expression in the female pool. Expression of actin was not gender specific in spot 41 but shown to be higher in male pool (spot 83). There was variability between the repeats of spot 41 although the same trend was followed and the expression of spot 83. There no published proteomic studies to date investigating gender differences in saliva.

Gene expression studies have shown that some genes exhibit gender differences in their expression with both the female showing higher expression in some genes and the male of others (Srivastava *et al* 2008).

5.4.2.2 The Effect of Age on Saliva Protein Expression

Figure 5.7 showed that each spot identified as ZA2G showed a general trend of decreasing in the age group between 24 - 27 and then increasing again in the age group 28 - 31. A general increase with age was shown for cystatins SN (spot 81) and SA (spot 66) as well as Lipocalin-1. The trend shown for IgA differed between the gel repeats as an increase was shown with age for one repeat whereas the second repeat showed a decrease in the highest age pool. Proteomic analysis of two age range pools of parotid saliva showed an increase in IgA expression in the pool of the older donor's saliva (Ambatipudi 2009). With increasing age the parenchyma of salivary glands is replaced by connective tissue and fat (Nagler 2004). Salivary secretion has also been seen to decrease with age (Yaegaki 1985) which could be a result of the deterioration of the salivary glands.

5.4.2.3 The effect of Sample Donation Time on Protein Expression in Saliva

Before potential biomarkers can be identified, any expression variation due to time of day expression variation needs to be excluded or taken into consideration. For example identifying a protein as a saliva specific forensic marker would be useless if fluctuations in its expression throughout the day were considerable. Ideally a marker used for identification should have consistent expression levels unaffected by changes in time of day or by age or sex of the individual.

If particularly low expression levels were shown at a certain time point during the day this could be mistaken for a small amount of saliva deposited at a scene or on an exhibit. The abundance of markers Histatins 1, 3 and 5 as well as Statherin have been shown to vary during the day (Gusman, Leone *et al*. 2004). Histatin levels have also shown to decrease with age (Johnson, Yeh *et al*. 2000). These fluctuations should be taken into account if Histatin 3 or statherin are to be used as a saliva markers as proposed by Juusola and Ballantyne. (2003).

The proteome of human saliva is highly variable dependent on time and susceptible to a variety of factors. Secretion is controlled by the autonomic nervous

system which can be affected by different stimuli such as the sight and smell of food. Secondly once glandular secretions enter the oral cavity proteins are subjected to further modifications leading to proteolytic cleavage by bacterial or host enzymes. Examples of such proteins are histatins and statherin (Helmerhorst 2007). For these reasons it is sensible to study saliva at the proteome level rather than the mRNA level where these changes would not be discovered (Helmerhorst 2007).

Although salivary cystatins are partially degraded in the oral cavity (decreased by 8 residues) they are still capable of exhibiting cysteine protease activity (Bobek 1994). For all proteins identified in this study expression was highest in the pool of samples taken after 6pm. This would follow the same trend as shown by α-amylase (Ferguson, Fort *et al*. 1973). However this pool only contained two male samples so could be biased. Ideally a repeat would be performed with a pool comprised of more samples preferably from both sexes. Protein expression was shown to be lowest in the afternoon (Figure 5.8c) for, Lipocalin-1 (Spot 59), Cystatin SA (Spot 66), Cystatin SN (Spot 81) and Zinc-α-2 glycoprotein (Spots 70 and 76).

Three spots were identified as the Zinc-alpha-2-glycoprotein (70,76 and 89). When looking at the gel image (Figure 5.4) these spots appear in a line which can be referred to as a spot “train” in the top left of the gel at approximately 39kDa. This term is used to describe spots with approximately the same molecular weight with varying isoelectric points. Spots form a horizontal line on the gel, characteristic of protein isoforms with different post translational modifications for example, an increasing number of phosphorylations (Sackmann-Sala, Ding *et al*. 2009).

5.4.2.4 The Effect of Food Consumption on Protein Expression in Saliva

Brushing teeth without toothpaste has shown that mechanical stimulation can increase salivary flow and change the composition of saliva. An increase in albumin and a decrease in amylase and IgA has been seen after brushing (Hoek, Brand *et al*. 2002). Although this could also be considered to be relevant with the time of day and saliva expression, the mechanical aspect of tooth brushing would be considered more relevant to the effect than of eating on saliva.

During food consumption the proportion of parotid saliva in whole saliva can increase to 50% (Humphrey and Williamson 2001). Thus the composition of saliva changes at this time. The mechanical stimulation of chewing food could also increase the amount of saliva within the oral cavity aiding with digestion and bolus formation. As

saliva is continually being deposited and removed by swallowing it was thought that the protein levels potentially elevated during food consumption would be short lived in the oral cavity. Cystatin SA (spot 66) expression increased up to an hour after food consumption and then decreased (Figure 5.9b). A similar trend was seen with ZA2G (spots 76 and 89). A decrease with time since eating was seen with actin (spot 83) and Lipocalin-1 (spot 59).

5.4.3 Protein Expression Analysis of Vaginal Fluid Samples

The composition and volume of vaginal fluid can vary with different stages of the menstrual cycle (Wilson 2005) and with age. At menopause the vaginal epithelium thins and cervico-vaginal secretions become sparse (Farage 2006). The vaginal fluid proteome has not been studied to the extent that the saliva proteome has been. Of those studies many have used samples obtained from pregnant women (Gravett *et al*. 2007; Pereira *et al*. 2007; Klein *et al*. 2008) whose protein expression may differ from non-pregnant women. Previous work has showed that Serpin B3, Fatty acid binding protein, Calgranulin B and immunoglobulin κ light chain were also positively identified using samples from a range of women aged 24-48 (Tang *et al*. 2007). Once again difficulties are posed when trying to make comparisons between gel images from previous work and this study due to different running conditions of the 1st dimension. Tang *et al*. (2007) used 13cm strips with a wider pH range (pH 3-11) than those that were used in this study. Bearing this in mind the protein spot locations identified in this study are supported by those presented by Tang *et al*. (2007).

5.4.3.1 The Effect of Age on Protein Expression in Vaginal Fluid

Peaks in IgG expression (spots 85 and 86) were shown in the teens and the 40s age groups with a decrease in the 50s age group. Serpin B3 showed an increase in expression in the 20s age group (spot 17) and 30s age group (spot 19). Calgranulin B peaked in the 20s group and tailed off finally increasing in the 50s age group. Physiological changes occur throughout the lifetime being linked to puberty, the menstrual cycle, pregnancy and menopause (Farage and Maibach 2006) and ultimately these changes are due to hormonal control. In post-menopausal women the pH rises in the vagina and there is an increase in microbial colonisation, this could

explain the increase in proteins associated with protection of the vagina from infection at this age for example, calgranulin B.

5.4.3.2 The Effect of Contraception on Protein Expression in Vaginal Fluid

Gene expression and hence protein expression can be affected by hormones, for this reason hormonal contraceptives were thought to affect the proteins expressed within vaginal fluid. When asked their method of contraception some donors only listed the pill without stating whether that was progesterone only or the combined pill. For this reason pools were produced for the donors stating just pill on their consent form and for those stating combined pill. As well as the pill, other forms of hormonal contraception studied were the progesterone only implanon™ implant and Depo-provera injection. In comparison, samples donated from individuals using condoms and those not using any contraception were also studied. An increase in expression of Serpin B3 (spot 19) was seen in the pools from donors not using hormonal contraception compared with those taking hormonal contraceptives (Figure 5.12). The opposite was seen with leukocyte elastase inhibitor (spot 59) where a decrease in expression was seen in the pools where no hormonal contraception was being used. A decrease in expression was seen for Serpin B3 (spot 17) and calgranulin B (spot 74) when implanon was being used compared with the other pools tested. It was thought that if protein expression was affected by contraceptive hormones, differences would be visible between those samples and the samples donated from individuals not taking hormonal contraception. For most pills taken daily, the level of hormones would be kept relatively constant throughout the month. Although there would be a constant low level of progesterone released with the implant or the injection, differences in the hormone levels would be seen depending how far through the course of medication the donor was. The lifespan of an implant is 3 years with the release rate of etonogestrel at approximately 60–70 µg/day in the first 5–6 weeks, decreasing to 35–45 µg/day by the end of the first year of use, to 30–40 µg/day by the end of the second year and to 25–30 µg/day by the end of the third year (The Electric Medicine Compendium a [online]). The injection has a lifespan of 3 months; it contains Parenteral medroxyprogesterone acetate (MPA) and is a long acting progestational steroid. The long duration of action results from its slow absorption from the injection site. Immediately after injection of

150 mg/ml MPA, plasma levels were 1.7 ± 0.3 nmol/l. Two weeks later, levels were 6.8 ± 0.8 nmol/l (The Electric Medicine Compendium b [online]).

5.4.3.3 The Effect of the Menstrual Cycle on Protein Expression in Vaginal Fluid

Calgranulin B expression was higher in the absence than in the presence of menstruating women (Figure 5.13). As mentioned previously, the majority of women in this pool were post-menopausal age and hence the volume of vaginal fluid produced by them was expected to be much lower. Calgranulin is a protein associated with protection against pathogens and hence a higher expression could be expected. Lower expression was seen with Serpin B3, epidermal fatty acid binding protein and one of the spots representing IgG (spot 86) than in the pools of menstruating women. IgG levels were reported previously to decrease from the proliferative phase to the luteal phase (Usala, Usala *et al*. 1989)

A previous study has demonstrated that the concentration of Leukocyte elastase inhibitor has reached a maximum at the middle of the menstrual cycle (Ovulatory Phase). Shot gun proteomics using samples from pregnant women (Klein *et al*. 2008) have shown this increase to be 5 times that shown in the follicular and luteal phases of the menstrual cycle. This was found to be untrue for the results using 2D proteomics, there was a difference between the duplicates for the ovulatory phase with the second repeat showing a greater expression than in the follicular phase. The highest expression was seen in the luteal phase. It could be proposed that differences in expression of Leukocyte elastase inhibitor are noted between pregnant and non pregnant women.

Of those pools looking at the menstruating women and cyclical changes, there seems to be a trend for a peak in expression during the ovulatory phase, which is seen with Fatty acid binding protein, calgranulin and Serpin B3.

5.5 Chapter Summary

A total of 268 and 193 spots were identified in the saliva and vaginal fluid samples respectively (Appendix VIII). Of those spots 19 saliva and 16 vaginal fluid spots were analysed by LC-MS/MS. Approximately half the spots cut for analysis yielded good protein hits in the database.

The quality of MASCOT search results obtained is dependent upon the size and intensity of the spot selected from the 2D gel.

Cyststain SN (spot 81) consistently showed the highest expression across all the saliva gel pools. Whilst Fatty acid binding protein (spot 73) showed the highest expression in all vaginal pools except of those samples from women in their 50s.

Lower protein expression was generally shown in the saliva samples from females compared with males with Cystatin SN (spot 81) Zinc-α-2 Glycoprotein (spots 70 & 89) and Immunoglobulin-α (Spot 79) actin-γ (spot 83) following this trend.

Some saliva proteins were seen to have an increasing expression with age (Immunoglobulin-α, Cystatins SA and SN) whilst Actin-γ (spots 83 and 41) showed a decrease.

Daytime variation in protein expression was seen with the majority of saliva proteins, showing reasonably high expression in the morning with a decrease in the afternoon between 2pm and 5pm then an increase again in the evening except for actin-γ (spots 41 and 83) that showed the opposite trend starting low and increasing in the afternoon then decreasing in the evening.

An inverse relationship was seen between expression and time since eating with Cystatin SN and Lipocalin. Immunoglobulin-α expression increased as the time since eating also increased. Expression changes in the other protein spots were contradictory between the repeated gels and no clear conclusions could be drawn from them.

The general trend shown for vaginal proteins when pooled by age was that expression decreased as age increased. This was not true for epidermal fatty acid binding protein (spot 69) and calgranulin (spot 74).

Expression of immunoglobulin seemed to be independent of contraceptive method. SerpinB3 (spots 17 and 19) was highest in the non hormonal pools (no contraceptive or condoms) whereas leukocyte elastase inhibitor (spot 59) and calgranulin (spot 64) were lowest in these groups. The different forms of hormonal contraceptives did not seem to affect the protein expression in the same way. Hiigher

levels were seen with the majority of the proteins in the pools with the pill or combined pill compared with the depo-provera injection or the implanon implant.

The stage of menstrual cycle showed a peak in expression of the majority of the proteins in the ovulatory phase then decreasing in the luteal phase. Expression was shown to be highest after menstruation in the follicular phase and lowest in the luteal phase for the majority of proteins (leukocyte elastase inhibitor (spot 59), superoxide dismutase (spot 64), calgranulin B (spot 74) and immunoglobulin (spot 85)). Whlist two proteins opposed ths trend (immunoglobulin (spot 86), Serpin B3 (spot 19), epidermal fatty acid binding protein (Spots 73 and 69)) with a lower expression after menstruation in the follicular phase.

From this evaluation, candidate biomarkers for saliva are cystatins SA and SN, lipocalin-1 and zinc-α-2 glycoprotein. For vaginal fluid candidate biomarkers are serpin B3 and leukocyte elastase inhibitor. Further testing for the specificity of these proteins would determine their suitability for biomarkers for forensic purposes.

Chapter 6
Confirmation of Candidate Biomarkers and their Body Fluid Specificity

CHAPTER 6 – Confirmation of Candidate Biomarkers and their Body Fluid Specificity

6.1 Introduction

The aim of the work described in this chapter was to determine whether the proteins identified in chapter 5 would be suitable markers for either saliva or vaginal fluid. Of the six protein identifications made for both saliva and vaginal fluid, four saliva proteins (Zinc-α-2 Glycoprotein, Lipocalin-1 Cystatin SA and Cystatin SN) and two vaginal proteins (Leukocyte Elastase Inhibitor and Serpin B3) were chosen for further analysis.

6.1.1 Verification of Protein identification

It is important to verify the identity of the proteins that could be candidates for body fluid identification. There are several ways of doing this. Ideally this would be performed using a 2D Western blot demonstrating that the spot identified is the exact spot cut from the gel. Furthermore it would also highlight multiple protein spots of the same protein (Lubec *et al*. 2003). In practice the Western blot spots may vary due to the exact positioning of the gel onto the transfer membrane especially in areas with a high density of spot coverage.

An alternative is to use simpler immunological techniques with the potential marker proteins. The resulting data can then be used in the development of a diagnostic method for body fluid testing.

ELISA and dot blots were chosen initially to test for body fluid specificity of the proteins previously identified by LC-MS/MS (Chapter 5). Both techniques have been used previously to identify body fluids for forensic purposes. ELISA has been used to identify semen, (Graves *et al*. 1985; Kyurkchiev *et al*. 1989; Simich *et al*. 1999; Sato *et al*. 2004), blood (Cattaneo *e*t *al*. 1992; Sato *et al*. 1999) and saliva (Quarino *et al*. 2005). Dot blots have been used for ABO blood typing in saliva (Rao and Kashyap 1992; Hamada *et al*. 2002) and for the identification of semen (Rao and Kashyap 1992; Sato *et al*. 2001).

6.1.1.1 Dot Blot

Dot blots have been used previously in forensic science. Antibodies against semenogelin a marker in seminal plasma showed no cross reactivity with saliva, urine, vaginal fluid sweat or serum (Sato *et al*. 2001) and were also used for species identification. Anti-human IgG antibodies were tested against blood from a range of animal species including human (Matsuzawa *et al*. 1993). ABO antigens were identified in saliva stains using as little as 0.00005 µl of saliva sample and offering a simple and non invasive method for ABO typing (Hamada *et al*. 2002). A simple Dot-ELISA was performed with a heat step introduced blocking non-specific reactions of the samples with secondary antibody.

6.1.1.2 ELISA

ELISA has been used for the detection of salivary amylase (Quarino *et al*. 2005) with monoclonal anti-human salivary amylase antibodies. However false positives were obtained with some semen, urine and sweat samples at low concentrations. For this reason a threshold value is needed for saliva to eliminate false positives. The ELISA could also be adapted to decrease sensitivity.

Blood was tested for, with an ELISA using IgG and albumin (Cattaneo *et al*. 1992) in buried blood samples over a period of 65 weeks. This was not to determine the presence of blood but to test the protein degradation in blood over that time.

The P30 antigen in semen, although found to be male specific was also present in male urine (Graves *et al*. 1985). Monoclonal antibody (mAb) 4E6 a sperm coating antigen, has successfully been used to identify both ejaculated spermatozoa and seminal plasma by ELISA. Although this antigen is a sperm protein it has also been detected in azoospermic samples (Kyurkchiev *et al*. 1989). PSA, the most common antigen used for detection of semen has been shown using ELISA that levels present in saliva (0.16 and 0.11 ng/ml) are close to those of semen samples (0.20 ng/ml) (Simich *et al*. 1999). In more recent years a commercial ELISA (SEMA test, Humagen fertility diagnostics) has become available to test for the presence of semen using mouse anti-human sperm no. 5 (MHS-5) antibodies which recognise the seminal vesicle specific antigen (SVSA) in the post ejaculated fragmented semenogelin.

For forensic identification purposes an ELISA would not be suitable, due to the high sample throughput required for each ELISA plate. The semenogelin antibodies described by Sato *et al.* (2001) were used to develop an immunochromatographic

assay for the detection of semen (Sato *et al*. 2004). Later commercialised as RSID-Semen (Independent Forensics). As described in Chapter 1 Independent Forensics also manufacture RSID immunochromatographic test cassettes for blood and saliva.

6.1.2 Salivary Proteins Identified by 2DGE and LC-MS/MS

6.1.2.1 Salivary Cystatins

In human saliva cystatins are abundant proteins (Dickinson 2002a). Salivary cystatins are extracellular, secreted proteins (Turk *et al*. 2008). They are members of a group of proteins known as cysteine protease inhibitors. Cysteine proteases are proteolytic enzymes which cleave peptide bonds through a reactive cysteine residue at the catalytic site of the protein (Bobek and Levine 1992). Cystatins are reversible competitive inhibitors of Cysteine proteases (Hiltke *et al*. 1999) responsible for the proteolytic destruction of cysteine proteinases from the host, bacteria or viruses. Many bacteria use cysteine proteases for colonisation and proliferation (Hiltke *et al*. 1999) which is of particular importance in the oral cavity where saliva plays a protective role against pathogens. The absence of cystatins in saliva would lead to infection of the soft tissue in the mouth leading to the onset of periodontal disease.

Cystatins also play a minor role in regulation of salivary calcium levels (Humphrey and Williamson 2001). It has been proposed that cystatins may participate in the regulation of proteinase activity (Henskens *et al. 1994*) from bacteria involved with dental plaque formation but this has yet to be demonstrated. Investigations have centred on correlations between the levels of salivary cystatins and peridontal disease states (Bobek and Levine 1992). Elevated levels of salivary cystatins were reported in patients with gingivitis and peridontal disease due to inflammation and bleeding of the gums (Henskens *et al*. 1994).

Cystatins were first isolated from human saliva in 1979 (Juriaanse and Booij 1979) but their cysteine protease inhibitory function and hence their name was not given until 1984 (Isemura *et al*. 1984) when Cystatin S was isolated and sequenced.

6.1.2.1 (i) Cystatin SN

After cystatin S the next salivary cystatin isolated by Ion-exchange chromatography was a neutral cysteine proteinase inhibitor. This protein was identified as Cystatin SN, having an immunological cross reactivity and thus some homologous

structure to Cystatin S and a p*I* value near neutrality (Isemura *et al*. 1986). Confirmation is seen in Figure 5.4 where the position of spot 81 (Cystatin SN) lies between pH 6 and pH 8 on the gel. This structural similarity was further supported by amino acid sequencing (Isemura *et al.*1987) which showed 10 amino acid substitutions present in Cystatin SN compared with Cystatin S.

6.1.2.1(ii) Cystatin SA

Cystatin SA was another cysteine proteinase inhibitor isolated from human saliva by Ion-exchange chromatography (Isemura *et al*. 1987). This protein was named Cystatin SA because it had an acidic p*I* value, confirmation of this was shown in Figure 5.4 where the position of spot 66 (Cystatin SA) is on the acidic, left hand side of the gel. Sequence homology was shown between this protein and previously identified Cystatins, 90.10% to Cystatin S 87.40% to Cystatin SN and 57.70% to Cystatin C.

6.1.2.2 Zinc-α-2 glycoprotein (ZA2G)

ZA2G is a single chain polypeptide with a molecular mass of 41 kDa. It's name is derived from its action in precipitating zinc ions (Shibata and Miura 1982; Schenkels *et al*. 1995). After the initial discovery in human plasma by ion-exchange chromatography (Burgi and Schmid 1961). ZA2G has been identified in a number of different body fluids and tissues including forensically relevant types such as urine (Shibata and Miura 1982), seminal plasma (Frenette *et al*. 1987), breast milk, sweat, saliva (Ohkubo *et al*. 1990) and skin (Tada *et al*. 1991). A recent review article (Hassan *et al*. 2008) confirms that the function of ZA2G is still unknown. It has been suggested that ZA2G plays a role in lipid metabolism (Rolli *et al*. 2007). ZA2G knockout mice have been used to test this theory (Rolli *et al*. 2007) where mice with both depleted ZA2G genes gained more weight than the wild type mice fed on the same diet. This function also supports the presence of ZA2G in semen where it may act as a factor involved in sperm motility regulation (Ding *et al*. 2007).

6.1.2.3 Lipocalin-1/ Von-Ebner's Gland Protein

Lipocalin-1 or Von Ebner's gland protein (VEGh) is named due to its secretion from the Von Ebner's gland contained within the tongue (Li and Snyder 1995). Lipocalin has a molecular weight of approximately 20 kDa and a p*I* of approximately 5 (Schenkels *et al*. 1995). This was confirmed in Figure 5.4 where the position of the

protein identified (spot 59) lay within this range on the gel. Lipocalins are small secreted extracellular proteins with small lipophilic molecules. In saliva lipocalin function is thought to be associated with the taste function potentially binding bitter compounds (Schmale *et al.* 1990). Initially expression was thought to be localised to saliva but amino acid sequence homology to tear prealbumin has shown they are the same protein (Redl 2000) thus expression is not localised exclusively in saliva.

6.1.3 Vaginal Fluid Proteins Identified by 2DGE and LC-MS/MS

6.1.3.1 Serpins

Serpins are a family of proteins with tertiary structure homology each consisting of nine α-helices and three β-sheets. The proteins were initially named due to their **ser**ine **p**roteinase **in**hibitor function. Since then serpins within the group have been identified without protease function. The serpin family has been categorised into clades (Gettins 2002), the two serpins identified by proteomic methods within this study are members of the clade B serpins with 82% amino acid sequence homology (Remold-O'Donnell 1993).

6.1.3.1 (i) Serpin B3 – Squamous Cell Carcinoma Antigen 1 (SCCA1)

First identified as a tumour antigen TA-4 (Kato and Torigoe 1977) from a cervical squamous cell carcinoma. Previous electrophoretic studies resulted in a single band with an approximate molecular weight of 45 kDa (Cataltepe *et al.* (2000). Separation of this single band by isoelectric focussing resulted in two major groups with differing pH ranges; acidic (p*I* 5.9-6.2) and neutral (p*I* 6.3-6.6) (Kato *et al*. 1984). The squamous cell carcinoma antigen (SCCA) has a misleading name as the neutral form of the antigen (SCCA1) is detected in the cytoplasm of normal and some malignant squamous cells, whereas the acidic form (SCCA2) is found exclusively within malignant tumours. SCCA1 is localised within squamous cells whereas SCCA2 is released from the cell and found in circulating plasma (Kato 1992).

Recent findings support the theory that SCCA has a function in programmed cell death, a process critical for autoimmunity. SCCA is typically found overexpressed in cancer cells with epithelial origin. Studies have shown that in its absence cells had an increased susceptibility to drug induced apoptosis, supporting the role of Serpin B3 in apoptosis resistance (Suminami *et al*. 2001).

SCCA has been used as a plasma marker for the identification of patients with cervical SCC. Post treatment levels have also been monitored as an indicator of therapeutic response (Cataltepe *et al*. 2000).

Immunohistochemistry positively identified SCCA within the squamous epithelium of the tongue and vaginal as well as the skin epidermis of normal tissues (Suminami *et al*. 2001).

6.1.3.1(ii) Serpin B1 Leukocyte Elastase Inhibitor

Serpin B1 is a 42 kDa (Cooley *et al*. 2001) protein that inhibits neutrophil elastase and Cathepsin G (Vidalino *et al*. 2009) in cells. It degrades bacterial components during phagocytosis. Small amounts of serpin B1 can be released from the cell protecting it from proteases released into the cytoplasm (during an inflammatory stress response), (Gettins 2002). Serpin B1 has been found in high amounts in neutrophils and monocytes (Cooley *et al*. 2001).

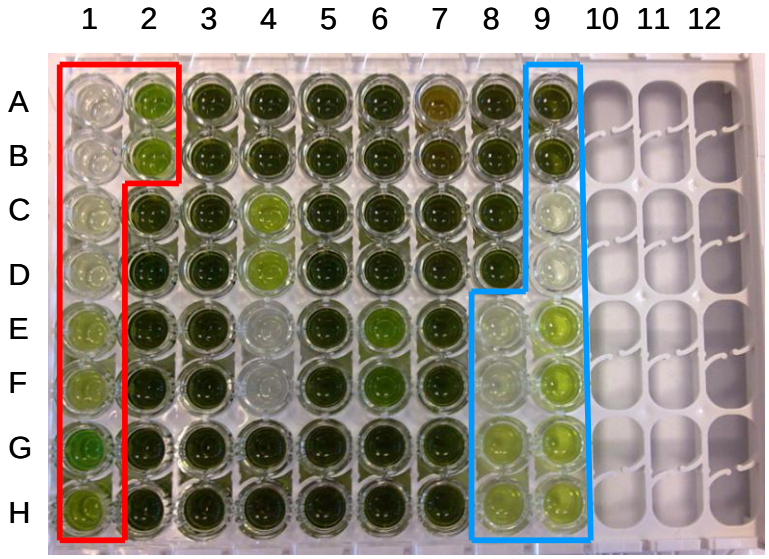
6.2 Results

6.2.1 Confirmation of Body Fluid Specificity using ELISA

Positively identified protein markers were tested for identity and specificity using commercially available ELISA kits. The CanAg SCC EIA kit (Fujirebio™) was used to test for the presence of squamous cell carcinoma antigen/leucocyte elastase inhibitor (vaginal fluid sample spots 17 and 19) and the Human Zinc-α -2 Glycoprotein ELISA kit (BioVendor) was used to test for the presence of Human Zinc-α-2 Glycoprotein (saliva sample spots 70, 76 and 89).

6.2.1.1 Confirmation of Leucocyte Elastase Inhibitor Specificity using CanAg SCC EIA Kit (Fujirebio™)

Initial results for this ELISA test showed that the samples assayed using a 1:50 dilution were too concentrated to recover any quantitative data regarding SCC expression for vaginal fluid samples. All other body fluids tested gave values within the standard curve at 1:50 dilution, except for menstrual blood which also was too high. The ELISA plate was photographed for reference to show the intensity of each well assayed (Figure 6.1a). Readings were only obtained for vaginal samples 9, 10 and 18 at this dilution. Schematic diagrams were drawn to show the ELISA plate layout showing which samples were placed into which wells. The calibration standards (Cal) are outlined in red whilst the body fluid comparison samples are outlined in blue, vaginal fluid samples (Vag) are left without an outline.



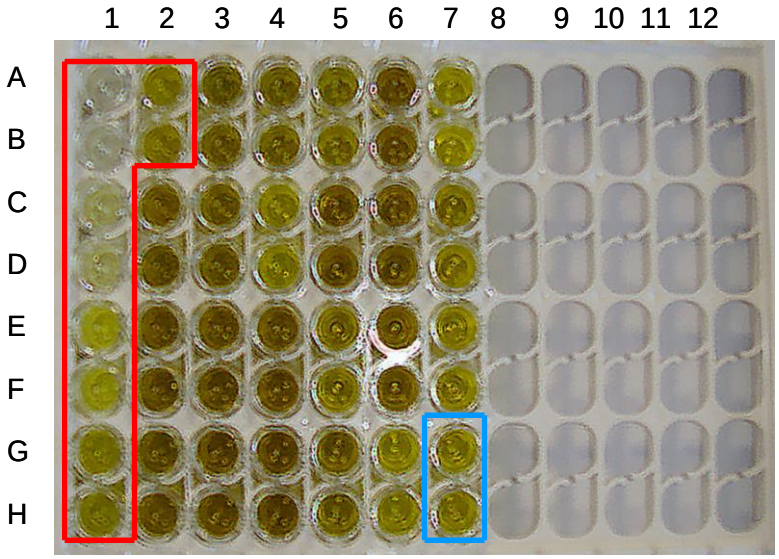
a)

	1	2	3	4	5	6	7	8	9	10	11	12
A	Cal A 0 µg/L	Cal E 46 µg/L	Vag 4	Vag 8	Vag 12	Vag 16	Vag 20	Vag 24	Menstrual blood			
B	Cal A 0 µg/L	Cal E 46 µg/L	Vag 4	Vag 8	Vag 12	Vag 16	Vag 20	Vag 24	Menstrual blood			
C	Cal B 1.1 µg/L	Vag 1	Vag 5	Vag 9	Vag 13	Vag 17	Vag 21	Vag 25	Breast milk			
D	Cal B 1.1 µg/L	Vag 1	Vag 5	Vag 9	Vag 13	Vag 17	Vag 21	Vag 25	Breast milk			
E	Cal C 5.1 µg/L	Vag 2	Vag 6	Vag 10	Vag 14	Vag 18	Vag 22	Blood	Saliva			
F	Cal C 5.1 µg/L	Vag 2	Vag 6	Vag 10	Vag 14	Vag 18	Vag 22	Blood	Saliva			
G	Cal D 24 µg/L	Vag 3	Vag 7	Vag 11	Vag 15	Vag 19	Vag 23	Semen	Urine			
H	Cal D 24 µg/L	Vag 3	Vag 7	Vag 11	Vag 15	Vag 19	Vag 23	Semen	Urine			

b)

Figure 6.1: a) A digital photograph of the 96 well CanAg SCC EIA ELISA plate taken immediately after readings were taken for samples with a 1:50 dilution, b) schematic diagram showing CanAg SCC EIA ELISA plate layout showing the calibrator standards outlined in red and the body fluid comparison samples outlined in blue.

The ELISA was repeated on the remaining vaginal fluid samples and the menstrual blood sample using a dilution of 1:200. The ELISA plate was photographed (Figure 6.2) and a schematic diagram was drawn.



a)

	1	2	3	4	5	6	7	8	9	10	11	12
A	Cal A 0 µg/L	Cal E 46 µg/L	Vag 4	Vag 8	Vag 14	Vag 19	Vag 23					
B	Cal A 0 µg/L	Cal E 46 µg/L	Vag 4	Vag 8	Vag 14	Vag 19	Vag 23					
C	Cal B 1.1 µg/L	Vag 1	Vag 5	Vag 11	Vag 15	Vag 20	Vag 24					
D	Cal B 1.1 µg/L	Vag 1	Vag 5	Vag 11	Vag 15	Vag 20	Vag 24					
E	Cal C 5.1 µg/L	Vag 2	Vag 6	Vag 12	Vag 16	Vag 21	Vag 25					
F	Cal C 5.1 µg/L	Vag 2	Vag 6	Vag 12	Vag 16	Vag 21	Vag 25					
G	Cal D 24 µg/L	Vag 3	Vag 7	Vag 13	Vag 17	Vag 22	Menstrual blood					
H	Cal D 24 µg/L	Vag 3	Vag 7	Vag 13	Vag 17	Vag 22	Menstrual blood					

b)

Figure 6.2: a) A digital photograph of the 96 well CanAg SCC EIA ELISA plate taken immediately after readings were taken for samples with a 1:200 dilution, b) schematic diagram showing CanAg SCC EIA ELISA plate layout showing calibrator standards outlined in red and the menstrual blood sample outlined in blue.

Bar graphs were drawn to show the SCC concentration of each vaginal fluid sample (Figure 6.3) and for other body fluid samples (Figure 6.4). SCC was positively identified in all vaginal fluids ranging from 812.75 µg/l in sample 9 to 17464 µg/l in sample 13. Sample 10 was an exception, where the SCC concentration was

determined as being below the threshold of the standard calibrators. The optical density at 450nm for the 0 µg/l was read as 0.08 and the reading for sample 10 was determined as 0.08. Therefore a concentration for that sample was determined as 0 µg/l. Future work would include another ELISA to be run with more diluted samples since some of the sample concentration values were calculated by extrapolation of the standard curve. The upper limit of the ELISA kit standards is shown on Figure 6.3 represented by a red line.

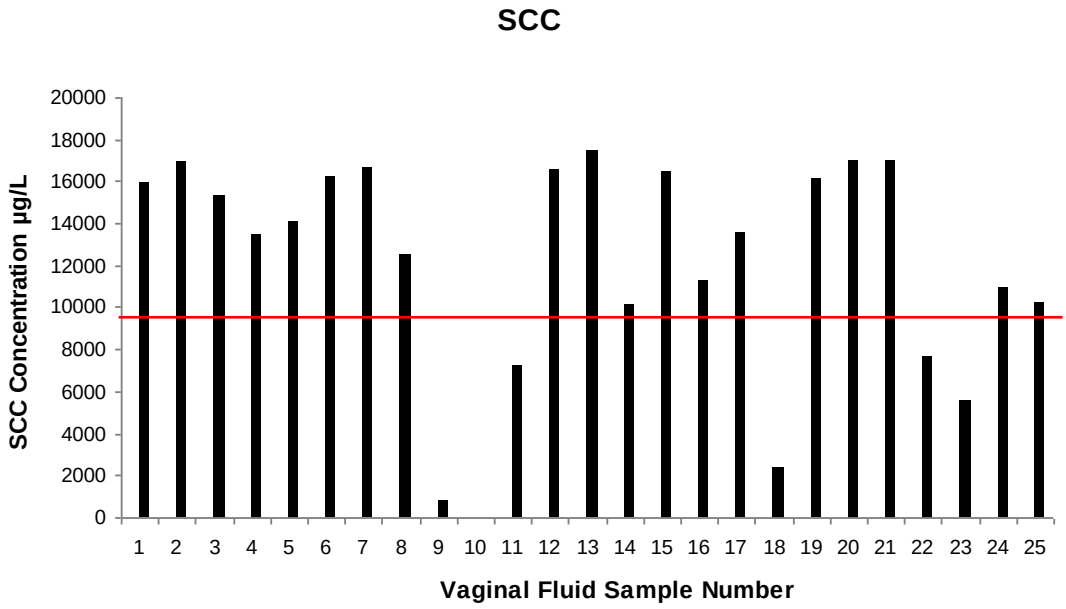


Figure 6.3: SCC concentration of each vaginal fluid sample measured by CanAg SCC EIA ELISA kit using optical density readings at 405nm. The red line represents the level above which sample concentrations were extrapolated.

Figure 6.4 displays results for the other body fluid samples assayed for SCC. Although all body fluid samples showed positive results for SCC the levels detected by the ELISA kit were much lower for the majority of the fluids tested in comparison with vaginal fluid. The lowest level of SCC was detected in the breast milk sample (0.65 µg/l) with the next lowest SCC concentration was seen in the blood sample (2.50 µg/l). These two readings are hardly visible in Figure 6.4 as they are so low in comparison to the other readings determined from the assay. The SCC concentration determined for the semen sample tested was 351.15 µg/l. The SCC concentration found in urine was 664.25 µg/l just under double that seen in semen with the next highest SCC concentration of 845.95 µg/l found in saliva. These body fluid samples were determined using a 1:50 dilution of the body fluid sample. The menstrual blood sample was seen to be too concentrated at that dilution and the concentration of this sample could not be

determined without a further dilution of the sample. A 1:200 dilution of the menstrual blood sample provided a SCC concentration of 9309.20 µg/l. This was the only body fluid sample that showed a SCC concentration in a similar range to that of the vaginal fluid samples. From Figure 6.3 it can clearly be seen that there are six vaginal fluid samples with SCC concentrations that are below that of the menstrual blood sample.

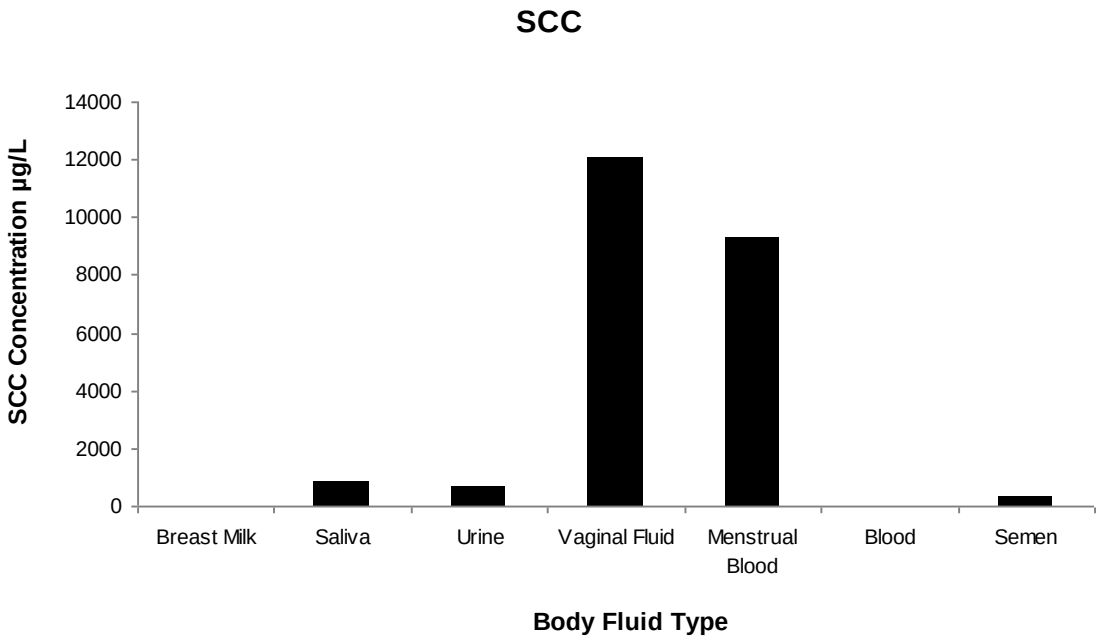


Figure 6.4: SCC concentration of each body fluid sample measured by CanAg SCC EIA ELISA kit using optical density readings at 405nm.

The sensitivity and specificity (Tze-Wey Loong. 2003) of the CanAg SCC EIA ELISA was calculated (Table 6.1). A total of 31 samples were tested, 25 vaginal fluid and 6 other body fluid samples. The cut off level for this calculation was set at 2000 µg/l. The sensitivity was defined as the number of vaginal fluid samples positively identified by ELISA divided by the total number of vaginal fluid samples tested. The specificity was defined as the number of other body fluid samples to give a negative result divided by the total number of samples tested by the ELISA that were not vaginal fluid.

$$\text{Sensitivity} = \frac{\text{Number of True Positives (TP)}}{\text{Number of True Positives (TP)} + \text{Number of False Negatives (FN)}}$$

$$\text{Specificity} = \frac{\text{Number of True Negatives (TN)}}{\text{Number of True Negatives (TN)} + \text{Number of False Positives (FP)}}$$

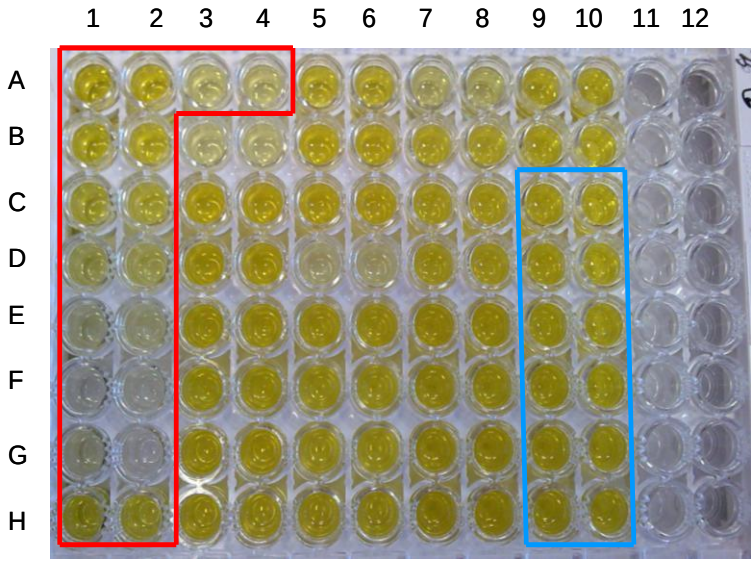
Table 6.1 Sensitivity and Specificity of the CanAg SCC EIA ELISA kit.

	Vaginal Fluid samples		
CanAg EIA ELISA kit Results		Positive	Negative
	Positive	23 (TP)	1(FP)
	Negative	2 (FN)	5 (TN)
		(23/25) x 100 = 92%	(5/6)x 100 = 83%
		Sensitivity	Specificity

This ELISA was shown to be 92% sensitive and 83% specific (Table 6.1) with the range of samples tested.

6.2.1.2 Confirmation of Human Zinc-Alpha-2-Glycoprotein (ZA2G) Specificity using ZA2G ELISA (BioVendor)

Initial results for the ZA2G ELISA test showed that the undiluted samples assayed were too concentrated to recover any quantitative data from saliva samples. A second ELISA was performed after diluting the samples 1:2. A digital photograph of the ELISA plate was taken for reference to show the intensity of each sample well (Figure 6.5). A schematic diagram was also drawn showing sample placement. The standards (std) are outlined in red whilst the body fluid comparison samples are outlined in blue. Saliva samples (Sal) are left without an outline. Figure 6.5 showed that every sample well gave a positive result for ZA2G.



a)

	1	2	3	4	5	6	7	8	9	10	11	12
A	Std 1 100 ng/mL	Std 1 100 ng/mL	QC Low	QC Low	Sal 8	Sal 8	Sal 16	Sal 16	Sal 24	Sal 24		
B	Std 2 50 ng/mL	Std 2 50 ng/mL	Sal 1	Sal 1	Sal 9	Sal 9	Sal 17	Sal 17	Sal 25	Sal 25		
C	Std 3 25 ng/mL	Std 3 25 ng/mL	Sal 2	Sal 2	Sal 10	Sal 10	Sal 18	Sal 18	Menstrual Blood	Menstrual Blood		
D	Std 4 12.5 ng/mL	Std 4 12.5 ng/mL	Sal 3	Sal 3	Sal 11	Sal 11	Sal 19	Sal 19	Breast Milk	Breast Milk		
E	Std 5 6.25 ng/mL	Std 5 6.25 ng/mL	Sal 4	Sal 4	Sal 12	Sal 12	Sal 20	Sal 20	Urine	Urine		
F	Std 6 3.125 ng/mL	Std 6 3.125ng/mL	Sal 5	Sal 5	Sal 13	Sal 13	Sal 21	Sal 21	Blood	Blood		
G	Blank	Blank	Sal 6	Sal 6	Sal 14	Sal 14	Sal 22	Sal 22	Semen	Semen		
H	QC High	QC High	Sal 7	Sal 7	Sal 15	Sal 15	Sal 23	Sal 23	Vaginal Fluid	Vaginal Fluid		

b)

Figure 6.5: a) A digital photograph of the 96 well Human Zinc-α-2-Glycoprotein ELISA plate taken immediately after readings were taken, b) schematic diagram showing Human Zinc-Alpha-2-Glycoprotein ELISA plate layout showing the calibrator standards outlined in red and the body fluid comparison samples outlined in blue.

Bar graphs were drawn to show the ZA2G concentration of each saliva sample (Figure 6.6) and for other body fluid samples (Figure 6.7). ZA2G was positively identified in all saliva samples assayed. The range that the standards cover and hence the concentration range calculated with confidence is shown by the two red lines on Figure 6.6 indicating the upper and lower limits of the calibrator standards. Samples 1,

11 and 16 showed low concentrations of ZA2G compared to the other 22 samples assayed (5.94 ng/ml, 2.45 ng/ml and 17.72 ng/ml respectively). The range of ZA2G concentrations for the other samples was between sample 13 (126.22 ng/ml) and sample 2 (216.38 ng/ml).

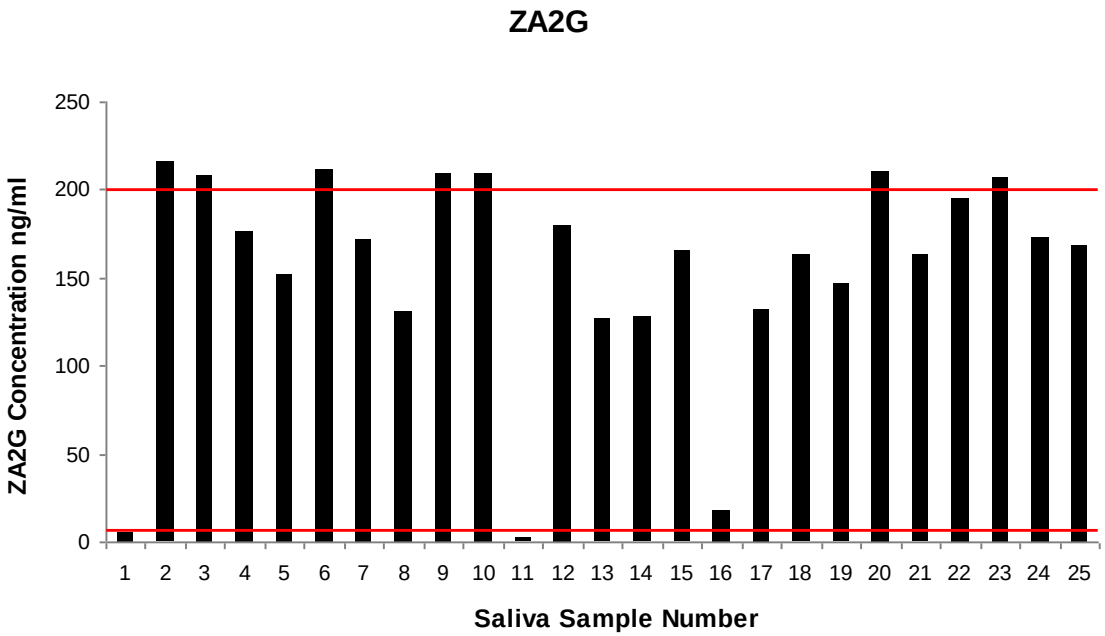


Figure 6.6: ZA2G concentration of each saliva sample measured by Human Zinc-α-2-Glycoprotein ELISA kit using optical density readings at 450nm. The red lines represent the range of the calibrator standards, the concentration of samples outwith this range were determined by extrapolation.

Looking at the other body fluid samples, (Figure 6.7) it can be seen that ZA2G is present within all the body fluid samples tested. The levels of expression were similar for each sample. The highest concentration of ZA2G was seen in menstrual blood (195.72 ng/ml) and the lowest concentration other than for saliva was breast milk (169.20 ng/ml). Since the levels of ZA2G seen in each fluid were similar and within the range of the saliva samples tested, it was not possible to identify sample type (Figure 6.7) from this ELISA test.

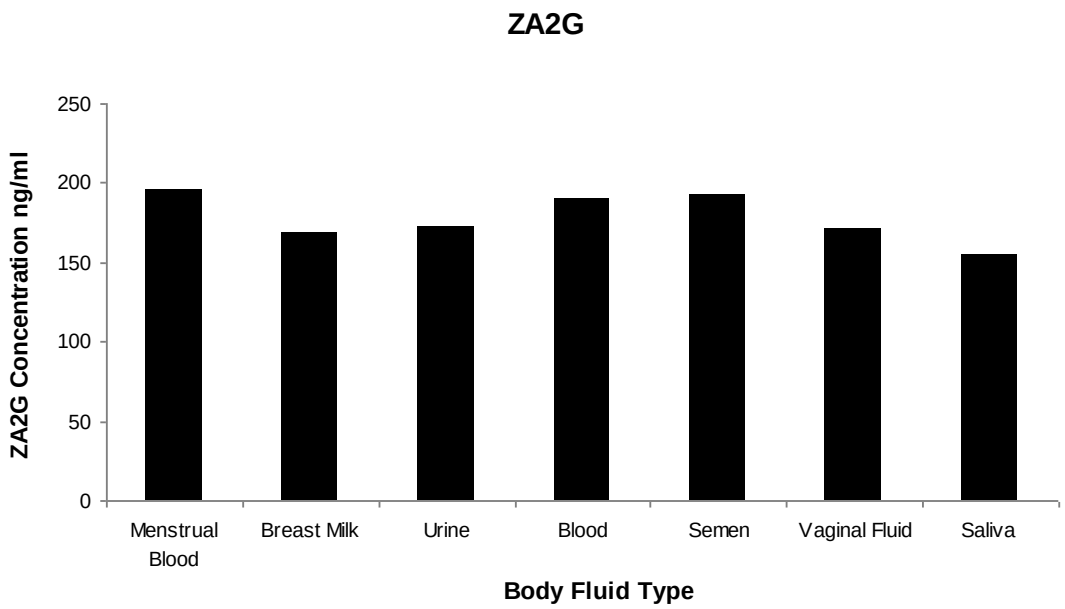


Figure 6.7: ZA2G concentration of each body fluid sample measured by Human Zinc-α-2-Glycoprotein ELISA kit using optical density readings at 450nm.

The sensitivity and specificity (Tze-Wey Loong. 2003) of the Human Zinc-α-2-Glycoprotein ELISA kit was calculated (Table 6.2). A total of 31 samples were tested, 25 saliva and 6 other body fluid samples. The cut off level for this calculation was set at 50 ng/ml. The sensitivity was defined as the number of saliva samples positively identified by the ELISA divided by the total number of saliva samples tested. The specificity was defined as the number of other body fluid samples to give a negative result divided by the total number of samples tested by the ELISA that were not saliva

$$\text{Sensitivity} = \frac{\text{Number of True Positives (TP)}}{\text{Number of True Positives (TP)} + \text{Number of False Negatives (FN)}}$$

$$\text{Specificity} = \frac{\text{Number of True Negatives (TN)}}{\text{Number of True Negatives (TN)} + \text{Number of False Positives (FP)}}$$

Table 6.2 Sensitivity and Specificity of the Zn-α-2-Glycoprotein ELISA kit.

	Saliva samples		
Human Zn-α-2-Glycoprotein ELISA kit Results		Positive	Negative
	Positive	22 (TP)	6 (FP)
	Negative	3 (FN)	0 (TN)
		(22/25) x 100 = 88%	(0/6)x 100 = 0%
		Sensitivity	Specificity

Ideally a test should be both sensitive and specific; this ELISA was 88% sensitive and 0% specific (Table 6.2) for the range of body fluid samples tested.

6.2.2 Confirmation of Specificity using a Dot Blot

6.2.2.1 Testing the Detection Limits of the Dot Blot with Recombinant Protein

Four protein markers were tested for body fluid specificity in the same range of body fluids as the ELISA kits. Cystatin SA, Cystatin SN, and Lipocalin-1 identified in saliva and SERPINB1 in vaginal fluid, were tested with antibodies specific to each protein (Figure 6.8). A serial dilution (Table 6.3) of commercial recombinant protein, was performed to determine the antibody limits for each protein tested (Figure 6.9). The volume of sample loaded to the membrane was 10 µl.

Each dot in the dot blots performed appeared as a narrow slot type band due to the loading device used. It can be seen that the background was relatively high in all the photographs. Bands were seen up to a 1:10 dilution for panels Cystatin SA (Figure 6.9 panel a), Cystatin SN (Figure 6.9 panel b), and SERPINB 1 (Figure 6.9 panel d). There were no bands visible with Lipocalin-1 (Figure 6.9 panel c). This experiment was repeated and the same results observed.

Table 6.3: Recombinant protein concentrations of each serial dilution.

Dilution	Recombinant Protein Concentration (µg/ml)			
	Cystatin SA	Cystatin SN	Lipocalin-1	SERPINB1
Neat	100µg/mL	100µg/mL	132µg/mL	90µg/mL
1:2	50µg/mL	50µg/mL	66µg/mL	45µg/mL
1:10	10µg/mL	10µg/mL	13.2µg/mL	9µg/mL
1:50	5µg/mL	5µg/mL	6µg/mL	4.5µg/ml
1:100	1µg/mL	1µg/mL	1.32µg/mL	0.9µg/mL

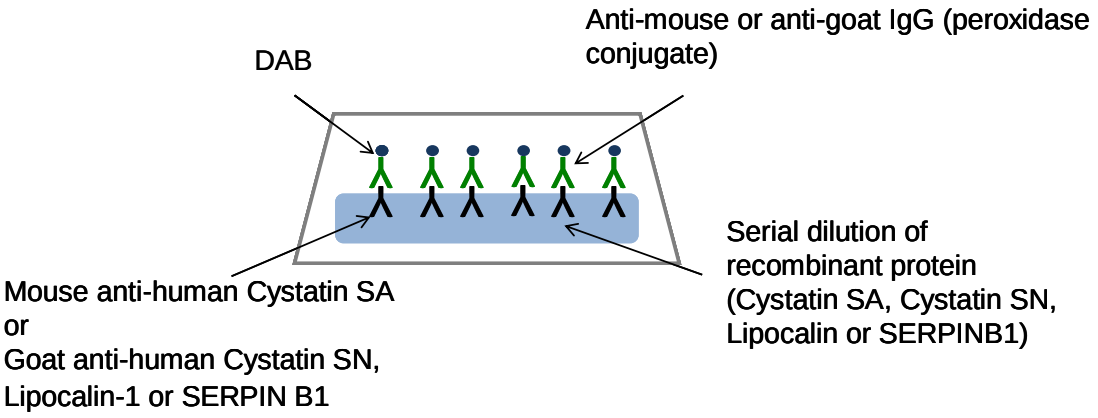


Figure 6.8: A schematic diagram of dot blot of recombinant protein in serial dilution

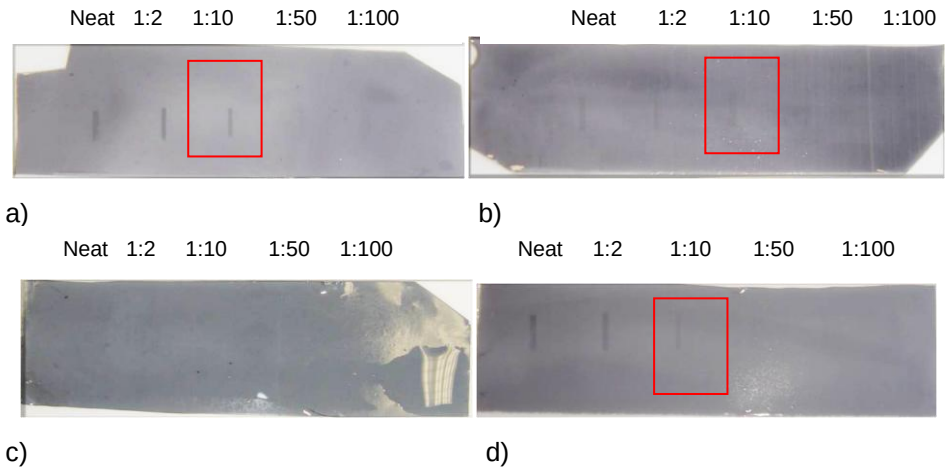


Figure 6.9: Detection limits of a) Cystatin SA, b) Cystatin SN, c) Lipocalin-1, and d) SERPINB1. Each antibody was tested against recombinant proteins in serial dilution (neat, 1:2, 1:10, 1:50 and 1:100).

6.2.2.2 Testing the Detection Limits of the Dot Blot with a Serial Dilution of Body Fluids

Each antibody was tested with the body fluid where it was initially isolated by 2DGE (Figure 6.10). Cystatin SA, Cystatin SN and Lipocalin-1 were tested against a saliva sample in serial dilution and SERPINB1 was tested with a vaginal fluid sample in serial dilution (Figure 6.11). The range of dilutions tested were undiluted, 1:2, 1:10, 1:50 and 1:100. The volume of sample loaded to the membrane was 10 µl.

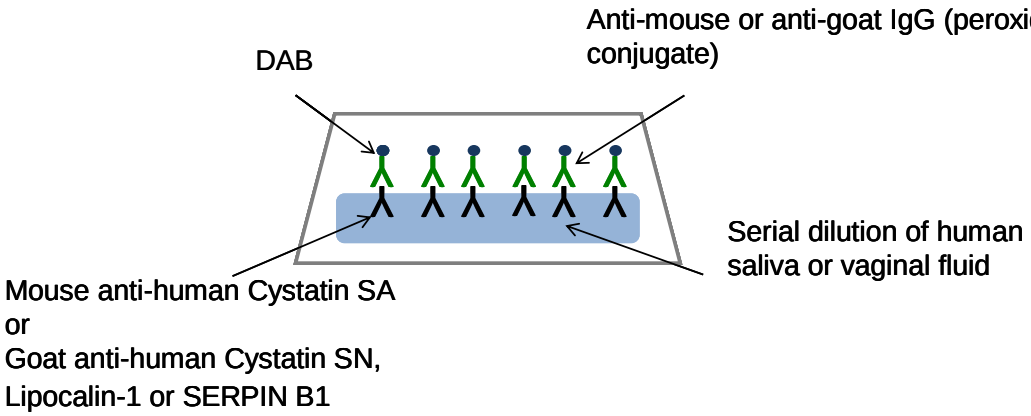


Figure 6.10: A schematic diagram of dot blot of saliva or vaginal fluid in serial dilution

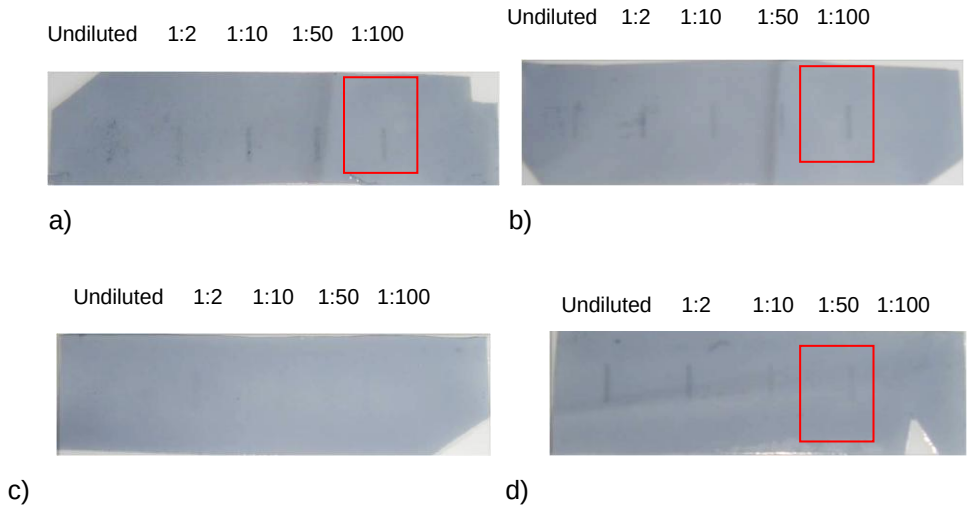


Figure 6.11: Detection limits of a) Cystatin SA, b) Cystatin SN, c) Lipocalin-1 with saliva in serial dilution (neat, 1:2, 1:10, 1:50 and 1:100), and d) SERPINB1 with vaginal fluid in serial dilution (neat, 1:2, 1:10, 1:50 and 1:100).

From these results it can be seen that Cystatin SA (Figure 6.11 a) and Cystatin SN (Figure 6.11 b) can be detected in up to a 1:100 dilution of saliva. The Lipocalin antibody failed to detect saliva at any of the dilutions tested including the undiluted

saliva (Figure 6.11 c). SERPINB1 detected vaginal fluid up to a 1:50 dilution (Figure 6.11 d).

6.2.2.3 Testing the Detection of the Dot Blot with Lipocalin Primary Antibody Direct to the Membrane

No bands were visible with either recombinant Lipocalin-1 protein or saliva in the dot blot. The lipocalin dot blot failure might have been caused by the primary or secondary antibody functioning incorrectly. To test the performance of the secondary antibody, a dot blot was performed with the primary goat anti-Lipocalin antibody (10 µl) spotted onto a membrane and the secondary antibody applied (Figure 6.12). The results for this dot blot are shown in Figure 6.13. No dilution was performed for this experiment.

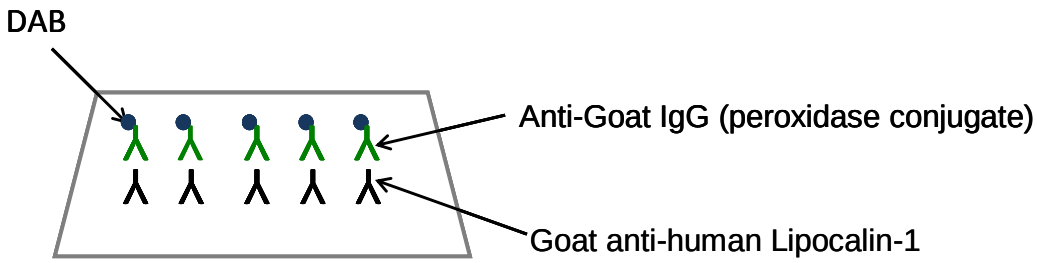


Figure 6.12: A schematic diagram of dot blot of Lipocalin-1 antibody

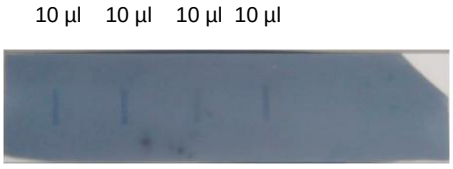


Figure 6.13: Dot blot of Goat anti- Lipocalin-1 antibody directly added to the membrane.

Bands were visible when the goat anti-Lipocalin was directly applied to the membrane and treated with secondary antibody and DAB as in previous experiments, although the background signal was high making the bands hard to visualise. Thus confirming the primary-secondary antibody reaction to be working.

However due to no positive reactions being shown with the positive control (recombinant protein) or the saliva sample no further dot blots were performed using this antibody.

6.2.2.4 Dot Blot Detection using a Range of Body Fluids

Each antibody was tested with a range of body fluids; menstrual blood, blood, breast milk, semen, saliva, vaginal fluid and urine in order to test for protein specificity (Figure 6.14). 10 µl of undiluted body fluid was used for the dot blot with each antibody.

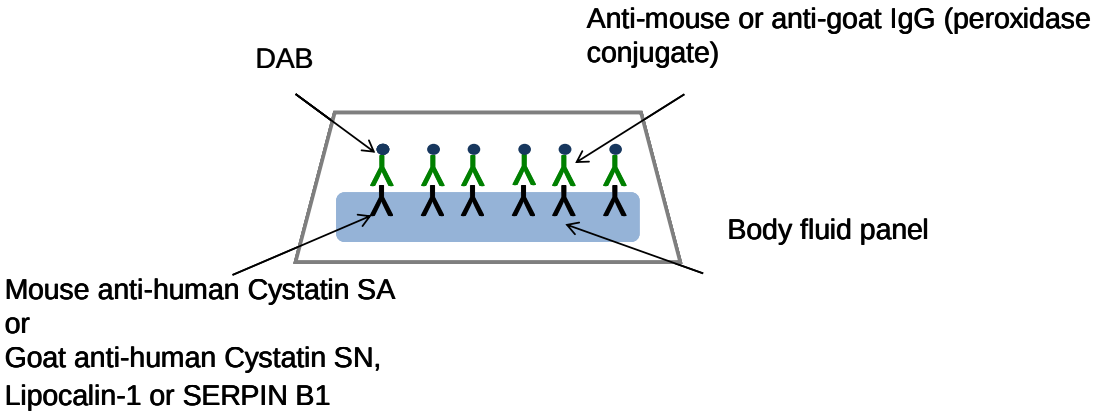


Figure 6.14: A schematic diagram of dot blot of body fluid panel with primary and secondary antibodies

A positive reaction was seen for all body fluids (Figure 6.15). All the antibodies exhibited positive signals with the saliva samples. However, the antibody for Cystatin SA (Figure 6.15 a) showed strong positive signals with menstrual blood and semen samples. Weak signals were obtained with blood, breast milk, urine and vaginal fluid. Cystatin SN (Figure 6.15 b) showed strong signals with blood and menstrual blood that appeared stronger than saliva. Signals with a similar intensity to the band observed in the saliva sample were shown with semen and vaginal fluid, whereas weak positive signals were shown with breast milk and urine. SERPINB1 that was originally detected in vaginal fluid showed strong signals with saliva, semen, menstrual blood (Figure 6.15 c) and as was expected, with vaginal fluid. Weak signals were also shown with blood, breast milk and urine.

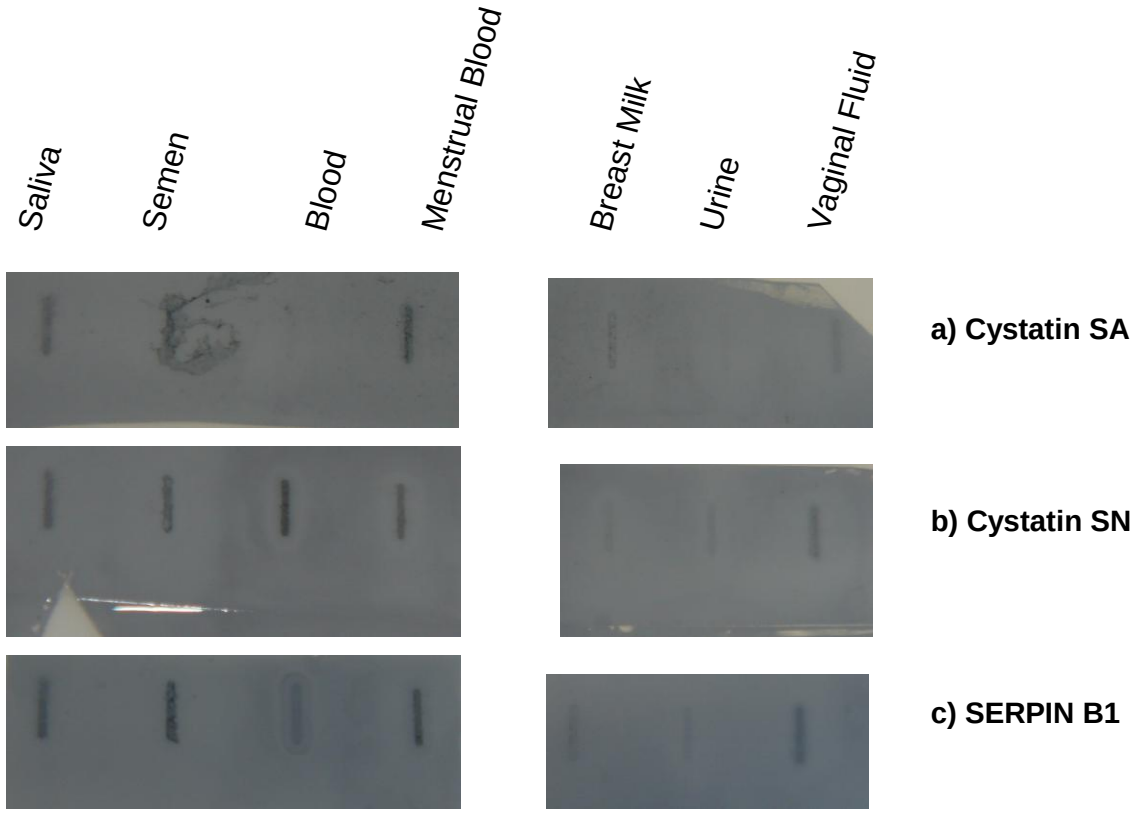


Figure 6.15: A dot blot to show antibody specificity tested with 10 µl of a range body fluids using a) Cystatin SA, b) Cystatin SN and c) SERPINB1 antibodies.

6.2.2.5 Dot Blot Detection using Blood Samples with Direct DAB Development

It was noted that in the previous experiment bands were visible on the membrane prior to DAB antibody detection (Figure 6.17). It was thought this might be due to haemoglobin staining the membrane from the blood samples. To test this a dot blot was performed using blood and menstrual blood in serial dilution (neat, 1:2, 1:5, 1:10, 1:50, 1:100, 1:200, 1:500 and 1:1000), treating the membrane immediately with DAB without antibody addition (Figure 6.16). This would determine whether the signals shown for blood are present without antibody addition, thus testing whether the haemoglobin levels present in the blood samples are high enough to react with DAB creating false positive results. The results from this reaction are shown in Figure 6.18.

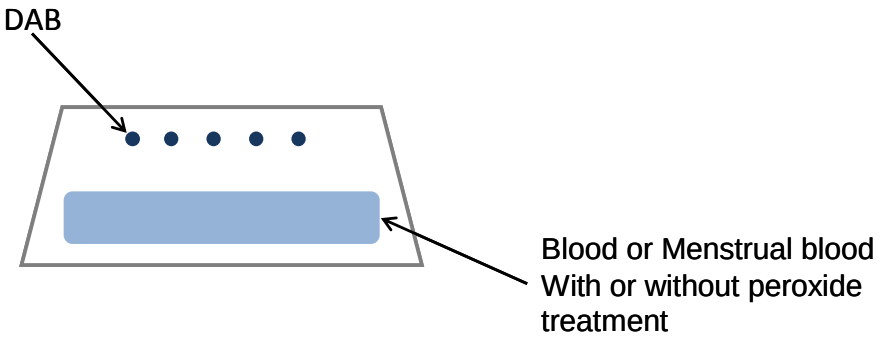


Figure 6.16: A schematic diagram of dot blot of blood and menstrual blood with or without peroxidase treatment

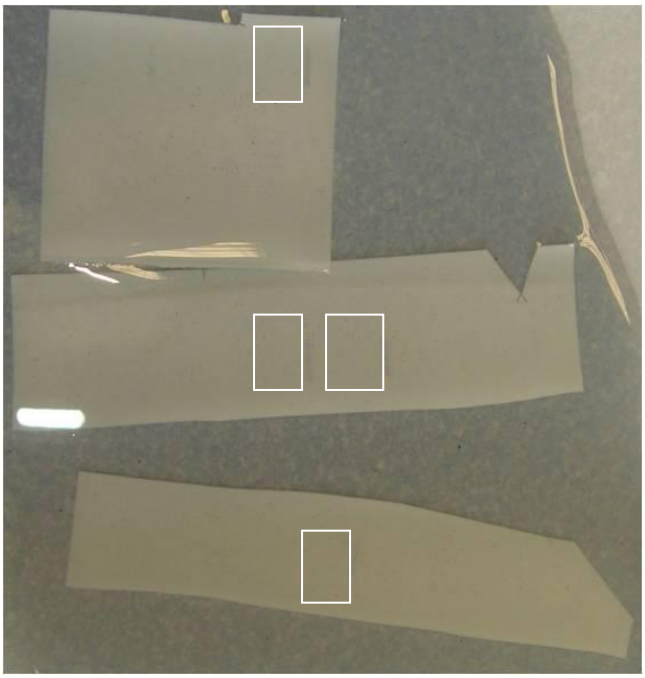


Figure 6.17: A digital photograph to show haemoglobin staining on the dot blot membranes (outlined in white) prior to DAB development.

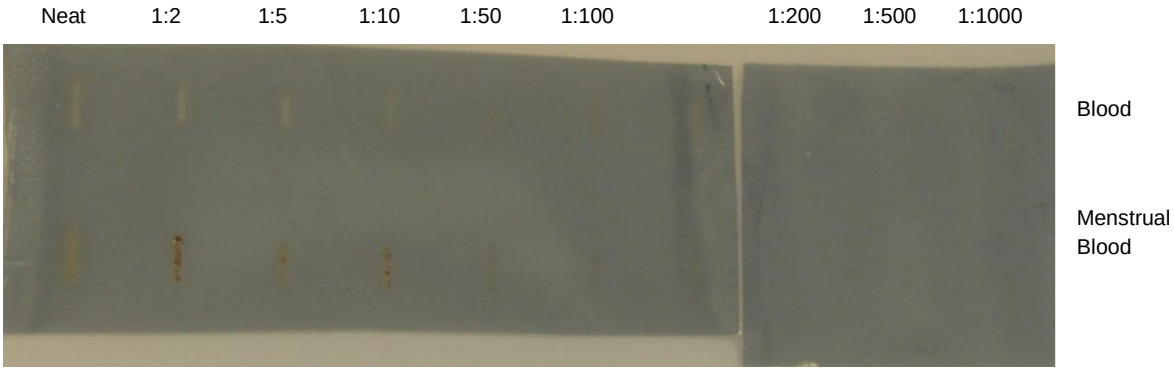


Figure 6.18: A DAB stained membrane loaded with a serial dilution (neat, 1:2, 1:5, 1:10, 1:50, 1:100, 1:200, 1:500 and 1:1000) of blood and menstrual blood samples without antibody addition.

Negative staining (appearing as white bands) was observed for the blood samples up to a 1:10 dilution. No blue staining, a characteristic of the previous positive signals, was seen with the menstrual blood samples (Figure 6.18) although brown stains were observed on the membrane.

6.2.2.6 Dot Blot Detection with a Peroxidase Treatment of Blood Samples

In an attempt to try and eliminate the background from the haemoglobin and DAB reaction. Endogenous peroxidase activity can be inhibited using methanolic hydrogen peroxide (Andrew and Jasani 1987). The experiment was repeated with both the blood and the menstrual blood samples. This time the samples were treated with 3% hydrogen peroxide in methanol, at 37°C for 1 hour before the dot blot was performed. A negative control of PBS and a positive control of untreated blood and menstrual blood were also blotted (Figure 6.19).

Figure 6.19: A DAB stained membrane loaded with a serial dilution (neat, 1:2, 1:5, 1:10, 1:50, 1:100, 1:200, 1:500 and 1:1000) of peroxidase treated blood and menstrual blood samples without antibody addition as well as a positive control of untreated blood and menstrual blood and a negative control of PBS.

In Figure 6.19, the positive control of untreated blood and menstrual blood gave strong positive blue reactions with DAB. Only the neat blood and menstrual blood samples exhibit a positive reaction when treated with hydrogen peroxide prior to performing the dot blot. This positive result is much weaker than that shown with the untreated blood samples. PBS showed a negative result as expected, which was consistent with the most diluted samples of blood and menstrual blood on the membrane.

6.2.2.7 Testing the Detection of the Dot Blot with a Range of Body Fluids after Protein Quantification

It was thought that the concentration of protein within each body fluid may have had an effect upon the results obtained. With this in mind a protein assay was performed in order to ensure that the same amount of protein was loaded in each body fluid tested (Figure 6.20).

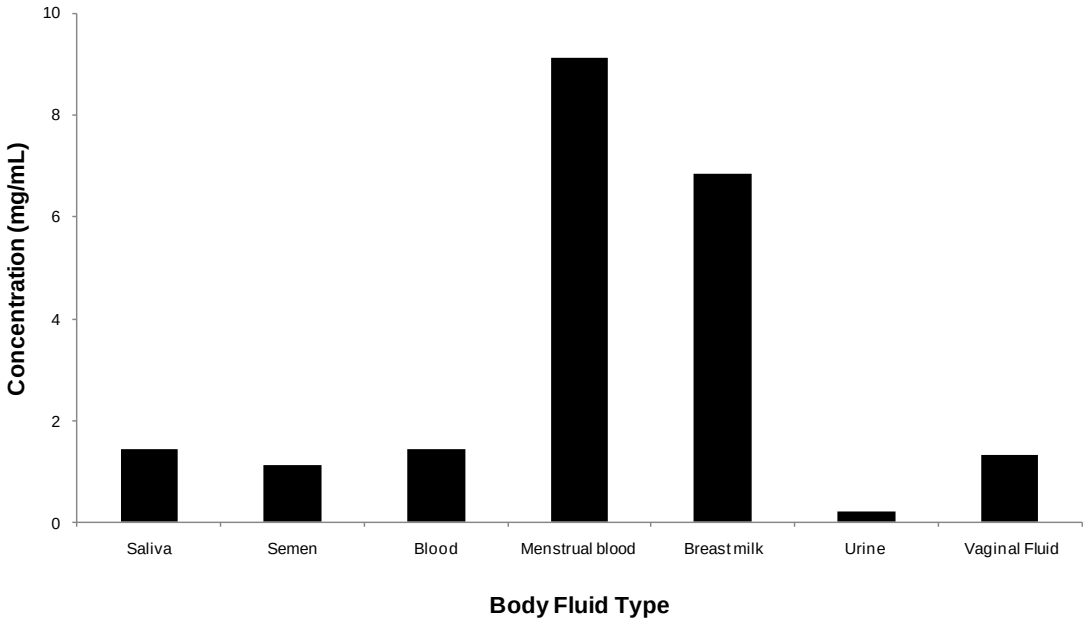


Figure 6.20: The concentration of each body fluid sample measured by *RCDC* protein assay.

The results demonstrate that the variation in protein concentration among body fluid samples was high, ranging from 0.181mg/ml for urine to 9.09 mg/ml for menstrual blood. It seemed possible that variation obtained could be due to different total protein loadings for each fluid.

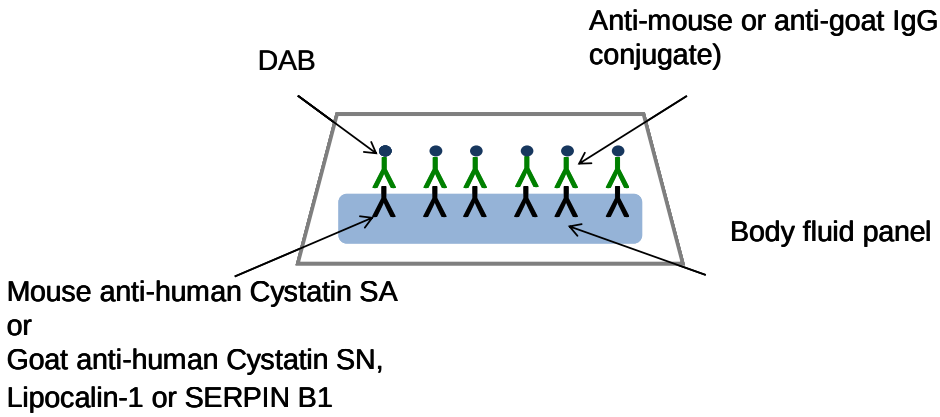


Figure 6.21: A schematic diagram of dot blot of body fluid panel

The body fluid dot blots were repeated with a protein loading of 10 µg/dot. Blood and menstrual blood samples were pre-treated with peroxidise (Figure 6.21).

These results (Figure 6.22) show that on each the PBS negative control gave no signal with DAB. The results for Cystatin SA antibody (Figure 6.22 a) gave a positive result for saliva, but semen, menstrual blood, breast milk and vaginal fluid were also positive. As before, a weak positive signal was shown with blood. It would appear that treating the samples with peroxidase has enhanced the signal produced on the membrane for the blood sample while the menstrual blood sample gave a similar intensity with or without peroxidase treatment.

For Cystatin SN (Figure 6.22 b), unlike the previous results in section 6.2.2.4, the fluids producing strong signals other than saliva were vaginal fluid and the peroxidase treated blood sample. The rest of the body fluids yielded weak signals. Strong signals were produced using the SERPINB1 antibody (Figure 6.22 c) with saliva as well as vaginal fluid. Semen and menstrual blood gave a medium strength signal with blood and breast milk producing the weakest signals. The peroxidase treatment appeared to enhance the result for the menstrual blood sample but reduced the blood sample signal on the membrane.

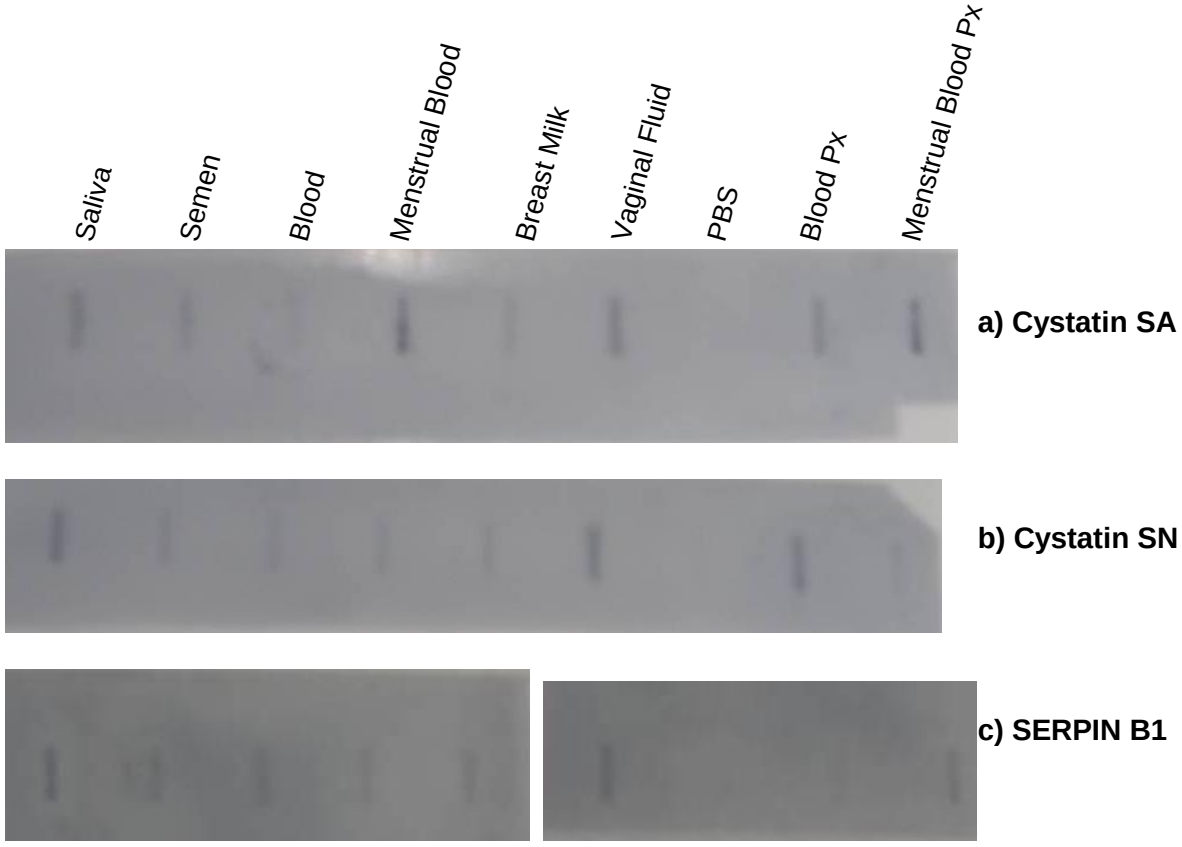


Figure 6.22: Dot blots to show antibody specificity tested with a range body fluids using a) Cystatin SA, b) Cystatin SN and c) SERPINB1 antibodies. Samples treated with peroxide have Px after them.

6.2.2.8 Testing the Detection of the Dot Blot for Secondary Antibody Specificity

As a result of unexpected positive results in blood samples after treatment with hydrogen peroxide and by body fluid samples in the dot blots (section 6.2.2.6) the secondary antibodies were tested directly for cross reactivity with each body fluid (Figure 6.23).

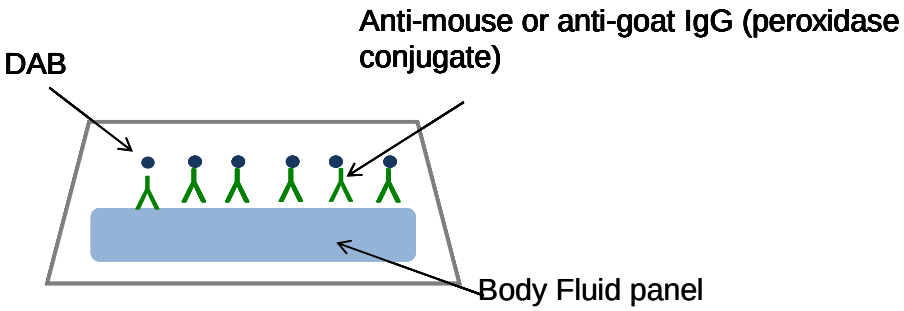


Figure 6.23: A schematic diagram of dot blot of body fluid panel with secondary antibodies

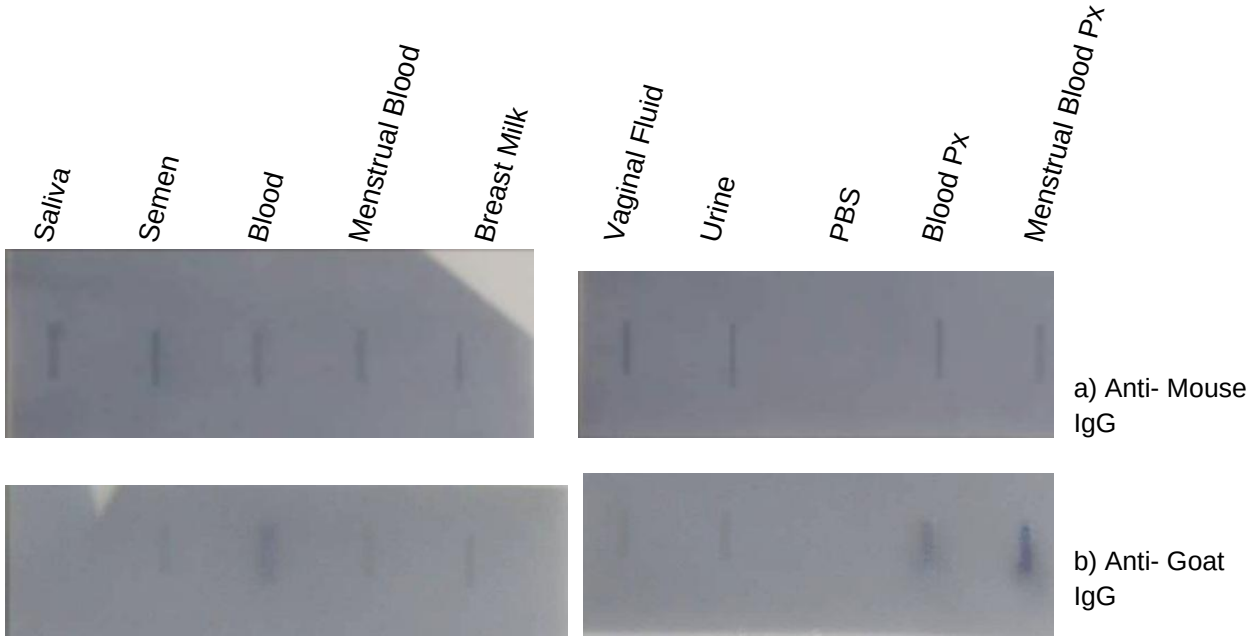


Figure 6.24: A dot blot to show secondary antibody specificity tested with a range body fluids using a) Anti-Mouse IgG and b) Anti-Goat IgG. Samples treated with peroxide have Px after them.

Weak positive results can be seen with Anti-Mouse IgG (Figure 6.24 a) for all body fluids. The PBS negative control remained negative in both secondary antibody dot blots. Weak positive results can be seen with Anti-Goat IgG (Figure 6.24 b) for semen, menstrual blood, breast milk, vaginal fluid and urine. Strong positive reactions were observed with the blood sample and hydrogen peroxide treatment seemed to enhance the positive reaction signal produced from both the blood and the menstrual blood sample. The menstrual blood sample in particular showed a dramatic change in intensity from a weak positive (without peroxide) to a strong positive (with peroxide).

The only sample not to show a positive result with Anti-Goat IgG was the saliva sample.

6.2.2.9 Testing the Detection of the Dot Blot for Secondary Antibody Specificity Reacting with Human IgG

Since it was also noted that the secondary antibodies were not specific to either Mouse IgG or Goat IgG, Human IgG was blotted and tested with both Anti-Goat IgG and Anti-Mouse IgG secondary antibodies and the colour developed with DAB (Figure 6.25). Human IgG was blotted with and without incubation with hydrogen peroxide at three different protein loads; 10 µg, 5 µg and 1 µg.

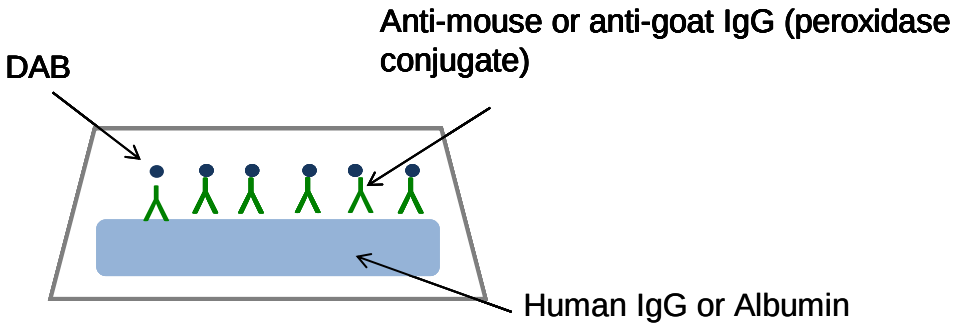


Figure 6.25: A schematic diagram of dot blot of Human IgG or albumin with secondary antibodies

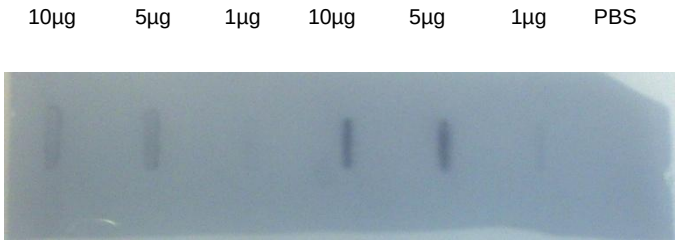


Figure 6.26: A digital photograph to show a DAB stained membrane loaded with human IgG protein, incubated with secondary Anti-Goat IgG, developed with DAB

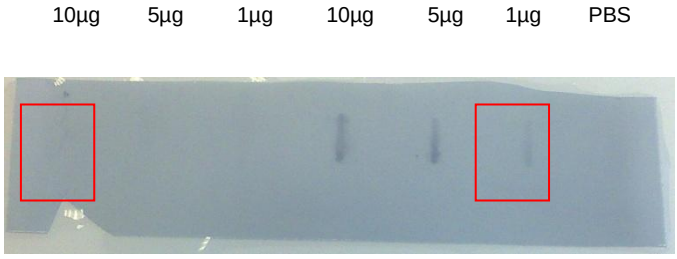


Figure 6.27: A digital photograph to show a DAB stained membrane loaded with human IgG protein, incubated with secondary Anti-Mouse IgG, developed with DAB

The negative PBS control was not detected by Anti-Goat (Figure 6.26) or Anti - Mouse IgG (Figure 6.27) secondary antibodies. Anti-Goat IgG (Figure 6.26) gave a weak positive reaction with each of the protein loads tested. This signal was enhanced in the samples that were incubated with hydrogen peroxide prior to blotting. A weak positive result can be seen for the 10 µg protein load when incubated with Anti-Mouse IgG but the other loads showed negative results. The samples incubated with hydrogen peroxide prior to blotting gave positive results for each protein load down to 1 µg.

6.2.2.10 Detection for DAB Specificity by Dot Blot

It was concluded that DAB was not binding specifically to the labelled antibodies but reacting with other proteins within the human body fluid samples. For this reason human albumin protein was tested with and without hydrogen peroxide treatment (Figure 6.28). An attempt was made to block the membrane with milk protein (1hour) in order to reduce the background signal. This would distinguish whether positive results were true positives or due to the loading buffer staining the membrane prior to DAB development (Figure 6.29)

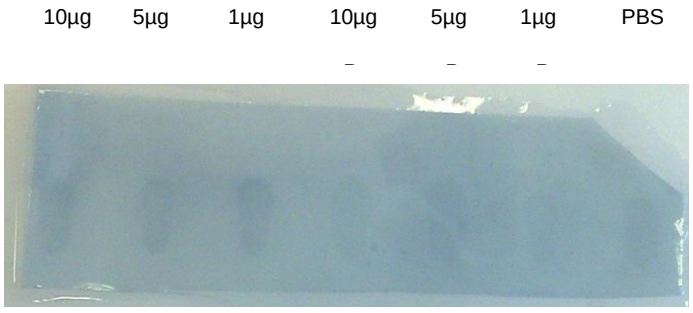


Figure 6.28: A digital photograph to show a DAB stained membrane loaded with human albumin protein without blocking developed with DAB (Peroxidase treated samples marked Px).

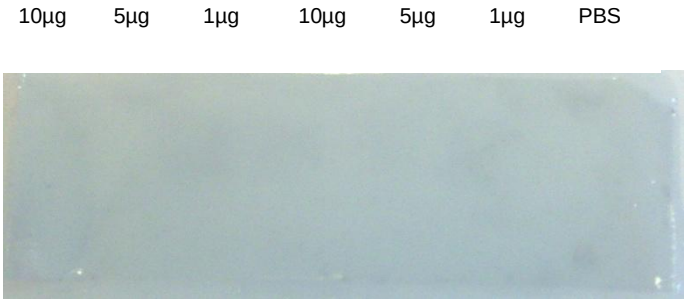


Figure 6.29: A digital photograph to show a DAB stained membrane loaded with human albumin protein with milk blocking prior to development with DAB (Peroxidase treated samples marked Px).

The results from Figure 6.28 show that an outline of each sample blotted can be seen on the membrane, including the negative control PBS. The background of this membrane is also relatively high. In the replicate membrane (Figure 6.29) (incubated in milk for one hour prior to DAB development), the sample outlines had disappeared and no positive bands were shown with albumin and DAB development.

Table 6.4: A table to summarise the findings of the dot blot experiments of primary (1º) and secondary (2º) antibodies before and after peroxide (Px) treatment. Staining intensities ranged from light (+) to medium (++) through to dark (+++). Negative (-ve) and precipitate (ppt) stainng was also observed.

Body Fluids	1° SERPINB 1 2° Goat DAB	1° Cystatin SN 2° Goat DAB	1° Cystatin SA 2° Mouse DAB	2° Goat DAB	2° Mouse DAB	DAB
Saliva	++	+++	+++	-	+	
Vaginal Fluid	++	+++	+++	+	+	
Semen	++	+	++	+	+	
Urine				+	+	
Breast Milk	+	+	++	+	+	
Blood	+	+	++	++	+	-ve staining 1:100
Menstrual Blood	+++	+	+	+	+	ppt staining 1:100
Blood H_2O_2	++	+++	+	+++	+	Stain only on neat
Menstrual Blood H_2O_2	+++	+	++	+++	+	Stain only on neat
PBS	-	-	-	-	-	-
Human Albumin						-
Human IgG				+ (+ Px)	+ (Px only)	

6.3 Discussion

6.3.1 Selection of Biomarkers for Testing Specificity

It was not viable to test all the proteins identified. In some cases this was due to the lack of specificity of the protein itself e.g. Actin, immunoglobulins and superoxide dismutase. These proteins are expressed in all body fluids and cell types and would therefore not be suitable biomarkers of either saliva or vaginal fluid. Epidermal fatty acid binding protein, as its name suggests, seemed an unlikely candidate as it is present within skin and likely to have been deposited onto the swab during vaginal fluid sample donation it would probably contaminate menstrual blood, breast milk, semen and blood also.

Another problem encountered when assessing the specificity was the difficulty in obtaining commercial ELISA kits or antibodies for the proteins identified at the time of searching. Those available were tested, however since this time, an ELISA to detect calgranulin B has become available and so further work would involve incorporation of this ELISA. This ELISA is primarily used for the detection of colon cancer by testing stools and therefore Calgranulin would be present in faeces. Although it is likely that this ELISA may have low specificity, if the levels present in the stool samples differ considerably from the levels in the other body fluid samples tested, this marker could still be used for vaginal fluid identification.

6.3.2 Confirmation of Protein Specificity - ELISA

6.3.2.1 Confirmation of Leucocyte Elastase Inhibitor Specificity using CanAg SCC EIA kit (Fujirebio™)

Spots 17 and 19 in vaginal fluid were positively identified as SCCA1 (Table 5.8) in vaginal fluid. CanAg SCC EIA kit was used to test the vaginal fluid samples individually. Sample 10 was the only sample to give a reading below the 0 µg/l calibrator and hence negative for SCC. If sample 10 does not contain SCC yet the pools containing sample 10 show expression of both spots 17 and 19 the other samples contributing to the pool could be showing the expression for the spots.

Sample 10 was provided by an individual not using any form of contraception and taken just before menstruation; no other samples were taken at this stage of the cycle. But samples from other donors not using any contraception showed positive ELISA results. The result could be due to the stage of the cycle, or genetic differences.

Although positive reactions were shown for body fluids other than vaginal fluid, namely, semen, saliva and urine the levels were lower than those seen in the vaginal fluid samples thus the ELISA could be deemed suitable for distinguishing vaginal fluid from these other body fluids if a lower limit was set for the test. The lowest concentration seen in a vaginal fluid sample (Figure 6.4) was 812.75 µg/l, due to its similarity to the saliva sample this could be considered a false negative. Further testing would need to be performed on a larger number of samples to determine the range of SCCA concentration for vaginal fluid and the other body fluids yielding a positive ELISA result. Menstrual blood was the only fluid to give a concentration in the region of the majority of the vaginal fluid samples. This could be due to menstrual blood being composed of vaginal secretions, blood and endometrium.

When calculating the sensitivity and specificity of the CanAg SCC EIA kit (Table 6.1) a threshold of 2000 µg/l was used. At this threshold, the ELISA was shown to be 92% sensitive and 83% specific within the range of the samples tested. The test shows an appropriate level of sensitivity to discount the false positives and negatives produced and is specific enough to differentiate between vaginal fluid and menstrual blood compared with the other body fluids tested.

The ELISA could not be used to differentiate between vaginal fluid and menstrual blood as the menstrual blood concentration determined in the ELISA was within the range of concentrations determined for vaginal fluid. Unfortunately the upper limits of the calibrator standards in this kit were below the concentration of many of the sample. If more time and kits were available this would be repeated once more diluting those samples where the concentrations were extrapolated. The SCC antigen is present in normal squamous cell epithelia which explains the positive results obtained for vaginal fluid in this ELISA. Elevated SCC levels may also be present in skin disorders such as psoriasis (Cataltepe *et al.* 2000). The SCC ELISA is routinely used for processing serum samples where the normal range for SCC concentration is 0.16 µg/l – 1.5 µg/l. This is 4 orders of magnitude lower than the average concentration found in vaginal fluid. The normal blood concentration obtained in this assay was 2.5 µg/l which was higher than the stated range from the manufacturers (see above). It must be noted that this was in whole blood rather than the serum concentrations noted above. The ELISA does not distinguish SCCA2 from SCCA1 so false positives could be identified due to the detection of SCCA2

6.3.2.2 Confirmation of Human Zinc-α -2-Glycoprotein (ZA2G) Specificity using ZA2G ELISA (BioVendor)

Human ZA2G ELISA was used to test the saliva samples individually. All saliva samples tested positive for ZA2G using the ELISA although some of the samples had concentrations that were determined by extrapolation from the standards within the assay. Once again this ELISA was manufactured for use with serum and therefore the range of standards were appropriate to that sample type. The manufacturers advise samples with a concentration higher than 500 ng/ml to be diluted and retested. If another kit was purchased those samples with concentrations higher than the 100 ng/ml standard would be diluted and repeated. Those below the 3 ng/ml standard could not be concentrated and thus are false negatives; the ELISA gave positive results for each of the body fluids tested. The ELISA showed that ZA2G was not specifically expressed in saliva. When calculating the sensitivity and specificity of the ZA2G ELISA (Table 6.2) using a lower level cut off of 50 ng/ml to discount the low saliva sample readings. The ELISA was shown to be 88% sensitive and 0% specific within the range of the samples tested. The sensitivity of the test could not be adjusted to account for only saliva samples producing positive results as the concentrations of ZA2G identified in the body fluid samples (Figure 6.7) were within the range of values given for the saliva samples (Figure 6.6). ZA2G was therefore eliminated as a candidate protein for characterising saliva.

6.3.3 Confirmation of Specificity using a Dot Blot

Where ELISA kits were unavailable, dot blots were used. The dot blot is a simple method for testing the specificity of antigens in different samples. Unlike a Western blot where proteins are separated based on molecular weight prior to incubation with antibodies, the exact protein band being detected cannot be ascertained using a dot blot, only whether or not the antigen is present in the sample or not. Advantages for this method are that is quicker and simpler to use than a Western blot.

Positive reactions were seen with both blood and menstrual blood for all primary antibodies tested. There are three factors which could be responsible for causing false positives within this dot blot experiment: 1) low specificity or non specificity of the primary antibody, 2) low specificity or non specificity of the secondary

antibody 3) reaction of endogenous enzymes within the body fluids reacting with the colour development substrate.

6.3.3.1 Testing the Detection Limits of the Dot Blot with Recombinant Protein

The lipocalin antibody gave problems throughout the dot blot procedures. It neither bound to lipocalin protein or to any of the proteins from body fluids, primary and secondary antibodies. The reagents were all working well. It was concluded that the antibody was not functional.

6.3.3.2 Testing the Detection Limits of the Dot Blot with a Serial Dilution of Body Fluids

In subsequent experiments the primary antibody was diluted with blocking buffer in order to lower the background of the blot. A blot was performed using either saliva (Cystatin SA, Cystatin SN and Lipocalin-1) or vaginal fluid (SERPIN B1) in serial dilution (Figure 6.11). Once again all antibodies reacted except Lipocalin-1 (Figure 6.11c) up to either a 1:50 (Figure 6.11d, SERPINB1) or a 1:100 (Figure 6.11a and b, Cystatin SA and SN respectively) dilution.

6.3.3.3 Testing the Detection of the Dot Blot with Lipocalin Primary Antibody Direct to the Membrane

The Lipocalin-1 antibody was raised in Goat, as was the SERPIN B1 antibody. Positive result bands were observed when SERPIN B1 was tested with both the recombinant protein (Figure 6.9d) and vaginal fluid (Figure 6.11d) in serial dilution. For these reasons it was thought that the primary-secondary antibody reaction was working efficiently but was further tested in order to be certain. 10 µL anti-human Lipocalin-1 antibody was blotted against the anti-Goat secondary antibody (Figure 6.13). Bands were seen on the membrane confirming that there was not a problem with the primary/secondary antibody reaction. As no bands were identified with either the recombinant protein or saliva when tested with Lipocalin-1 antibody it was decided not to use this antibody any further as none of the positive controls revealed any bands.

6.3.3.4 Dot Blot Detection using a Range of Body Fluids

When each antibody was tested against a panel of body fluids, numerous positive bands were observed of differing intensities for each blot (Figure 6.14). The volume of body fluid was initially kept constant at 10 µl as it was thought in a forensic scenario body fluids would more likely be in their native concentrated form. It was difficult to determine whether the positive reactions produced in the dot blot were due to the presence of the antigen in the body fluid itself or whether due to the same volume of each fluid being used, a bias was added to the experiment. So, although the antigen may be present it may be present in much smaller amounts in one fluid compared to another, as was demonstrated by the SCC ELISA (section 6.3.2.1). In order to determine the effects of the differing body fluid protein concentrations on the dot blot a protein assay was performed and the dot blots were repeated using 10 µg of protein in each body fluid (section 6.3.3.7) as opposed to a constant volume.

6.3.3.5 Dot Blot Detection using Blood Samples with Direct DAB Development

Prior to DAB addition, staining shown on the membrane was thought to be caused by Haemoglobin (Figure 6.17). When DAB was added directly to a membrane blotted with blood and menstrual blood samples in serial dilution without prior antibody incubation, negative staining and brown staining was shown (Figure 6.18). A reaction was being seen between the DAB and the blood samples. Blood samples are known to exhibit peroxidase activity due to the haemoglobin within them (Ahlquist and Schwartz. 1975). The peroxidase activity of the haem group catalyses the change in oxidation state of a substrate within both blood presumptive tests LMG and Phenolphalein causing a colour change (Webb *et al.* 2006).

Endogenous peroxidase within the blood and menstrual blood samples were thought to cause a false position reaction with DAB causing a colour change on the membrane (Figure 6.18).

6.3.3.6 Hydrogen Peroxide Treatment of Blood Samples

Endogenous peroxidase activity was inhibited in menstrual blood and blood samples using a 3% hydrogen peroxide solution in methanol and incubating at 37°C for 1 hour with the lids off the Eppendorf tubes in order for any liberated oxygen to be

released (Li, Fan *et al*. 2007). Samples were blotted once again in serial dilution without antibody incubation (Figure 6.19) and only the neat samples showed positive reactions and these appeared less intense than the blood samples where no treatment had been performed.

6.3.3.7 Testing the Detection of the Dot Blot with a Range of Body Fluids after Protein Quantification

Quantification of body fluids showed that the protein concentration in the panel of fluids tested ranged from 0.181 mg/ml for urine to 9.09 mg/ml for menstrual blood. When the body fluid blot was repeated with 10 µg of each fluid, positive bands were once again shown in all of the body fluids for each antigen tested. Some of the antigens have been positively identified in other body fluids, for example Cystatin SN has been identified in tear fluid by immunoblot (Barka, Asbell *et al*. 1991) Lipocalin-1 has also been found within tears by studying endonuclease activity (Yusifov, Abduragimov *et al*. 2008) but not in the fluids tested in this study. For this reason it is unclear whether the biomarkers are not specific to saliva or vaginal fluid and present within the other body fluids tested or whether the antibodies used are not specific themselves.

6.3.3.8 Testing the Detection of the Dot Blot for Secondary Antibody Specificity

As positive results were still given for all the body fluids tested with each antibody the specificity of the secondary antibody was tested. When the secondary antibodies were blotted directly with the body fluid panel positive bands were visible for all body fluids with anti-mouse IgG and for semen, menstrual blood, breast milk, vaginal fluid and urine with anti-goat IgG. Peroxide treatment seemed to enhance the blood and menstrual signal produced with anti-goat IgG.

6.3.3.9 Testing the Detection of the Dot Blot for Secondary Antibody Specificity Reacting with Human IgG

Human IgG was blotted and tested with both anti-mouse IgG and anti-goat IgG to test for specificity and cross reactivity with human IgG. Human IgG is present within all body fluids although at differing levels. In serial dilution with and without peroxide

treatment anti-goat IgG showed positive reactions with up to 1 µg of human IgG protein. The anti-mouse IgG was less reactive only producing a band with 10 µg of human IgG untreated but peroxide treatment enhanced the reaction with the antibody detecting as little as 1 µg of human IgG thus this secondary antibody was reliable if the samples were not treated with peroxidase prior to blotting.

6.3.3.10 Testing the Detection of the Dot Blot for DAB Specificity

No reaction was seen with DAB directly added to the membrane blotted with human albumin protein. This shows that DAB will not react to any given protein within a sample and is specifically reactive to peroxide.

6.4 Chapter Summary

In summary the findings from this chapter show that the SCC ELISA showed high levels of sensitivity and specificity whereas the Zn-α-2-Glycoprotein ELISA kit only showed high sensitivity, and no specificity in the body fluid samples tested.

The Lipocalin-1 primary antibody gave no reaction with Lipocalin antigens in saliva samples or the recombinant Lipocalin-1 protein. Positive results were seen for the other three antibodies when tested against their fluid of origin and the recombinant protein specific for the antibody.

Although positive results were obtained for each antibody with the body fluid of origin when tested alongside a panel of body fluids false positives were also shown for nearly every body fluid type, due to cross species reactivity of the secondary antibody. When blood and menstrual blood samples were reacted with DAB alone without any antibodies a positive reaction was seen, this was overcome when the same experiment was repeated with the blood and menstrual blood samples undergoing a peroxide treatment.

False positive reactions were shown with both secondary antibodies when blots were performed without the primary antibody incubation.

The Goat secondary antibody was shown to react with human IgG showing a degree of cross reactivity which was not shown with the secondary mouse antibody. PBS was shown to be consistently negative throughout all the blots performed.

A summary of the Dot Blot results is shown in Table 6.4.

Chapter 7
Discussion

Chapter 7 – Discussion

7.1 Biomarker Suitability

The ideal biomarker should be expressed exclusively within one body fluid type. No cross reactivity should be seen with other body fluids and the chosen marker should be expressed population wide. Biomarker expression should not be affected by external factors.

7.1.1 Cystatin SA

Although positive bands were seen on the dot blot membrane for all the body fluids tested (Figure 6.22a) with the Cystatin SA antibody, weak positive results were also visible on the membrane when only the secondary Anti-Mouse IgG (Figure 6.24 a) was tested with the body fluids. This implies that the secondary antibody was binding non-specifically to a component of the body fluid, probably IgG. When blotted with human IgG this theory was confirmed as 10 µg of protein produced a positive band with the anti-mouse IgG antibody (Figure 6.27). The signal was enhanced by peroxide treatment showing a positive band with 1 µg of protein. Isemura *et al.* (1991) have shown immunoblots to produce bands representative of Cystatin SA in tears and seminal plasma but stated these could be non-phosphorylated cystatin S. The specificity of Cystatin antibodies within this study may have affected the results as the anti-human Cystatin SA antibody shows 100% cross reactivity with Cystatin SN and Cystatin S (R&D systems).

7.1.2 Cystatin SN

Although positive bands were seen on the dot blot membrane for all the body fluids tested (Figure 6.22b) with the Cystatin SN antibody, weak positive results were also visible on the membrane when only the secondary Anti-Goat IgG (Figure 6.24 b) was blotted. When the secondary antibody was tested with the body fluid panel, positive bands were seen for semen, menstrual blood, breast milk, vaginal fluid and urine suggesting that the antibody was exhibiting non specific binding to a component of the body fluids. When blotted with human IgG protein this was confirmed as 5 µg of protein produced a positive band with the anti-goat IgG antibody (Figure 6.26). This showed that the secondary antibody exhibited cross reactivity with human IgG and

some of the signal produced in the body fluid dot blot (Figure 6.24b) could be due to this cross reactivity of the antibody. Once again this effect was enhanced by peroxide treatment. Cystatin SN secretion has been found exclusively in saliva (Hiltke, Lee *et al*. 1999) but immunoblots have shown a positive reaction to anti-human cystatin SN in tears (Barka, Asbell *et al*. 1991). As mentioned previously (section 7.1.1) the specificity of Cystatin antibodies within this study may have affected the results. The anti-human Cystatin SN antibody showed 30% cross reactivity with Cystatin SA and 15% with Cystatin S.

7.1.3.Lipocalin-1

As the Lipocalin-1 antibody did not produce positive bands with either recombinant Lipocalin-1 (Figure 6.9c) protein or with saliva (Figure 6.11c) the specificity of the antibody could not be tested further with other body fluids. Lipocalin has also been identified in tears (Yusifov, Abduragimov *et al*. 2008).

7.1.4 ZA2G

Although ZA2G was identified successfully by ELISA in each saliva sample within the study, levels were variable. Cross reactivity was also seen in each of the body fluids within the panel tested. For this reasons ZA2G would not be a suitable forensic marker for saliva. Due to its universal expression this protein could be considered as a positive control for any test that was developed. Previous studies have confirmed the presence of ZA2G within human serum (Burgi and Schmid 1961), urine (Shibata and Miura 1982), semen, breast milk, sweat, and saliva (Ohkubo, Niwa *et al*. 1990).

7.1.5 SCCA /SERPIN B3

SCCA was identified in all but one of the vaginal fluid samples within this study by ELISA although the levels within each vaginal sample were variable. When the body fluid panel was used with the ELISA low levels were shown with saliva, urine and semen. Of these, the only fluid to show a reaction above the threshold seen with vaginal fluid was saliva. The only body fluid to give a reading similar to the vaginal fluid samples was menstrual blood which, due to its composition, was expected. These results suggest that SCCA could be used as a marker for a fluid of vaginal origin. A low

level cut off would be recommended if this protein was selected as a marker for vaginal fluid to discount the low level positive results shown by the other body fluids tested. SCCA is expressed in normal squamous epithelia (Cataltepe, Gornstein *et al*. 2000) and hence likely to be found in body fluids containing these cells such as saliva, vaginal fluid. The acidic form of SCCA (SCCA1) has been found in the saliva (Kato 1996). The presence of squamous epithelia in urine could be explained by contaminating skin cells deposited during collection. Epithelia cells have also been identified within semen samples and this could also be due to contamination from skin cells of the mucosal skin at the opening of the male urethra upon ejaculation. In order for SCCA1 to be used as a vaginal fluid marker, menstrual blood markers would have to be used in conjunction with it, as currently all that can be deduced from this ELISA is that the samples are of vaginal origin or not. Menstrual blood is easy to distinguish from vaginal fluid due to the presence of haemoglobin so this could be used a blood marker in conjunction with SCCA to distinguish between vaginal fluid and menstrual blood.

7.1.6 Leukocyte Elastase Inhibitor/SERPIN B1

Although positive bands were seen on the dot blot membrane for all the body fluids tested (Figure 6.22c) with the SERPIN B1 antibody, weak positive results were also visible on the membrane when only the secondary Anti-Goat IgG (Figure 6.24b) was tested with the panel of body fluids. This suggests that the antibody was exhibiting non specific binding to a component of the body fluid. When the secondary antibody was tested with the body fluid panel positive bands were seen for semen, menstrual blood, breast milk, vaginal fluid and urine. As mentioned above (section 7.1.2). Cross reactivity with human IgG could account for some of the signal produced in the body fluid dot blot (Figure 6.22c).

7.2 Future Work

7.2.1 Expression Analysis

In this study saliva and vaginal fluid were selected for protein expression analysis using 2DGE. Nine protein spots were successfully identified for each fluid type relating to 6 different proteins in both saliva and vaginal fluid. These protein spots were selected based on their position within the 2D gel and their universal expression across each of the saliva or vaginal fluid samples. It is impossible to make comparisons between the two different fluid types in this study through software analysis due to the different protein profiles of the samples and a lack of identical protein vectors between them.

Difference Gel Electrophoresis (DIGE) is a method used to make comparisons between up to three samples, each labelled with a fluorescent dye and run at the same time on one 2D gel (Marouga, David *et al*. 2005). It is capable of comparing two samples plus a control comprising both samples combined in equal amounts. Samples are pre-labelled with fluorescent cyanine dyes (Cy2, Cy3 and Cy5). Using DIGE would mean that two body fluids could be analysed in parallel together and comparisons between them would be easily made determining which proteins were unique to each fluid. As this is a relatively new technique the imaging software and scanning instruments are not easily accessible and the analysis would need to be outsourced. Finding differences between two body fluids is a small step in forensic body fluid identification, ideally body fluid markers for the whole range of body fluids encountered in forensic investigations would need to be made. Initially saliva and vaginal fluid were chosen for their applicability to a rape case, for this to be complete, semen would need to be added to this analysis.

Another gap in body fluid identification is determining the differences between venous blood and menstrual blood. It can be unclear in rape cases with vaginal bleedings being attributed to menstruation by the perpetrator which could actually be evidence of a violent assault (Bauer and Patzelt 2008). Although a lateral flow strip is available for blood identification (Independent Forensics) there is no published data for the results when tested with menstrual blood.

Other pH range strips could be tested to determine whether any potential markers lie outwith the pH range used in this study such as acidic proline-rich proteins (aPRPs). aPRPs have been identified as having expression specific to saliva (Schenkels, Veerman *et al*. 1995; Hardt, Thomas *et al*. 2005), therefore making them

suitable biomarkers. PRP's bind calcium ions maintaining mineral homeostasis and provide a protective and reparative environment for dental enamel which is important for the integrity of the teeth. These were not detected in this study as they have a p*I* of approx 4 calculated using the ExPASy Compute p*I*/Mw tool [online] and the most acidic *pI* used for spot analysis in this study was a strip with a lower limit of 5. Since staining with Coomassie blue R250 has also revealed that PRPs stain pink rather than blue (Hardt, Thomas *et al*. 2005), it would therefore be simple to identify these spots in a future experiment if run on another 2D gel with a more acidic p*I* range including p*I* 4.

7.2.2 Verification of Protein Specificity

Firstly not all the proteins identified were suitable biomarkers due to universal expression in all body fluids and therefore not used for verification of specificity. Other proteins did not have commercial antibodies or ELISA kits available at the time of searching. Since this time an ELISA detecting Calgranulin B (Cellsciences) has been identified, further work could be performed testing this ELISA with the vaginal fluid samples and the body fluid panel to determine its suitability as a biomarker for vaginal fluid. This ELISA is primarily used for the detection of colon cancer by testing stools and therefore Calgranulin would be present in faeces.

Of the verification tests performed, the commercial ELISA kits were more successful than the dot blots due to antibody specificity problems experienced with the dot blot. The SCCA ELISA could distinguish samples of vaginal origin (vaginal fluid and menstrual blood) from the other body fluids tested. To differentiate between vaginal fluid and menstrual blood further experiments would be required to identify menstrual blood markers which could be used in conjunction with the SCCA marker. Further experiments would need to look at whether the levels of SCCA decrease rapidly prior to menstruation as was shown with sample 10. This would require more samples to be collected from donors at this stage of the cycle and an ELISA being run with them. As the experiments determined both primary (cystatin SA and SN) and secondary antibody specificity was problematic in the dot blots performed.

Further investigations would be necessary with different primary and secondary antibodies if available. 1D western blotting would ensure that antibodies bound to the correct molecular weight protein band in the gel representative of the protein biomarker, but both cystatin SA and Cystatin SN have similar molecular weights therefore the only way to determine between them would be by 2D western blotting due to their different p*I*s.

7.3 Conclusion

There is a requirement for a sophisticated method of body fluid identification especially for vaginal fluid samples. Recent developments have produced a saliva test (RSID) with little cross reactivity with other body fluids although not commonly used in forensic laboratories (personal comment) whereas the more traditional and more commonly used method of Phadebas paper showed false positives with breast milk, pancreatic amylase and bacterial amylase.

From these tests a new methodology for body fluid identification should be identified. Although a saliva test has been identified ideally all body fluids should be identified in a one step procedure rather than using separate strip tests for individual body fluids. The first stage in this development would be to identify protein biomarkers of body fluids.

The optimisation of saliva and vaginal fluid proteomic methods provided suitable experimental conditions. Sample extraction was performed from source or in PBS followed by acetone precipitation followed by protein concentration being determined by the RCDC protein assay. Electrophoretic conditions, using 7cm pH 5-8 strips in conjunction and 12% polyacrylamide gels in the second dimension were used with SYPRO Ruby fluorescent gel stain (Invitrogen). Image analysis was performed by Prodigy samespots software.

A total of 268 spots were identified in the saliva samples and 193 spots were identified in the vaginal fluid samples. Of these spots 19 saliva spots and 16 vaginal fluid spots found in each of the sample pools were analysed by LC-MS/MS. In both saliva and vaginal fluid samples 9 protein biomarkers were identified within saliva and vaginal fluid. Of the proteins identified there were some that were considered universally expressed; IgA, actin, IgG, superoxide dismutase and epidermal fatty acid binding protein. Those that were thought to be specific to saliva; zinc-α-2 glycoprotein, Cystatin SA, Cystatin SN and Lipocalin, and those thought to be specific to vaginal fluid; SERPIN B1, Leukocyte elastase inhibitor and Calgranulin B were found.

The SCC ELISA showed high levels of sensitivity and specificity whereas the Zn-α-2-Glycoprotein ELISA kit only showed high sensitivity, and no specificity in the body fluid samples tested with expression being detected in each fluid tested.

The Lipocalin-1 primary antibody was unsuccessful in identifying Lipocalin antigens in saliva samples as well as the recombinant Lipocalin-1 protein. Although positive results were obtained for each antibody with the body fluid of origin when tested alongside a panel of body fluids, false positives were also shown for nearly

every body fluid type. False positive reactions with body fluids were shown with both secondary antibodies when blots were performed without the primary antibody incubation. The Goat secondary antibody was shown to react with human IgG showing a degree of cross reactivity which was not shown with the secondary mouse antibody. PBS was shown to be consistently negative throughout all the blots performed.

From this it can be seen that further investigations would need to be performed in order to confirm the specificity of the biomarkers where dot blots were used for verification due to the lack of specificity of the antibodies used. The SCC ELISA was capable of distinguishing samples of vaginal origin (vaginal fluid and menstrual blood) from the other body fluid samples. The SCC ELISA could be therefore used in association with menstrual blood markers to identify vaginal fluid.

Appendices

Appendix I

Research participant information sheet

"Identification of characteristic proteins for distinguishing between body fluids for forensic purposes"

You are being invited to participate in a research study. Please take time to read the following information carefully and discuss it with others if you wish. Feel free to ask if there is anything that is unclear or if you would like any further information. You may take as long as you would like to decide whether or not you wish to take part. Thank you for reading this.

What is the purpose of this study?

This study intends to investigate two body fluids, namely saliva and vaginal fluid, in order to determine a way to identify a body fluid stain. Your name will not be available from our records. It is often vital to know the source of a fluid in police work. By improving the current methods used by forensic scientists and police forces, it is hoped that crimes such as rape and murder would be easier to solve and prove in courts of law.

Why have I been chosen?

People chosen for this study are fit, healthy and living in the Aberdeen area.

Do I have to take part?

No. You are completely free to decide whether or not to take part in the study. If you decide not to take part, this will not affect your treatment in any way. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you do decide to take part you are still free to withdraw at any time without giving a reason. Should you wish to withdraw from the study any sample stored can be returned to you or destroyed on request.

What will happen to me if I take part?

If you decide to take part in this study, we will ask you to produce a sample of your saliva. The saliva sample will be less than a teaspoonful. Your participation in this study can end after one donation, however if you would like to donate again in the future, you can indicate this on the consent form overleaf. The research for this study will be carried out over a period of not less than three years, with all remaining samples being destroyed at the end of the study in not more than five years.

Will my taking part in this study be kept confidential?

Any sample you donate to this study will be treated confidentially and investigated anonymously. All data gathered from the study would be stored in accordance with the Data Protection Act (1998). The methods used for this study would not allow for any medical information to be gathered, and no information about your sample would be passed on to a third party under any circumstances.

Will the study benefit me?

You will not benefit.

What will happen to the samples I donate?
Once samples have been analysed and upon completion of this study, samples will be destroyed.

What will happen to the results of the research study?

The results of the study would form the bulk of a research degree (PhD) thesis with the possibility of publication within an appropriate scientific journal. No names will be included in any report of the research results. The results of the study will be available to any interested participant, such as yourself.

Contact for further information

Mrs Louisa Vincini

c/o The Robert Gordon University
School of Life Sciences
St Andrew Street
Aberdeen
AB25 1HG

Tel: 01224262847

Email: prs.vincini@rgu.ac.uk

You will be given a copy of this information sheet and a signed consent form to keep.

Thank you for taking part in this study.

Appendix II

Research participant information sheet

"Identification of characteristic proteins for distinguishing between body fluids for forensic purposes"

You are being invited to participate in a research study. Please take time to read the following information carefully and discuss it with others if you wish. Feel free to ask if there is anything that is unclear or if you would like any further information. You may take as long as you would like to decide whether or not you wish to take part. Thank you for reading this.

What is the purpose of this study?

This study intends to investigate two body fluids, namely saliva and vaginal fluid, in order to determine a way to identify a body fluid stain. Your name will not be available from our records. It is often vital to know the source of a fluid in police work. By improving the current methods used by forensic scientists and police forces, it is hoped that crimes such as rape and murder would be easier to solve and prove in courts of law.

Why have I been chosen?

You are attending the clinic Square 13 for an intimate examination.

Do I have to take part?

No. You are completely free to decide whether or not to take part in the study. If you decide not to take part, this will not affect your treatment in any way. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you do decide to take part you are still free to withdraw at any time without giving a reason. Should you wish to withdraw from the study any sample stored can be returned to you or destroyed on request.

What will happen to me if I take part?

If you decide to take part in this study, a sample of your vaginal fluid and/or saliva will be given. The vaginal fluid sample will be in the form of two swabs taken during your routine gynaecological examination and the saliva sample will be less than a teaspoonful. The research for this study will be carried out over a period of not less than three years, with all remaining samples being destroyed at the end of the study in not more than five years.

Will my taking part in this study be kept confidential?

Any sample you donate to this study would be treated confidentially and investigated anonymously. All data gathered from the study would be stored in accordance with the Data Protection Act (1998). The methods used for this study would not allow for any medical information to be gathered, and no information about your sample would be passed on to a third party under any circumstances.

Will the study benefit me?

You will not benefit.

What will happen to the samples I donate?

Once samples have been analysed and upon completion of this study, samples will be destroyed.

What will happen to the results of the research study?

The results of the study would form the bulk of a research degree (PhD) thesis with the possibility of publication within an appropriate scientific journal. No names will be included in any report of the research results. The results of the study will be available to any interested participant, such as yourself.

Contact for further information

Mrs Louisa Vincini

c/o The Robert Gordon University
School of Life Sciences
St Andrew Street
Aberdeen
AB25 1HG

Tel: 01224262847

Email: prs.vincini@rgu.ac.uk

You will be given a copy of this information sheet and a signed consent form to keep.

Thank you for taking part in this study.

Appendix III

Research participant consent form

Project title: "Identification of characteristic proteins for distinguishing between body fluids for forensic purposes"

Name of researcher : Louisa Vincini

Please initial box

1. I confirm that I have read and understood the information sheet dated.....(version.....) of the above study and have had the opportunity to ask questions. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my legal rights or medical treatment being affected. ☐

3. I agree to take part in the above study. ☐

☐

_____	_____	_____	
Name of participant	Date	Signature	M/F

_____	_____	_____
Age	time sample taken	time since eating/drinking/chewing gum

_____	_____	_____
Name of person taking consent (if different from the researcher)	Date	Signature

_____	_____	_____
Researcher	Date	Signature

_____
Sample Number

Appendix IV

Research participant consent form

Project title: "Identification of characteristic proteins for distinguishing between body fluids for forensic purposes"

Name of researcher : Louisa Vincini

Please initial box

4. I confirm that I have read and understood the information sheet dated.....(version.....) of the above study and have had the opportunity to ask questions. ☐

5. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my legal rights being affected. ☐

6. I agree to take part in the above study. ☐

_____	_____	_____
Name of participant	Date	Signature

_____	_____
Age	Stage of Menstrual Cycle

_____
Form of contraception

_____	_____	_____
Name of person taking consent	Date	Signature

_____
Sample Number

Appendix V

Research participant consent form

Project title: "Identification of characteristic proteins for distinguishing between body fluids for forensic purposes"

Name of researcher : Louisa Vincini

Please initial box

7. I confirm that I have read and understood the information sheet dated.....(version.....) of the above study and have had the opportunity to ask questions. ☐

8. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my legal rights being affected. ☐

9. I agree to take part in the above study. ☐

_____	_____	_____
Name of participant	Date	Signature

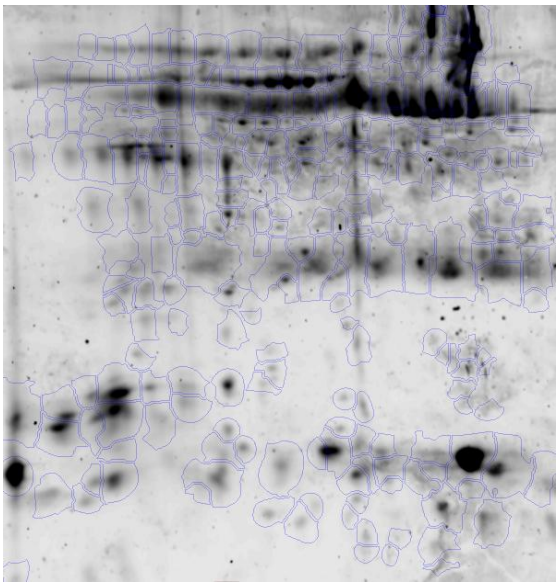
_____	_____
Age	Type of sample donated

_____
Sex

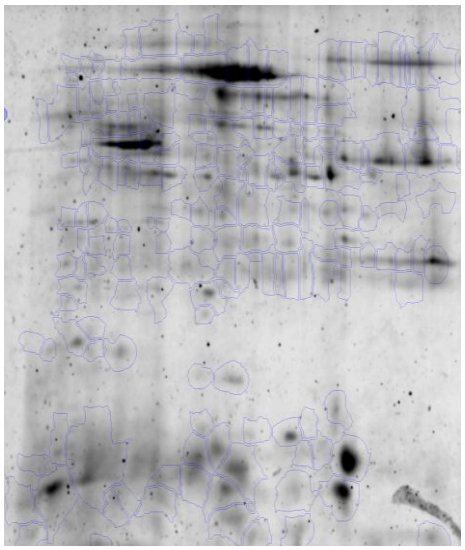
_____	_____	_____
Name of person taking consent	Date	Signature

_____
Sample Number

Appendix VI – Spot Count Images

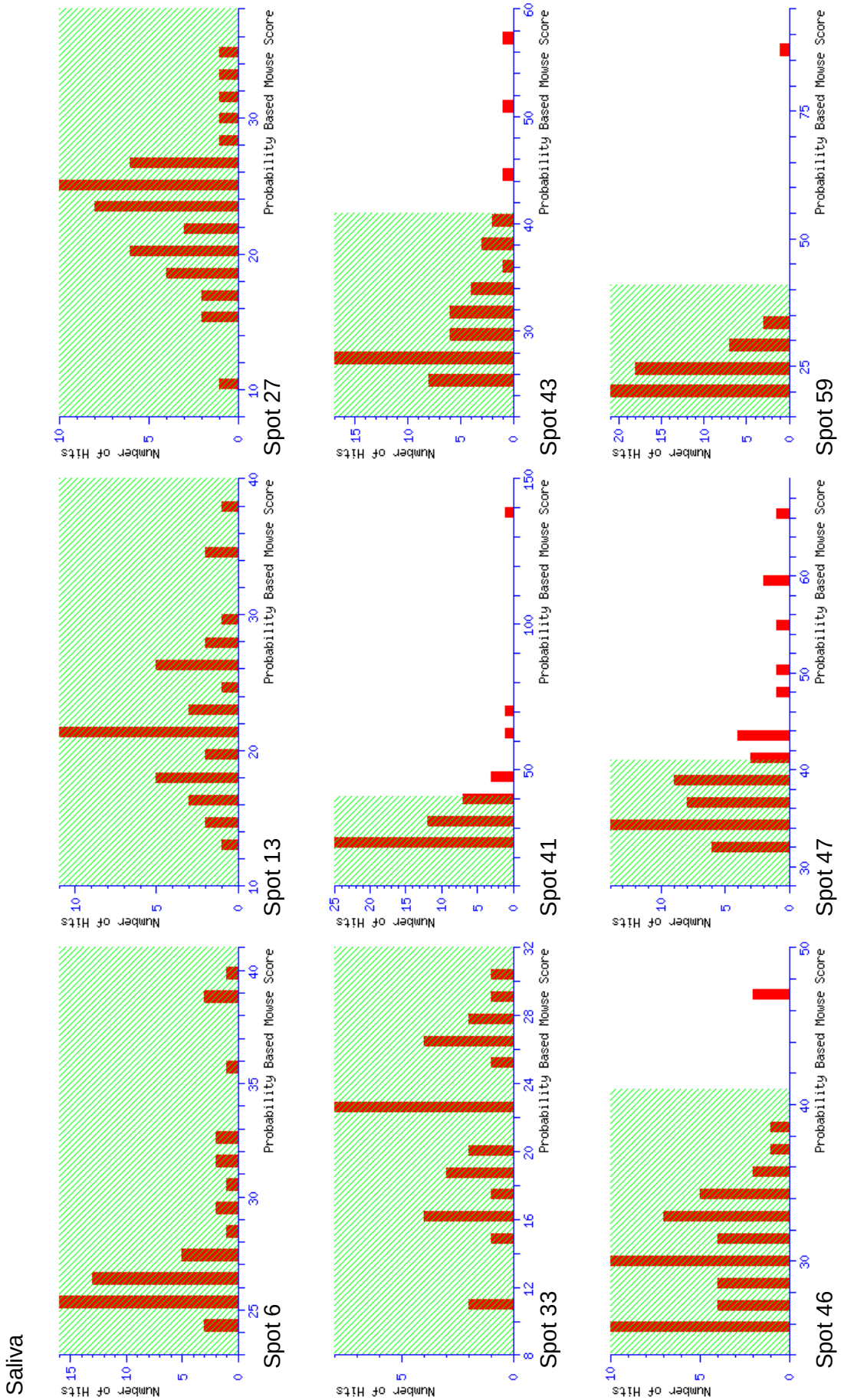


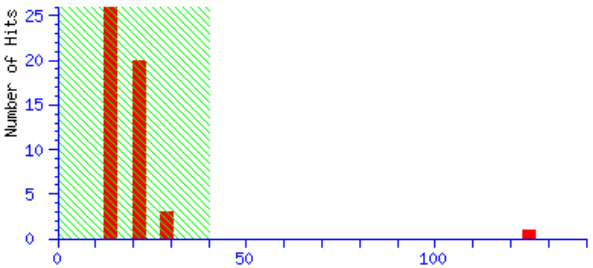
268 Saliva spots identified by SameSpots software



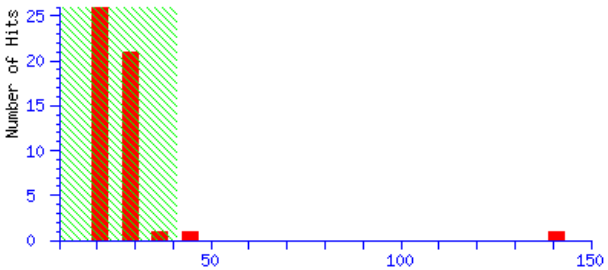
193 Vaginal spots identified by SameSpots software

Appendix VII – Hit Distribution Graphs

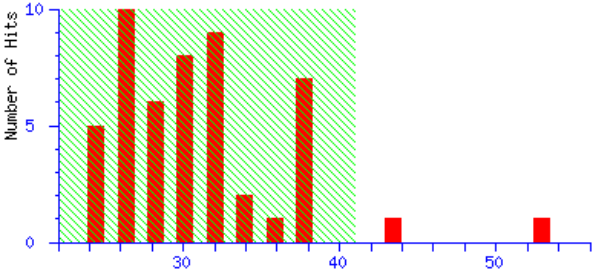




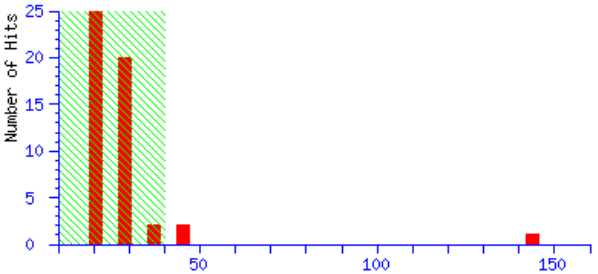
Spot 66



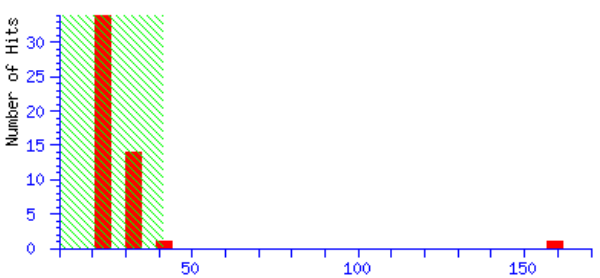
Spot 70



Spot 72



Spot 76



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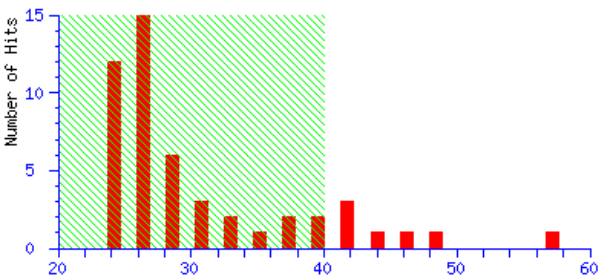

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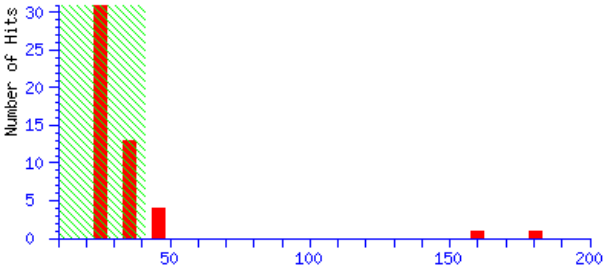

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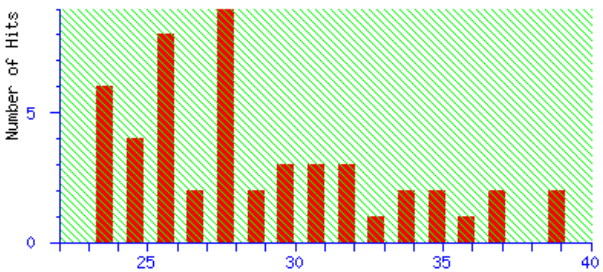

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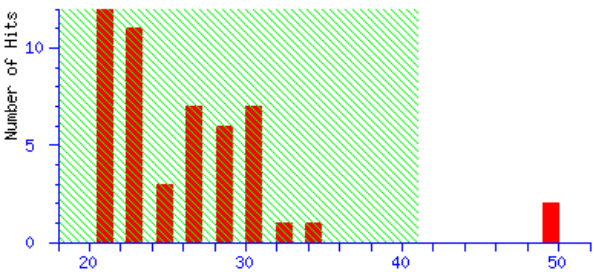
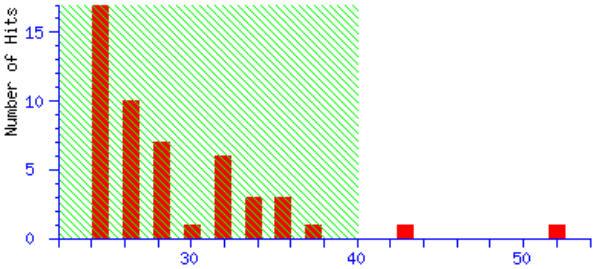
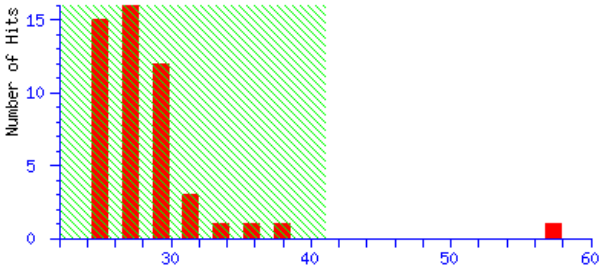


Spot 89

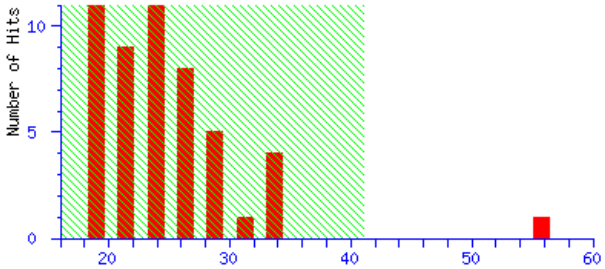
Vaginal Fluid



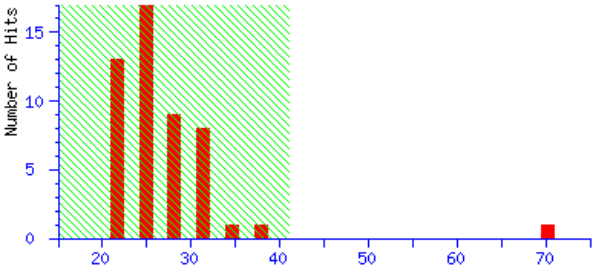
Spot 6



Spot 14



Spot 17



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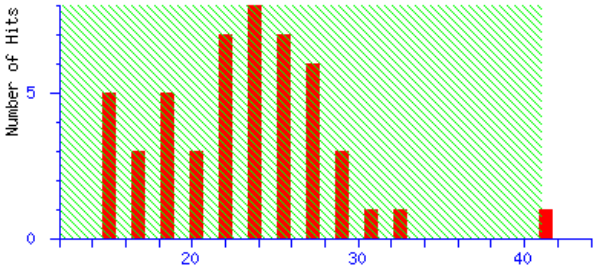

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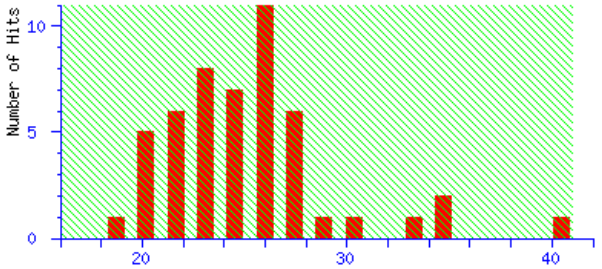

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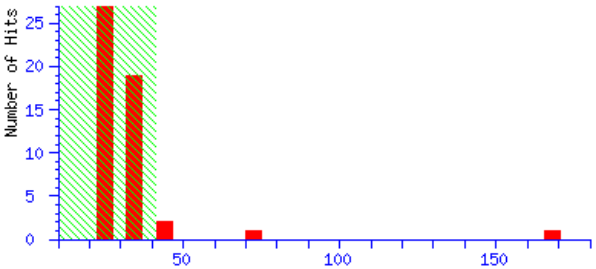
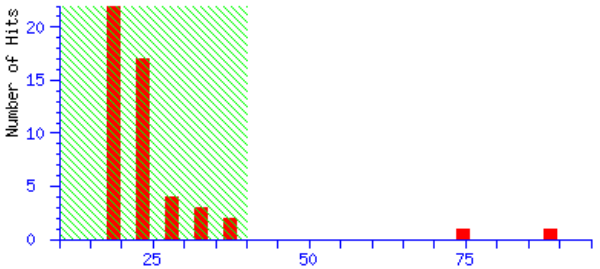


Spot 59



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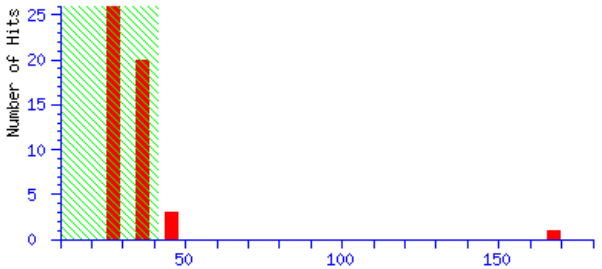
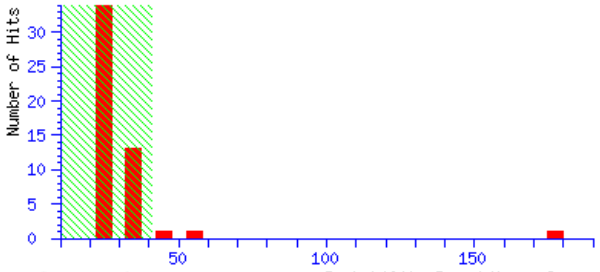
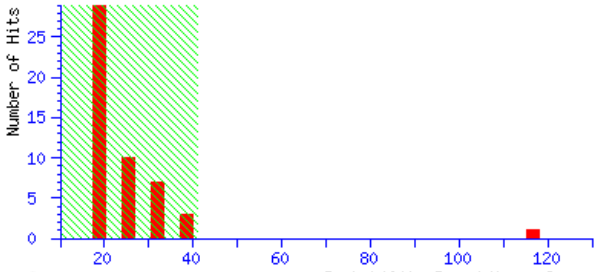


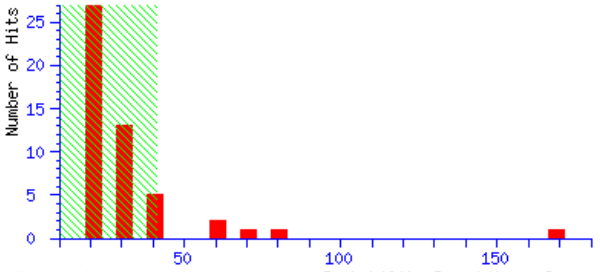
Spot 69



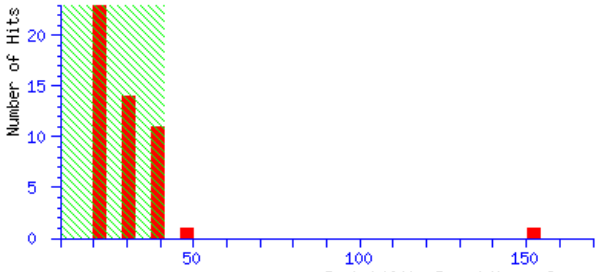
Spot 73



Spot 74



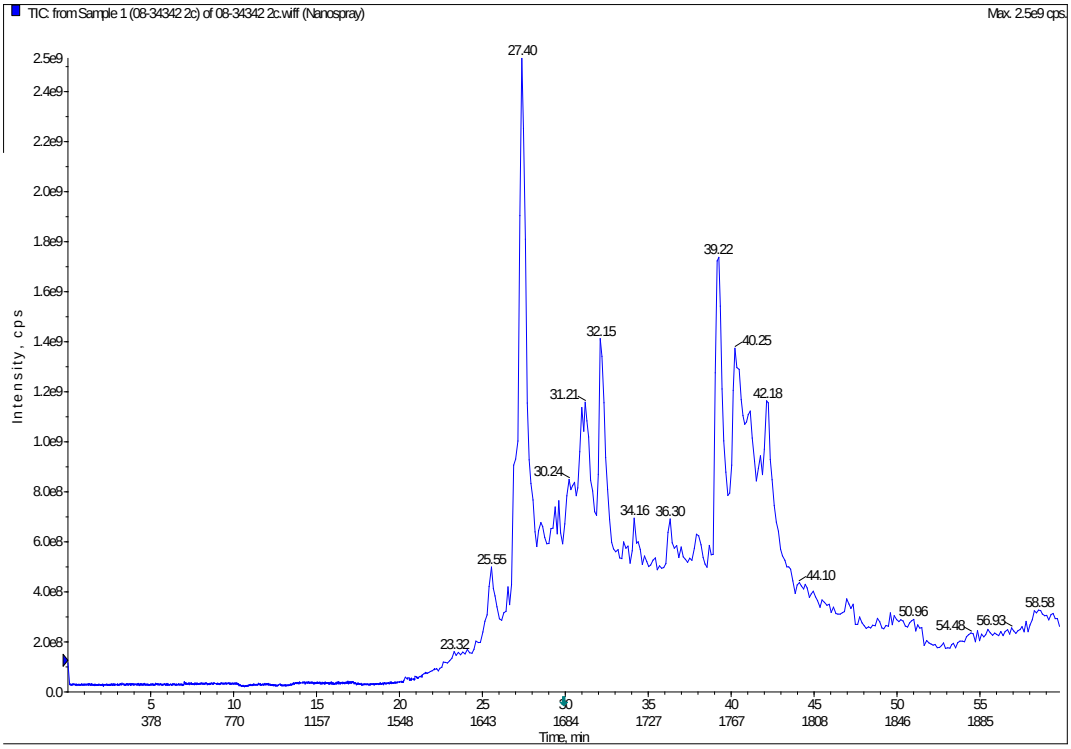
Spot 85



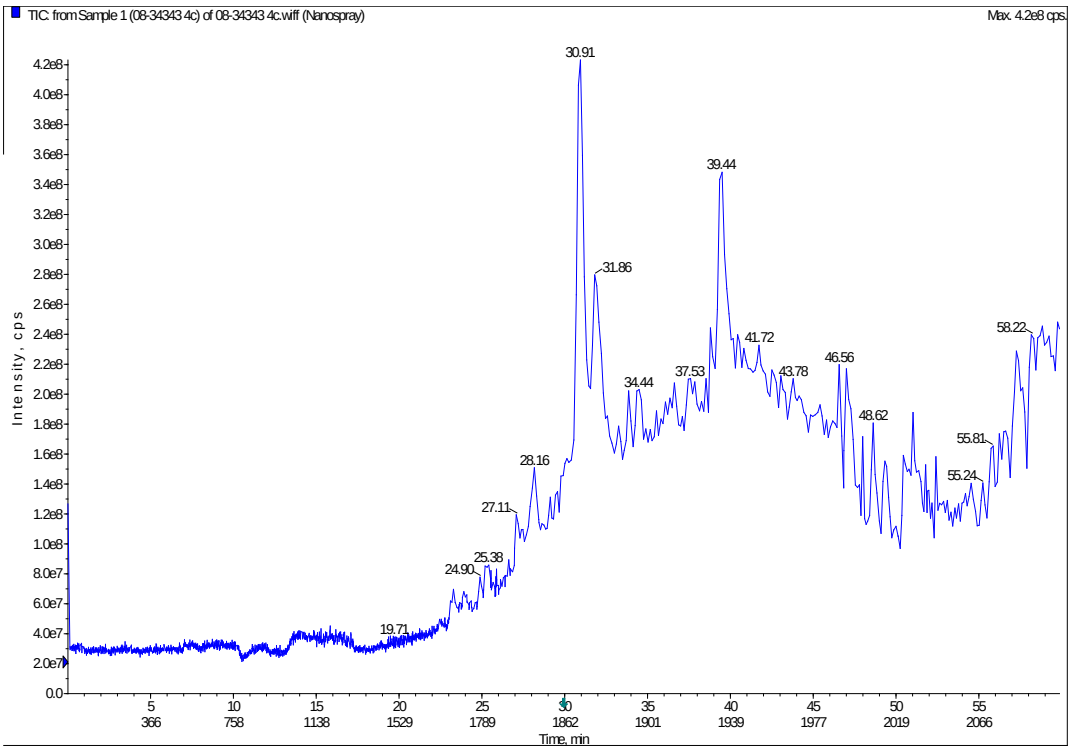
Spot 86

Appendix VIII – Total Ion Count Graphs

IgG Controls

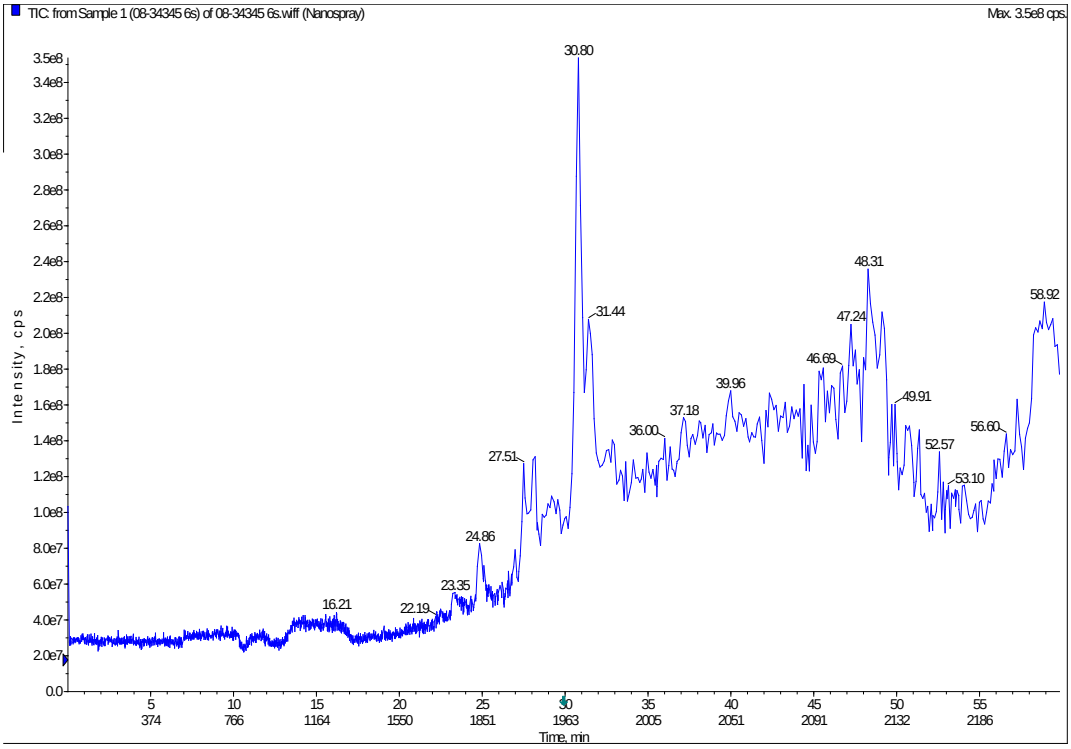


Spot 1C

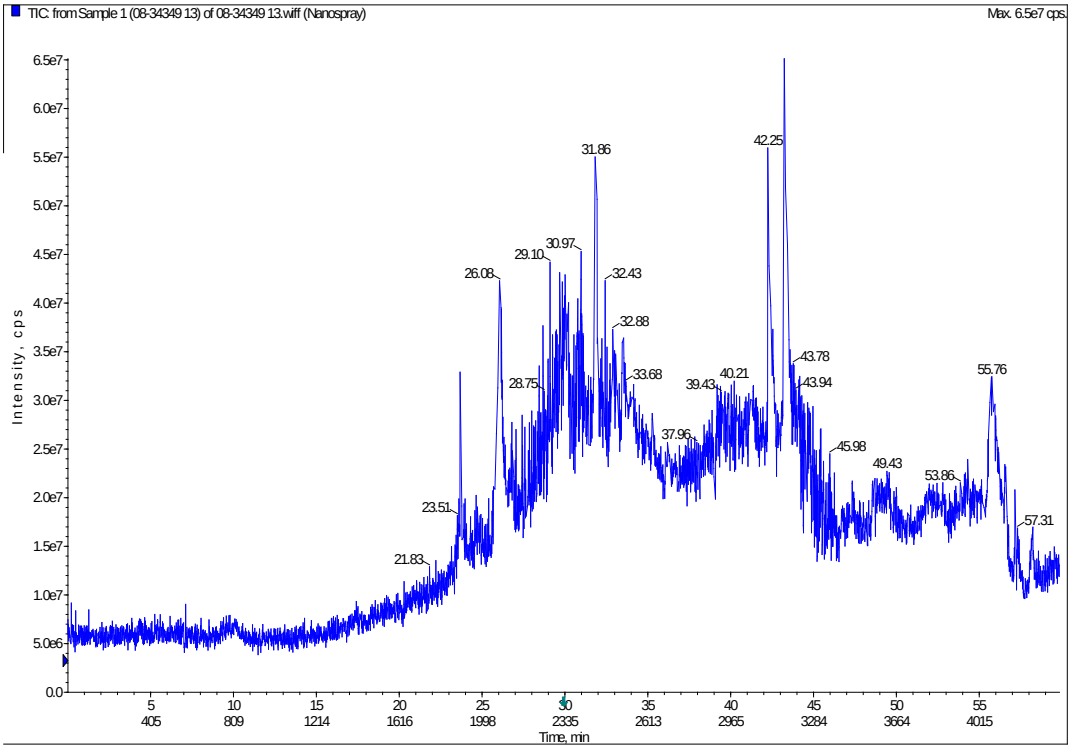


Spot 2C

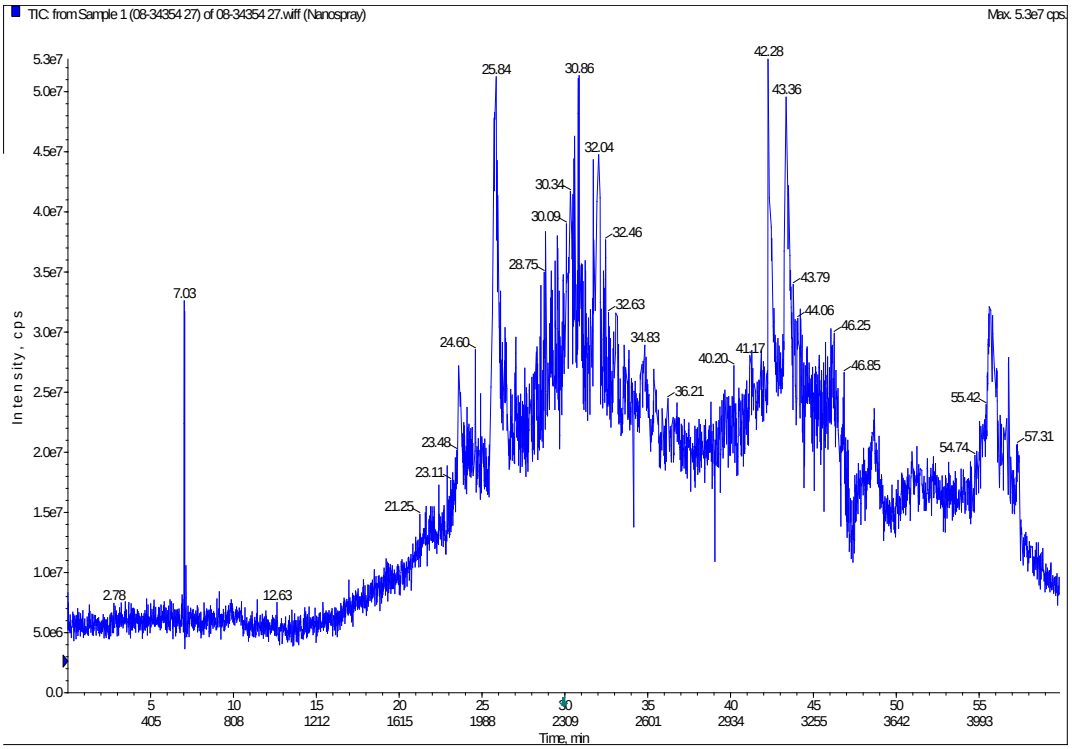
Saliva



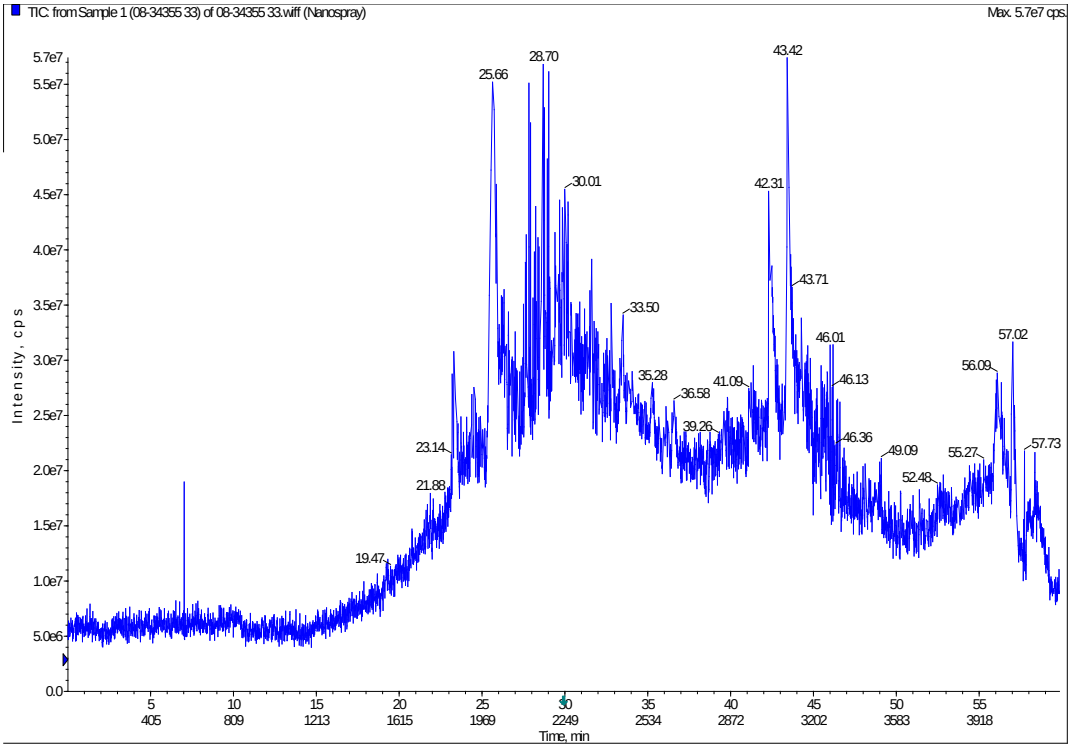
Spot 6



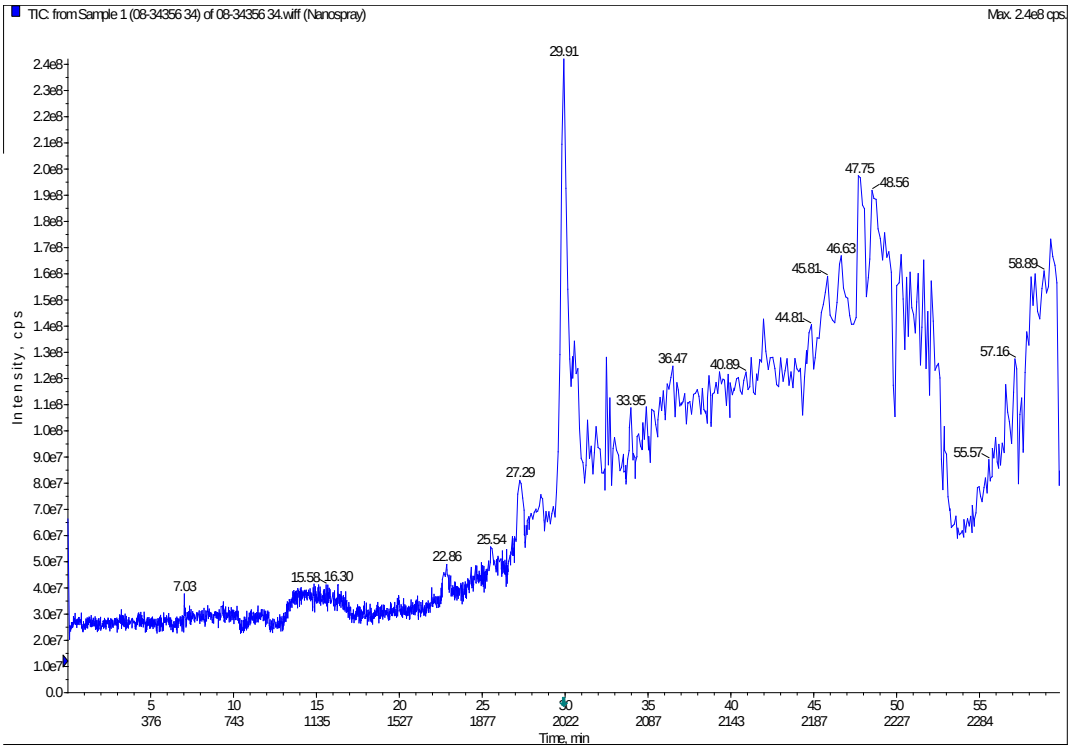
Spot 13



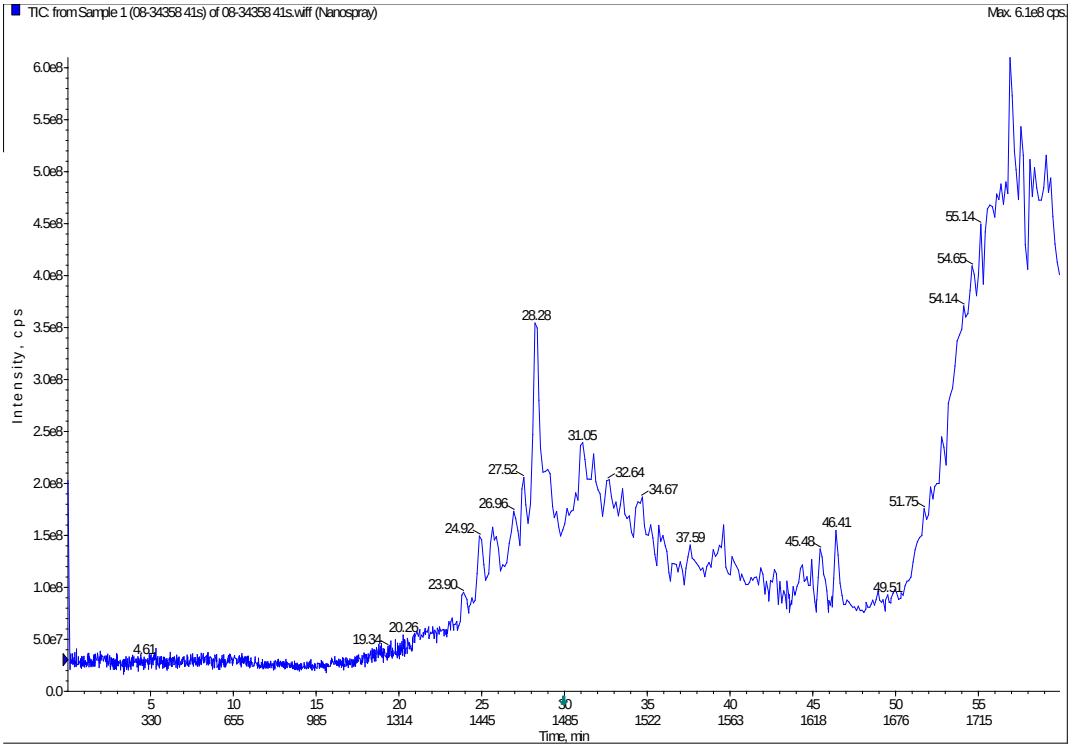
Spot 27



Spot 33



Spot 34



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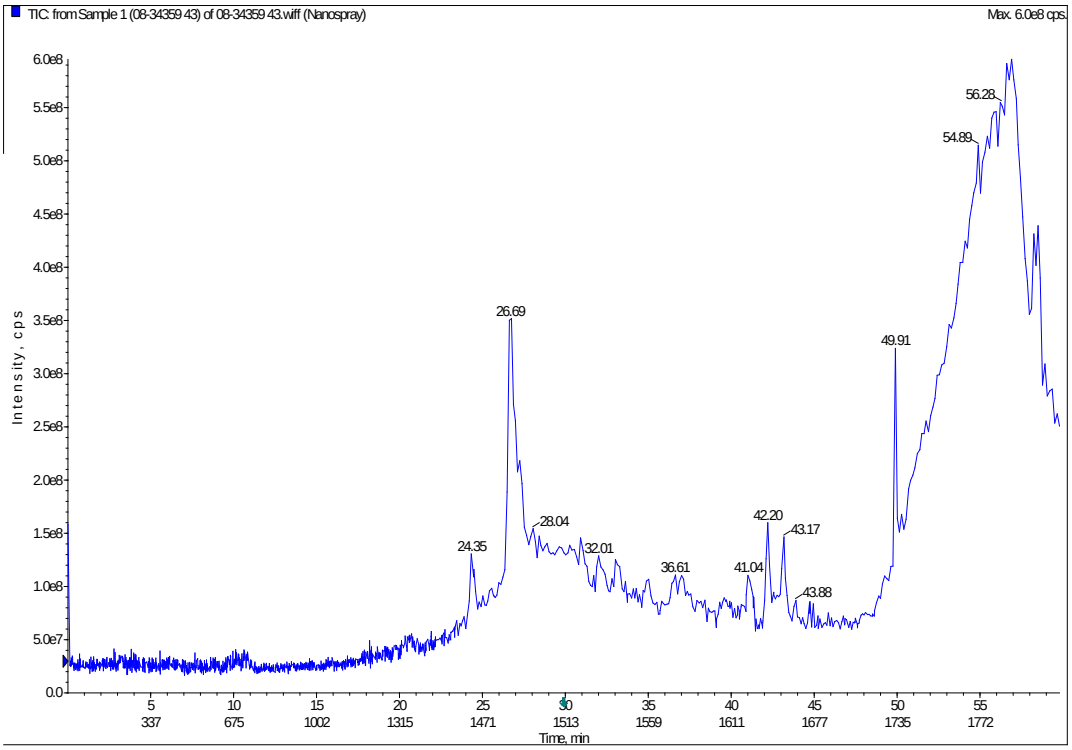

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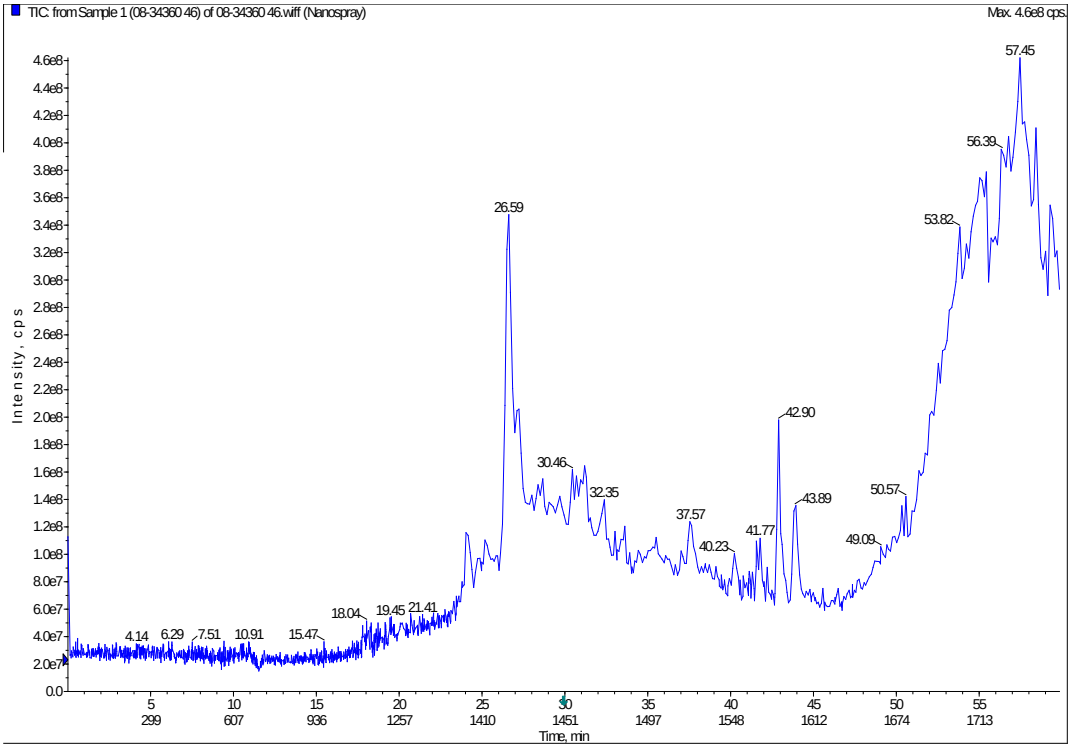

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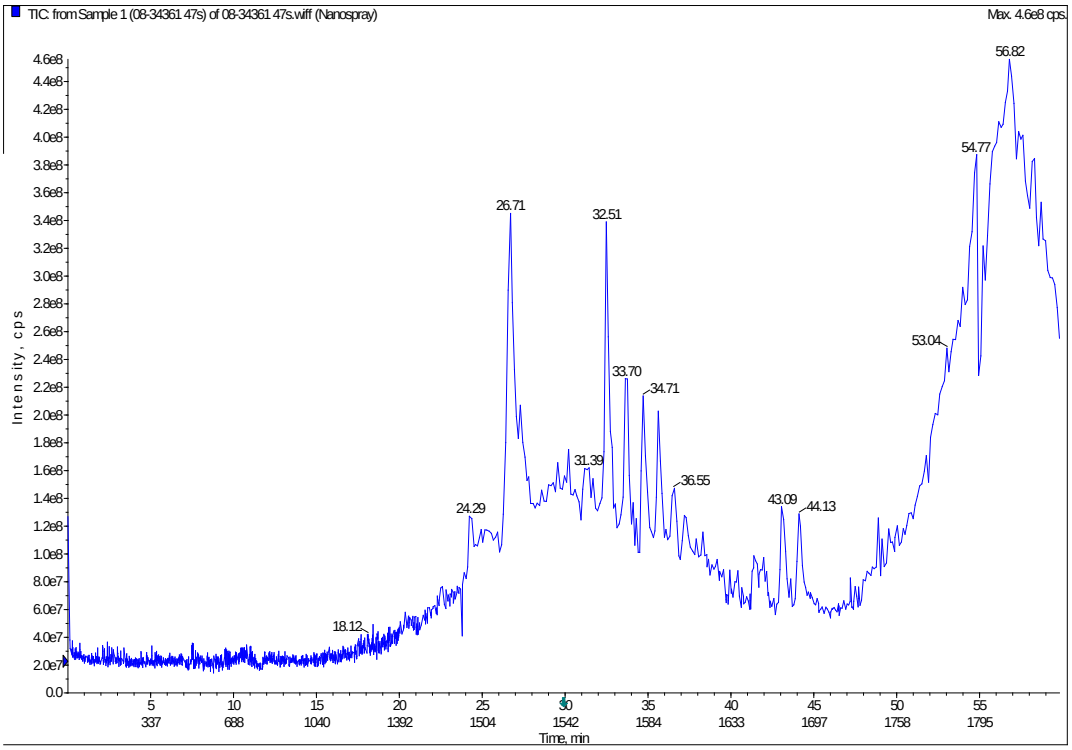

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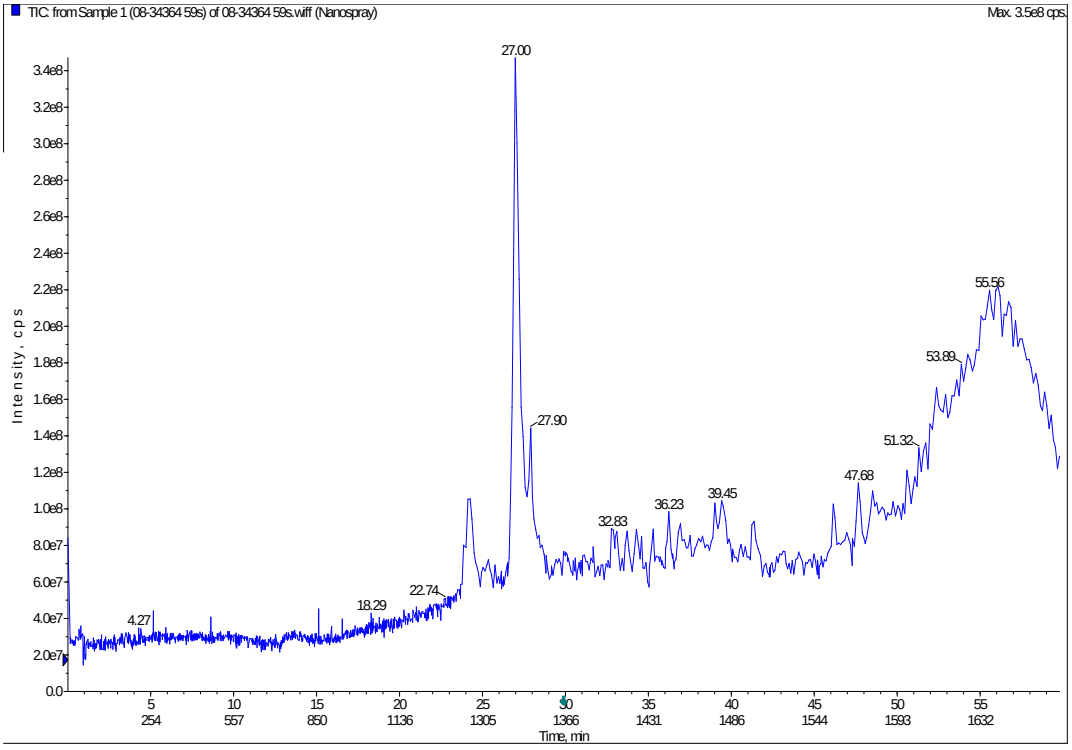
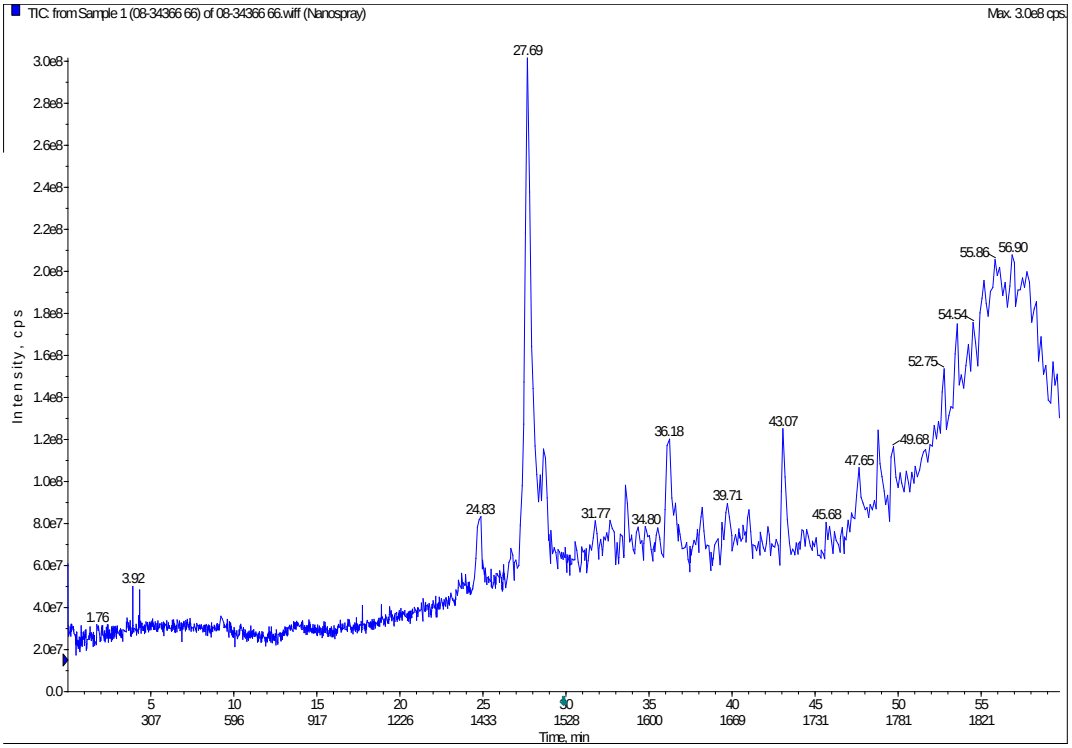
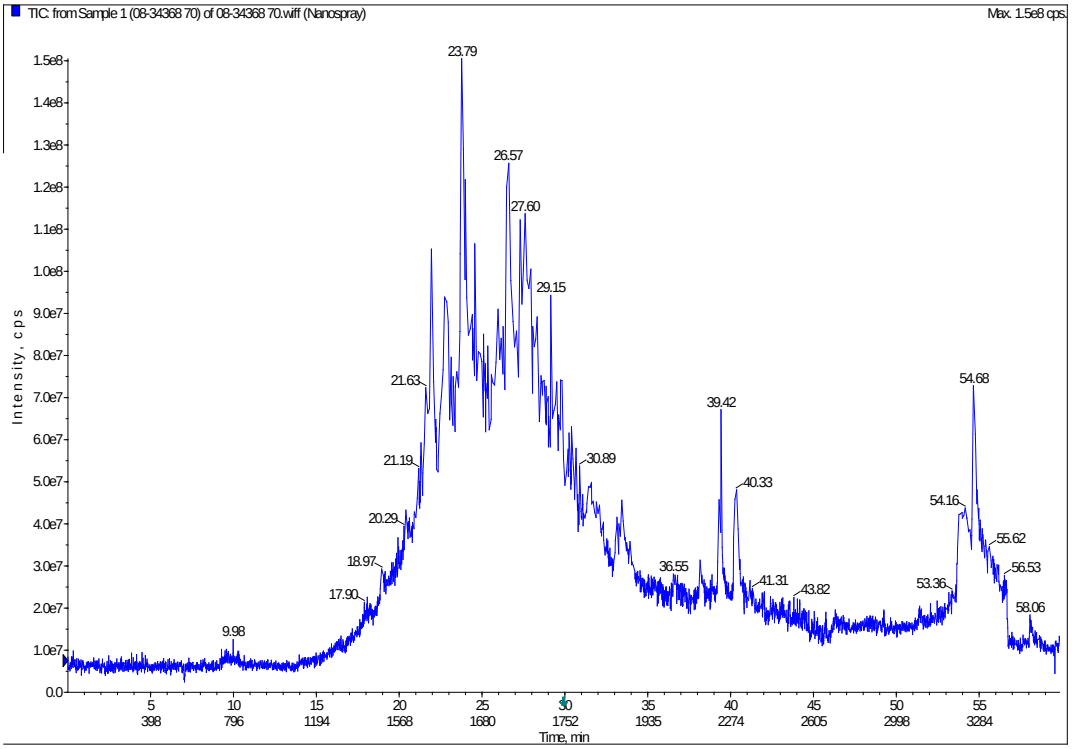


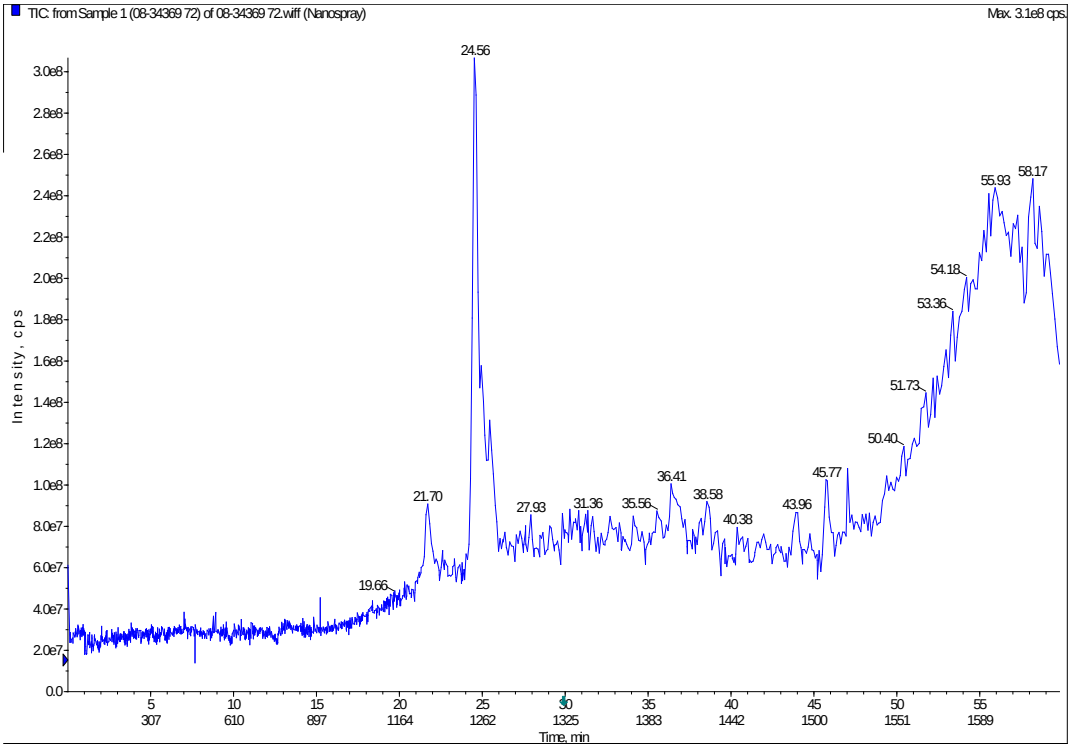
Spot 59



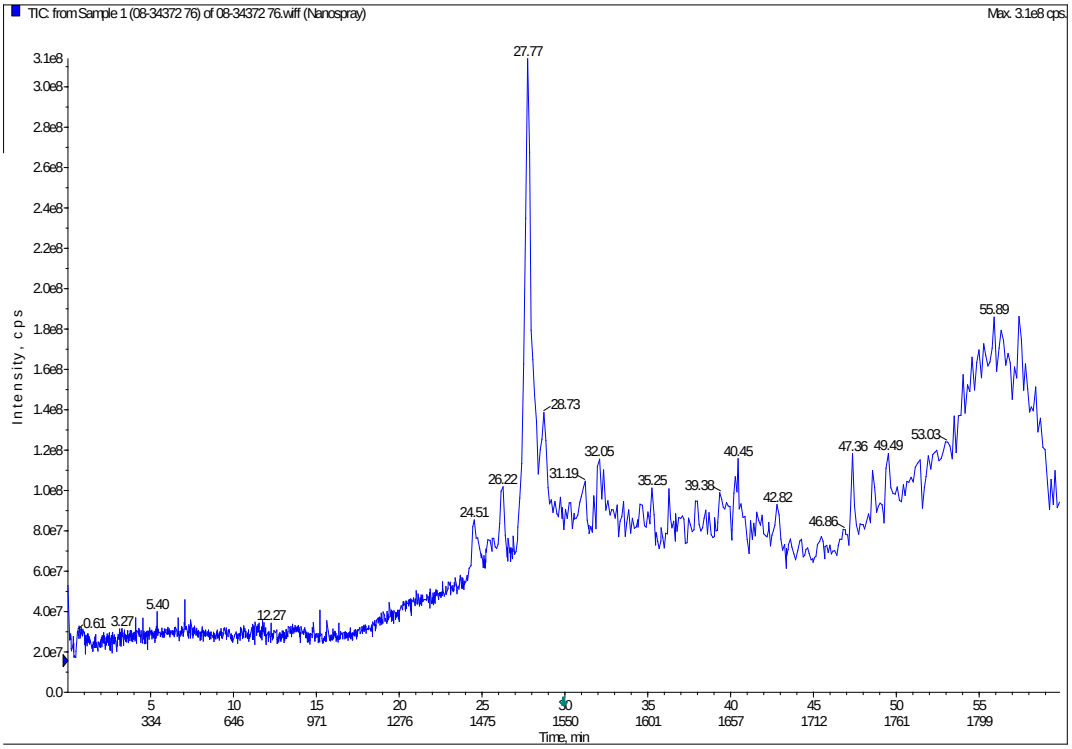
Spot 66



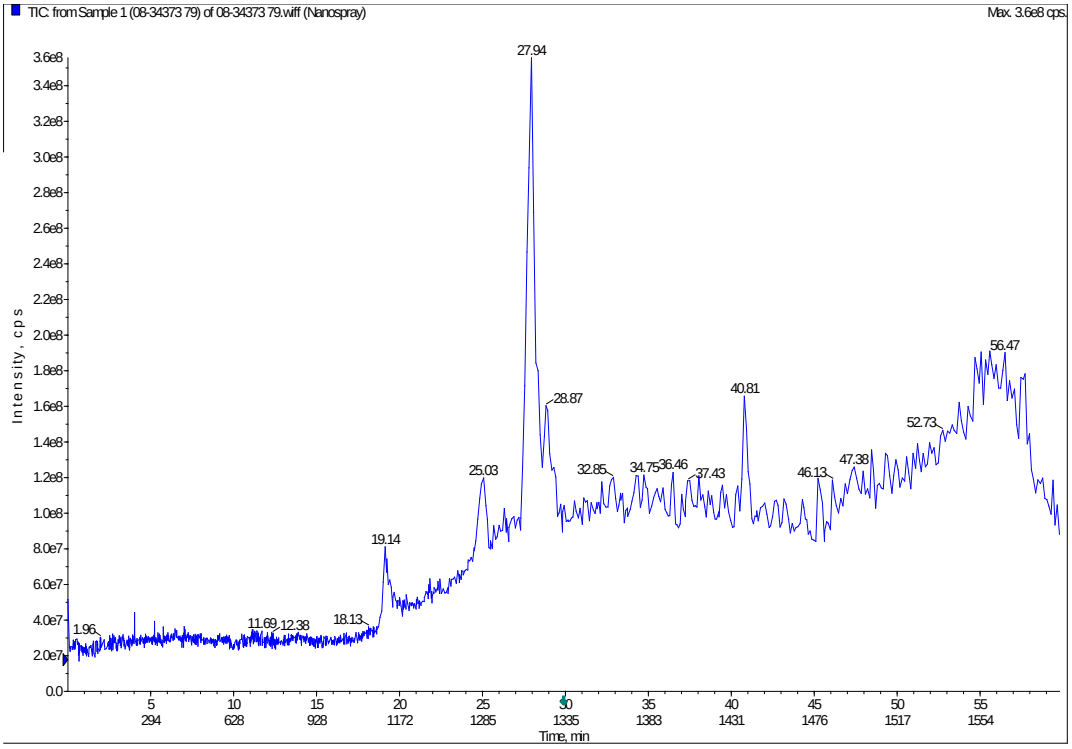
Spot 70



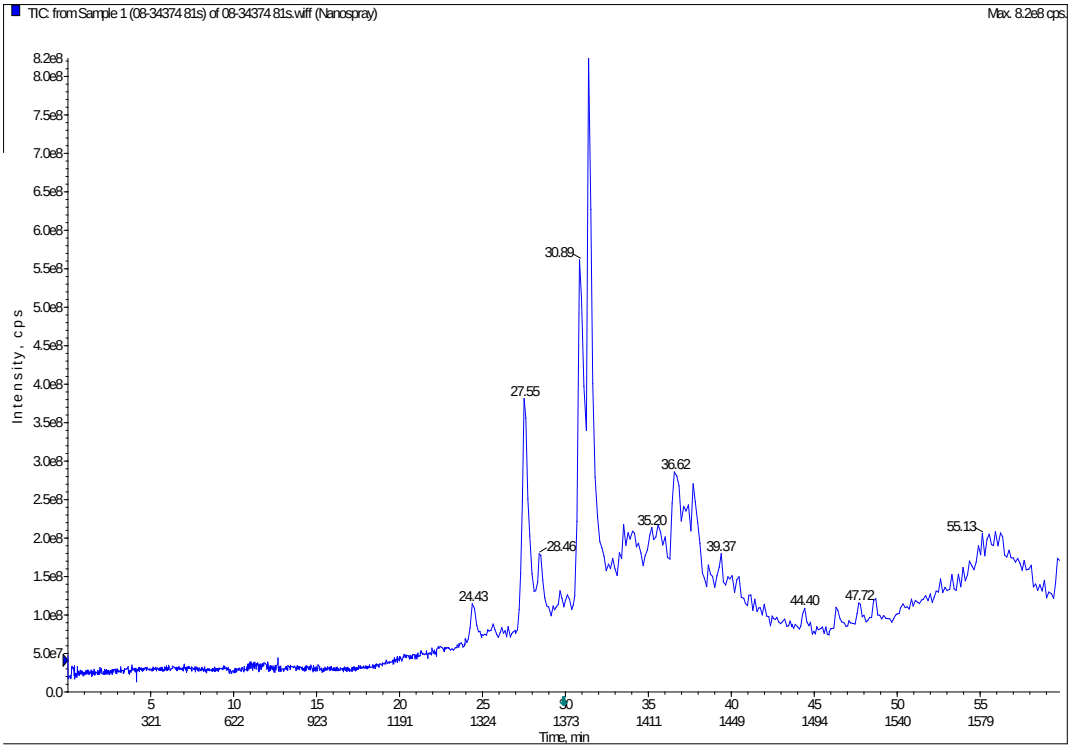
Spot 72



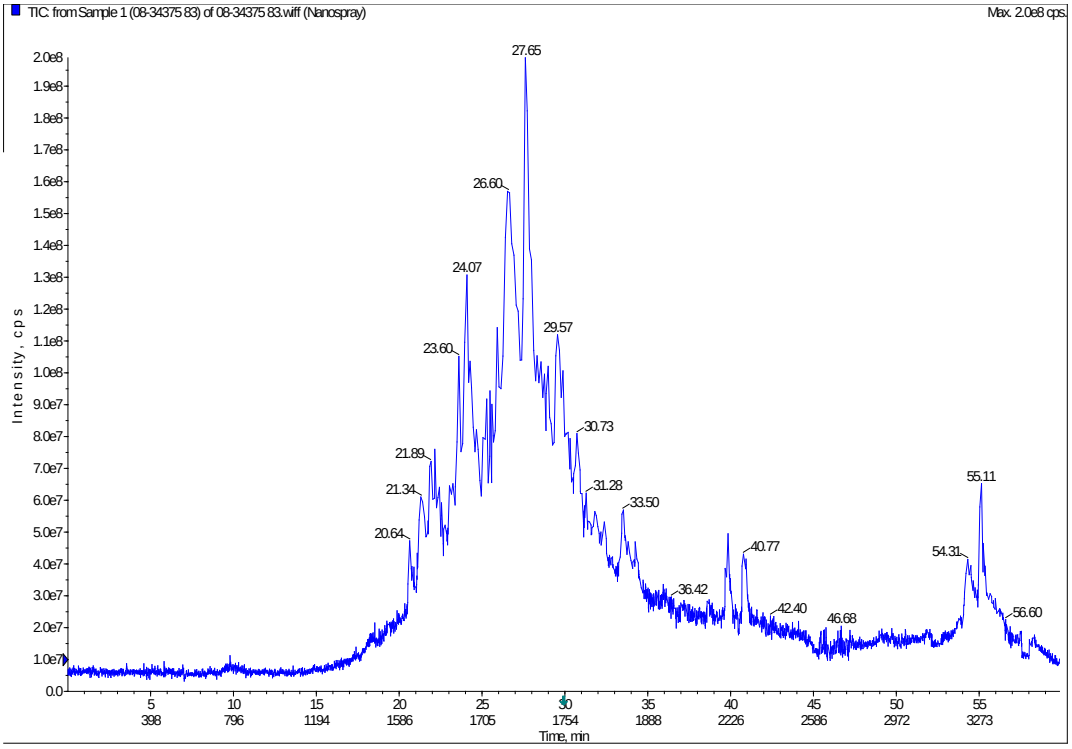
Spot 76



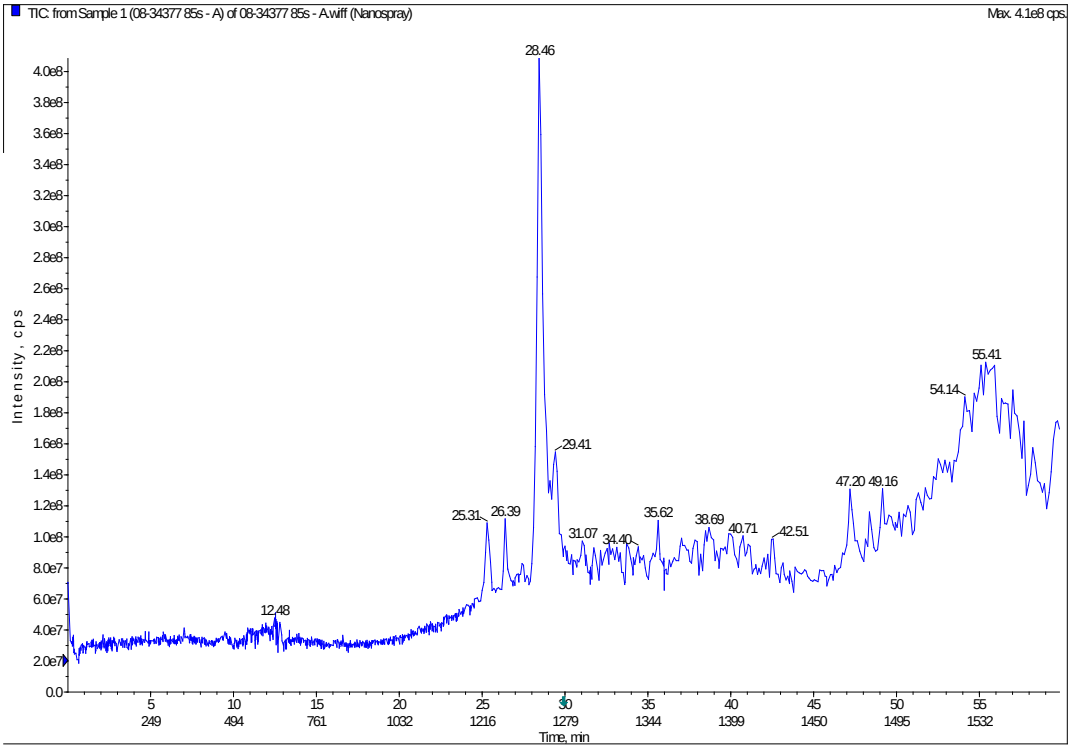
Spot 79



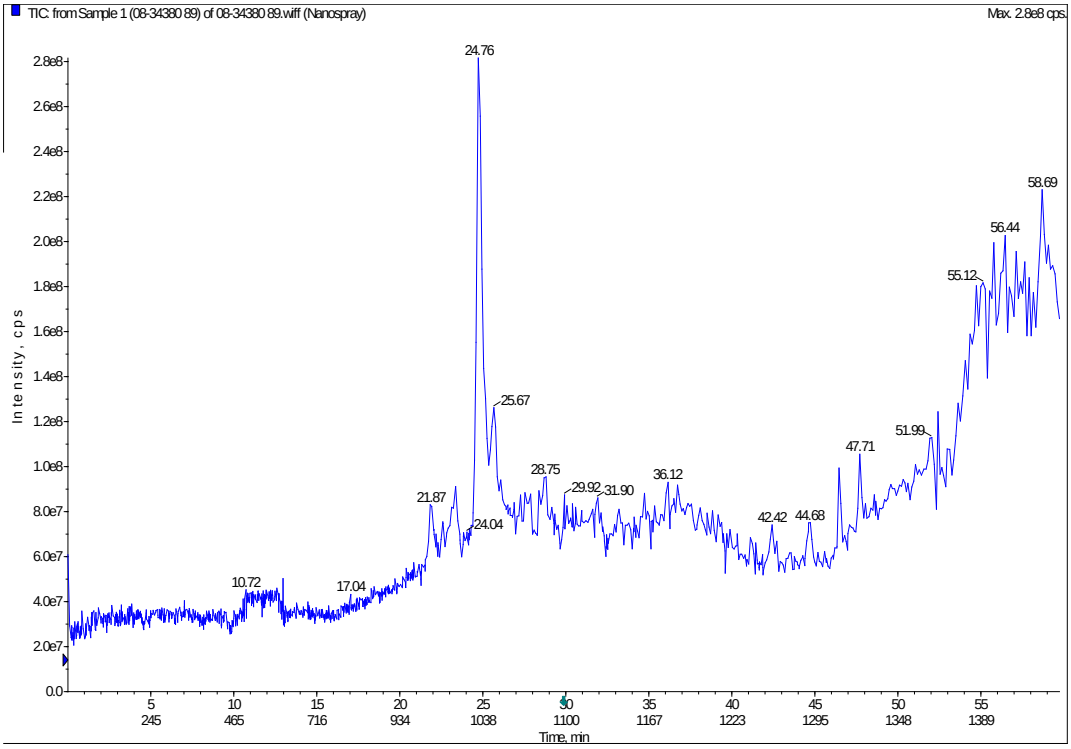
Spot 81



Spot 83

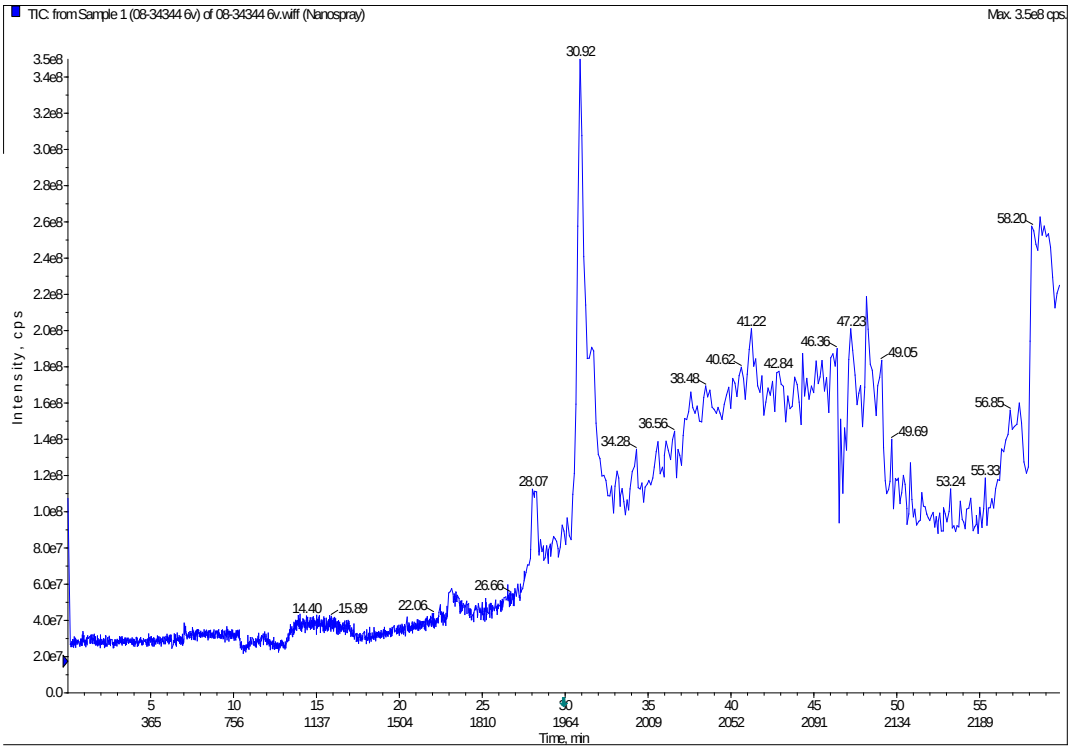


Spot 85

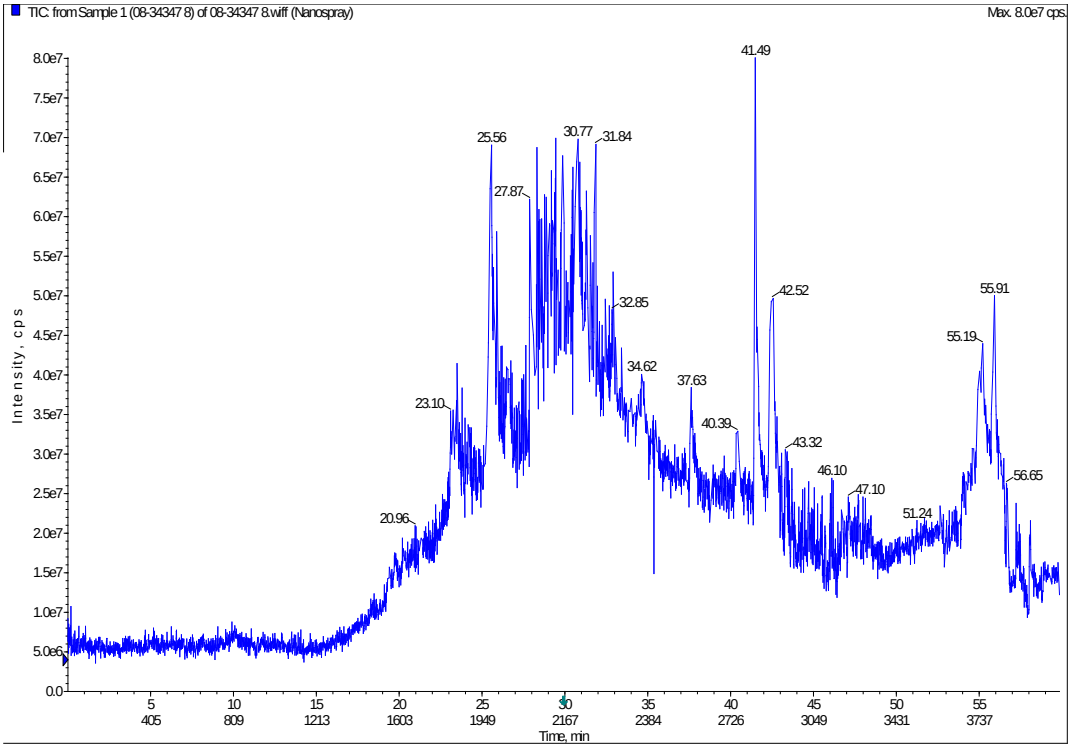

TIC from Sample 1 (08-34380 89) of 08-34380 89.wiff (Nanospray)
Max. 2.8e8 cps.
Intensity, cps
2.8e8
2.6e8
2.4e8
2.2e8
2.0e8
1.8e8
1.6e8
1.4e8
1.2e8
1.0e8
8.0e7
6.0e7
4.0e7
2.0e7
0.0
24.76
58.69
56.44
55.12
25.67
51.99
47.71
28.75
29.92
31.90
36.12
21.87
24.04
42.42
44.68
10.72
17.04
5
245
10
465
15
716
20
934
25
1038
30
1100
35
1167
40
1223
45
1295
50
1348
55
1389
Time, min

Spot 89

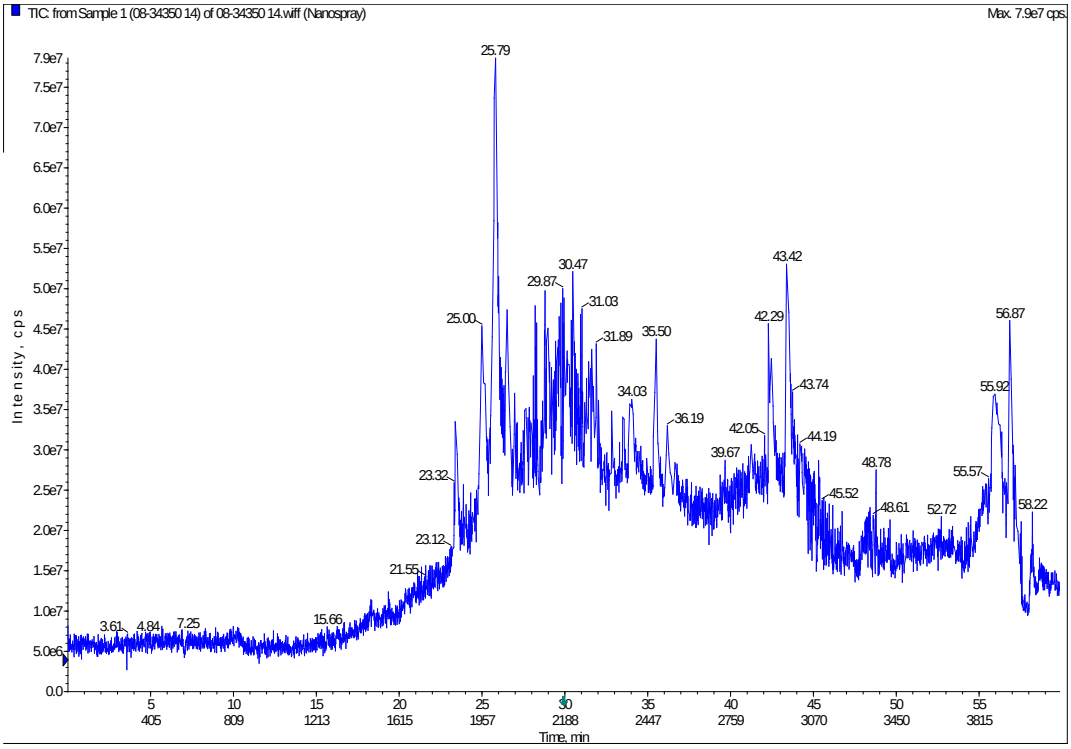
Vaginal Fluid



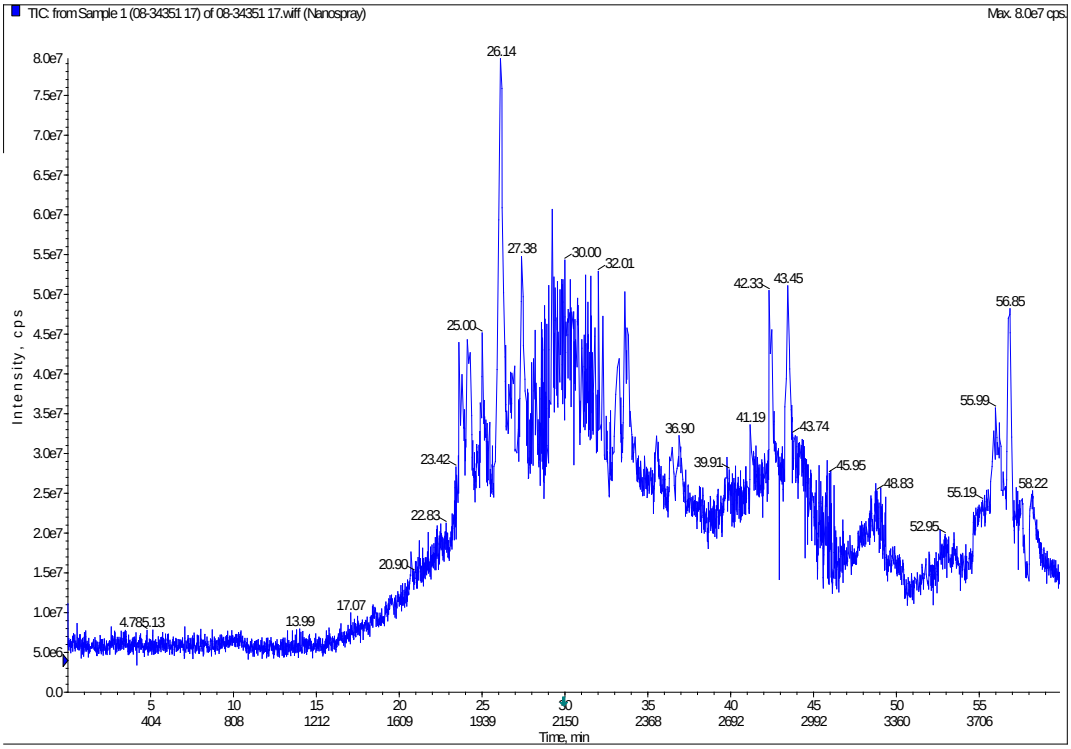
Spot 6



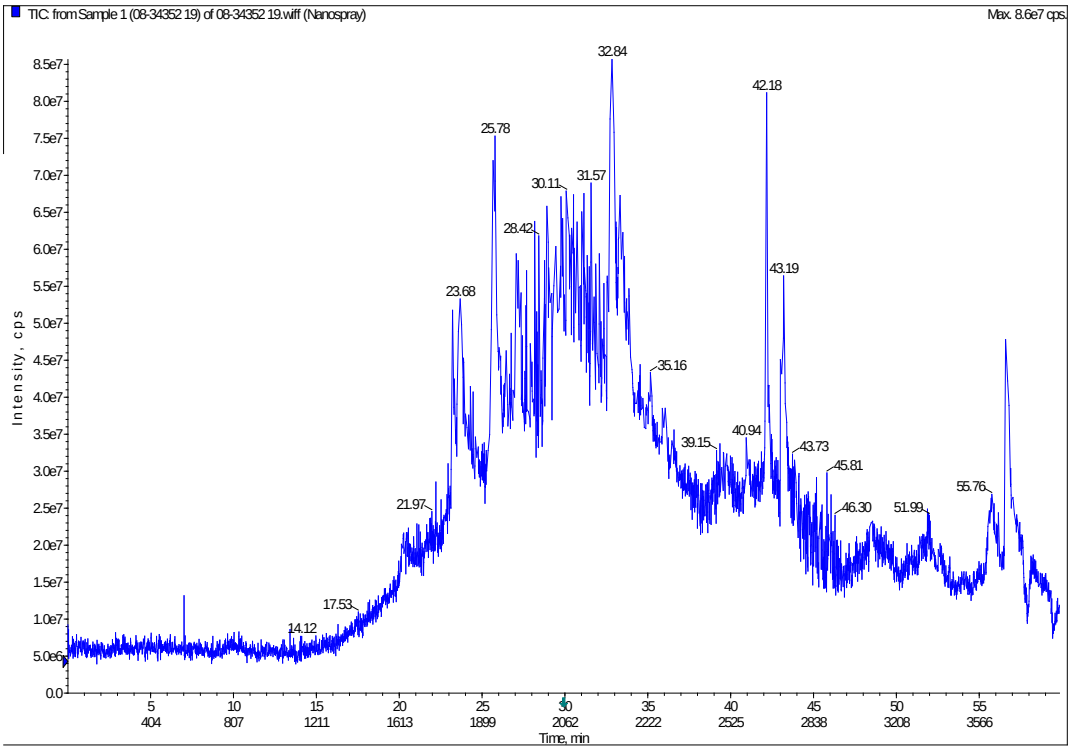
Spot 8



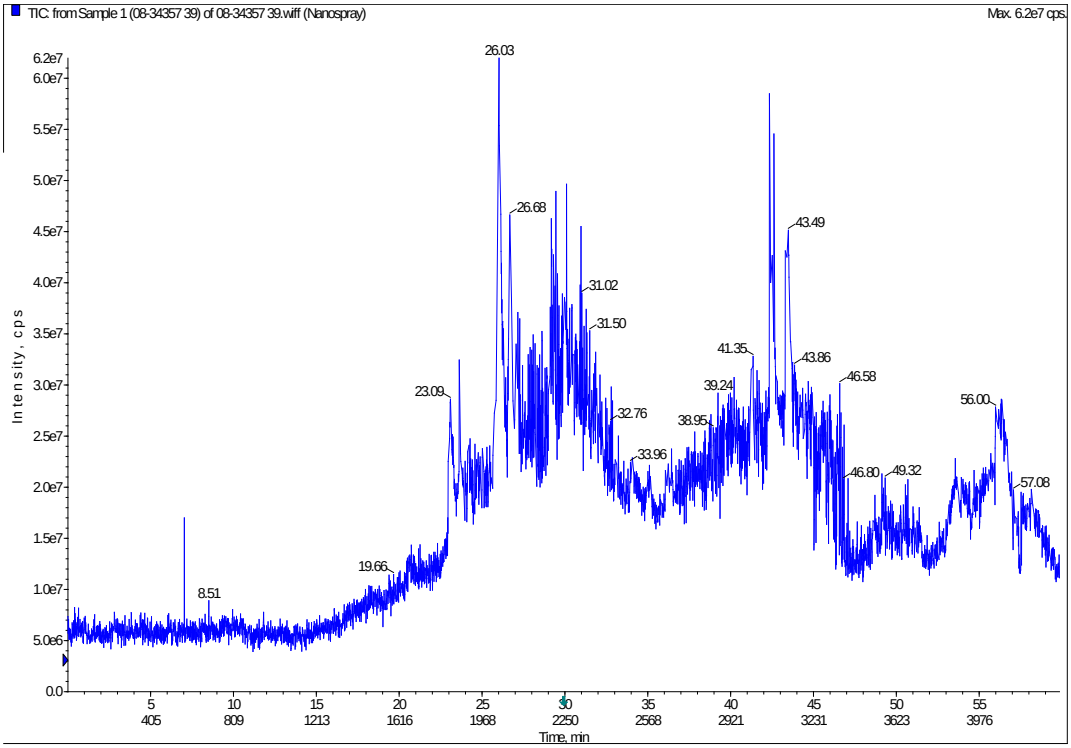
Spot 14



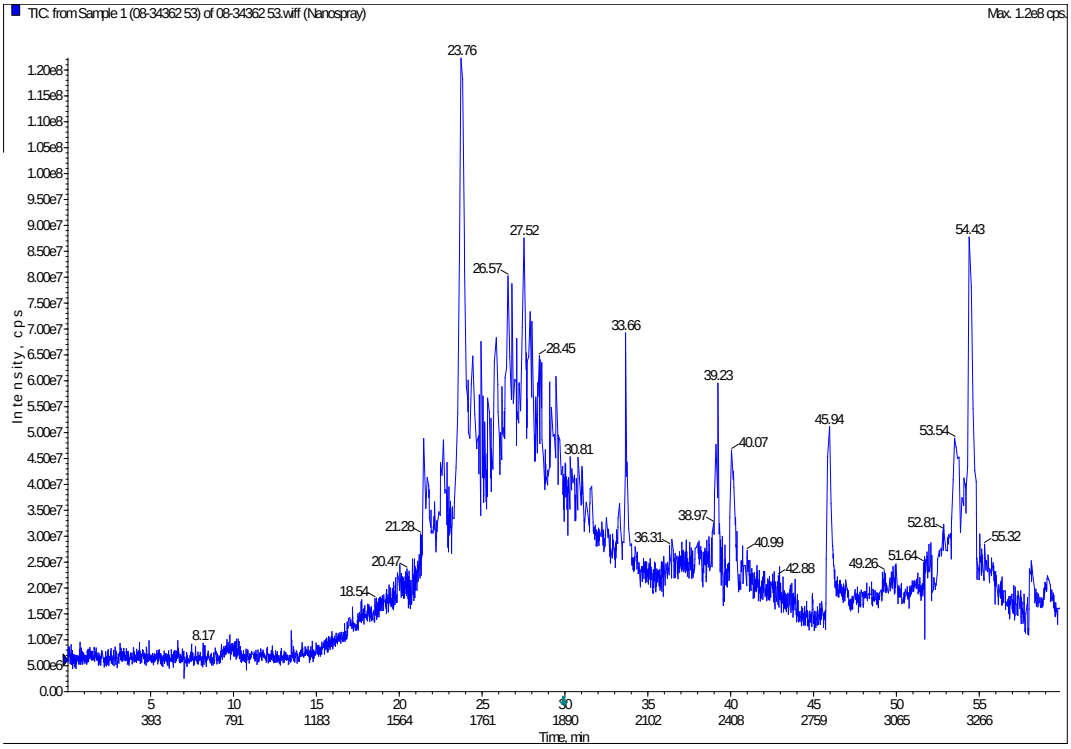
Spot 17



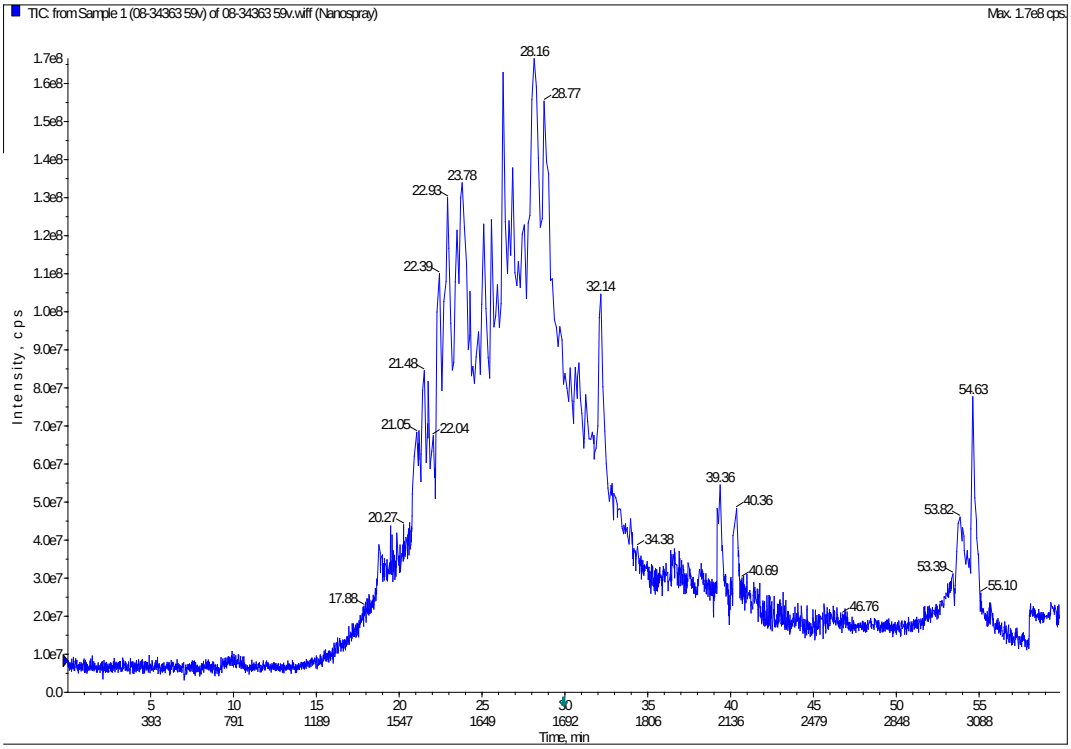
Spot 19



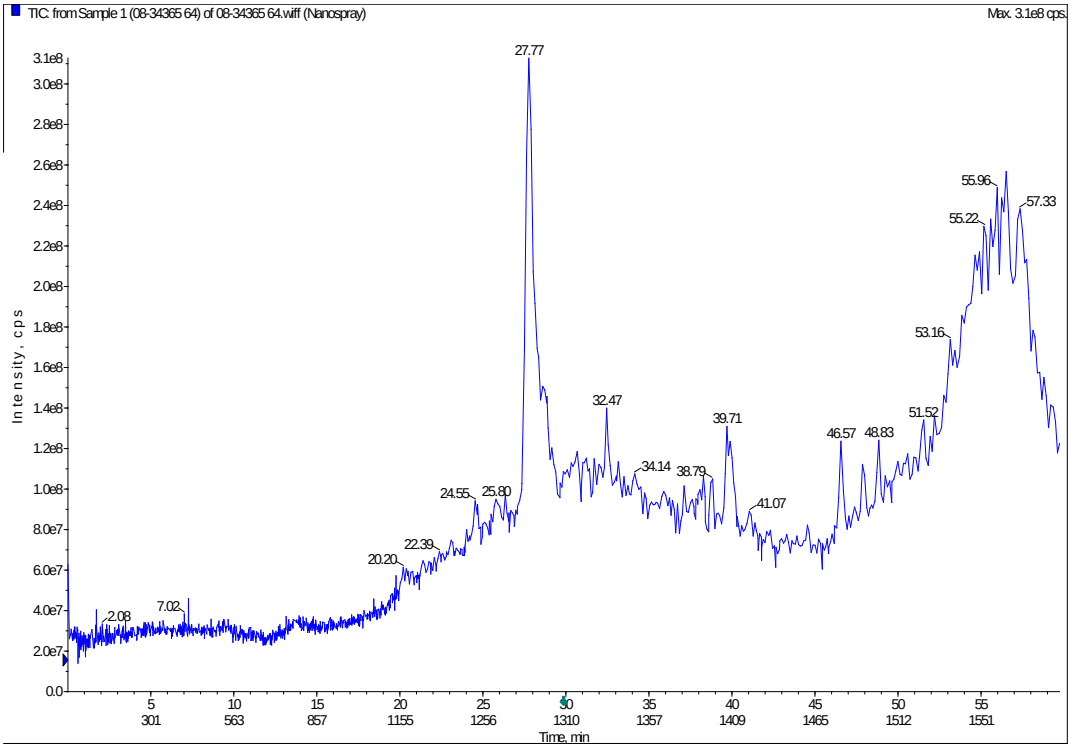
Spot 39



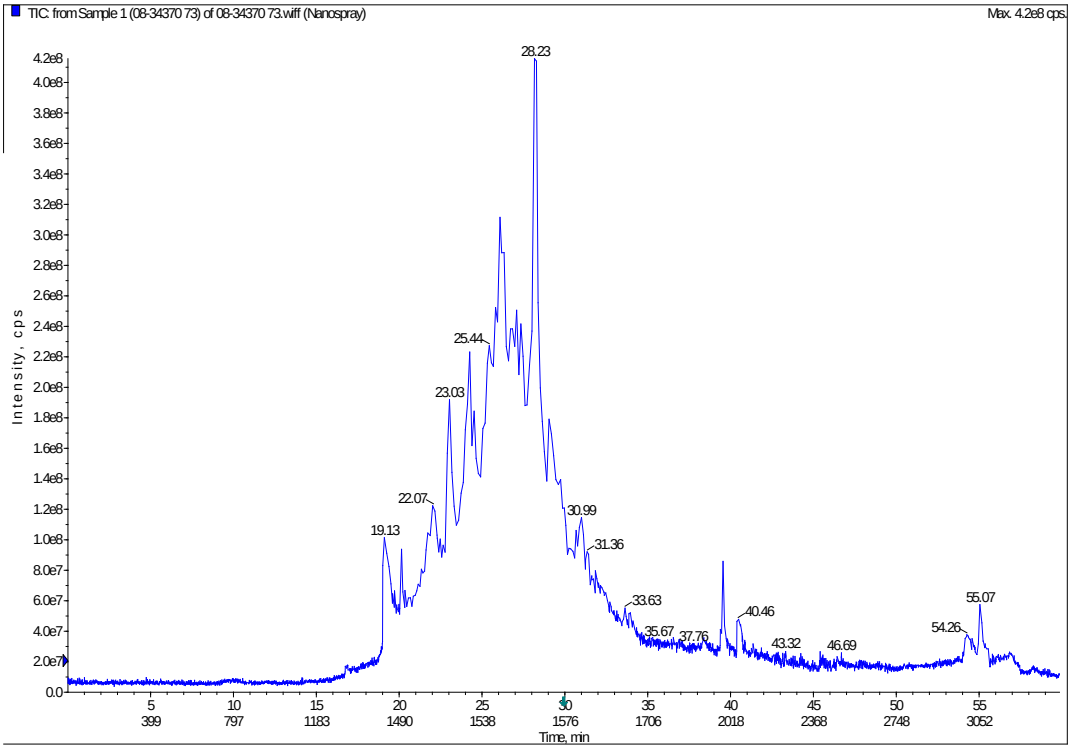
Spot 53



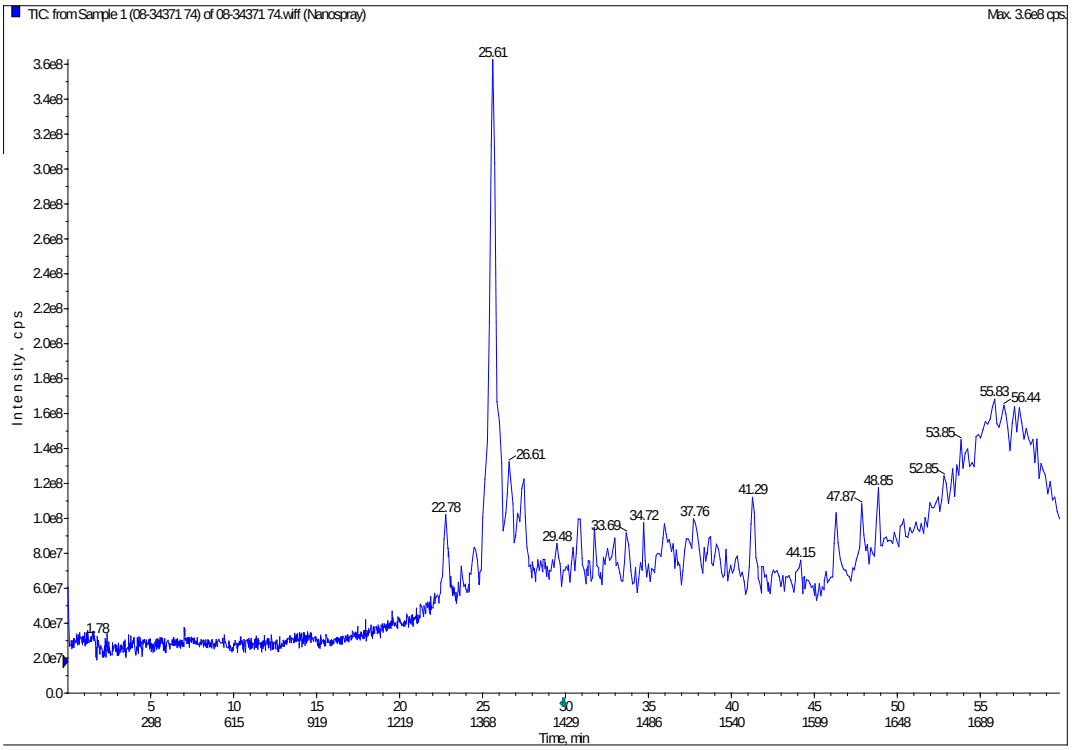
Spot 59



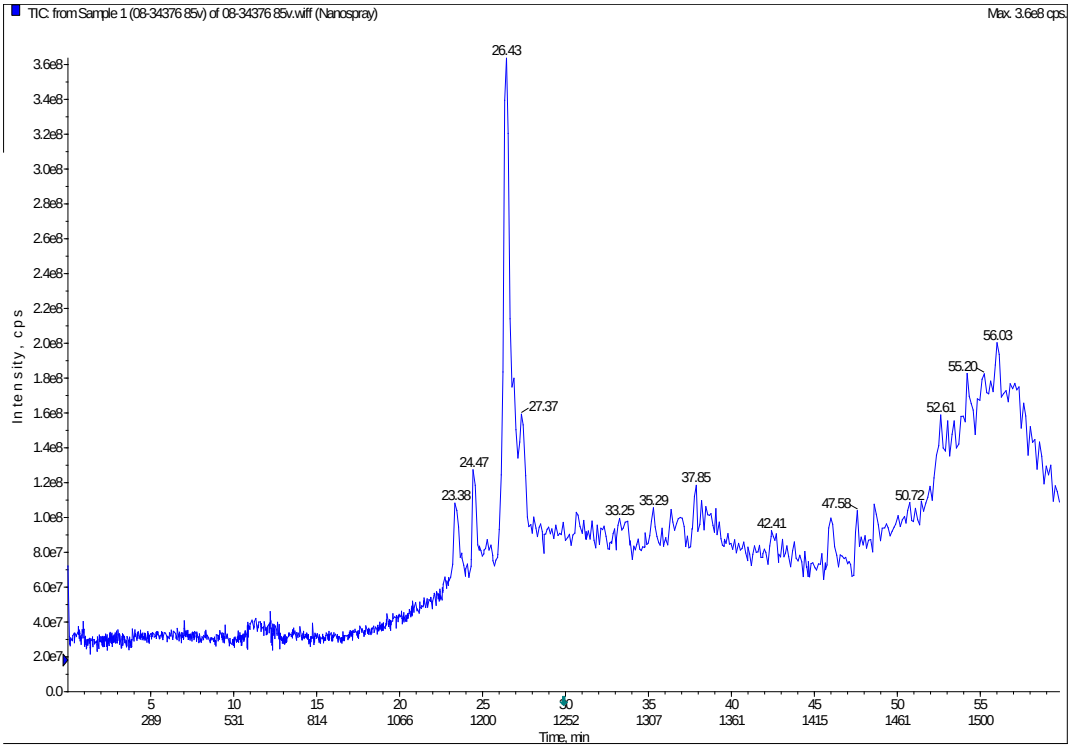
Spot 64



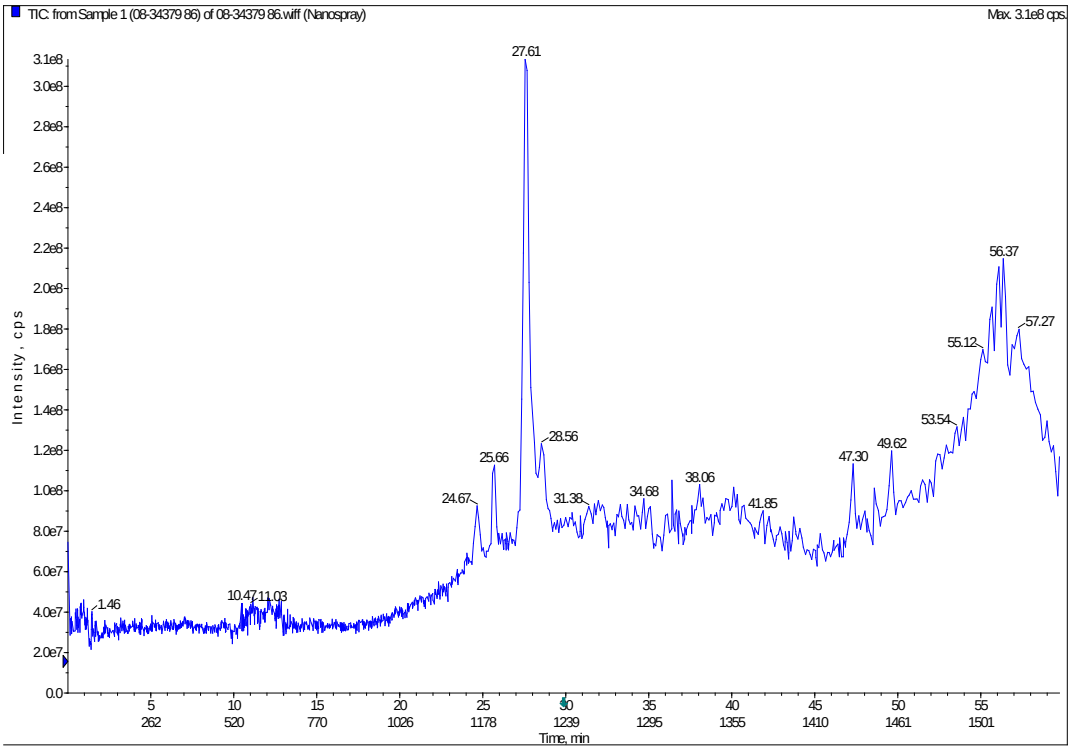
Spot 73



Spot 74

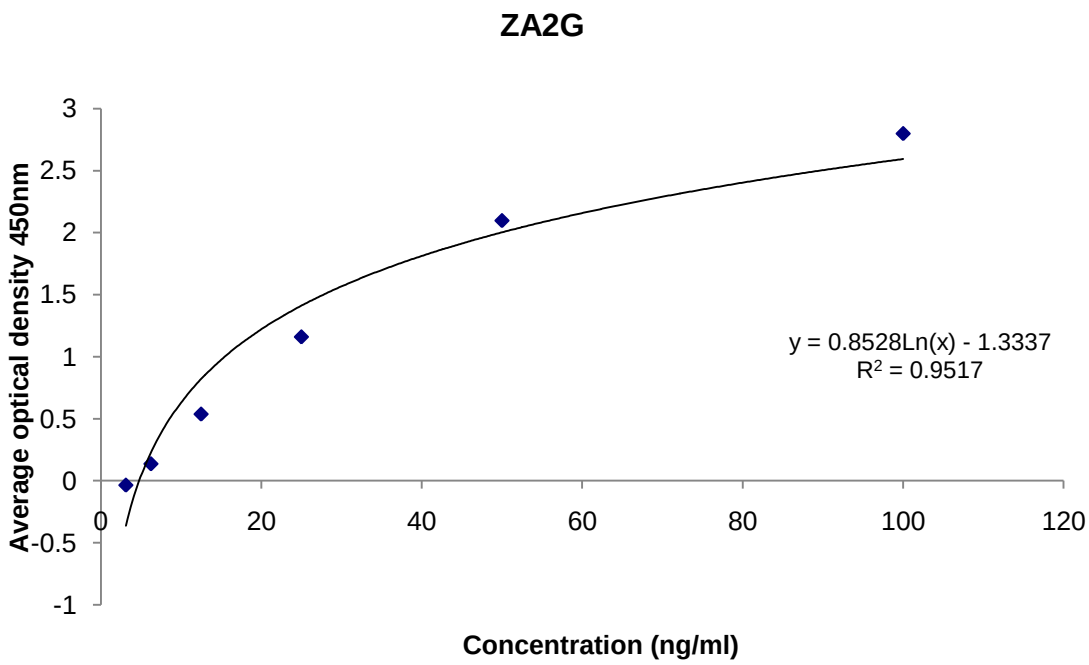


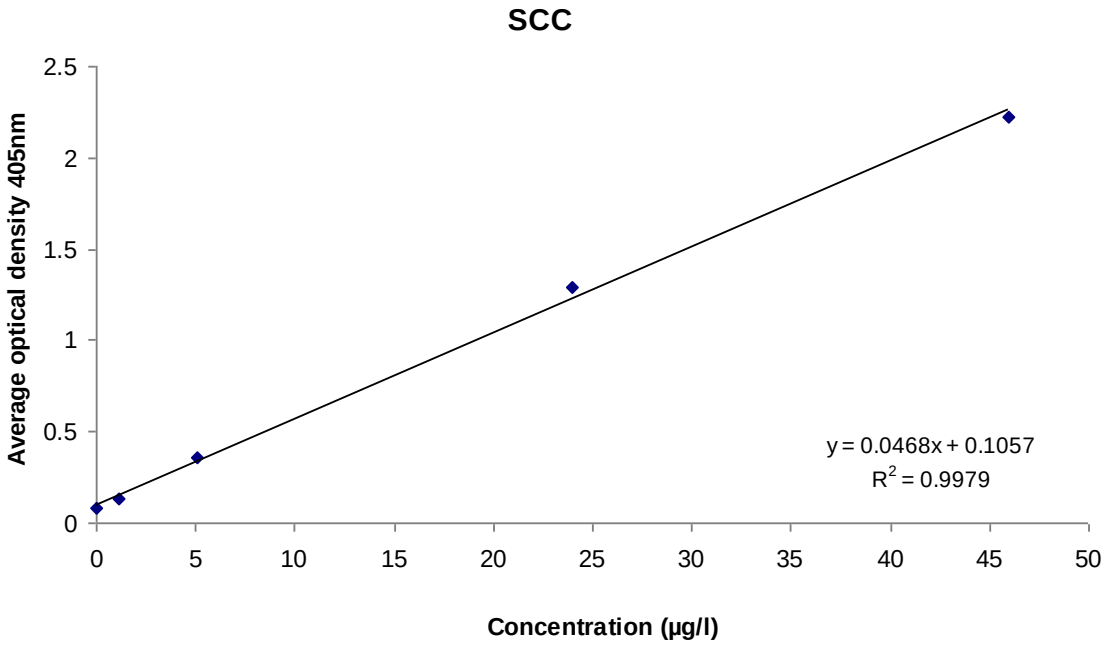
Spot 85


TIC from Sample 1 (08-34379 86) of 08-34379 86.wiff (Nanospray)
Max. 3.1e8 cps.
Intensity, cps
Time, min
27.61
56.37
57.27
55.12
53.54
49.62
47.30
28.56
25.66
24.67
31.38
34.68
38.06
41.85
1.46
10.47
11.03

Spot 86

Appendix IX- ELISA Standard Curves





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Public Output

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Identification of Body fluids using 2-D gel electrophoresis.

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With advances in DNA technology there has become a need for a sophisticated method of body fluid detection in forensic analysis. In this study, a two-dimensional proteomics approach to identification was investigated. Human body fluids differ dramatically in function and composition. As protein expression is tissue dependent a proteomics approach seems viable and should show variation between fluid types.
Body fluids studied were saliva, vaginal fluid and semen as these are most likely to be encountered in forensic labs. Clear differences were shown in the proteomic profiles of each body fluid when stained with SYPRO Ruby gel stain. These differences could be exploited in further experiments in order to find biomarkers for each fluid type.

Identification of Body fluids using 2D gel electrophoresis

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Background

- When evidence is collected from a crime scene, it is of paramount importance to preserve the DNA for analysis. However, it is often of vital importance to determine the biological source of the DNA.
- To make optimal use of this evidence, the area(s) of the exhibit most likely to yield DNA should be examined, involving locating and identifying body fluids.
- Traditional body fluid identification methods are not always conclusive and can yield false positives. Not all body fluids can be identified at present e.g. vaginal fluid
- Human body fluids differ dramatically in function and composition. As protein expression is tissue dependent proteomics would be a viable approach to show variation between fluid types and discover biomarkers to enable body fluid identification.

Aims

- Identify protein spots individual to body fluid types.
- Characterise protein spots by Mass spectroscopy.
- Use these proteins to develop a body fluid identification assay.

Methods

- Body fluid samples were collected from volunteer donors.
- Body fluids studied were vaginal fluid, semen, and saliva.
- Samples were pooled according to physical factors thought to have an effect upon protein expression (figure 1).
- Samples within each pool were run in duplicate using pH 5-8 IPG strips and 12% polyacrylamide gels.

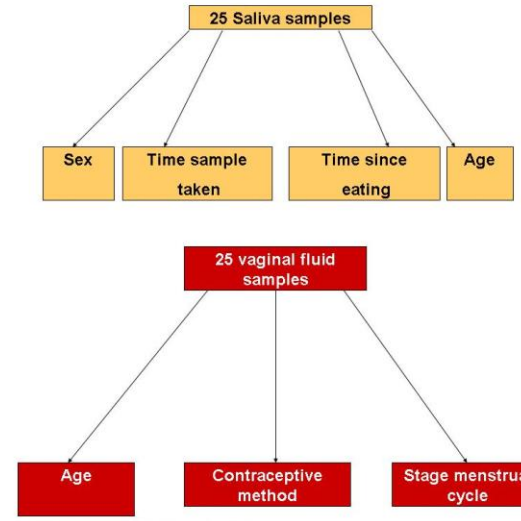

Figure 1. Schematic diagram of sample pools

Results

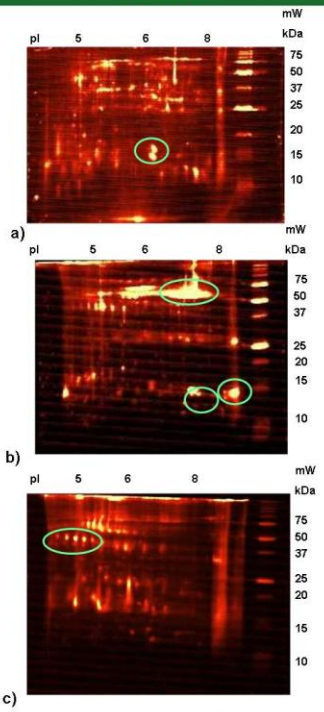

Figure 2. Two-dimensional gel images of a) vaginal fluid b) saliva and c) semen using pH5-8 IPG strips and 12% polyacryalamide gels stained with SYPRO Ruby gel stain. Proteins with body fluid type expression are highlighted in green.

Discussion

- Initial observations show that the individual sample pools show similar 2D protein patterns for each sample type.
- Clear differences can be seen between sample types (figure2).
- Each sample pool displays some differences in expression levels of individual proteins.
- These differences will be examined in more detail with additional analysis.

Conclusion

- Each body fluid type showed variation in the proteomic profile produced.
- Computational analysis of the gels will aid in biomarker identification for each fluid type.
- The results confirm that it is possible to use 2D electrophoresis to obtain distinct patterns from body fluids.
- It should be possible to identify proteins that can be used as markers for these fluids.

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University of Teeside Middlesborough.
A comparison of saliva detection methods.

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α-Amylase has traditionally been used to detect saliva stains using techniques such as Phadebas paper and the Iodine reaction. These tests cannot distinguish between isoforms of α-Amylase and hence cannot differentiate between salivary and pancreatic amylases. Recent developments have seen the introduction of detection kits which claim to offer a greater sensitivity and specificity; an immunochromatographic membrane strip test manufactured by Independent Forensics using antibodies specific to human salivary amylase, and the SALIgAE test from Abacus Diagnostics.

In this study we compared these kits with Phadebas paper, by analysing human body fluids such as saliva, semen, urine, blood, menstrual blood, sweat and vaginal secretions, saliva from several animals and mock casework samples as well as two commercially produced α-Amylase products. The data showed that the immunochromatographic strip test was the most sensitive yet showed some cross reactivity with sweat and semen stains. The SALIgAE test showed cross reactivity with urine and pancreatic α-Amylase. We concluded that neither of these tests demonstrate the specificity claimed.

A comparison of saliva detection methods

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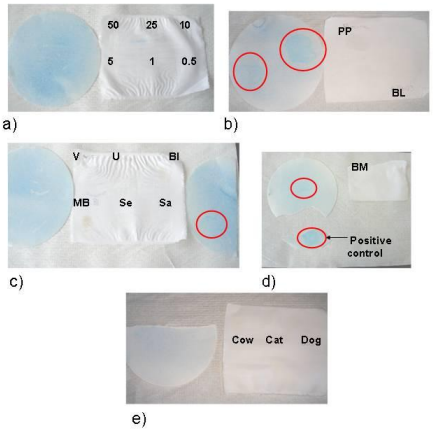
Background

- α-Amylase has traditionally been used to detect saliva stains using techniques such as Phadebas® paper and the Iodine reaction.
- These tests cannot distinguish between isoforms of α-amylase and hence cannot differentiate between salivary and pancreatic amylases.
- Recent developments have seen the introduction of detection kits which claim to offer a greater sensitivity and specificity; RSID® (independent forensics) SALIgAE® (Abacus diagnostics).

Aims

- To compare three modern techniques for saliva identification, RSID® SALIgAE® and Phadebas®.
- To identify the limits of saliva detection for each method.
- To test the specificity of each method comparing other human body fluids and animal saliva samples.
- To test the specificity of each method with commercially available α-amylases

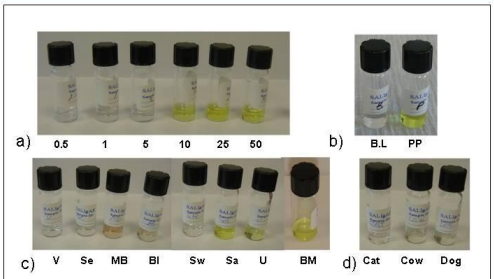
Methods & Results



Phadebas test results for a) sensitivity of saliva detection (µl). b) commercial α-amylases. c) & d) human body fluids cross reactivity and e) animal saliva cross reactivity.

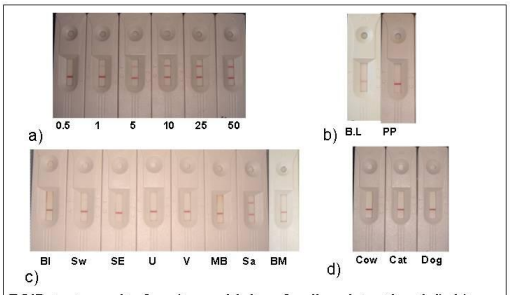
Figure 1. Phadebas test

Key			
V- Vaginal fluid	U – urine	Bl- Blood	MB – menstrual blood
Se – semen	Sa- Saliva	Sw- Sweat	BM – breast milk
PP – Porcine pancreatic amylase	BL – Bacillus Lichenformis amylase		



SALIgAE test results for a) sensitivity of saliva detection (µl). b) commercial α-amylases. c) human body fluids cross reactivity and d) animal saliva cross reactivity.

Figure 2. SALIgAE test



RSID test results for a) sensitivity of saliva detection (µl). b) commercial α-amylases. c) human body fluids cross reactivity and d) animal saliva cross reactivity.

Figure 3. RSID test

Discussion

- The detection limit of RSID® was shown to be 1 µl of saliva (figure 3a) which was 10 x as sensitive as the SALIgAE® test (figure 2a). Phadebas® paper was unable to detect 50 µl undiluted saliva on fabric (figure1a).
- The RSID® kit showed no immediate cross-reactivity with other human body fluids (figure 3c). After 10 minutes a weak positive result was observed on the sweat and semen sample strips. The SALIgAE® kit showed false positive results for urine and breast milk (figure 2c). Phadebas® paper also produced a weak positive result with the breast milk sample (figure 1d).
- No cross reactivity was observed with any of the tests with animal saliva samples (figures 1e, 2d and 3d).
- The RSID® kit showed no cross reactivity with the commercial amylases (figure 3b). The SALIgAE® test showed a false positive for porcine pancreatic amylase (figure 2b). Both commercial amylases gave positive results with Phadebas® paper (figure 1b).

Conclusion

RSID showed no cross reactivity with any of samples and also was found to be the most sensitive of the three tests.

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