Refinement of analytical technologies for detection of biomolecules of importance to the dairy sector

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Abstract

There is a continuous push on the dairy industry to enhance livestock productivity to meet with the demands of ever increasing human population. This demand can be achieved by developing rapid and early diagnostic aids to help curtail various problems encountered in the livestock production. The first study of the present thesis was focussed on standardizing initial steps towards development of Surface Plasmon Resonance for progesterone and oestradiol 17-β, both of which are critically implicated in animal reproduction. For progesterone, the binding response of two different length linkers, and the antigen-antibody binding response of two different source monoclonal antibodies (P1922 from Sigma vs SE-7720-1430 from Serotec) were evaluated. It was concluded that the long length linker had better binding response than the short length linker. The antibody obtained from Serotec (SE-7720-1430) had greater sensitivity but its binding response was inconsistent. On the other hand, the sensitivity of the monoclonal antibody from Sigma (P1922) was lower, although its binding response was consistent. For oestradiol 17-β, antibody procured from Bio-trend (BT70-1020-06) was tested and its binding response was consistently low on all the test days. This study thus suggests that careful testing and selection of antibodies to achieve desired antigen-antibody binding response is a critical step towards development of SPR for progesterone and oestradiol 17-β. The second study was undertaken to refine the currently existing fluorescent techniques to measure phytoporphyrin in the peripheral circulation of cows. Phytoporphyrin is implicated in facial eczema (FE), which is a photosensitization disease of high economical importance. This occurs due to disturbances in the chlorophyll metabolism as a result of liver damage and bile duct occlusions caused by fungal

toxicity. The present study described new modified fluorescent methods to measure phytoporphyrin in the serum of cows. Further, the absorption and emission spectra of phytoporphyrin were compared with that of other chlorophyll metabolites and thus a currently existing anomaly in the chemical structure of phytoporphyrin was rectified.

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Publications

Campbell, W. M.; Dombroski, G. S.; Sharma, I.; Partridge, A. C.; Collett, M. G. 2010: Photodynamic chlorophyll a metabolites, including phytoporphyrin (phylloerythrin), in the blood of photosensitive livestock: Overview and measurement. *New Zealand Veterinary Journal,* **58**: 146-54.

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List of Abbreviations

4TPH-P4	6-[3-[(pregn-4-ene-3, 20-dione-4-yl) thiopropanoyl] – amino] hexanoic acid
4TP-P4	3-(pregn-4-ene-3, 20-dione-4-yl) thiopropanoic acid
ALP	alkaline phosphate
AST	aspartate transaminase
CL	corpus Luteum
^{o}C	degree celsius
DMSO	dimethyl sulfo-oxide
E2	oestradiol 17-β
E2,C3	3 (propanoic acid)-17 β hydroxyl 1, 3, 5 (10) estratriene
EDC	N-ethyl-N-(3-dimethylaminopropyl)-carbodimide
ELISA	enzyme-linked immunosorbent assay
FC	flow cell
FE	facial Eczema
FSH	follicular stimulating hormone
GDH	glutamate dehydrogenase
GGT	γ-glutamyltransferase
GnRH	gonadotrophin releasing hormone
ID	Identification
L	litre
LH	luteinizing hormone
LOD	limit of detection
min	minute (s)
NHS	N-hydroxysuccinimide
NMR	nuclear magnetic resonance
OEG	oligoethylene glycol
OVA	ovalbumin
%	percent
P4	progesterone
RIA	radio-immuno assay
RU	response unit
sec	second (s)
SPR	surface plasmon resonance
vs	versus

Chapter 1 - Introduction

1.1 Economic impact of the dairy sector

The dairy sector contributes significantly to many economies around the globe. In New Zealand, dairy farming and animal production is the backbone of the national economy, with products of animal origin contributing around 50% of total exports (Encyclopaedia of the Nations, 2010), whereas in many fast developing economies, dairy farming is the source of livelihood for millions of farmers. Thus, there is a continuous push to enhance dairy productivity to meet the demands of ever increasing populations.

As part of that push, the focus of this thesis is toward the development of small molecule diagnostics for detecting the steroids associated with the oestrous cycle, and the chlorophyll metabolites associated with facial eczema (FE).

1.2 Steroids in the oestrous cycle

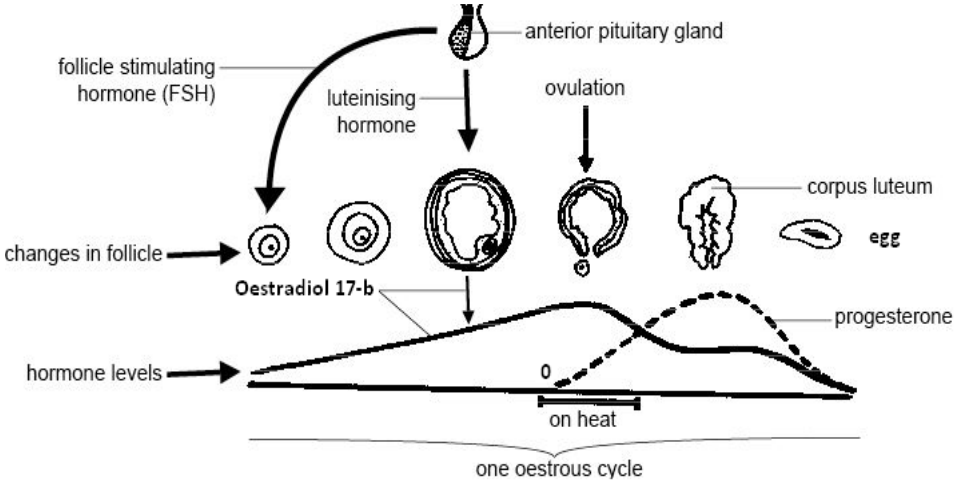


Figure 1.1. Concentration profile of progesterone and oestradiol during oestrous cycle (Posthuma-Trumpie *et al.*, 2009).

In a dairy herd, regular production of healthy calves is required to achieve the desired milk productivity, and to maintain adequate numbers of animals for replacement stock. This can be achieved by maintaining the cows in regular reproductive cycles, and adopting efficient and economical measures to curtail health and production disorders. One such measure includes the development of fast and reliable diagnostic tools to detect the right time of oestrus for insemination, and to evaluate the success or failure of insemination. Failure to achieve optimal timing of insemination results in poor conception rates, and renders animals infertile for a longer period of time.

The oestrous cycle is a highly coordinated biological event controlled by the interaction between the hypothalamus, pituitary and ovarian tissue (Figure 1.1). At the onset of puberty, the hypothalamus secretes gonadotrophin releasing hormone (GnRH), which acts on the pituitary to secrete follicular stimulating hormone (FSH) and luteinising hormone (LH). FSH acts on the granulosa and theca interna cells of the developing follicle (McNeilly *et al.*, 1991) and stimulates the ovarian cells to produce oestrogens. Of these, oestradiol 17-β has the highest activity and is of potential clinical use as an indicator for the time of mating (Noakes, 2001). After a follicle becomes fully matured, the concentration of oestradiol 17-β reaches a maximum, and exerts a positive feedback upon the pituitary body that results in a surge of LH being released and which results in ovulation and formation of the corpus luteum. Under the influence of LH, the secretory activity of the theca interna and theca externa cells shifts from oestradiol to progesterone (Scaramuzzi *et al.*, 1993). Progesterone concentration begins to increase after ovulation as at this stage the corpus luteum becomes active. During the luteal phase, the concentration of progesterone increases whilst that of oestradiol 17-β remains low until the development of the next wave of large follicles. If the animal has not conceived,

oxytocin is released from the corpus luteum which induces luteolysis by releasing prostaglandin F_2 alpha from the uterus (Flint *et al.*, 1989; Wathes *et al.*, 1989; Scaramuzzi *et al.*, 1993) and, hence, the concentration of progesterone falls. Thus, the follicular development is self-regulated by negative and positive feedback loops between the ovary and the hypothalamic-pituitary system (Baird *et al.*, 1991; McNeilly *et al.*, 1991).

The peripheral concentration of progesterone has been reported as a reliable indicator of ovarian activity and reproductive status in animals (Foote *et al.*, 1979; Waldmann, 1999). It is very low during pro-oestrus and oestrus as progesterone has inhibitory role on the oestrus behaviour. This means that once the concentration of progesterone reaches above the threshold level, the behavioural manifestation of oestrus disappears even if the hormone causing oestrus i.e. oestradiol is present in the optimal concentration (Allrich, 1994). Therefore, oestradiol 17-β and progesterone both play a critical role during the oestrous cycle and subsequently in pregnancy (Abeyawardene and Pope, 1990). An optimum concentration and ratio of these steroids is essential to ensure the manifestation of behavioural symptoms of oestrus and subsequent fertilization (Noakes, 2001).

1.2.1 Current methods for steroid detection

There are a number of common methods used on-farm for detecting oestrus; including observation of behavioural symptoms, use of heat mount detectors, pedometers, monitoring of electrical impedance of the genitalia and use of teaser males (Lehrer *et al.*, 1992; Lopez *et al.*, 2002). Whilst all of these methods have been used with greater or lesser degrees of success, all suffer from limited accuracy, the need for repeated handling of animals, or a high level of operator/observer skill.

Moreover, animals can have weak oestrus behaviour and, hence, the detection of oestrus becomes difficult (Yoshida and Nakao, 2005).

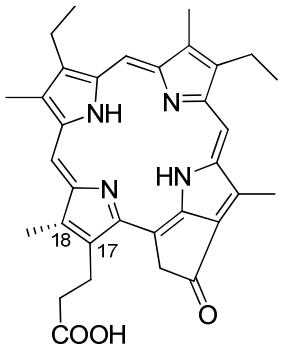
The current laboratory based methods used to evaluate the reproductive status of the animal include measuring the concentrations of oestradiol 17-β and progesterone by radio-immuno assay (RIA) and enzyme-linked-immune-sorbent assay (ELISA). Although ELISA is much safer than RIA, since radioactive substances are not involved in the former, both of these traditional methods are labour-intensive. Moreover, both ELISA and RIA generally require that batches of samples are processed simultaneously (Posthuma-Trumpie *et al.*, 2009). Thus, these techniques are not practical for development of a rapid diagnostic for the reproductive state of an animal. Furthermore, the concentrations of these steroids, and in particular those of oestradiol 17-β, are at the limit of the sensitivity of these existing techniques.

1.2.2 Surface Plasmon Resonance

Surface Plasmon Resonance (SPR) bio-sensing, based on a Biacore X100 instrument, is based upon the perturbation of a plasmon resonance by the target analyte and the subsequent change in the angle of the reflected polarised light. Its advantages over currently existing immunoassay techniques such as ELISA and RIA include: the speed of the response, label-free detection methodology and the detection of the bimolecular interaction in real time. The plasmon resonance occurs on a two dimensional glass/metal (gold or silver) interface, onto which the capture material is appended. Chapter 2 gives a more detailed description of the construction of the sensor and its application to steroid detection.

1.3 Facial Eczema

Facial eczema (FE) in dairy animals is caused by ingestion of a fungus during grazing, which releases the toxin spordesmin A and causes irreversible liver damage. This damage consequently hampers the metabolism of chlorophyll and results in the accumulation of its metabolites, predominantly phytoporphyrin, in the circulatory system of the cow. Phytoporphyrin (Figure 1.2) is known to cause skin cell eczema in the presence of light (Morris *et al.*, 2004; Collett *et al.*, 2008), and diseased animals become anorectic and experience great difficulty in ingestion.



Phytoporphyrin
$C_{33}H_{34}N_4O_3$
Exact Mass: 534.26
Mol. Wt.: 534.65

Figure 1.2. Chemical structure of the phytoporphyrin.

1.3.1 Phytoporphyrin detection

Earlier studies have described the use of florescence spectroscopy to detect phytoporphyrin levels in blood. However, there is a considerable inconsistency in the literature regarding a standard methodology for giving reproducible results. Moreover, there exists an ambiguity in the literature regarding the chemical structure of phytoporphyrin (Rimington and Quin, 1934; Moss, 1987; Scheie *et al.*, 2002; Ashby *et al.*, 2003; Scheie *et al.*, 2003a).

1.4 Thesis outline

The aim of this thesis was to develop analytical methods to detect steroids using SPR, and phytoporphyrin using fluorescence spectroscopy.

This includes:

- Chapter 2 comprises an introduction to SPR, and the development of an SPR based assay for progesterone and oestradiol 17-β. Due to the batch-to-batch variability of commercially available antibodies, a major emphasis of this chapter is a discussion on the importance of careful testing of antigen-antibody binding response, and optimisation of linker length prior to analysis.
- Chapter 3 is focussed on rectifying the existing ambiguity in the literature regarding the chemical structure of phytoporphyrin, and the development of a fluorescence based assay for phytoporphyrin detection. The outcome of this work is published in The New Zealand Veterinary Journal (Campbell *et al.*, 2010).
- Chapter 4 presents the conclusion of the thesis.

Chapter Two – Development of surface plasmon resonance steps for detection of steroids

2.1 Introduction and review of literature

2.1.1 Surface Plasmon Resonance

Surface plasmon resonance (SPR) is a surface–sensitive method used to detect bio-molecular interactions. The commonly-used surface plasmon resonance sensors are optical sensors that are based upon the principle of excitation of the surface plasmons (Phillips and Cheng, 2007). A surface plasmon resonance occurs when a photon incident on a glass coated noble metal surface causes the electrons on the metal to oscillate as a plasmon and consequently generate an electric field. Any change in the chemical environment adjacent to the metal surface causes a change in the angle of the incident light, making possible to detect small mass change on the sensor surface (Homola, 2003). The advantages of SPR include label free detection, surface sensitive response and real time measurement (Phillips and Cheng, 2007).

The phenomenon of anomalous diffraction due to the excitation of surface plasmon waves was first described at the beginning of the twentieth century (Wood, 1902). In general, SPR includes three different types of optical systems to excite the plasmons from the sensor surface. These include systems with prisms, gratings and optical waveguides. The system of prism coupler is known as the ‘‘Kretschmann configuration’’ which forms the basis of the majority of recently-developed commercialised SPR sensors. In these sensors, a prism couples the p-polarised light on the surface plasmon (gold) film and reflects the light onto a photodiode detector (Homola *et al.*, 1999). In instruments with a grating coupler, light is reflected at the lower refractive index substrate. In instruments with optical waveguide couplers, the

SPR wavelength shifts are measured as a result of the bio-molecular interaction process. Nonetheless, all of these systems follow the direct, label-free and real-time measurement of changes in the refractive index at the sensor surface (Tudos and Schasfoort, 2008).

Surface plasmon resonance involves attachment of the interacting partner (ligand) on the surface of the sensor chip and passing of the interacting partner (analyte) present in the sample over the surface (Figure 2.1). This interaction of the analyte with the ligand produces a response which is proportional to the mass of analyte bound to the chip. In 1990, the first commercial SPR biosensor was launched by Biacore International AB. Since then, Biacore sensor technology has been further refined in terms of speed, throughput and accuracy (Homola *et al.*, 1999). Other commercial biosensors include biosensors by British Windsor Scientific, Nippon Laser and Electronics laboratory and Texas Instrument and Analytical μ-Systems (Homola, 2003).

The Biacore X100 SPR system comprises of a detector, light source, flow channel and sensor chip (Figure 2.1). A beam of light of appropriate wavelength that is incident on the metal surface (gold; approximately 50 nm thickness) passes through the prism at an angle bigger than the critical angle for total internal reflection. The incident light excites the free electrons on the metal surface and causes a wave-like oscillation of these electrons and thus produces an exponentially decaying evanescent wave. The amplitude of the wave decays exponentially with increase in distance from the interface (Stenberg *et al.*, 1991). This wave travels a short distance from the metallic film (the effective depth for the purpose of sensing is around 300 nm into the medium) (Mitchell, 2010), and therefore causes reduction in the intensity of the reflected light. At a selected wavelength and angles, the photons of

the light wave react with the free electron cloud of the metal film causing a drop in the intensity of the reflected light. The angle of maximum loss of reflected light intensity is called the resonance angle or SPR angle (Tudos and Schasfoort, 2008). This angle is quite sensitive for the refractive index of the sample near the metal surface. The adsorption of the biomolecules on the metal surface causes the change in the refractive index near the metal surface and thus results a shift in the resonance angle. This response (angular shift) is measured in the response unit (RU) and 1000 RU corresponds to an angle change of approximately ~ 0.1^0. In the Biacore system, 1 RU corresponds to 1 pg/mm^2 surface binding of the biomolecules. Whilst for the majority of proteins, binding of ~ 1 ng/mm^2 of protein at the dextran surface is required to induce a 1000 RU signal change (Stenberg *et al.*, 1991; Kim *et al.*, 2007).

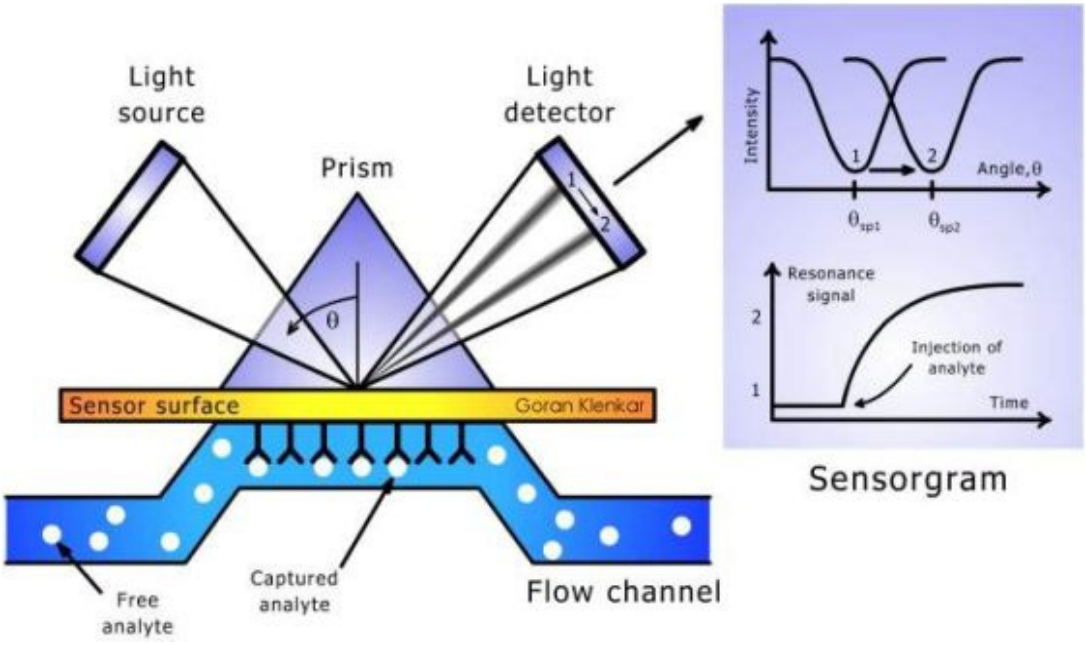


Figure 2.1. Representation of the Biacore Surface plasmon resonance phenomenon along with the change in the incident angle during the binding of the molecule on the surface.

The sample is supplied in a controlled manner to the sensor surface by a micro fluidic system which consists of a series of channels and valves in a plastic block (Integrated Micro fluidic Cartridge; IFC). The flow cell on the sensor surface forms when the sensor chip surface is pressed against a set of open channels on the surface of the IFC (GE Healthcare, 2008). Several types of sensor chips are used in the Biacore systems. Among the different sensor chips, CM5 is the most widely used sensor chip. It consists of a glass slide covered with the thin gold film (50 nm thickness) coated with a carboxymethylated dextran matrix layer (Kim *et al.*, 2007). The carboxymethylated dextran surface on the CM5 chip allows the covalent immobilization of molecules, with advantages including: high surface stability, high surface capacity, stability under a wide range of regeneration conditions, and providing a hydrophilic environment for bimolecular interaction (Kim *et al.*, 2007; Yuan *et al.*, 2007). Multiple use of the chip surface is also possible provided a careful regeneration of the surface after each measurement cycle has been performed. The ligand or analyte is immobilized onto the sensor surface and this can be achieved through electrostatic pre-concentration of the ligand. The pH for immobilization should lie between 3.5 and the isoelectric point of the ligand solution. Prior to immobilization, the sensor surface should be activated for covalent immobilization in order to establish a strong covalent bond between the ligand and the sensor chip surface and consequently to obtain high coupling yields. To achieve this, the carboxylic group of the carboxymethylated dextran surface is activated by using EDC/NHS ((1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (**1**))/N-hydroxysuccinimide (**2**)) as this results in the formation of reactive succinimide ester (Figure 2.2). These activated esters further react spontaneously with the amine and nucleophilic groups of the ligand (Schasfoort *et al.*, 2008).

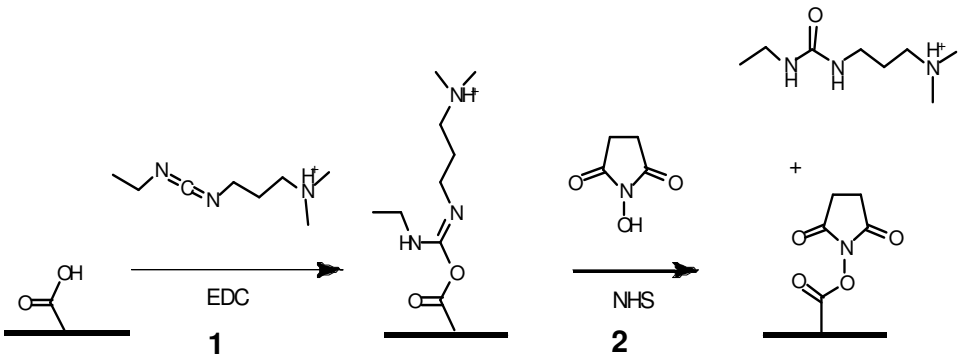


Figure 2.2. Activation of the sensor surface (Schasfoort *et al.*, 2008).

The applications of the SPR bio-sensing include rapid detection and identification of biological analytes, measurement of reaction kinetics, active concentration, affinity constant and thermodynamic information (Rich and Myszka, 2000; Homola, 2003). Moreover, the bio-molecular recognition for an analyte is achieved without any radioactive or fluorescence labelling (Homola, 2003). SPR is thus not only a powerful label-free detection method, but is also safer to use for various diagnostic procedures. Therefore, SPR can be considered advantageous over currently existing immunoassay techniques such as enzyme linked immunosorbent assay (ELISA) and radio immunoassay (RIA).

2.1.2 Steroids and oestrous cycle

Steroids are tetracyclic ring structured compounds (Figure 2.3). These are synthesized from cholesterol on the smooth endoplasmic reticulum (sER) and mitochondria of the cells of the adrenal cortex, gonads and placenta (Andrew, 2001). Steroids can be classified according to their structure or their biological actions. Steroids that are active in the reproductive system can be classified into three main structural categories: androstane, pregnane or oestrane ring structures (Figure 2.4),

reflecting biological activities as androgens, progestagens or oestrogens (Andrew, 2001).

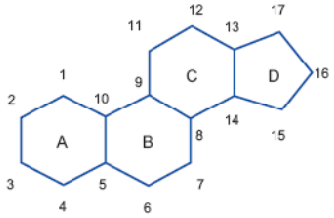
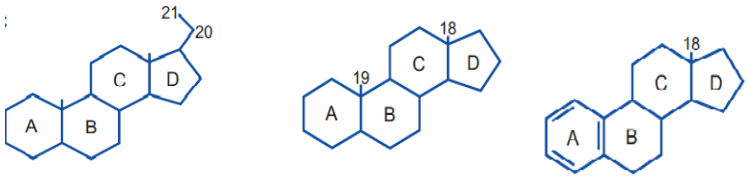


Figure 2.3. Basic molecular structure of the steroid (Andrew, 2001).



A (Pregnane) B (Androstane) **C** (Oestrane)

Figure 2.4. Chemical structure of different forms of steroids (Andrew, 2001).

Steroids play critical roles in development, sexual differentiation, growth, metabolism and reproduction (Meikle *et al.*, 2004). They are fat soluble and can pass directly through the cell membranes.

In animals, progesterone and oestrogen (Figure 1.1, 14 p) are the main steroid hormones involved in ensuring normal reproductive functions such as onset and manifestation of oestrus, fertilization and conceptus development (Conneely, 2001). The reproductive cycle (oestrous cycle) of the cow is normally of 21 days duration. Several biological events such as follicular growth, ovulation, formation of corpus

luteum and luteolysis occur during the oestrous cycle (Dhanda and Ravindra, 2005). The whole oestrous cycle is broadly divided into pro-oestrus, oestrus, metoestrus and dioestrus. The pro-oestrus (normally of 2 to 3 days) and oestrus (1 to 3 days duration) together are often termed as follicular phase as there is rapid follicular activity during this period. This is accompanied by an increase in the concentration of oestradiol 17-β in peripheral plasma. Whereas, metoestrus and dioestrus are collectively called as luteal phase due to presence of a functional corpus luteum (Noakes, 2001). During the oestrous cycle, two different peaks of oestradiol 17-β are observed in the blood plasma; one during oestrus (responsible for oestrus behaviour) and other 4-6 days later (smaller peak) which is due to secretion of oestradiol 17-β from the growing follicles. Similarly these two peak levels of oestradiol 17-β are also found in milk (Gyawu and Pope, 1983). Using radio-immunoassay studies in cattle, the concentrations of oestradiol 17-β in milk, plasma and in urine on Day 0 (onset of oestrus), were 84 ± 41 pg/mL, 31 ± 11 pg/mL and 39 ± 9 pg/mL, respectively. Subsequently, on Day 4 these concentrations in milk, plasma and urine had decreased to 34 ± 3 pg/mL, 14 ± 2 pg/mL and 8 ± 2 pg/mL, respectively (Monk *et al.*, 1975). Thus, the concentration of oestradiol 17-β can be used as a good indicator for detection of stage of oestrous cycle and potentially also the right time of insemination in order to achieve high conception rates.

In the cow ovulation occurs 12 h after the end of oestrus and results in the formation of corpus luteum which is the source of progesterone. The increase in the concentration of progesterone provides negative feedback upon the release of pituitary hormones. Thus, the progesterone-induced decrease in the plasma LH (luteinising hormone) pulse frequency means that LH concentrations are not sufficient to support further secretion of oestradiol 17-β (Abeyawardene and Pope,

1990) by developing follicles. The concentration of progesterone is very low from Day 1 to Day 3 of the oestrous cycle (Okuda *et al.*, 2001). It starts increasing from Day 4 to Day 12 in parallel with an increase in the size of corpus luteum, and then becomes constant until Day 16-18. If the animal does not conceive, luteolysis occurs, which results in a sudden decrease in the progesterone concentration (Rioux and Rajotte, 2004). Adequate concentrations of progesterone are required for preparation of uterus for implantation and subsequent development of conceptus (Bindon, 1971). The adequate concentration of progesterone is very important for successful pregnancy diagnosis as it is required for the survivability, implantation and early development of ovum and embryo (Khatun *et al.*, 2009). Therefore, oestradiol 17-β and progesterone (Figures 2.5 and 2.6, respectively) appeared the major hormones implicated at various stages of reproductive cycle in cows.

2.1.3 Measurement of progesterone concentration

Progesterone (CAS No.57-83-0) is a non-aromatic reproductive steroid hormone having empirical formula $C_{21}H_{30}O_2$ and molecular weight 314.46 (Chemical structure, Figure 2.5). Due to its small molecular weight, it is weakly antigenic unless complexed with an immunogenic carrier.

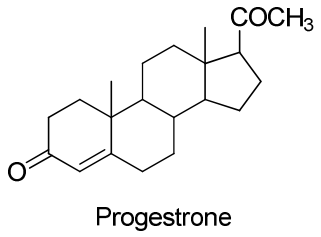


Figure 2.5. Chemical structure of progesterone.

The change in concentration of progesterone in milk can be used to study ovulation failure, abnormal secretion of progesterone and ovarian luteal function (Gyawu and Pope, 1983). Studies have shown an increased rate of conception if the insemination of an animal is performed when the concentration of progesterone in milk is less than 5 ng/mL (Laitinen and Vuento, 1996). In a study, Dobson and Fitzpatrick (1976) reported a strong positive relationship between the concentrations of progesterone in milk and blood (e.g., r=0.88). The majority of the procedures used for estimation of progesterone in serum are based upon ELISA and RIA (Bosch *et al.*, 1978; Deshpande, 1996; England and Concannon, 2002), in which immobilized antibody required to be discarded after single use. In addition, high performance liquid chromatography is also used by using ethanol–water solvent binary mixtures as mobile phase and by achieving the thermostatization of the column to 40^0C (Pucci *et al.*, 2003).

Studies have also measured the progesterone concentrations in milk using different biosensor techniques. Hart *et al.* (1996) used a screen printed amperometric sensor by immobilizing the monoclonal antibody on the sensor surface of screen–printed carbon electrode was used. However, the test was unreliable for milk samples as the interferent within the milk oxidized near the operating potential of the optical sensor. In another study, the enzyme substrate 4-aminophenylphosphate (which oxidises at a lower potential (+2VvsSCE)) was used. This minimized the interference from the milk sample and therefore allowed the measurement of progesterone concentration up to 5 ng/mL (Pemberton *et al.*, 1998). Further, naphthyl phosphate as an enzyme substrate was used and this allowed the detection limit between up to 2 ng/mL (Pemberton *et al.*, 2000). Carralero *et al.* (2007) developed an amperometric immunosensor to quantify progesterone using a colloidal gold–graphite–Teflon–

tyrosinase system. However, this sensor was unable to deliver on-the-spot diagnosis owing to its cumbersome procedure and longer testing time. An inhibition assay for progesterone in bovine milk was developed using a Biacore SPR instrument. The limit of detection was 3 ng/mL, which was later improved to 0.4 ng/mL (Gillis *et al.*, 2002; Gillis *et al.*, 2006). Furthermore, Arevalo *et al.* (2009) developed an electrochemical immunosensor coupled to a glassy carbon electrode and integrated to a microfluidic system to quantify low concentrations of progesterone in bovine serum.

The limit of detection (LOD) of a molecule in SPR primarily depends upon the sensor signal produced by antigen-antibody binding. Therefore, a weak antigen-antibody response or the nonspecific binding arising due to the presence of various other molecules in a biological sample or matrix component can interfere with the generation of SPR signals. This is because the bio-molecular recognition may exhibit the cross sensitivity to structurally similar non-target molecules. Further, a higher concentration of non-target molecules compared to that of the target molecule can also conceal the actual response produced by the target molecule (Homola, 2003). Moreover, the signal strength corresponds to the change in the refractive index, which can also vary due to a change in the concentration or composition of the buffer, or adsorption of the material on the sensor surface. To minimize this variation, recent studies have suggested injecting the original buffer solution used for baseline injection (Schasfoort *et al.*, 2008). Another hurdle in achieving the required levels of detection required for bio-molecules such as steroids might be their smaller molecular size, since the SPR generated is proportional to the mass of the molecule binding to the surface (Mitchell *et al.*, 2005). Therefore, it is essential to conjugate, small steroid molecules with bigger protein conjugates to enhance the antigen-

antibody binding. For example, in the SPR study of Yuan *et al.* (2007), progesterone was conjugated to ovalbumin (OVA) linked with oligoethylene glycol (OEG) to form protein conjugate (P4-OEG-OVA), which was subsequently immobilized onto the mixed self-assembled monolayer surface. This not only increased the sensitivity of the assay but also lowered the limit of detection to 4.9 ng/L. Whilst attaching a large nanoparticle to the small molecule could resolve this problem, it can also create a steric effect which can block the approach of the small antigen to the antibody; thereby affecting the regeneration of the sensor surface (Yuan *et al.*, 2007).

Achieving an effective antigen-antibody binding is the key to achieve desired signal strength in a SPR protocol. The careful selection, handling and storage of antibodies are critically important. Previous studies have attempted different procedures to achieve effective antigen-antibody binding to increase the sensitivity of the assay. Claycomb *et al.* (1998) achieved 0.1 ng/mL as the limit of detection but reported the rapid degradation of antibody as a big hurdle in making the test practically viable. Subsequently, a nitrocellulose membrane with a pore size of 12 µm was used as the solid surface to coat the antibodies and the coated antibody was placed in a reaction chamber prior to the testing (Delwiche *et al.*, 2001). This allowed the measurement in milk during various phases of oestrous cycle viz. luteal phase (LOD 2 to 10 ng/ml) and follicular phase (LOD ‹1ng/ml), respectively. Subsequently, Sananikone et al. (2002) developed an optical sensor based on an antigen competition assay in which the nitrocellulose membrane and the monoclonal antibody was immobilized at one end of the membrane and the entire surface was blocked with bovine serum albumin. The conjugate progesterone was labelled with horse radish peroxidase and 3, 3'5, 5'-tetramethylbenzidine dihydrochloride with hydrogen peroxide was used as substrate. This allowed detection of progesterone ranging from 5 to 10 ng/mL in the buffer and

1 to 50 ng/mL in milk. But due to its high variability and a longer test time meant that this test could not be put into practice as a field test.

Recent studies have reported the attachment of nanoparticles to the antibody instead of antigen to enhance the sensitivity. Jiang *et al.* (2009) developed a SPR assay for estriol-16-glucorunoid without any prior treatment of the sample. In this study, the conjugate of the estriol-16-glucorunoid (E3-16G-OEGOVA) (estriol-16-glucorunoid conjugated to the oligoethylene linker and ovalbumin) was synthesized and immobilized on the CM5 Biacore biosensor sensor surface. After immobilizing the conjugate, different assays such as a direct binding assay and an inhibition assay were performed, using either the polyclonal antibody alone or by conjugating nanogold particles to the polyclonal antibody. The advantage of conjugating the antibody was that it required less usage of antibody. These authors reported an enhanced sensitivity and lowered limit of detection (from 76 pg/mL to 14 pg/mL) by using a low concentration of nanogold particle conjugated antibody.

The specific binding of the antibody to the conjugated steroid depends upon many factors such as the nature of steroid link to the protein carrier molecule, the position at which the linker is attached to the steroid molecule and the orientation of the steroid to the antibody (Gani *et al.*, 1994). Moreover, the antigen-antibody binding capacity might also be dependent upon the type of antibody and the source of its origin. In general, monoclonal antibodies are expected to yield better test results than are polyclonal antibodies. However, not many studies in the literature have reported the use of monoclonal antibodies for detection of steroids such as progesterone, and even where they have been used, results are still variable or the assay seems to be unpractical due to arduous protocols (Groves *et al.*, 1990; Waldmann, 1999). Furthermore, the batch to batch variation of the antibody, linker

length and the handling of sample prior to the analysis might also be the critical factors that can affect the development of SPR for a biomolecule. In immunoassay procedures such as ELISA, difficulties have been reported in achieving required antigen-antibody binding specificity and sensitivity for low immunogenicity molecules such as progesterone (Grover and Odell, 1977; Basu *et al.*, 2006; Khatun *et al.*, 2009; Shrivastav *et al.*, 2010;). In a recent study of direct ELISA, Shrivastav *et al.* (2010) tested the efficacy of different combinations of antibodies and enzyme conjugates to measure progesterone in the cow milk, and reported significant variation in the results arising due variability of these combinations to bind with progesterone and due to the presence of different related steroids in the milk.

There is, however, a paucity of the literature that has studied the effects of different sources of antibodies upon the antigen-antibody binding capacity and consequently to achieve the desired signal strength in a SPR protocol for progesterone.

2.1.4 Measurement of oestradiol 17-β concentration

Oestradiol 17-β (CAS No. 50-28-2) is an aromatic reproductive steroid hormone. Its empirical formula is $C_{18}H_{24}O_2$ (Figure 2.6) and molecular weight is 272.38. The main different forms of oestrogen present in the animal body are oestradiol 17-β, oestrone and oestradiol 17-α, however oestradiol 17-β is the most active form of oestrogen and is synthesized by the granulosa cells of preovulatory dominant follicle cell (Okuda *et al.*, 2001).

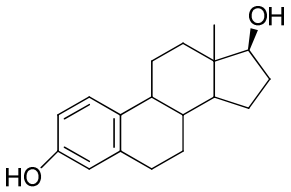


Figure 2.6. Chemical structure of oestradiol 17-β.

Oestrogens are readily soluble in milk; hence milk is a good medium in which to measure their concentration (Gyawu and Pope, 1983). A number of techniques have been reported for quantification of oestradiol: these include RIA, ELISA, gas chromatography–mass spectrometry, gas chromatography–tandem mass spectrometry, liquid chromatography–mass spectrometry, liquid chromatography–tandem mass spectrometry, fluorimetric enzymeimmunoassay, and enzyme inhibitor screening immunoassay (Snyder *et al.*, 1999; Huang and Sedlak, 2001; Farre *et al.*, 2007; Ou *et al.*, 2009). These methods have limitations, of which limited sensitivity and tedious and time consuming protocols are the most important. An electrochemical enzyme-linked immunosorbent assay for the measurement of oestradiol 17-β in extracted bovine serum has also been developed (Draisci *et al.*, 2000). In this test, the competition assay was performed in a conventional ELISA plate. After completion of the immunological and enzymatic reactions; the mixture contained in each well was injected into a flow injection analysis system coupled to an electrochemical cell. However due to a strong inhibition of the horse radish peroxidase enzyme, the test was not reliable for whole serum analysis. A screen-printed immunosensor for the detection of oestradiol 17-β in human serum extracts has also been described (Pemberton *et al.*, 2005). In another study a disposable electrochemical immunosensor for detection of oestradiol 17-β in non-extracted bovine serum was developed (Volpe *et al.*, 2006). In this experiment, the

immunosensor was developed using a polyclonal antibody and a screen-printed electrode as signal transducer. The whole procedure required small sample volumes and the total time for analysis was about 30 min (excluding time required for the precoating, coating and blocking steps).

In order to achieve efficient antigen-antibody binding and, consequently, greater signal strength, earlier studies attempted substitution at various positions on the oestradiol molecule for conjugation. Miyashita *et al*. (2005) used an inhibitive surface plasmon resonance assay, in which 17-β oestradiol–BSA conjugates were immobilized on SPR sensor chip and the binding achieved inhibition at concentrations of about 0.6–30 ng/mL. Mitchell *et al.* (2006) reported that oestradiol 17-β substituted at the 2 position (2-E2) is more sensitive than the corresponding 3 and 4 position analogues. The limit of detection using primary antibody alone in the SPR assay for oestradiol 17-β (2-E2) was 96 pg/mL, whereas after using a secondary antibody enhancement the LOD was 25 pg/mL. This is the lowest limit of detection so far achieved using an SPR assay for oestradiol 17-β. Mitchell *et al.* (2006) further reported 25 pg/mL as limit of detection for a surface plasmon resonance based oestradiol assay by covalently conjugating oligoethylene glycol linker to the oestradiol molecule. In a recent study, Ou *et al.* (2009) obtained 170 pg/mL as the lower limit of detection for oestradiol in serum by using a combination of SPR and a coupled online in-tube solid-phase microextraction system. However, as for progesterone, there is a dearth of studies that have examined the effects of different sources of antibodies on the antigen-antibody binding response.

2.2 Objectives

The present study was undertaken with the following objectives:

1. To study the SPR response of two different length linkers of progesterone 4 conjugate
2. To study the binding response of two different source monoclonal antibodies (P1922 from Sigma vs SE7720-1430 from Serotec) for small length linker progesterone 4 conjugate.
3. To study the binding response of monoclonal antibody BT70-1020-06 from Bio-trend for position 3 oestradiol 17- β conjugate.

2.3 Materials and Methods

2.3.1 Chemicals

Primary mouse anti-progesterone (SE7720-1430) monoclonal antibody (BGN/ 6-5E-10B IgG1) was obtained from Abd Serotec (Oxford, UK), and primary rat anti-progesterone (P1922) monoclonal antibody ($2H_4$) was procured from Sigma (Saint Louis, USA). Primary mouse anti-oestradiol 17-β (BT70-1020-06) monoclonal antibody was procured from Bio-trend (Chemikalien, GmbH, Germany). The amine coupling Kit (0.1 M N-hydroxysuccinimide (NHS), 0.4M N-ethyl-N-(3-dimethylaminopropyl)-carbodimide (EDC), 1 M ethanolamine hydrochloride pH (8.5) and HBS-EP+ running buffer pH 7.4 (10 mM HEPES (pH 7.4), 150 mM NaCl, 3 mM EDTA and 0.05% surfactant P-20) were purchased from Biacore International (Uppsala, Sweden). General reagents were diluted in Ultra pure Milli-Q water (Millipore Corporation, Billerica, MA, USA) and the progesterone for preparation of standards was cell culture tested (P-8783, ≥99% pure, Sigma).

2.3.2 Instrumentation

CM5 research-grade sensor chips were used for the immobilization and different measurements. The Surface plasmon resonance Biacore X100 was from Biacore AB (Uppsala, Sweden). All the measurements were carried out at 25^0C.

2.3.3 Preparation of the sensor surface for progesterone and oestradiol 17-β

2.3.3.1 Immobilization of progesterone-ovalbumin conjugates on the sensor surface

Two ovalbumin conjugates of progesterone namely progesterone substituted at 4-position and linked with thiopropanoic acid (4TP, P4 OVA (**1**)), and progesterone substituted at 4-position and linked with aminocaproic (6-amino hexanoic) acid (4TPH, P4 OVA (**2**)) (Figure 2.7) were synthesized (details in Appendix).

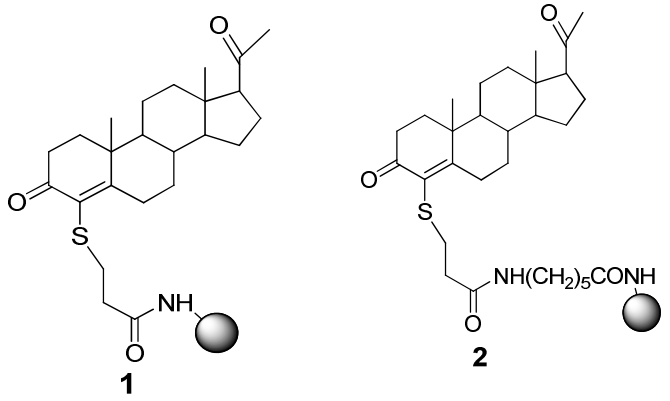
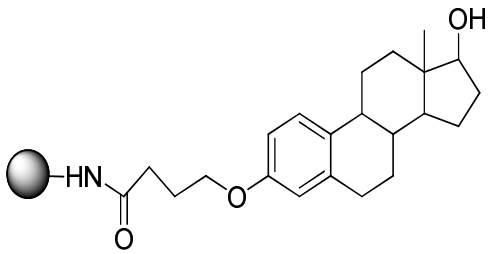


Figure 2.7. Different length linker (thiopropanoic acid (1) and (6-amino hexanoic acid) (2) of progesterone conjugated to ovalbumin (●) immobilized on the CM5 sensor surface: 4TP, P4 OVA (**1**), 4TPH, P4 OVA (**2**).

To assess the effect of the length of the linker, both of these progesterone-ovalbumin conjugates with different linker lengths (4TP, P4 OVA (**1**) and 4TPH, P4 OVA (**2**)) were immobilized covalently on the dextran layer of CM5 sensor surface using the same procedure. OVA (Ovalbumin) (2 mg/mL) was immobilized on Flow Cell 1 (the reference flow cell), whilst the progesterone-ovalbumin conjugate (5.5 mg/mL) was immobilized on Flow Cell 2, in accordance with the immobilization instructions of the instrument. To achieve this carboxyl groups of the carboxymethylated dextran layer were activated by 7 min injection of 70 µL, 390 mM EDC/100 mM NHS (mixed at a 1:1 ratio) at the rate of 10 µL/min. In Flow Cell 2, the progesterone-ovalbumin conjugate was immobilized using the target immobilization level of 10000 RU, at a rate of 5 µL/min (for 20 min). All conjugates were diluted in 10 mM sodium acetate solution (pH 4.0) and filtered through a 0.22 µm Phenomenex syringe filter (Phenomenex, Torrance CA, USA) to remove any insoluble material. Any remaining unreacted active ester groups were blocked by 7 min injection of 70 µL of 1 M ethanolamine hydrochloride pH 8.5 at the rate of 10 µL/min. The reference flow cell (Flow Cell 1) was immobilized in the same manner. Therefore, a sensor surface was produced having a difference of <1000 RU between the Flow Cell 2 and Flow Cell 1. For analysis of the results, the RU obtained in Flow Cell 1 was subtracted from that in Flow Cell 2 to eliminate nonspecific binding. Before starting binding studies, the surface of both flow cells was conditioned with ten successive injections of HBS buffer followed by a 20 µL injection of 50 mM NaOH (regeneration solution) at 10µL/min.

2.3.3.2 Immobilization of oestradiol 17–β ovalbumin conjugates on sensor surface



E2,C3 OVA

3

Figure 2.8. Oestradiol 17-β conjugated to linker propanoic acid and OVA (●) (E2, C3 OVA (**3**)) which was immobilized on the CM5 sensor surface.

Oestradiol 17-β ovalbumin conjugate (**3**) (Figure 2.8) was immobilized covalently on the dextran layer of CM5 sensor surface. The conjugate E2, C3 OVA (**3**) (100 µg/mL) was immobilized on Flow Cell 2 manually to an immobilization level of 6188.6 RU. In the same way, OVA (200 µg/mL) was immobilized on Flow Cell 1 (the reference flow cell) with an immobilization level of 6218.4 RU. This was achieved by adding separately E2, C3 OVA (**3**) and OVA to 10 mM sodium acetate solution (pH 4.0) and filtering through a 0.22 µm Phenomenex syringe filter (Phenomenex, Torrance CA, USA) to remove any insoluble material. Prior to the manual immobilization, the carboxyl groups of the carboxymethylated dextran layer were activated by 7 min injection of 70 µL of 391 mM EDC/100 mM NHS (mixed manually at a 1:1 ratio) at rate of 10 µL/min. In Flow Cell 2, E2, C3 OVA (**3**) conjugate was immobilized manually at 5 µL/min, total of 8 pulses in which the first 3 pulses were of 500 µg/mL and rest of the pulses were 100 µg/mL. The times for each pulse were; 1st pulse 60 sec, 2nd pulse 180 sec, 3rd pulse 360 sec, 4th pulse 60 sec, 5th pulse 420 sec, 6th pulse 180 sec, 7th pulse 360 sec, 8th pulse 420s sec and 9th pulse 180 sec, with a waiting time of 60 sec between each pulse. In Flow Cell 1,

OVA was also immobilized manually at 5 µL /min with a total of five pulses, where the first pulse was of 2 mg/mL whilst the rest of pulses were 200 µg/mL. The times for different pulses were: 1st pulse 60 sec, 2nd pulse 24 sec, 3rd pulse 24 sec, 4th pulse 24 sec, 5th pulse 60 sec, with a waiting time of 60 sec between each pulse. Any remaining unreacted active ester groups were blocked by 7 min injection of 70 µL of 1 M ethanolamine hydrochloride pH 8.5 at a flow rate of 10 µL/min. In this way, a sensor surface was produced having a difference (between FC2 and FC1) of <100 RU. To remove the nonspecific binding, the response from Flow Cell 1 was subtracted from that of Flow Cell 2. Prior to use the sensor surface in binding studies, the surface of both the flow cells was conditioned with ten successive injections of HBS buffer followed by a 20 µL injection of 50 mM NaOH (regeneration solution) at a flow rate of 10 µL/min. All the measurements were carried out at 25^0C.

2.3.4 Direct antibody–binding performance on the sensor surface for progesterone and oestradiol 17–β conjugates

2.3.4.1 Direct antibody–binding performance on the sensor surface for progesterone

Direct antibody binding assay using sigma monoclonal antibody (P1922)

Monoclonal antibody P1922 solution was serially diluted in the HBS-EP + running buffer and a plot of the response versus antibody concentration was measured by using single injection of 0, 1, 2.5, 5, 7.5,10, 20 µg /mL of antibody concentration on 4TP, P4 OVA (**1**) sensor surface. The flow rate for all sample injections was 30 µL/min for 2 min (60 µL), with a waiting time of 180 sec, before regeneration of the sensor surface using two pulses of NaOH (50 mM: each 20 µL, 10 µL/min).

Direct antibody binding assay using Serotec monoclonal antibody (SE7720-1430)

Monoclonal antibody SE7720-1430 was diluted as described for P1922 antibody. The antibody SE7720-1430, in concentrations ranging from 0 to 1 µg/mL (i.e. 0, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0 µg/mL), was injected in triplicate onto both of the CM5 sensor surfaces (4TP, P4 OVA (**1**) and 4TPH, P4 OVA (**2**)). Thereafter, 60 µL of sample was injected on the each sensor surface, with a waiting time of 180 sec before regeneration of the sensor surface using two regeneration pulses of NaOH (50 mM: each 20 µL, 10 µL/min).

2.3.4.2 Inhibitive immunoassays (standard curves) for progesterone conjugate using SPR

The inhibition assay was designed such that the antibody was mixed with a progesterone standard prior to injection over the sensor surface. The principle is that the progesterone present in the standard binds to the antibody, and residual antibody binds to the progesterone in the 4TPH, P4 OVA (**2**) conjugate that was immobilized on the sensor surface. The greater the amount of progesterone in the standard, the lower the quantity of antibody available to bind on the sensor surface and therefore lower the binding response.

Progesterone vs Serotec antibody

Standard progesterone solutions were prepared in HBS buffer at concentrations ranging from 0.2 to 200 ng/mL, (i.e. 0, 0.2, 2, 20, 200 ng/mL). To make the final concentrations (i.e. for progesterone 0, 0.1, 1, 10, 100 ng/mL and for antibody 0.4 µg /mL), 100 µL of each solution of progesterone (i.e. from 0.2 to 200 ng/mL) was incubated with an equal volume of antibody solution (0.8 µg/mL) in HBS buffer,

and from the resulting mixture, 105 µL was injected over the sensor surface for 120 sec at a flow rate of 30 µL/min. The regeneration of all biosensor surfaces was performed by two pulses of 50 mM NaOH, as previously described. The progesterone concentration scouting standard curves were obtained using OriginPro graphing software (OriginLab Corporation, Northampton MA, USA) Figures 2.12 (a and b).

2.3.4.3 Surface plasmon resonance analysis on sensor surface (4TP, P4 OVA (1))

There was variability in the response of antibody binding between each test day on the 4TP, P4 OVA (**1**) sensor surface. On close observation, the baseline of the 4TP, P4 OVA (**1**) sensor surface appeared to have been modified due to nonspecific binding on the sensor surface. To remove this, the regeneration of the 4TP, P4 OVA (**1**) sensor surface was attempted using different regeneration solutions: ((i) 50 mM NaOH, (ii) 100 M NaOH, (iii) glycine (pH 3.5), (iv) glycine (pH 2), (v) 10% CH_3CN+100 mM NaOH, (vi) 150 mM NaOH, (vii) 10% CH_3CN+150 mM NaOH, (viii) 200 mM NaOH, or (ix) 20% CH_3CN+200 mM NaOH). However, the baseline did not settle. Therefore, it was inferred that the baseline might have changed due to the deposition of salt on the sensor surface.

Hence, a new 4TP, P4 OVA (**1**) sensor surface, with the same short conjugate, was immobilized, this however also resulted in variable responses. Therefore the following alternative procedures were attempted to aid in understanding the observed results:

1. 50 µL aliquots (1 µg/mL) of the supplied stock solution (1 mg/mL) were prepared. These aliquots were tested for homogeneity using the SPR instrument and were found to be homogenous (data not shown). These aliquots were then frozen at -20^{0}C and were thawed immediately preceding

the analysis. The aliquots were also maintained on ice after removing from the freezer in order to avoid denaturing the proteins (antibody).

2. To avoid the adsorption of the antibody on the surface of the glass tubes; plastic tubes were used for serial dilution.
3. To test the homogeneity of the standard solution and adsorption of the antibody on the surface of the tube, ultrasonication with heating was attempted for two minutes immediately prior to the analysis.
4. Prior to use, all the plastic tubes were washed three times with buffer containing surfactant (HBS-EP+ running buffer pH 7.4 (10 mM HEPES (pH 7.4), 150 mM NaCl, 3 mM EDTA and 0.05% surfactant P-20)) and then thrice with Milli-Q water and subsequently dried in air. These tubes were then used for serial dilution of the antibody and also for subsequent experiments. This procedure was considered necessary to rule out any adsorption of antibody on to the surface of the plastic tubes.
5. The sensor surface was preconditioned using five injection cycles of 0.5 µg/mL and three injection cycles of 1 µg/mL antibody before progesterone concentration scouting.
6. A total of four blank runs were injected in between the actual sample run in order to keep the baseline stable (i.e. to remove any non-specific binding).
7. Fresh running buffer was filtered and then degassed daily prior to use in the experiment. This was done to curtail any problems arising due to buffer precipitation.
8. The process of system checking, calibration, desorption and sanitization was also performed to check any conformity of the surface plasmon resonance instrument.

9. The SPR machine was also found correct when tested using another antibody which was stable and consistent.

2.3.4.4 Direct antibody binding performance on the chip surface for oestradiol 17–β conjugate

Monoclonal antibody BT70-1020-06 solution was serially diluted in filtered and degassed HBS-EP+ running buffer. A plot of the response versus antibody concentration was measured by injecting 0, 5, 7.5, 10, 12.5, 15, 20 µg/mL concentrations of antibody on to the E2, C3 OVA (**3**) sensor surface. The flow rate for all sample injections was 30 µL/min for 120 sec each, with a waiting time of 180 sec before regeneration of the sensor surface which was achieved using two regeneration pulses of 50 mM NaOH.

2.4 Results and Discussion

2.4.1 Progesterone

2.4.1.1 Progesterone antibody binding assay

The results showed that a signal of 110 RU was achieved by using 0.5 µg/mL antibody concentration of SE7720-1430 monoclonal antibody (Serotec antibody), whilst a concentration of 5 µg/mL was required to produce the same signal by the P1922 monoclonal antibody procured from Sigma (Figures: 2.9 and 2.10(a)).

There are number of explanations for the difference in the antibody binding behaviours of the two antibodies:

1. It may result from the fact that the primary anti-progesterone monoclonal antibody (P1922; 2H4 IgG1) from Sigma was of rat origin, and the primary anti-progesterone monoclonal antibody (SE7720-1430; BGN/ 6-5E-10B IgG1) from Serotec was of mouse origin.

2. Another possible reason could be the different sources of the antibody procurements. The primary mouse anti-progesterone monoclonal antibody (SE7720-1430; BGN/ 6-5E-10B IgG1) was obtained from Abd Serotec (Oxford, UK) while primary rat progesterone monoclonal antibody (P1922; 2H4 IgG1) was procured from Sigma (Saint Louis, USA). Thus, this difference can also be attributed to a possible different protocol that might have been used to raise these antibodies by two different sources.

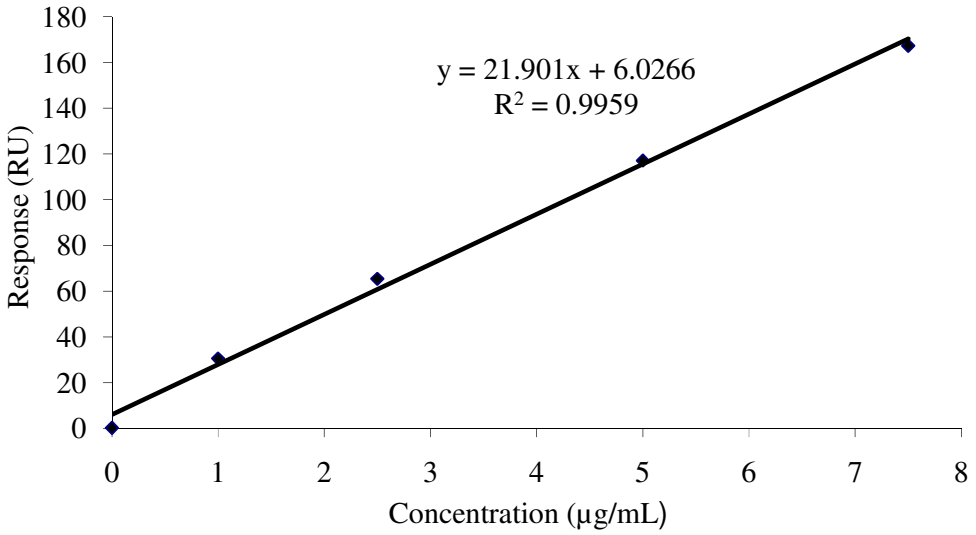


Figure 2.9. Binding assay of P1922 monoclonal antibody (Sigma) on sensor surface immobilized with short length linker progesterone conjugate (4TP, P4 OVA **(1)**).

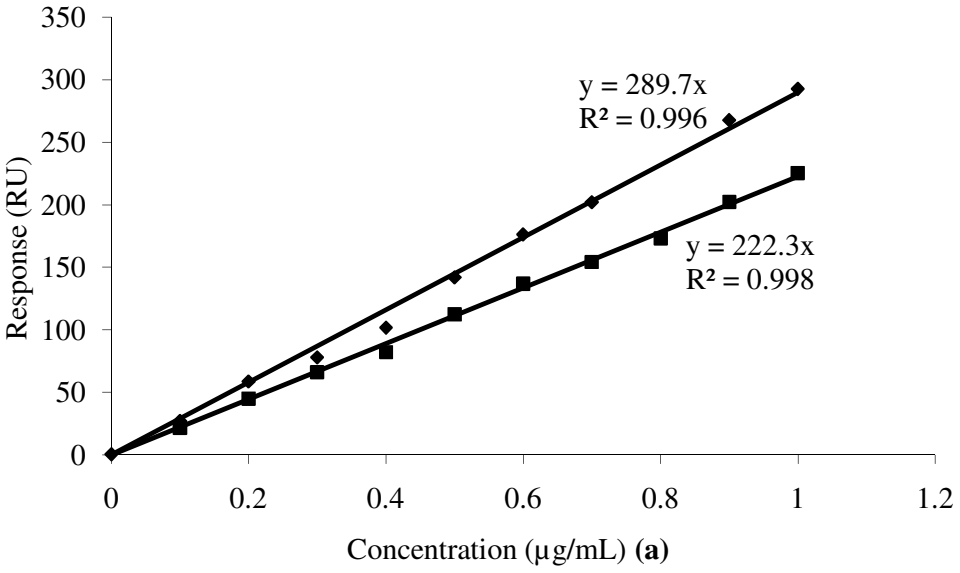


Figure 2.10 (a). Binding assay of SE7720-1430 monoclonal antibody (Serotec) on different days on the sensor surface immobilized with short length linker progesterone conjugate (4TP, P4 OVA **(1)**).

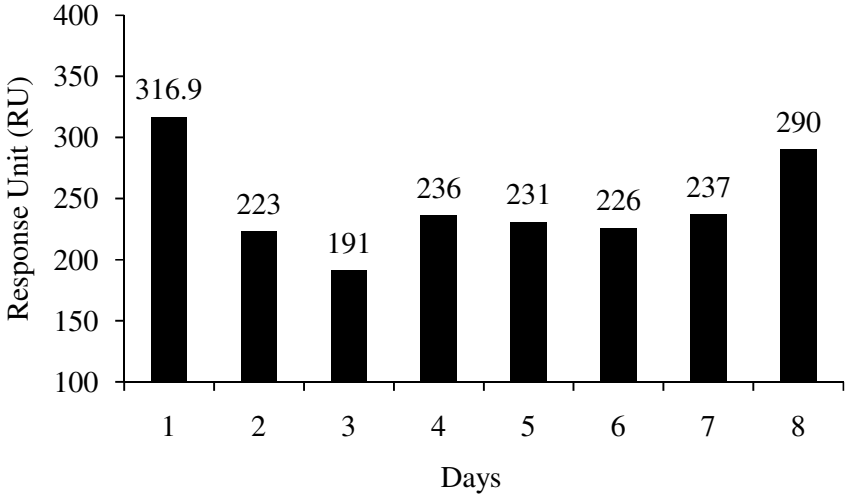
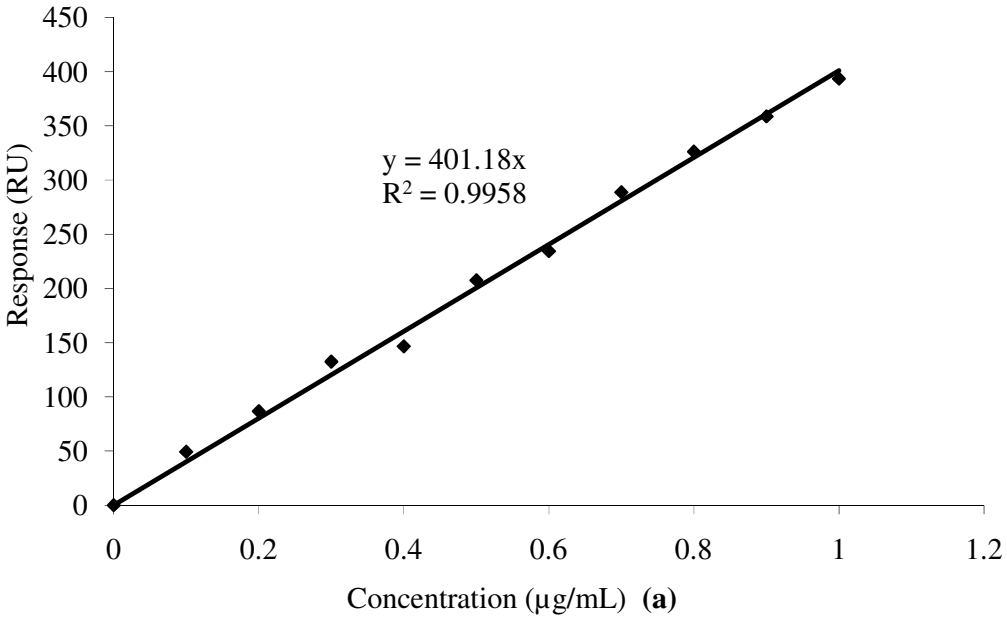
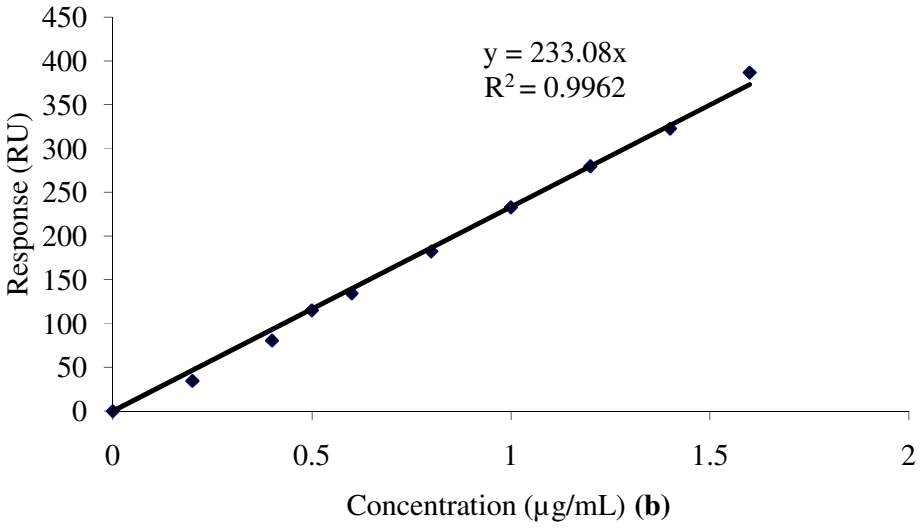


Figure 2.10 (b). Variation in SE7720-1430 (Serotec) mouse monoclonal antibody response on different days at 1 µg/mL.

Interestingly, although the primary mouse anti-progesterone monoclonal antibody (SE7720-1430), procured from Serotec was relatively sensitive; however, the response of this antibody was variable such that the percentage deviation (coefficient of variation) in the response on different test days at 1 µg antibody/mL concentration was 16% (Figure 2.10 (b)). Thus, the response of the antibody binding assay was linear on the same day; it was variable on different test days (Figure 2.10(a)).

Progesterone is a small molecule and hence is weakly immunogenic and therefore might require highly specific monoclonal antibodies. Further, it is emphasised that a very low concentration of the SE7720-1430 monoclonal antibody (Serotec antibody) was used compared to the P1922 monoclonal antibody (Sigma antibody). Therefore this inconsistent behaviour of the former antibody on both progesterone conjugate chips could be due to its low concentration. It further shows that the progesterone is a weakly immunogenic molecule**.** Therefore, this warrants testing the binding of this molecule by altering the assay conditions in future studies such as the concentration of antibody and incubation time. This suggests procuring of a highly immunogenic antibody from another source in such future studies. The present research thus highlighted the difficulty in achieving a consistent antigen-antibody binding response for progesterone.

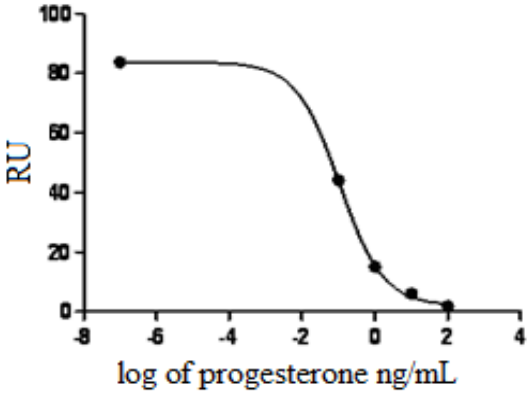




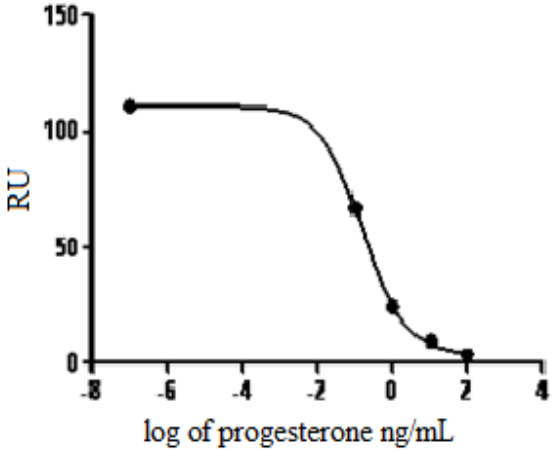
Figures 2.11 (a and b). Binding assay of SE7720-1430 monoclonal antibody (Serotec) on sensor surface immobilized with: (**a**) long length linker progesterone conjugate 4TPH, P4 OVA **(2),** and (**b**) short length linker progesterone conjugate 4TP, P4 OVA **(1)**, respectively.

The antibody binding response for long length linker progesterone conjugate (4TPH, P4 OVA (**2**)) was around 207 RU at 0.5 µg/mL of antibody concentration (Figure 2.11 (a)). This was greater than the small length linker progesterone conjugate (4TP, P4 OVA (**1**)) which was around 110 RU at the same concentration (Figure 2.11 (b)) using a primary mouse anti progesterone (SE7720-1430) monoclonal antibody. This showed that the antibody response was increased by increasing the linker length. The present study clearly described a better antigen-antibody binding response by using a long length liker when conjugated at 4-position on the progesterone molecule. The protein conjugation is a convenient and widely used method for steroids to achieve solid phase immobilization (Wu *et al.*, 2002). However, this requires conjugation at specific sites by attaching the linkers of appropriate length to these conjugates to achieve maximum binding with the antibody. Earlier studies have reported that the capacity of antibody to bind with an antigen increases when a large protein antigen is attached to the molecule through a linker (Kennel, 1982). The results of the present study were in accord with those of earlier studies. For example, (Wu *et al.*, 2002) have shown that, in a rapid flow biosensor surface on a Biacore surface plasmon resonance instrument, the response rate for progesterone-4 conjugate in terms of its binding capacity with antibody increases with an increase in the length of the linker from 4 atoms to 11 or 18 atoms.

2.4.1.2 Standard curve for progesterone concentration scouting on sensor surface 4TPH, P4 OVA (2)



(a)



(b)

Figures 2.12 (a and b). Progesterone concentration scouting standard curve using a sensor surface immobilized with 4TPH, P4 OVA **(2)** at different test days.

The progesterone concentration scouting standard curves gave the expected sigmoidal shape, but the response at the lowest concentration (0.4 µg/mL) of SE7720-1430 monoclonal antibody (Serotec) on different test days was inconsistent. There was a marked variation, even at equal concentrations of both progesterone and

antibody, and hence this assay was not reproducible (Figures 2.12 (a and b)). Therefore it was not possible to make the reliable standard curve for the detection of concentration of progesterone. Thus, it was suggested that a using a highly specific antibody in further assays might be helpful in getting consistent results.

2.4.2 Oestradiol 17-β

2.4.2.1 Oestradiol 17-β antibody binding assay

The concurrent study in our laboratory has reported poor yield of oestradiol 17-β conjugated at position 2 to OVA following the protocol described earlier (Mitchell *et al.*, 2006). Hence, in the present study, oestradiol 17-β substituted in the 3 position (**3**) was used (Figure 2.8).

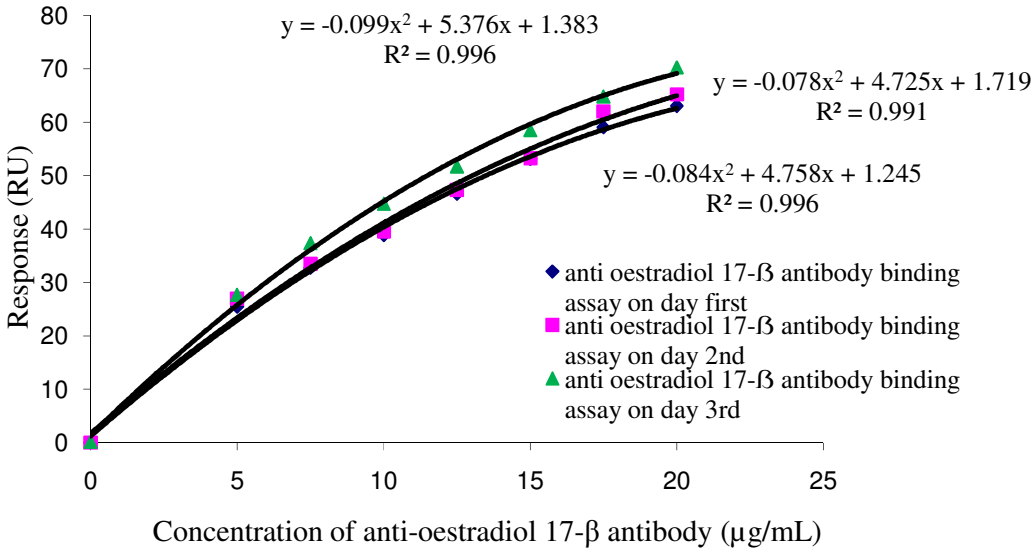


Figure 2.13. BT70-1020-06 monoclonal antibody binding assay on chip immobilized with E2, C3 OVA **(3)**.

A concentration of 20 µg/mL primary mouse anti-oestradiol 17-β (BT70-1020-06) monoclonal antibody produced a signal of 70 RU (Figure 2.13). On repeating the antibody concentration binding assay on different days, the antibody response for oestradiol 17-β was much less variable (6% coefficient of variation) than for progesterone antibody. This suggests that consistent response can also be achieved if the oestradiol is substituted at 3 position. However, the binding response of the antibody was not linear (Figure 2.13). This perhaps can be due to the less availability of the steroid conjugates for the antibody on the sensor surface, which can be due to the small length of the linker or can be due to the small amount of the steroid on the sensor surface.

From these results one can predict that the use of a long-length linker conjugated steroid may relieve the steric effects, and might better exposure of the steroid to bind with the antibody on the sensor surface.

Conclusion

In the present study, progesterone conjugates with different length linkers substituted at the 4-position on the progesterone molecule were immobilized on sensor surfaces of SPR followed by antibody binding assays. It was concluded that a long linker gave a better binding response, in terms of antigen-antibody binding efficacy, than a short linker. The result of variable response of different source antibodies in the present study not only highlights the importance of careful selection and testing of antibodies procured but also testing the antigen-antibody binding response of different source antibodies under different assay conditions such as different concentrations of the antibody and incubation time. This study thus indicates that achieving of effective antigen-antibody binding

response is a critical factor in achieving desired surface plasmon resonance for progesterone molecule.

Similarly, the result of antigen-antibody binding response of oestradiol 17-β, not only emphasize on testing other monoclonal antibodies from different sources, but also that a long-length linker might be of help in improving the binding response by relieving the steric effect on the sensor surface.

It is suggested that care must be taken over sourcing the antibodies and their subsequent materials handling. Further studies are required to test more different source monoclonal antibodies, linkers and substitution position to optimise the surface plasmon resonance protocols for progesterone and oestradiol 17-β.

Chapter Three -Facial Eczema

3.1 Introduction

Facial eczema in cattle and sheep is a hepatogenous photosensitive disease which results in heavy economical losses to the farming community. Facial eczema (FE) primarily occurs during autumn in New Zealand (Collett *et al.*, 2008). The cause of facial eczema is a fungus, *Pithomyces charatarum,* which produces the Spordesmin A toxin. Even a low intake of sporidesmin A can cause marked liver damage and significantly reduce the milk production. The production losses due to FE in New Zealand have been variously estimated up to $126 million for sheep and beef cattle (Smith and Towers, 2002). The symptoms of FE include marked swelling of eyes, ears, and face, and hyperaemia of skin, udder and teats. Sometime a secondary bacterial infection occurring underneath the scab further aggravates the lesion. General treatment of facial eczema includes parentral administration of non-steroidal anti-inflammatory drugs (NSAIDs), antihistamines, topical application of zinc oxide ointment and even steroid injections (Smith *et al.*, 1978; Collett *et al.*, 2008).

3.1.1 Photosensitization

Photosensitization is characterised by abnormal blistering and sloughing of the skin due to sunlight sensitivity. In severe cases, permanent effects may be observed such as scarring of skin, deformed ears and blindness. Photosensitization is classified depending upon the source of photodynamic pigment viz. primary or Type I photosensitivity, Type II photosensitivity (aberrant endogenous pigment synthesis), Type III or secondary (hepatogenous) photosensitivity and Type IV photosensitivity facial eczema is a secondary or Type III photosensitivity (Anonymous, Merck & Co., 2008a). The source of sensitivity in primary photosensitization is external e.g. the

animal feed (Ivie, 1982)), and occurs when the photodynamic agent is absorbed either directly through the skin or the digestive tract. After distribution to the skin through the circulation, this photodynamic agent reacts with the light of appropriate wavelength and results in skin photosensitization reaction. Examples of the agents that cause primary photosensitivity are hypericin (from *Hypericum perforatum)* and fagopyrin (from *Fagopyrum esculentum).*

Secondary photosensitization or hepatogenous sensitivity (Figure 3.1) is the most common cause of photosensitivity in livestock. Examples of hepatogenous sensitivity include common bile duct occlusion, facial eczema and lupinosis (Mycotoxic Lupinosis) (Anonymous, Merck & Co., 2008a). In fact, a number of photodynamic agents are involved in inducing secondary photosensitization, although the chemical nature of many of them is still not fully known (Ivie, 1982). Chlorophyll metabolites are the primary photosenitizers in facial eczema, as shown in Figure 3.2, resulting in the formation of phytoporphyrin (earlier known as phylloerythrin) along with many other intermediate metabolic products such as pyropheophorbide-a and pheophorbide-a (Rimington and Quin, 1934; Louda *et al.*, 1998). In healthy animals, phytoporphyrin is absorbed into the circulation and undergoes conjugation in the liver and consequently excreted through the faeces (Tennant, 1998; Scheie *et al.*, 2002). However, in a sick animal with liver damage, phytoporphyrin is not excreted and accumulates in the liver and is then distributed through the circulation system to reach the skin, where it causes photosensitization. As little as 0.1 µg/mL of phytoporphyrin in the peripheral circulation is enough to induce the photosensitization in the animal (Ivie, 1982).

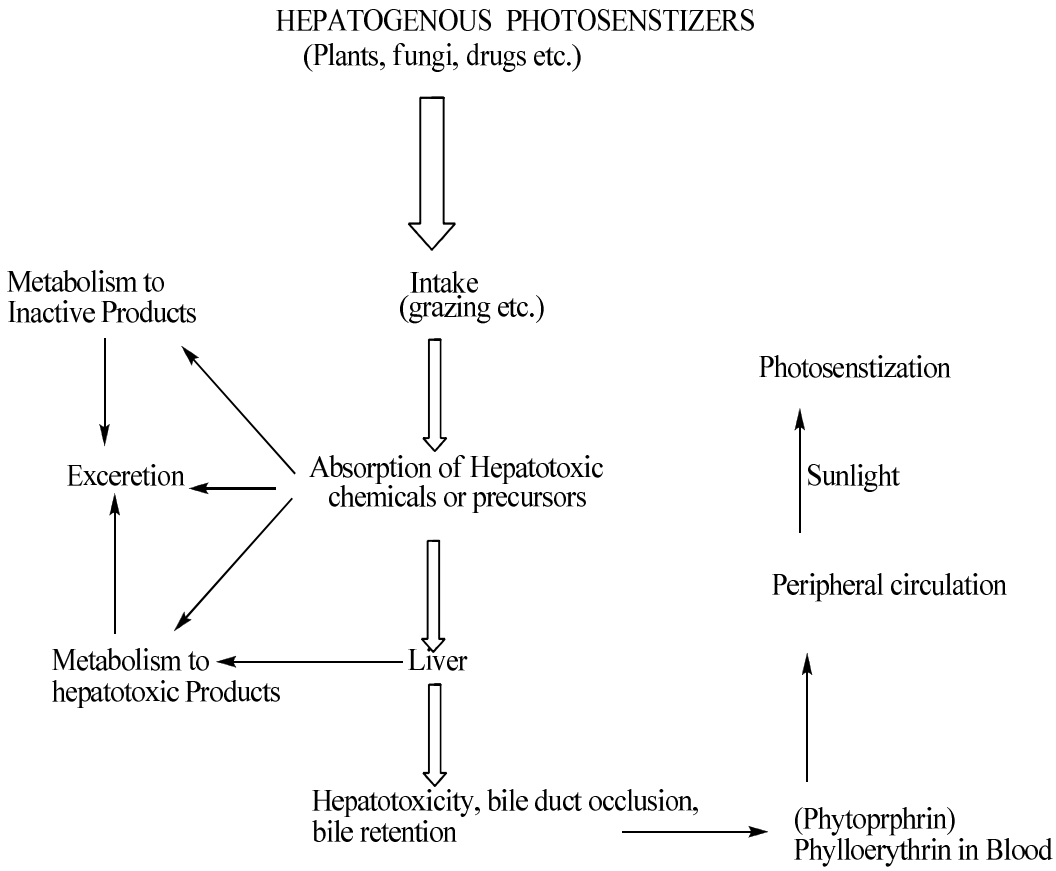


Figure 3.1. Diagrammatic representation of hepatogenous photosensitization (Ivie, 1982).

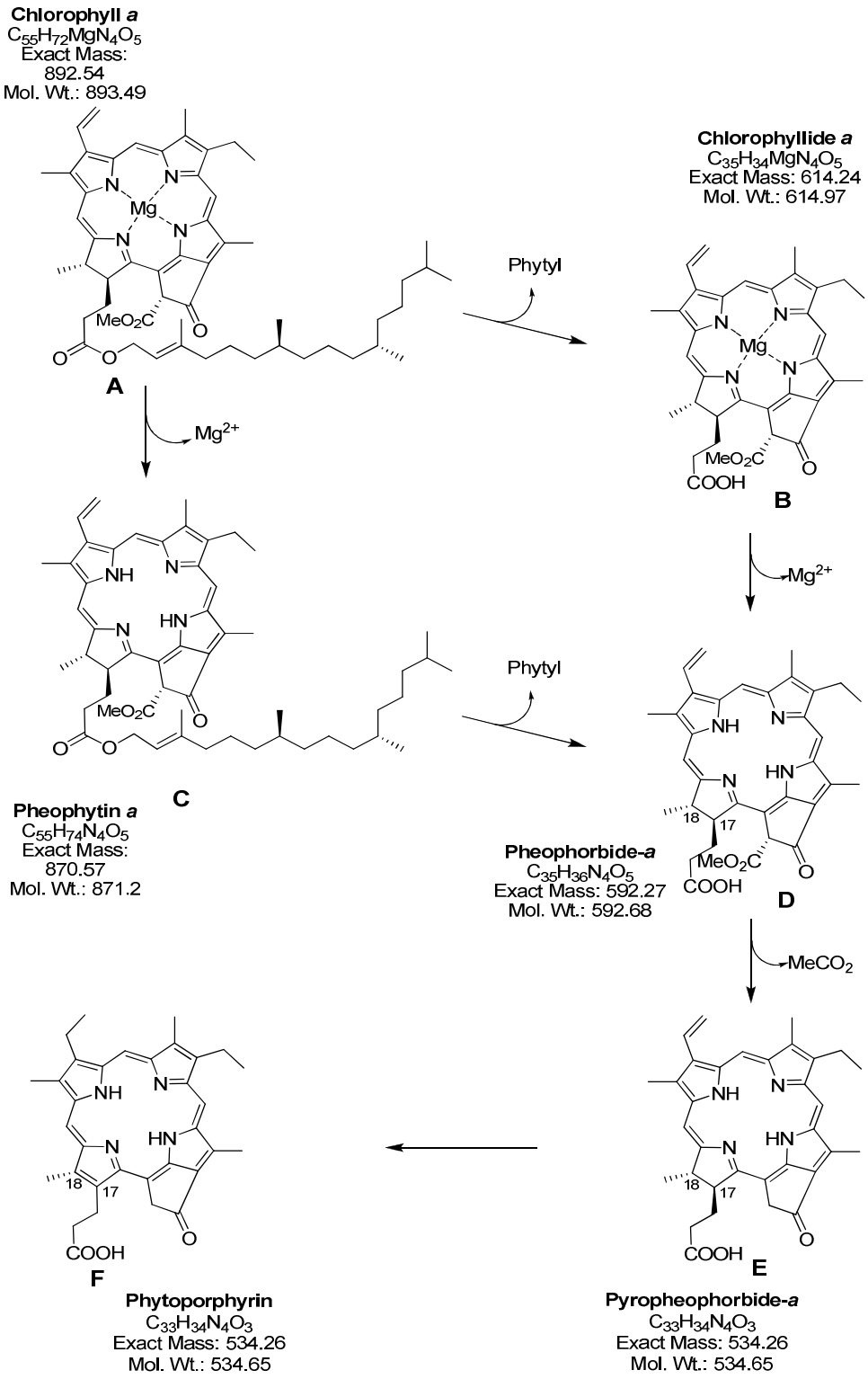
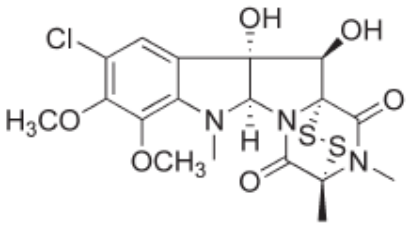


Figure 3.2. Schematic representation of chlorophyll metabolism (showing the structures, exact mass and molecular weight for each) (Campbell *et al.*, 2010).

3.2 Pathophysiology of facial eczema

As mentioned above, facial eczema is hepatogenous sensitivity of the animals and the etiological agent is a fungus *Pithomyces charataru* which produces the sporidesmin A toxin, (Figure 3.3). This fungus grows in the warm humid conditions on the dead litter. Once ingested by the grazing animals, the toxin is released into the circulation and subsequently reaches to liver (Morris *et al.*, 2004).



Sporidesmin A
$C_{18}H_{20}ClN_3O_6S_2$
Mol. Wt. 474

Figure 3.3. Chemical structure of Sporidesmin A (Morris *et al.*, 2004).

In the liver, the reduction of the disulfide bond of Sporidesmin A, in the presence of thiols (e.g. glutathione) results in the formation of dithiol (Figure 3.4). Dithiol undergoes auto-oxidation and consequently mediates the reduction of molecular oxygen to superoxide radical anion ($O_2^{\cdot-}$) (Morris *et al.*, 2004). One molecule of Sporidesmin A is capable of producing many molecules of superoxide. These superoxides subsequently form hydrogen peroxide. Consequently, in the presence of catalytic transition metals, superoxide radical anion reacts with hydrogen peroxide and results in the formation of hydroxyl radicals, as explained in the following chemical reaction:

$$2O_2^{\bullet -} + 2H^+ \longrightarrow H_2O_2 + O_2$$

$$O_2^{\bullet -} + H_2O_2 \longrightarrow O_2 + 2OH^{\bullet}$$

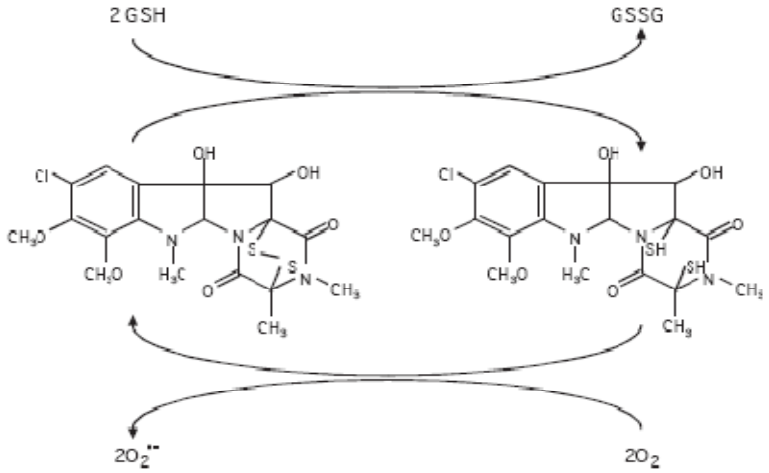


Figure 3.4. Diagrammatic representation of the formation of superoxide radical anion (Morris *et al.*, 2004).

All of these molecules viz. hydroxyl radical, superoxide radical anion and hydrogen peroxide (together known as active oxygen species), damage the liver and billiary system, consequently leading to cholestasis (Morris *et al.*, 2004). Therefore, the normal hepatic metabolism of chlorophyll is interfered.

Facial eczema results when phytoporphyrin undergoes a photo induced reaction with oxygen, resulting in the formation of singlet oxygen ($^1O_2$) and possibly other reactive oxygen species. $^1O_2$ is believed to be the main cytotoxic product formed during photochemical treatment of cells (Weishaupt *et al.*, 1976; Moan and Sommer, 1985). $^1O_2$ is short-lived in cells and has a diffusion length of 10–20 nm (Moan and Berg,

1991). Thus, the primary cytotoxic effect of a photosensitizer after exposure to light occurs near to the site of $^1O_2$ formation. Hence, an acute inflammatory response of the skin cells is induced due to the excitation of phytoporphyrin by sunlight, which consequently results in photosensitization of the skin.

3.2.1 Diagnosis of facial eczema using liver enzyme

Spordesmin A toxin damages liver and bile ducts, and the extent and degree of liver damage is generally used to measure the severity of the sporidesmin intoxication. Earlier studies have used a graduated scale of 1-5 (McFarlane *et al.*, 1959) or 0-5 (Campbell and Sinclair, 1968.; Smith *et al.*, 1978) to evaluate the severity of liver damage. In either scale, the graduations represent an increase in the severity of the liver damage with the ascending order of scale, such that a scale of 0 shows no signs of the disease, 1 reflects mild injury with no clinical signs, 2 reflects more severe disease but no clinical signs, 3 represents marked injury with mild clinical diseases, 4 shows severe injury with marked clinical disease, whilst 5 represents a severe fatal form of the disease (Di Menna *et al.*, 2009).

Ford (1974) reported an increase in the gamma glutamyl transferase (GGT) concentrations in sheep that were experimentally administered with spordesmin A. These authors further found that GGT concentration was directly related to liver damage. In adult cattle, serum concentrations of liver enzyme such as GGT, alkaline phosphate (ALP), aspartate transaminase (AST), glutamate dehydrogenase (GDH), L-iditol dehydrogenase are useful indicators for indentifying hepatic diseases. Similarly the concentration of billirubin also increases with increase in the severity of liver damage due to bile duct occlusion (Pearson *et al.*, 1995). However, the problem with using liver damage enzymes as a measure of facial eczema is that these

enzymes are not specific to spordesmin A toxin, as their concentrations can also increase in a variety of other diseases (Anonymous, Merck & Co., 2008b; Di Menna *et al.*, 2009). This necessitates developing an efficient analytical method to measure the specific photosensitive agents such as phytoporphyrin that are implicated in facial eczema.

3.3 Structural ambiguity with phytoprphyrin

Porphyrin contains a tetrapyrrol ring molecule (Figure 3.5) found in both green leaves and red blood cells. It is tautomeric with respect to the location of the two hydrogen atoms that are involved in the peripheral conjugated system. However, for nomenclature purposes, the name porphyrin implies that saturated nitrogen atoms are at positions 21 and 23 unless specifically indicated otherwise. In porphyrins, the positions 2, 3, 7, 8, 12, 13, 17, 18 are referred as beta positions, whilst 1, 4, 6, 9, 11, 14, 16, 19 are alpha positions and 5, 10, 15, 20 positions are meso-positions (Moss, 1987).

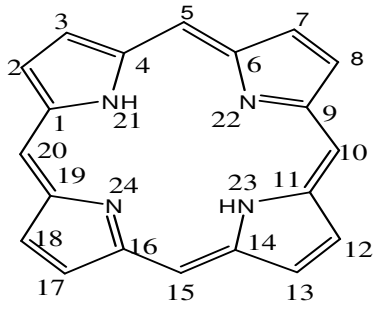


Figure 3.5. Structure of porphyrin (Moss, 1987).

Similarly, the large heterocyclic aromatic ring containing three pyrroles and one pyrroline in the core coupled through four methine linkages is referred as chlorin (Figure 3.7b). In chlorins, saturated carbon atoms are located at the non-fused

carbon atom of one of the pyrrol rings examples include pyropheophorbide-a and pheophorbide-a (Moss, 1987).

Porphyrins and chlorins have characteristic similarities and differences in their spectra. They have similar patterns in their absorption spectra, being made up of two distinct regions. One is a 'B' (soret) band at about 390-430 nm, and another is a 'Q' band region, made up of up to four bands in the range of 480-700 nm. However, they are easily distinguished by the significant differences in their relative intensities. Porphyrins have a very high B-band to Q-band ratio (as high as 50:1), whereas chlorins have a low ratio (as low as 5:1), with the highest Q band being red-shifted by up to 25 nm, and with a greatly increased intensity (Milgrom, 1997). The emission spectra also show a similar red-shift from porphyrin to chlorin (Milgrom, 1997). This is well illustrated by contrasting pyropheophorbide-*a* (a chlorin) and phytoporphyrin, both of which have the same chemical formula and molecular weight ($C_{33}H_{34}N_4O_3$ and 534.65), yet different spectra (Figure 3.10).

Phytoporphyrin (Figure 3.6a) was earlier known as phylloerythrin, but recently the International Union of Pure and Applied Chemists have recommended its preferred name as phytoporphyrin (Moss, 1987). It is a naturally occurring porphyrin having the empirical formula $C_{33}H_{34}N_4O_3$ with a molecular weight 534.65. It is formed by the breakdown of the chlorophyll molecule (Hynninen, 1991). Phytoporphyrin is a lipophilic compound which is located on the plasma, mitochondrial, endoplasmic reticulum and nuclear membranes. It is absorbed into the cells through a diffusion controlled process (Scheie *et al.*, 2002).

There is, however, some confusion regarding the structure and chemical formula of phytoporphyrin. The correct structure of the phytoporphyrin has a double bond at the

17, 18 position i.e. a fully aromatic compound (Rimington and Quin, 1934; Fischer, 1937; Clare, 1944; Moss, 1987) and its empirical formula is $C_{33}H_{34}N_4O_3$ (Sci Finder Scholar Registery Number is 26359-43-3). However, some authors (Scheie *et al.*, 2002; Ashby *et al.*, 2003; Scheie *et al.*, 2003a) have reported phytoporphyrin (porphyrin) as a partially aromatic compound (single bond at the 17, 18 positions); whilst some others have classified this as a chlorin (Moss, 1987). Its empirical formula is $C_{33}H_{36}N_4O_3$ (Scholar Registry number is 56145-41-6) and this is a mesopyropheophorbide-a (Figure 3.6b) which is a metabolite of pyropheophorbide-a and precursor of phytoporphyrin (Louda *et al.*, 1998). Therefore, besides developing an analytical method to detect phytoporphyrin, it is pivotal to confirm its correct chemical structure.

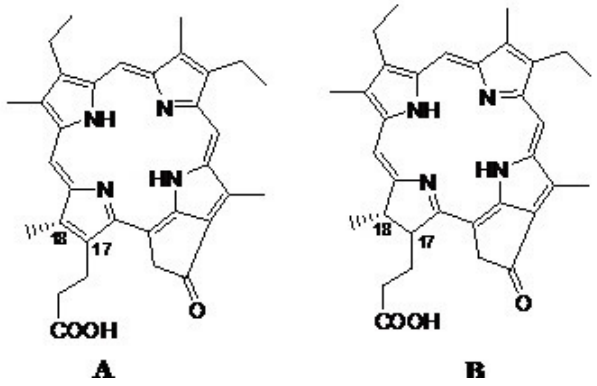


Figure 3.6. (**a**) Chemical structure of porphyrin (phytoporphyrin) (**b**) chlorin (mesopyropheophorbide-a).

3.3.1 Fluorescent methods to detect phytoporphyrin

Perrin (1958) developed a method to quantify phytoporphyrin in the blood based upon the absorption spectra of phytoporphyrin in ether. This assay successfully measured the phytoporphyrin concentrations in blood plasma within a range of

0.05 mg to 0.5 mg/100mL. However, during this protocol some phytoporphyrin was lost during the extraction and washing steps. Moreover, this method appears very long and arduous analytical procedure.

Recently, a fluorescence assay has been described for the quantification of phytoporphyrin using a standard solution of phytoporphyrin either in dimethyl sulfo-oxide (DMSO) or in methanol (Scheie *et al.*, 2002; Scheie *et al.*, 2003b). In those studies, phylloerythrin was diluted in DMSO to a concentration of 1 µg/mL and the fluorescence was detected by exciting samples at 418 nm (Soret band) and detecting emission at 500–800 nm (Scheie *et al.*, 2002). In another study, these authors used methanol to dissolve phytoporphyrin to a concentration of 1 mg/mL stored this solution at –20°C, and then re-diluted it to a concentration of 0.1 mg/mL on the day of the florescence examination. The resulting solution was then added to plasma and serum samples from adult animals to generate the standard curves (Scheie *et al.*, 2003a). In an attempt to avoid any discrepancy in the results due to any pre-existing phytoporphyrin in the standard plasma (when using these samples for generating standard curves), these authors diluted the stock solution (concentration 2 mmol/L) of phytoporphyrin to working solution concentrations of 0.1 µmol/L and 0.2 µmol/L using neonatal plasma (Scheie and Flaoyen, 2003). In yet another study, these authors diluted the stock solution of phytoporphyrin to the concentrations of 0.02 to 0.2 mmol/L, using methanol and generated blank samples by adding plasma to the methanol solution. No phytoporphyrin solution was added to the blanks, so the area under the spectral curve was calculated by subtracting the florescence of blanks from that of the test samples (Scheie *et al.*, 2003b).

3.4 Objectives

Due to the inconsistency in the literature regarding the chemical structure of phytoporphyrin, and the variability of the spectrofluorometric methods currently used to evaluate facial eczema, the focus of the present chapter is:

1. To determine the correct chemical structure of the phytoporphyrin.
2. To develop a simple and reliable spectroscopic method for the quantification of the phytoporphyrin in serum samples of the diseased animals.

3.5 Materials and Methods

Chemicals

Pheophorbide-*a* {15664-29-6} (Batch # LY04-184), pyropheophorbide-*a* (Louda *et al.*) (~95% pure; Batch # 012705), and phytoporphyrin {26359-43-3} (~95% pure; Batch # JB04-233) were procured from Frontier Scientific Inc. (Logan, UT, USA). These were stored at -20°C. AnalaR methanol (99.8% pure) was supplied by BDH (VWR International Ltd, Poole, England).

Preparation of standard solutions

Stock solutions (25 µM) of pheophorbide-*a* and pyropheophorbide-*a* in methanol were prepared and frozen at -20^0C until required. The dissolution of the pheophorbide-*a* and pyropheophorbide-*a* was checked by using ultraviolet and fluorescence spectrophotometery. For phytoporphyrin, it was found that the optimum procedure for the preparation of a stock solution in methanol, with a concentration no higher than 3.7 µM was achieved by dissolving the material at 50^0C for 45 min, with the aid of ultrasonication. Solutions prepared in this manner were stable for several weeks when stored at room temperature in the dark. At these low concentrations, adsorption of phytoporphyrin on to glass surfaces can affect solution concentration;

therefore the stock solution was prepared and stored in polypropylene containers. Absorption and emission spectra of all the standard stock solutions were measured daily prior to any testing to ensure quality, and new solutions were prepared as required.

Samples

Samples were supplied by the Pathobiology Group of Institute of Veterinary, Animal and Biomedical Sciences, Massey University, Palmerston North, New Zealand. Blood samples from diseased and healthy cows were collected in evacuated glass collection tubes (vacutainers) without using any anticoagulant. Following blood collection, the vacutainers were wrapped with aluminium foil to protect from sunlight and subsequently serum was extracted from these samples. Thereafter, these serum samples were stored at -20^0C for later on analysis.

Fluorescent measurement

Fluorescence was measured using a Perkin Elmer spectrometer in a semi-micro quartz cuvette (0.8 mL, 4.75 x 4.75 x 33 mm; Perkin Elmer Inc., Beaconsfield, Bucks, UK) in which 200 µL of serum sample, 200 µL of Milli-Q water and 350 µL of methanol were mixed. The fluorometer was set for an excitation wavelength of 425 nm and the emission spectra were collected between 425-750 nm. Slit widths were set at $15_{excitation}$ and $20_{emission}$ nm, with a scan speed of 100 nm min^{-1}. An emission cut-off filter of 515 nm and a red-sensitive photomultiplier tube were employed to increase the red response. Intensities and peak areas for phytoporphyrin were obtained by measuring the peak at 644 nm. The conformity of the instrument was checked routinely with a standard calibration block. The standard emission

spectra for pheophorbide-*a* and pyropheophorbide-*a* were recorded by excitation at 408 and 409 nm, respectively, using the standard photomultiplier detector.

A number of calibration curves were generated from control sera of species and age cohorts to establish an average calibration curve (Figure 3.7). Different ratios of methanol and stock phytoporphyrin solution in methanol (5, 10, 20, 40, 80, 160, 320, 350 µL) were added to give the desired concentration in a total volume of 750 µL. There was a significant deviation from the linearity when adding the stock solution to the serum due to a significant degree of coagulation caused by the methanol. This was overcome by making a 1:1 dilution of serum with Milli-Q water. It was important to record the spectra quickly and clean the cuvettes thoroughly after each measurement as phytoporphyrin adsorbs strongly onto the quartz cuvette walls**.**

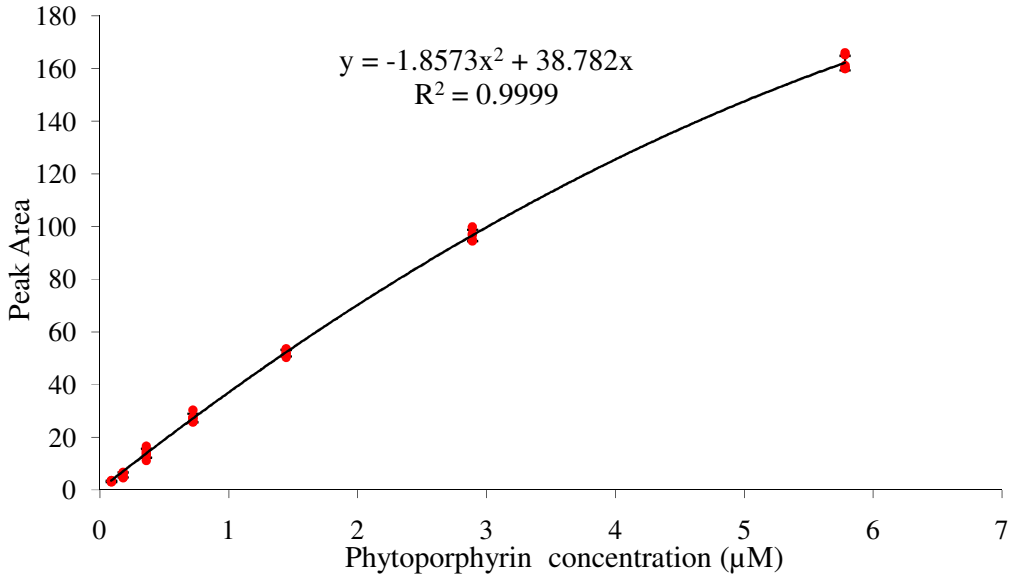
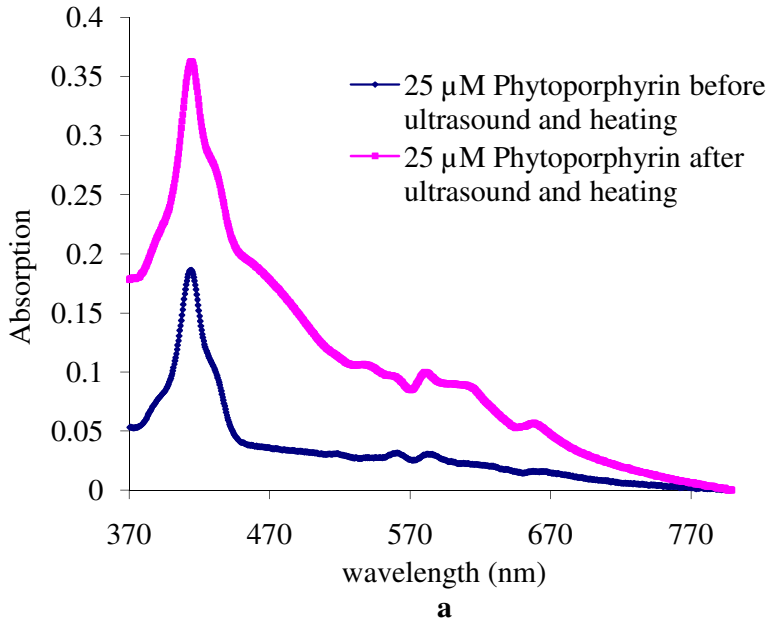


Figure 3.7. An average calibration curve by addition of standard phytoporphyrin solution in the non-diseased cow serum.

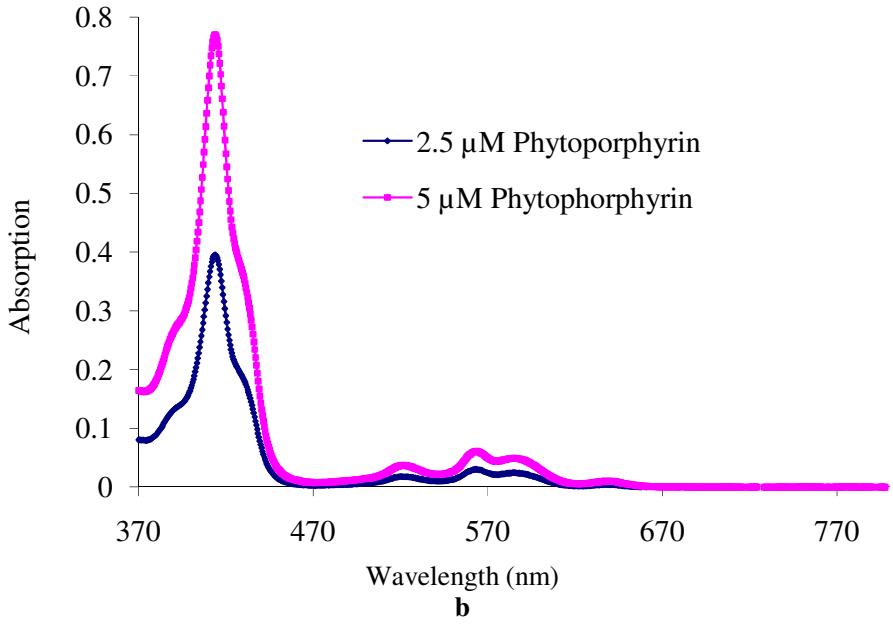
3.6 Results and Discussion

In order to measure the phytoporphyrin in the test samples, the methodology described in earlier studies (Scheie and Flaoyen, 2003; Scheie *et al.*, 2003b) was followed. However, this gave inconsistent results, as the required concentration of phytoporphyrin in methanol (25 μM) would not fully dissolve, and in fact precipitated out over time (as verified in the changes in the absorption spectra: Figure 3.8a). To overcome this problem, a standard solution of 5 μM was prepared which was further diluted to 2.5 μM with the aid of ultrasonication and heating. This resulted in a consistent absorption spectrum (less than 2% change in absorption: Figure 3.8b) which suggested that the resultant solution was fully dissolved.

Limited dissolution was observed when using a 25 μM stock solutions of pyropheophorbide-a or pheophorbide-a, as verified by their absorption spectra (Figure 3.9) carried out before and after ultrasonication with heating. Changes of 1% for pyropheophorbide-a and 2% in the pheophorbide-a were deemed acceptable for use of the solutions as a standard.



a



b

Figure 3.8 (a) Absorption spectra of the 25 µM phytoporphyrin solution before and after the aid of ultrasonication and heating showing the change in the absorption **(b)** Half dilution of the 5 µM phytoporphyrin solution after the aid of ultrasonication and heating.

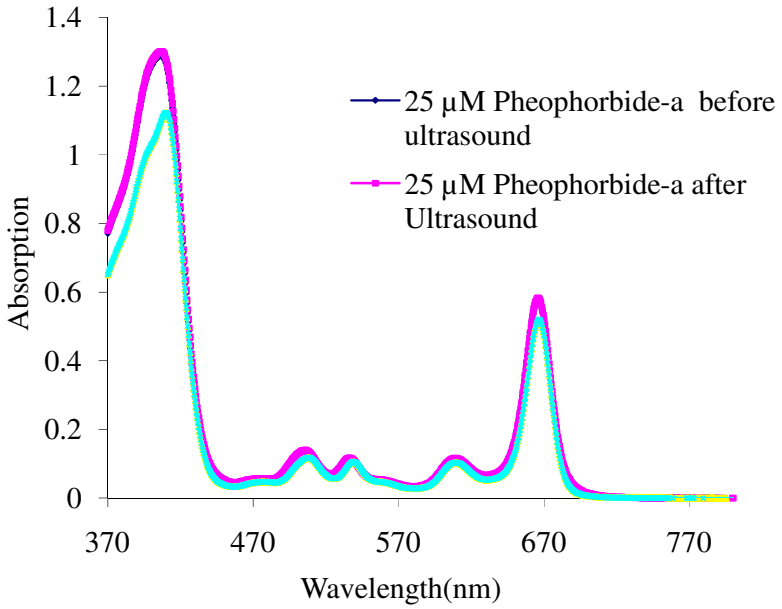


Figure 3.9. Absorption spectra of chlorins (25µM pheophorbide-a and 25µM pyropheophorbide-a) before and after the aid of ultrasonication and heating.

A comparison of the absorption spectra (i.e. relative intensities of ratio of B band to Q band) of phytoporphyrin and pyropheophorbide-a (Chlorins) (Figure 3.10) and also of the red shifted emission spectra of the pyropheophorbide-a showed that phytoporphyrin (phylloerythrin) is a porphyrin. In the present study, the commercially supplied "phylloerythrin (phytoporphyrin)" was confirmed to be porphyrin, showing a typical characteristic porphyrin spectrum with excitation at 425 nm and maximum emission at 644 nm (Figure 4.5). By contrast, pheophorbide-a and pyropheophorbide-a produced typical chlorin spectra and had excitation/emission maxima of 408/669 nm and 409/669 nm, respectively (Figure 4.6). Thus, this spectral analysis clearly showed that whilst pyropheophorbide-a is a chlorin; phytoporphyrin is a porphyrin despite the fact that pyropheophorbide-a has the same molecular weight and chemical formula as that of phytoporphyrin.

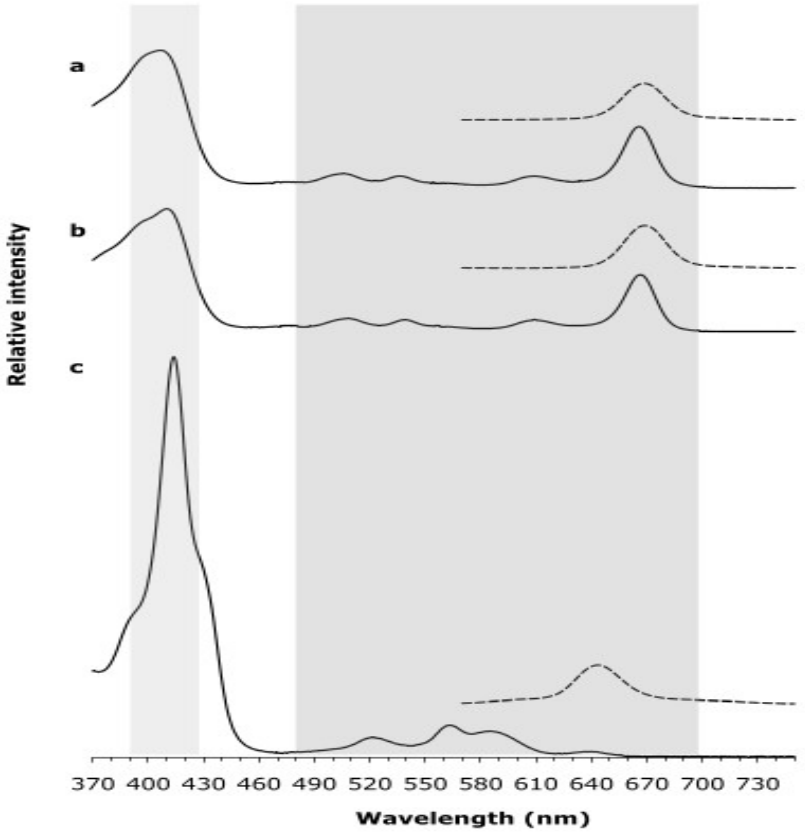


Figure 3.10. Standard absorption (——) and emission (---) spectra for the chlorins (**a**) pheophorbide *a*, molecular weight 592.68, and (**b**) pyropheophorbide *a*, molecular weight 534.65, and (**c**) the porphyrin, phytoporphyrin, molecular weight 534.65. Narrow shaded band at ~ 390-430 nm = 'B' (Soret) band. Wide shaded band at ~ 480-700 nm = 'Q' band region.

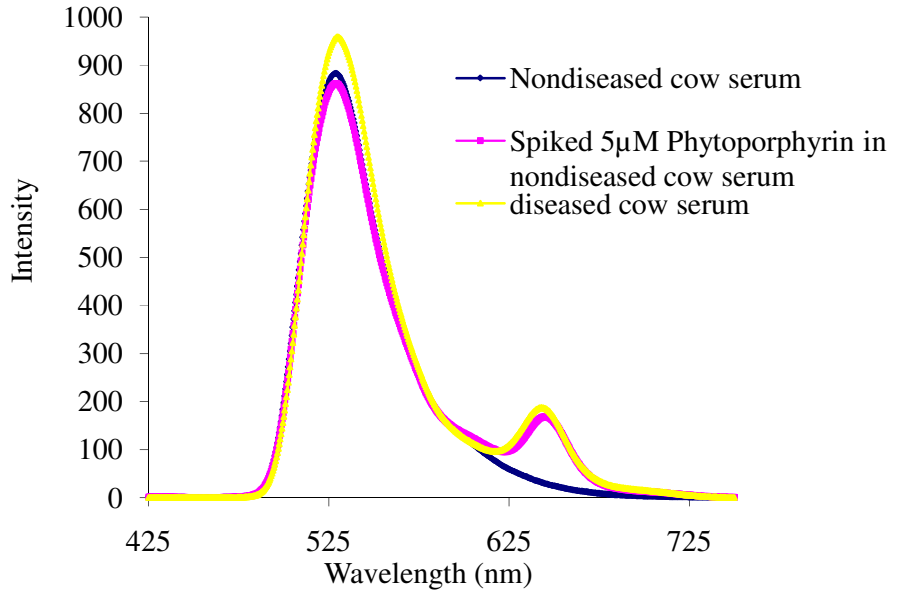


Figure 3.11. Emission spectra of phytoporphyrin in diseased, nondiseased and phytoporphyrin-spiked nondiseased cow serum.

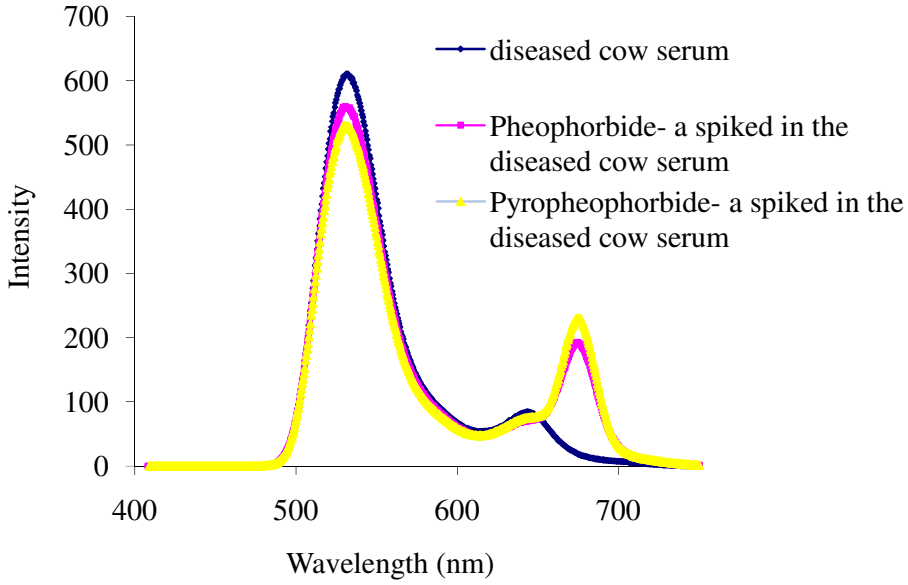


Figure 3.12. Emission spectra of pheophorbide-a and pyropheophorbide-a in diseased cow serum and pheophorbide-a and pyropheophorbide-a spiked diseased cow serum.

A simultaneous work undertaken in this laboratory showed that pyropheophorbide-a and phytoporphyrin had very similar chromatographic retention times, the same chemical formula and the same mass, but were distinguishable based on the UV-vis spectrum. In sera from photosensitive animals, the fluorescence emission at 644 nm was solely due to the presence of phytoporphyrin and not of any other chlorophyll-*a* metabolites (Figure 3.11). Neither of the chlorins produced spectral peaks in any of the photosensitive animals (Figure 3.12).

Thus the present study, along with a simultaneous study in the same laboratory, further confirmed that the molecule present in the sera of diseased animals was phytoporphyrin, but that chlorins (e.g. pheophorbide-a or pyropheophorbide-a) were not present. Consequently, it was concluded that phytoporphyrin is the molecule responsible for photosenstization resulting in facial eczema in cows.

Moreover, using the present fluorescent procedure, the standard curve of control sera resulted in a reliable measurement of the phytoporphyrin concentration in the cow serum which ranged from 0.4 to 6 μM. The concentration of phytoporphyrin in the sera of animals affected by facial eczema ranged from 0.4 μM to 1.8 μM (Table 3.1). These data further suggested that the fluorescent procedure used in the present study is a reliable method to measure the phytoporphyrin concentration in the sera of cows.

Table 3.1. Liver enzyme (GGT = γ-glutamyltransferase, and GDH = glutamate dehydrogenase) activities, total bilirubin concentration, and phytoporphyrin* concentration for animals with clinical facial eczema.

Animal/ID	GGT[a] (U/L at37^{0}C)	GDH[b] (U/L at37^{0}C	Bilirubin[c] (μM)	Phytoporphyrin* (μM)
Cow/5634	2,389	6,800	150	0.4
Cow/3634	1,293	511	6	1.8
Cow/4055	3,865	918	14	1.4
Cow/4267	1,789	432	3	0.7
Cow/4700	1,388	391	4	1.2

[a] Normal range 0-36 U/L (cattle), [b] normal range 8-41 U/L (cattle), [c] normal range 0-13 μM (cattle).

*Only phytoporphyrin concentration was measured by the fluorescent method developed in the present study.

3.7 Conclusion

Facial eczema is a disease of considerable economical importance. The toxin produced by the fungus causes liver damage and thus limits the ability of an affected animal to efficiently eliminate phytoporphyrin. The presence of phytoporphyrin in the peripheral circulation can thus be used as a biomarker to determine the manifestation of this disease. Therefore, in a herd, regular screening of animals for phytoporphyrin concentration can help to develop preventive measures against facial eczema. Moreover, this can also help treating such animals to minimize the severity of the disease.

In the present research, it was verified that the photosensitive agent, phytoporphyrin involved in facial eczema is a porphyrin and not a chlorin. Thus, the ambiguity regarding the chemical structure of the phytoporphyrin was rectified. A new spectrofluorometric method for detection of phytoporphyrin in the sera of diseased animals was also developed with the limit of detection 0.4 μM.

Chapter Four – Conclusion

The studies described in this thesis were a part of a larger study being carried out at Massey University, New Zealand, with the focus of developing novel sensing technologies, and improving the current analytics of various biomolecules implicated in the animal health and production. The rearing of animals to obtain different products and by-products to meet human needs is a much older practice. A rapid increase in the human population is exerting a continuous push on the farming sector to enhance livestock productivity. Various diseases of livestock and problems of the animal production system can render the rearing of animals inefficient and uneconomical. The key factors of a successful dairy industry include maintaining the cows in a healthy condition and ensuring their normal reproductive function. These can be achieved by developing methodologies to measure various biomolecules that are critically implicated in animal physiology. Further, this might be of help in identifying and diagnosing various livestock problems.

4.1 Development of surface plasmon resonance steps for progesterone and oestradiol 17-β

This study was focussed to develop SPR protocols for measuring progesterone and oestradiol 17-β. Both of these hormones are critically involved in the reproductive cycle of cows. Oestradiol 17-β is associated with the manifestation of behavioural oestrus and, thus, its peripheral concentrations can be used to detect the time of oestrus and to optimize the timing of insemination. Progesterone is responsible for preparing the uterus for pregnancy and development of the conceptus (Noakes, 2001). Its concentrations decline precipitously ~18 days after oestrus if conception has not occurred, a phenomenon that has been used to achieve either an early

pregnancy diagnosis or to predict the timing of re-insemination of animals that have not conceived. Currently various methods are used to measure the concentration of oestradion 17-β and progesterone to predict the time of insemination and to achieve early pregnancy diagnosis, however these have limited practical viability under routine farming practices.

Surface plasmon resonance has the potential to provide a new laboratory method for the measurement of reproductive steroids. To do so, the key fundamental step is to achieve an effective SPR signal strength. The essential steps to achieve this are: a high-affinity antibody for the test molecule, and the correct linker length, which is substituted at the appropriate site on the molecule (Wu *et al.*, 2002). In the present study, two different length linkers were synthesized for progesterone, and the long length linker appeared to exhibit a better binding response than the short length linker. Simultaneously, two different monoclonal antibodies from two different sources i.e P1922 from Sigma and SE7720 -1430 from Serotec, were tested. The P1922 antibody which was of rat origin yielded consistent results but had lower binding response. On the other hand, SE7720 -1430 antibody which was of mouse origin had greater binding response but this was inconsistent on different test days. For oestradiol 17-β, the monoclonal antibody of mouse origin (BT70-1020-06) from Bio-trend had a consistently low binding response. The present research thus indicates that the proper testing and careful selection of antibodies and the appropriate length of the linker is pivotal to achieve the required signal strength. It is suggested that further studies are required to evaluate the efficacy of other source antibodies along with testing different length linkers in order to achieve an effective SPR for progesterone and oestradiol 17-β.

4.2 Facial eczema

The second study was undertaken to refine the currently existing analytical methodologies to measure phytoporphyrin, which is a molecule associated with FE in cows. Facial eczema is a disease of high economic importance that is of particular importance to New Zealand (Smith and Towers, 2002). It occurs as a manifestation of the disturbance in the chlorophyll metabolism of grazing animals which is itself a sequel to liver damage and bile duct occlusion caused by *Pithomyces charatarum* toxicity. This increases the concentrations of the chlorophyll breakdown product, phytoporphyrin, in the peripheral circulation, which consequently results in photosensitization of the skin (Morris *et al.*, 2004). Therefore, phytoporphyrin can be used as biomarker for facial eczema.

Using phytoporphyrin as biomarker for FE necessitates developing efficient and repeatable analytical tests to detect the concentration of phytoporphyrin in blood. Although a few studies (Scheie and Flaoyen, 2003; Scheie *et al.*, 2003b) have reported analytical protocols for the measurement of phytoporphyrin, these seems to have limited feasibility for use as rapid routine tests due to difficulties of assay methodology. Not only this, but the literature is also anomalous about the chemical structure of phytoporphyrin (Rimington and Quin, 1934; Fischer, 1937; Clare, 1944; Moss, 1987).

The present study described a new and viable protocol for dilution of phytoporphyrin standard solution such that it can be consistently used for the routine florescent procedures without giving any variability in the absorption spectra. In this study, the absorption and emission spectra of various chlorophyll metabolites such as pheophorbide-a, pyropheophorbide-a and phytoporphyrin were compared.

Subsequently, by evaluating their P-band to Q-band ratio, it was concluded that phytoporphyrin is a porphyrin, whereas pheophorbide-a and pyropheophorbide-a are chlorins. These findings thus clarified the anomaly about the chemical structure of phytoporphyrin. Moreover, with the new fluorescence protocol, it was possible to measure phytoporphyrin with a limit of detection of 0.4 μM, which is well above the minimal detectable dose required to differentiate between normal and diseased animals. Thus, the present analytical protocol can be regarded as a viable, repeatable and efficient method for detecting phytoporphyrin in the peripheral circulation of animals, which has the potential to be an effective means of diagnosis of facial eczema. Moreover, this can be potentially adopted for a routine screening of animals in a herd to measure phytoporphyrin concentrations in order to predict the likelihood of onset of facial eczema. This might be of help in adopting efficient therapeutic or preventive measure to curtail prevalence of facial eczema.

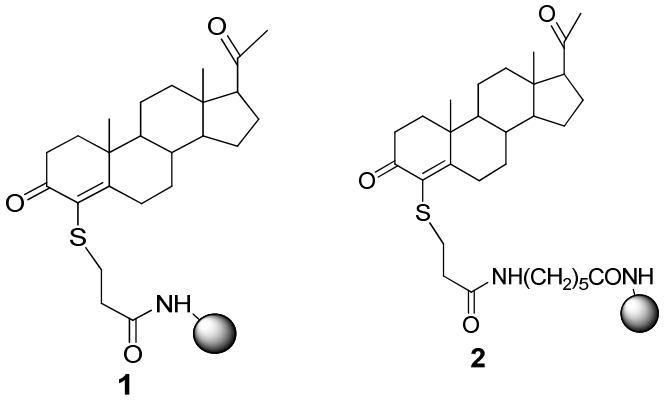
Appendix

Synthesis of steroid conjugates

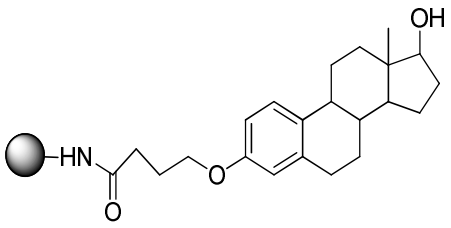
The present study is a part of a larger study carried by the research group at Massey University. Those compounds that were synthesised by the candidate are marked using an asterisk (*), and synthetic procedures are detailed below.

Surface plasmon resonance detection of progesterone and oestradiol was carried out by attaching the conjugate comprising of ovalbumin (OVA ( )), a linker and the desired steroid onto the surface of a CM5 chip. Assessment of the sensitivity and selectivity of the sensor chip was then determined by a series of competitive assays.

Preparation of the conjugate required modifying the steroids and their coupling with OVA, which was achieved as described in the earlier studies (Wu *et al.*, 2002; Mitchell *et al.*, 2006). The 4 substituted progesterone derivatives (**1** and **2**) (Appendix Figure 1) and 3 substituted oestradiol 17-β (**3**) (Appendix Figure 2) were conjugated to OVA as below:



Appendix Figure 1. Progesterone conjugates with OVA ( ) via thiopropanoic acid (4TP, P4 OVA) (**1**) and 6-amino hexanoic acid (4TPH, P4 OVA) (**2**).



3

Appendix Figure 2. Oestradiol 17-β conjugated via linker propanoic acid to OVA (●) (E2, C3 OVA) (**3**).

Synthesis

Reagents and Materials

Progesterone (P8783), succinic anhydride, 3-mercaptopropanoic acid, and 4, 7, 10-trioxa- 1, 13-tridecanediamine were obtained from Sigma Aldrich, USA. Analytical grade methanol and Milli-Q water were used to synthesize all the compounds. The solvents used in chromatography were distilled from the laboratory grade reagents.

Synthesis of OEG **(oligoethylene glycol) linker (5)**

The synthesis of the OEG (oligoethylene glycol) linker is depicted in Appendix Figure 3.

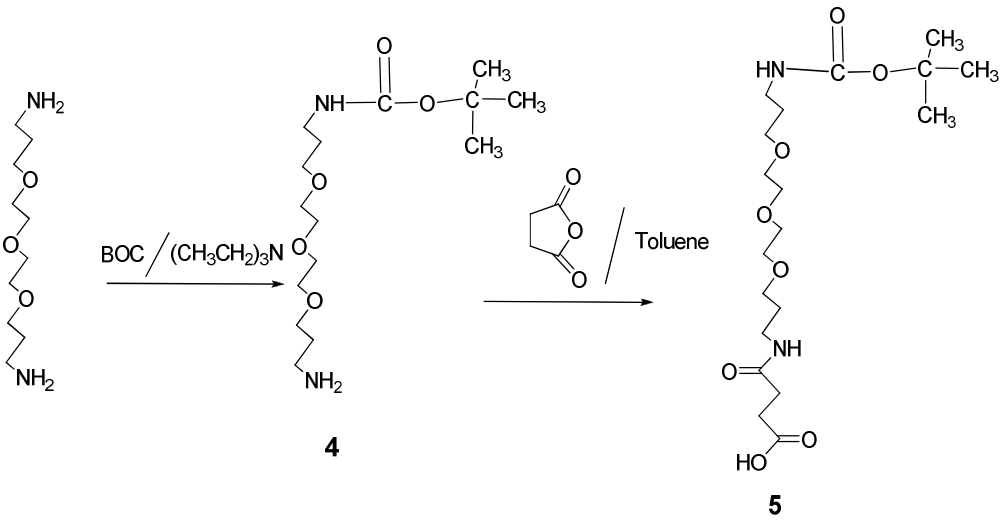
N-(t-Butoxycarbonyl)-4, 7, 10-trioxa-1, 13-tridecanediamine* (**4**)

This compound was prepared as described by Mitchell *et al.* (2005). 4, 7, 10-trioxa-1, 13 tridecanediamine (1.9013 g, 8.6 mmol, 1.88 eq)) was dissolved in 13 mL of MeOH and Et_3N (1mL) was added with continuous stirring. To this mixture di-tert-butyldicarbonate ((1.0388 g, 4.58 mmol, 1 eq) dissolved in 10 mL of MeOH) was added drop wise over 30 min with continuous stirring, and the mixture was left to stir

overnight under argon. The solution was then concentrated under reduced pressure, and purified by silica gel chromatography using DCM/MeOH/TEA (10/1/0.1). The resulting product was pale yellow oil (4) in a 36% yield. ^{1}H NMR (400MHz, MeOH-d_4): δ 4.9 (s, 1H), 3.1-3.3 (m, 13H), 2.7 (m, 3H), 2.5 (m, 2H), 1.3-1.5 (m, 4H), 1.1 (s, 9H).

Carboxylilate Linker* (**5**)

Compound 5 was prepared according to the method of Yuan *et al.* (2007). Compound (4) (600.3 mg, 1.87 mmol, 1 eq) was dissolved in toluene (7mL). To this solution, succinic anhydride (300.4 mg, 2.98 mmol, 1.59 eq) was added and the mixture was refluxed under argon for one hour. The solution was then concentrated under reduced pressure and purified by silica gel chromatography using DCM/MeOH/HCOOH 10/1/0.1. This resulted in the formation of a pale yellow coloured solid (5), yield 60% (Appendix Figure 3). ^{1}H NMR (400MHz, MeOH-d_4): δ 4.9 (s, 1H), 3.1-3.3 (m, 15H), 2.7 (m, 3H), 2.5 (m, 4H), 1.3-1.5 (m, 4H), 1.1 (s, 9H).

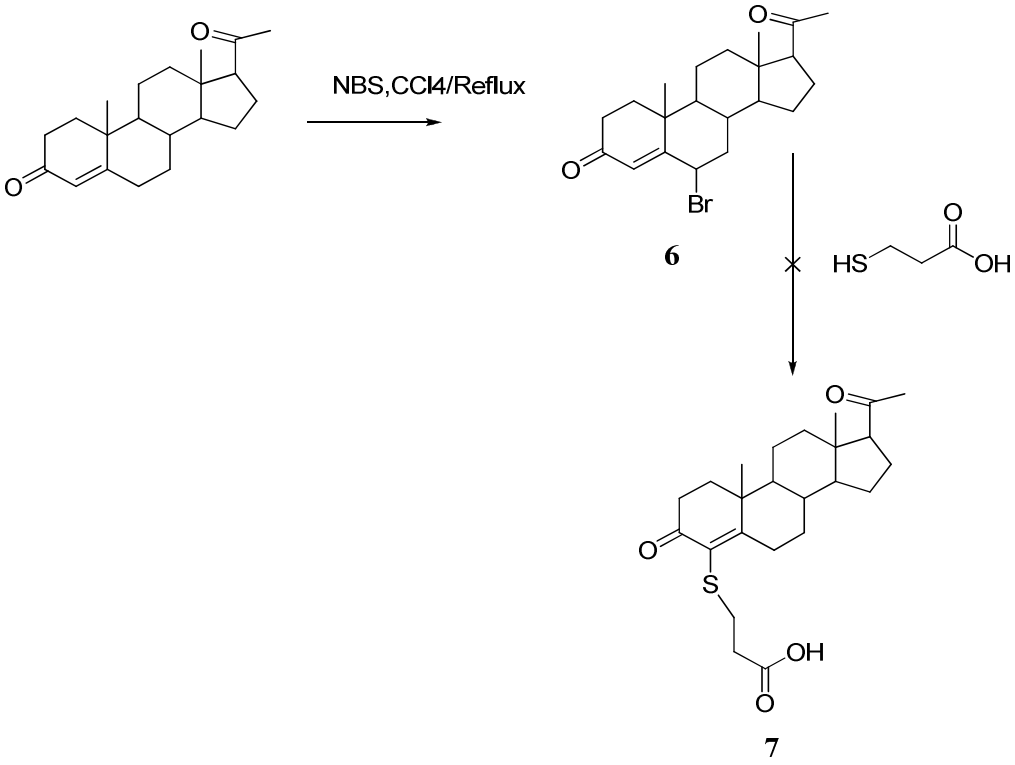


Appendix Figure 3. Synthetic route for the oligoethylene linker.

Synthesis of progesterone derivative for attachment with linker

To facilitate the coupling of the linker molecule onto progesterone, a pendant mercaptopropanoic acid group was attached at the 4 position. Various synthetic approaches were attempted to synthesize compound 7, including attachment via the bromo (Appendix Scheme 1) and epoxy (Appendix Scheme 2) analogues.

Scheme 1



Appendix Scheme 1. Attempted synthesis of 4-(2'-carboxyethylthio)-pregn-4-ene-3, 20-dione (**7**) from the bromo analogue.

6-Bromo-pregn-4-ene-3, 20-dione* (**6**)

Compound (**6**) was synthesized according to the protocol reported by Sondheimer *et al.* (1953). Progesterone (1.0089 g, 3.20 mmol, 1 eq) was dissolved in carbon tetrachloride (30 mL). N-bromosuccinimide (601 mg, 3.37 mmol, 1.05 eq) was

added and the mixture was refluxed for 1 hour. The white precipitate that formed was filtered off and the filtrate was evaporated under reduced pressure. The filtrate was crystallized from acetone/n-hexane to give compound **6** in a yield of 49%. ^{1}H NMR (400MHz, $CDCl_3$): δ.0.74(s,3H,18-CH3),1.55(s,3H,19-CH_3),2.14 (s,3H,21-CH_3), 4.97(m,1H, 6 α -H),5.91(s, 1H,4-H).

Attempted synthesis of 4-(2'-carboxyethylthio)-pregn-4-ene-3,20-dione* (**7**)

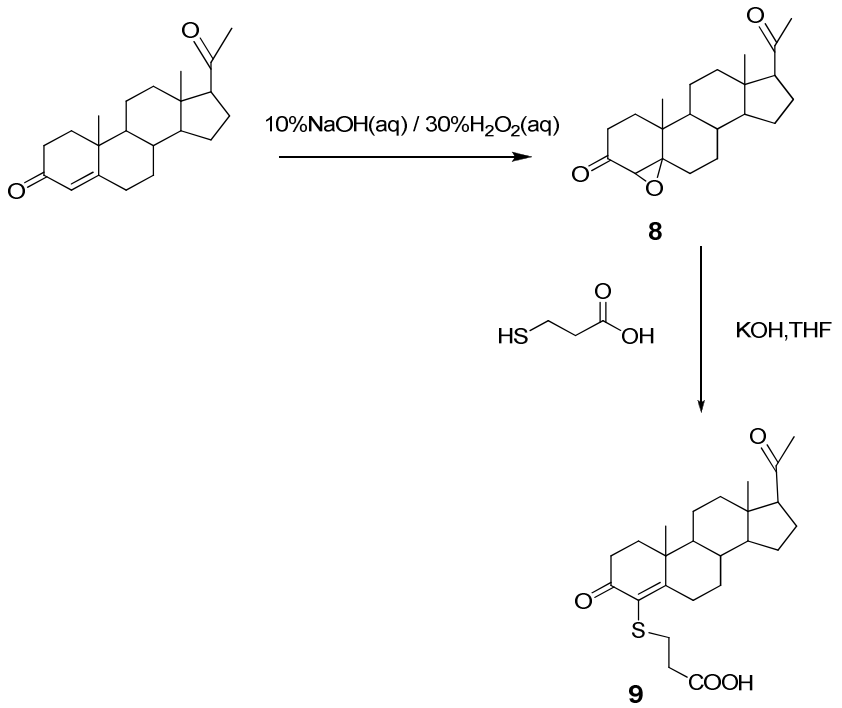
To compound **(6)** (160.3 mg, 0.407 mmol, 1 eq), 7 mL of 1% methanolic KOH solution was added and the mixture was stirred, and 3-mercaptopropanoic acid (100 μL, 122 mg, 1.15 mmol, 3 eq) was added. The mixture was refluxed for 3 hours under argon atmosphere. The reaction mixture was cooled at room temperature and concentrated under reduced pressure. To this concentrate, Milli-Q water was added and acidified to pH 1.5 by addition of diluted aqueous HCl. This solution was then extracted with Et_2O and the organic layer was dried over $MgSO_4$. The solution was concentrated under reduced pressure and the compound was purified by the silica gel chromatography using EtOAc/n-hexane (1:1) and then $CHCl_3$/MeOH (15:1) as eluents. However, the procedure resulted in the formation of only trace amounts of the desired product 7 as indicated by ^{1}H NMR.

Attempts to modify the procedure as listed in Appendix Table 1, also proved unsuccessful, and as a result efforts were turned to the synthesis of **7** via the epoxide analogue **8** (Appendix Scheme 2).

Appendix Table 1. Different reaction conditions used to synthesise 4-(2'-carboxyethylthio)-pregn-4-ene-3-20-dione* (**7**).

S. N.	Base	Equivalent	Mercaptopropanoic acid	Equivalent
1.	KOH	3 eq	Mercaptopropanoic acid	3 eq
2.	KOH	30 eq	Mercaptopropanoic acid	3 eq
3.	Potassium-tertiary butoxide	3 eq	Mercaptopropanoic acid	1.2 eq
4.	Sodium methoxide	5 eq	Mercaptopropanoic acid	1.5 eq

Scheme 2



Appendix Scheme 2. Synthesis of 4-(2'-carboxyethylthio)-pregn-4ene-3, 20-dione (**9**) from the corresponding epoxide.

4, 5-epoxypregnane-3, 20-dione* (**8**)

Compound **8** was synthesized according to the procedure described by Yoshida *et al.* (1989), Progesterone (123.5 mg, 0.392 mmol) was dissolved in methanol (8 mL), and 10% NaOH (0.5 mL) and 30% H_2O_2 (0.5 mL) were added. The mixture was stirred at 0^0C and, after 2 hours, further aliquots of 10% NaOH (0.5 mL) and 30% H_2O_2 (0.5 mL) were added and the solution stirred for a further 2 hours. The solution was neutralized using dilute acetic acid, and concentrated to one third under reduced pressure. Ethyl acetate was added to the white precipitate and extracted with Milli-Q water and saturated aqueous NaCl solution. The organic layer was concentrated under reduced pressure and purified by silica gel chromatography using ethyl acetate/n-hexane (1:1) as eluent. A white coloured powder of the desired product **8** was formed in a yield of 58%. ^{1}H-NMR (400MHz, $CDCl_3$): δ 0.61, 0.62, 1.03, 1.12, 2.08 and 2.09(each s, 3H, 18-, 19-and 21 –CH3 respectively) and 2.95, 3.00 (s, 1H, 4-H).

4-(2'-carboxyethylthio)-pregn-4-ene-(3, 20)-dione (9)

Compound **9** was synthesized according to the procedure of Yoshida *et al.* (1989). A solution of 3-mercaptopropanoic acid (37.6 mg, 1.06 mmol, 4.9 eq) and 25% KOH aqueous solution (0.104 mL) was stirred at room temperature for 10 min. 4, 5-Epoxy progesterone (70.5 mg, 0.213 mmol, 1 eq) in EtOH (1 mL) was added to the mixture and was stirred overnight at room temperature. 3-Mercaptopropanoic acid (37.6 mg, 1.06 mmol, 4.9 eq) was added and the mixture was then stirred at 80°C for 5 hours. Additional 3-mercaptopropanoic acid (37.6 mg, 1.06 mmol, 4.9 eq) was added to the mixture and stirred overnight at 80°C. Milli-Q water (73.9 mL) was added to this solution at room temperature. The pH of this solution was adjusted to 4 with diluted aqueous HCl at 0^0C, and the acidified solution was extracted with EtOAc. The

organic layer was washed with Milli-Q water and saturated aqueous NaCl solution, and then dried over anhydrous $MgSO_4$. The dried organic layer was evaporated under reduced pressure. The residue was purified using silica gel column chromatography using EtOAc/n-hexane (3: l) as eluent. However, the desired product **9** was present only in trace amounts as shown by ^{1}H NMR spectroscopic analysis, and as such was difficult to isolate and purify.

Modification of the procedure by using tetrahydrofuran (THF) instead of EtOH resulted in the desired product **9** as a white solid in 77% yield. ^{1}H-NMR (400MHz, C_5D_5N): δ 0.67 (s, 3H, 18-CH_3), 1.20 (s, 3H, 19-CH_3), 2.12 (s, 3H, 2l-CH_3), 3.08 (s, 1H, 7-H), 5.80 (s, 1H, 4-H).

References

Encyclopaedia of the Nations 2010 – Animal Husbandry http://www.nationsencyclopedia.com/Asia-and-Oceania/India-ANIMAL-HUSBANDRY.html *accessed on 05.05.2010*.

Abeyawardene, S. A.; Pope, G. S. 1990: Concentrations of estradiol 17-beta in plasma and milk and progesterone in plasma during the estrus cycle and in early-pregnancy in goats. *British Veterinary Journal,* **146**: 101-105.

Allrich, R. D. 1994: Endocrine and neural control of estrus in dairy -cows. *Journal of Dairy Science,* **77**: 2738-2744.

Andrew, R. 2001: Clinical measurement of steroid metabolism. *Best Practice & Research Clinical Endocrinology & Metabolism,* **15**: 1-16.

Anonymous, Merck & Co., I. W. S., NZ USA. 2008a: Photosentisation. *The Merck Veterinary Manual.* Merial Limited.

Anonymous, Merck & Co., I. W. S., NZ USA. 2008b: Hepatic diseases in large animals. *The Merck Veterinary Manual.* Merial Limited.

Arevalo, F. J.; Messina, G. A.; Molina, P. G.; Zon, M. A.; Raba, J.; Fernandez, H. 2009: Determination of progesterone (P4) from bovine serum samples using a microfluidic immunosensor system. *Talanta,* **80**: 1986-1992.

Ashby, K. D.; Wen, J.; Chowdhury, P.; Casey, T. A.; Rasmussen, M. A.; Petrich, J. W. 2003: Fluorescence of dietary porphyrins as a basis for real-time detection of fecal contamination on meat. *Journal of Agricultural and Food Chemistry,* **51**: 3502-3507.

Baird, D. T.; Campbell, B. K.; Mann, G. E.; McNeilly, A. S. 1991: Inhibin and estradiol in the control of FSH secretion in the sheep. *Journal of Reproduction and Fertility,* **43**: 125-38.

Basu, A.; Shrivastav, T. G.; Maitra, S. K. 2006: A direct antigen heterologous enzyme immunoassay for measuring progesterone in serum without using displacer. *Steroids,* **71**: 222-230.

Bindon, B. M. 1971: The role of progesterone in implantation in the sheep. *Australian Journal of Biological Science,* **24**: 149-58.

Bosch, A. M. G.; Hell, H. V.; Brands, J.; Schuurs, R. H. W. M. 1978: *Proceedings of the International Symposium on Enzyme Labeled immunoassay of Hormones and Drugs,* Berlin, Walter de Gruyter.

Campbell, A. G.; Sinclair, D. P. 1968: Control of facial eczema in lambs by use of fungicides. *Proceedings of the Ruakura Farmers' Conference,* 148-157.

Campbell, W. M.; Dombroski, G. S.; Sharma, I.; Partridge, A. C.; Collett, M. G. 2010: Photodynamic chlorophyll a metabolites, including phytoporphyrin (phylloerythrin), in the blood of photosensitive livestock: Overview and measurement. *New Zealand Veterinary Journal,* **58**: 146-54.

Carralero, V.; Gonzalez-Cortes, A.; Yanez-Sedeno, P.; Pingarron, J. M. 2007: Nanostructured progesterone immunosensor using a tyrosinase-colloidal gold-graphite-Teflon biosensor as amperometric transducer. *Analytica Chimica Acta,* **596**: 86-91.

Clare, N. T. 1944: Photosensitivity diseases in New Zealand. III. The photosensitizing agent in facial eczema. *New Zealand Journal of Science and Technology, **A***: 202-220.

Claycomb, R. W.; Delwiche, M. J.; Munro, C. J.; BonDurant, R. H. 1998: Rapid enzyme immunoassay for measurement of bovine progesterone. *Biosensors & Bioelectronics,* **13**: 1165-1171.

Collett, M.; Sharma, I.; Bullock, S.; Officer, D.; Partridge, A. 2008: Spring Eczema. *Proceeding of the Society of Dairy Cattle Veterinarians of NZVA,* **268***:* 71-76

Conneely, O. M. 2001: Perspective: Female steroid hormone action. *Endocrinology,* **142**: 2194-2199.

Delwiche, M.; Tang, X.; BonDurant, R.; Munro, C. 2001: Estrus detection with a progesterone biosensor. *Transactions of the ASAE,* **44**: 2003-2008.

Deshpande, S. S. 1996: *Enzyme Immunoassays, from Concept to Product Development.* Chapman & Hall, New York.

Dhanda, P.; Ravindra, J. P. 2005: Ovarian hormones and their role in oestrous cycle in animals: A review. *Indian Journal of Animal Sciences,* **75**: 885-892.

Di Menna, M. E.; Smith, B. L.; Miles, C. O. 2009: A history of facial eczema (pithomycotoxicosis) research. *New Zealand Journal of Agricultural Research,* **52**: 345-376.

Dobson, H.; Fitzpatrick, R. J. 1976: Clinical application of the progesterone-in-milk test. *British Veterinary Journal,* **132**: 538-542.

Draisci, R.; Volpe, G.; Compagnone, D.; Purificato, I.; delli Quadri, F.; Palleschi, G. 2000: Development of an electrochemical ELISA for the screening of 17 beta-estradiol and application to bovine serum. *Analyst,* **125**: 1419-1423.

England, G.; Concannon, W. 2002: Recent Advances in Small Animal Reproduction. *International Veterinary Information Service*. Ithaca, New York, USA www.ivis.org.

Farre, M.; Kuster, M.; Brix, R.; Rubio, F.; de Alda, M. J. L.; Barcelo, D. 2007: Comparative study of an estradiol enzyme-linked immunosorbent assay kit, liquid chromatography-tandem mass spectrometry, and ultraperformance liquid chromatography-quadrupole time of flight mass spectrometry for part-per-trillion analysis of estrogens in water samples. *Journal of Chromatography, **A*** **1160**: 166-175.

Fischer, H. 1937: Chlorophyll. *Chemical Reviews,* **20**: 41-68.

Flint, A. P.; Sheldrick, E. L.; Jones, D. S.; Auletta, F. J. 1989: Adaptations to pregnancy in the interactions between luteal oxytocin and the uterus in ruminants. *Journal of Reproduction Fertility, **Suppl*** **37**: 195-204.

Foote, R. H.; Oltenacu, E. A.; Kummerfeld, H. L.; Smith, R. D.; Riek, P. M.; Braun, R. K. 1979: Milk progesterone as a diagnostic aid. *British Veterinary Journal,* **135**: 550-8.

Ford, E. J. H. 1974: Activity of gamma glutamyl-transpeptidase and other enzymes in the serum of sheep with liver of kidney damage. *Journal of Comparative Pathology,* **84**: 231-243.

Gani, M.; Coley, J.; Piron, J.; Humphreys, A. S.; Arevalo, J.; Wilson, I. A.; Taussig, M. J. 1994: Monoclonal antibodies against progesterone- effect of steroid carrier coupling position on antibody apecificity *Journal of Steroid Biochemistry and Molecular Biology,* **48**: 277-282.

GE Healthcare. 2008: Biacore Sensor surface Handbook (Edition AB). Amersham Place, Buckinghamshire, UK. pp 10-13.

Gillis, E. H.; Gosling, J. P.; Sreenan, J. M.; Kane, M. 2002: Development and validation of a biosensor-based immunoassay for progesterone in bovine milk. *Journal of Immunological Methods,* **267**: 131-138.

Gillis, E. H.; Traynor, I.; Gosling, J. P.; Kane, M. 2006: Improvement to a surface plasmon resonance based immunoassay for the steroid hormone progesterone. *Journal of the Association of Official Analytical Chemists International,* **89**: 838-842.

Grover, P. K.; Odell, W. D. 1977: Specificity of antisera to sex steroids I. The effect of substitution and stereochemistry. *Journal of Steroid Biochemistry and Molecular Biology,* **8**: 121-126.

Groves, D. J.; Sauer, M. J.; Rayment, P.; Foulkes, J. A.; Morris, B. A. 1990: The preparation of an ovine monoclonal antibody to progesterone. *Journal of Endocrinology,* **126**: 217-22.

Gyawu, P.; Pope, G. S. 1983: Oestrogens in milk. *Journal of Steroid Biochemistry,* **19**: 877-82.

Hart, J. P.; Pemberton, R. M.; Luxton, R.; Wedge, R. 1996: Studies towards a disposable screen-printed amperometric biosensor for progesterone. *4th World Congress on Biosensors (Biosensors 96).* Bangkok, Thailand, Elsevier Advanced Technology.

Homola, J. 2003: Present and future of surface plasmon resonance biosensors. *Analytical and Bioanalytical Chemistry,* **377**: 528-539.

Homola, J.; Yee, S. S.; Gauglitz, G. 1999: Surface plasmon resonance sensors: review. *Sensors and Actuators B-Chemical,* **54**: 3-15.

Huang, C. H.; Sedlak, D. L. 2001: Analysis of estrogenic hormones in municipal wastewater effluent and surface water using enzyme-linked immunosorbent assay and gas chromatography/tandem mass spectrometry. *Environmental Toxicology and Chemistry,* **20**: 133-139.

Hynninen, P. H. 1991: Chemistry of chlorophylls: modifications. *Chlorophylls*: 145-209.

Ivie, G. W. 1982: Chemical and biochemical aspects of photosensitization in livestock and poultry. *Journal of the National Cancer Institute,* **69**: 259-62.

Jiang, X.; Waterland, M.; Blackwell, L.; Wu, Y.; Jayasundera, K. P.; Partridge, A. 2009: Sensitive determination of estriol-16-glucuronide using surface plasmon resonance sensing. *Steroids,* **74**: 819-824.

Kennel, S. J. 1982: Binding of monoclonal- antibody to protein antigen in fluid phase or bound to solid supports *Journal of Immunological Methods,* **55**: 1-12.

Khatun, S.; Nara, S.; Tripathi, V.; Rangari, K.; Chaube, S. K.; Kariya, K. P.; Kumar, S.; Shrivastav, T. G. 2009: Development of ELISA for Measurement of Progesterone Employing 17--OH-P-HRP as Enzyme Label. *Journal of Immunoassay and Immunochemistry,* **30**: 186-196.

Kim, M.; Han, S. H.; Shin, Y. B.; Chung, B. H. 2007: Surface plasmon resonance biosensor chips. *Biochip Journal,* **1**: 81-89.

Laitinen, M. P.; Vuento, M. 1996: Immunochromatographic assay for quantitation of milk progesterone. *Acta Chemica Scandinavica,* **50**: 141-5.

Lehrer, A. R.; Lewis, G. S.; Aizinbud, E. 1992: Estrus detection in cattle-recent developments. *Animal Reproduction Science,* **28**: 355-361.

Lopez, H.; Bunch, T. D.; Shipka, M. P. 2002: Estrogen concentrations in milk at estrus and ovulation in dairy cows. *Animal Reproduction Science,* **72**: 37-46.

Louda, J. W.; Li, J.; Liu, L.; Winfree, M. N.; Baker, E. W. 1998: Chlorophyll-a degradation during cellular senescence and death. *Organic Geochemistry,* **29**: 1233-1251.

McFarlane, D.; Evans, J. V.; Reid, C. S. 1959: Photosensitivity diseases in New Zealand. XIV. The pathogenesis of facial eczema. *New Zealand Journal of Agricultural Research,* **2**: 194-200.

McNeilly, A. S.; Picton, H. M.; Campbell, B. K.; Baird, D. T. 1991: Gonadotropic control of follicle growth in the ewe. *Journal of Reproduction and Fertility*, ***Suppl.*** **43**: 177-186.

Meikle, A.; Tasende, C.; Sosa, C.; Garofalo, E. G. 2004: The role of sex steroid receptors in sheep female reproductive physiology. *Symposium on Reproduction in Small Ruminants.* Colonia del Sacramento, Uruguay, CSIRO Publishing.

Milgrom, L. R. 1997: *The Colors of Life: an Introduction to the Chemistry of Porphyrins and Related Compounds*. Oxford University Press, Oxford, UK. pp 84–88.

Mitchell, J. 2010: Small molecule immunosensing using surface plasmon resonance. *Sensors,* **10**: 7323-7346.

Mitchell, J. S.; Wu, Y. Q.; Cook, C. J.; Main, L. 2005: Sensitivity enhancement of surface plasmon resonance biosensing of small molecules. *Analytical Biochemistry,* **343**: 125-135.

Mitchell, J. S.; Wu, Y. Q.; Cook, C. J.; Main, L. 2006: Estrogen conjugation and antibody binding interactions in surface plasmon resonance biosensing. *Steroids,* **71**: 618-631.

Miyashita, M.; Shimada, T.; Miyagawa, H.; Akamatsu, M. 2005: Surface plasmon resonance-based immunoassay for 17beta -estradiol and its application to the measurement of estrogen receptor-binding activity. *Analytical and Bioanalytical Chemistry,* **381**: 667-673.

Moan, J.; Berg, K. 1991: The photodegradation of porphyrins in cells can be used to estimate the lifetime of singlet oxygen. *Journal of Photochemistry and Photobiology,* **53**: 549-53.

Moan, J.; Sommer, S. 1985: Oxygen dependence of the photosensitizing effect of hematoporphyrin derivative in NHIK 3025 cells. *Cancer Research,* **45**: 1608-10.

Monk, E. L.; Erb, R. E.; Mollett, T. A. 1975: Relationships between immunoreactive estrone and estradiol in milk, blood, and urine of dairy-cows *Journal of Dairy Science,* **58**: 34-40.

Morris, C. A.; Towers, N. R.; Hohenboken, W. D.; Maqbool, N.; Smith, B. L.; Phua, S. H. 2004: Inheritance of resistance to facial eczema: a review of research findings from sheep and cattle in New Zealand. *New Zealand Veterinary Journal,* **52**: 205-215.

Moss, G. P. 1987: Nomneclature of tetrapyroles. *Pure and Applied Chemistry,* **59**: 779-832.

Noakes, D. E. 2001: Endogenous and exogenous control of ovarian cyclicity. In Noakes, D. E. P., T. J.; England, G. C. W. (Eds.). *Arthur's Veterinary reproduction and obstetrics.* Saunders, Edinburgh, UK. pp 5-28.

Okuda, K.; Uenoyama, Y.; Berisha, B.; Lange, I. G.; Taniguchi, H.; Kobayashi, S.; Miyamoto, A.; Schams, D. 2001: Estradiol-17 beta is produced in bovine corpus luteum. *Biology of Reproduction,* **65**: 1634-1639.

Ou, H. C.; Luo, Z. F.; Jiang, H.; Zhou, H. M.; Wang, X. P.; Song, C. X. 2009: Indirect Inhibitive Immunoassay for Estradiol Using Surface Plasmon Resonance Coupled to Online In-Tube SPME. *Analytical Letters,* **42**: 2758-2773.

Pearson, E. G.; Dirksen, G.; Meyer, J.; Seitz, A.; Rowe, K. E. 1995: Evaluation of liver function tests in neonatal calves. *Journal of American Veterinary Medical Association,* **207**: 1466-1469.

Pemberton, R. M.; Hart, J. P.; Foulkes, J. A. 1998: Development of a sensitive, selective electrochemical immunoassay for progesterone in cow's milk based on a disposable screen-printed amperometric biosensor. *Electrochimica Acta,* **43**: 3567-3574.

Pemberton, R. M.; Hart, J. P.; Mottram, T. T. 2000: An electrochemical immunosensor for milk progesterone using a continuous flow system. *6th World Congress on Biosensors (Biosensors 2000).* San Diego, California, Elsevier Advanced Technology.

Pemberton, R. M.; Mottram, T. T.; Hart, J. P. 2005: Development of a screen-printed carbon electrochemical immunosensor for picomolar concentrations of estradiol in human serum extracts. *Journal of Biochemical and Biophysical Methods,* **63**: 201-212.

Perrin, D. D. 1958: Determination of phylloerythrin in blood *Biochemical Journal,* **68**: 314-318.

Phillips, K. S.; Cheng, Q. 2007: Recent advances in surface plasmon resonance based techniques for bioanalysis. *Analytical and Bioanalytical Chemistry,* **387**: 1831-1840.

Posthuma-Trumpie, G. A.; van Amerongen, A.; Korf, J.; van Berkel, W. J. H. 2009: Perspectives for on-site monitoring of progesterone. *Trends in Biotechnology,* **27**: 652-660.

Pucci, V.; Bugamelli, F.; Mandrioli, R.; Luppi, B.; Raggi, M. A. 2003: Determination of progesterone in commercial formulations and in non conventional micellar systems. *Journal of Pharmaceutical and Biomedical Analysis,* **30**: 1549-1559.

Rich, R. L.; Myszka, D. G. 2000: Advances in surface plasmon resonance biosensor analysis. *Current Opinion in Biotechnology,* **11**: 54-61.

Rimington, C.; Quin, J. I. 1934: Studies on the photosensitisation of animals in South Africa. VII The nature of the photosensitising agent in geeldikkop. *Onderstepoort Journal of Veterinary Science and Animal Industry,* **3**: 137-57.

Rioux, P.; Rajotte, D. 2004: Progesterone in milk: a simple experiment illustrating the estrous cycle and enzyme immunoassay. *Advances in Physiology Education,* **28**: 64-67.

Sananikone, K.; Delwiche, M. J.; BonDurant, R. H.; Munro, C. J. 2002: Quantitative lateral flow immunoassay for measuring progesterone in bovine milk. *ASAE International Meeting.* Chicago, Illinois, American Society of Agricultural Engineers, **47**: 1357-1365

Scaramuzzi, R. J.; Adams, N. R.; Baird, D. T.; Campbell, B. K.; Downing, J. A.; Findlay, J. K.; Henderson, K. M.; Martin, G. B.; McNatty, K. P.; McNeilly, A. S.; Tsonis, C. G. 1993: A model for follicle selection and the determination of ovulation rate in the ewe. *Reproduction Fertility and Development,* **5**: 459-478.

Schasfoort, R. B. M.; Tudos, A. J.; 2008: SPR instrumentation. *Handbook of Surface Plasmon Resonance*. pp 35-80.

Scheie, E.; Flaoyen, A. 2003: Fluorescence spectra and measurement of phylloerythrin (phytoporphyrin) in plasma from clinically healthy sheep, goats, cattle and horses. *New Zealand Veterinary Journal,* **51**: 191-193.

Scheie, E.; Flaoyen, A.; Moan, J.; Berg, K. 2002: Phylloerythrin: Mechanisms for cellular uptake and location, photosensitisation and spectroscopic evaluation. *New Zealand Veterinary Journal,* **50**: 104-110.

Scheie, E.; Ryste, E. V.; Flaoyen, A. 2003a: Measurement of phylloerythrin (phytoporphyrin) in plasma or serum and skin from sheep photosensitised after ingestion of Narthecium ossifragum. *New Zealand Veterinary Journal,* **51**: 99-103.

Scheie, E.; Smith, B. L.; Cox, N.; Flaoyen, A. 2003b: Spectrofluorometric analysis of phylloerythrin (phytoporphyrin) in plasma and tissues from sheep suffering from facial eczema. *New Zealand Veterinary Journal,* **51**: 104-110.

Shrivastav, T. G.; Chaube, S. K.; Charu; Rangari, K.; Kariya, K. P.; Singh, R.; Nagendra, A. 2010: Enzyme linked immunosorbent assay for milk progesterone. *Journal of Immunoassay and Immunochemistry,* **31**: 301-313.

Smith, B. L.; Coe, B. D.; Embling, P. P. 1978: Protective effect of zinc sulphate in a natural facial eczema outbreak in dairy cows. *New Zealand Veterinary Journal,* **26**: 314-315.

Smith, B. L.; Towers, N. R. 2002: Mycotoxicoses of grazing animals in New Zealand. *New Zealand Veterinary Journal,* **50**: 28-34.

Snyder, S. A.; Keith, T. L.; Verbrugge, D. A.; Snyder, E. M.; Gross, T. S.; Kannan, K.; P., G. J. 1999: Analytical methods for detection of selected estrogenic compounds in aqueous mixtures. *Environmental Science and Technology,* **33**: 2814-2820.

Sondheimer, F.; Kaufmann, S.; Romo, J.; Martinez, H.; Rosenkranz, G. 1953: Steroids. XLVII. Synthesis of steroidal hormone analogs hydroxylated at C-2. *Journal of American Chemical Society,* **75**: 4712-15.

Stenberg, E.; Persson, B.; Roos, H.; Urbaniczky, C. 1991: Quantitative determination of surface concentration of protein with surface plasmon resonannce using radiolabeled proteins. *Journal of Colloid and interface Surface,* **143**: 513-526.

Tennant, B. C. 1998: Lessons from the porphyrias of animals. *Clinics in Dermatology,* **16**: 307-315.

Tudos, A. J.; Schasfoort, R. B. M. 2008: Introduction to surface plasmon resonance. *Handbook of Surface Plasmon Resonance.* pp 1-14.

Volpe, G.; Fares, G.; delli Quadri, F.; Draisci, R.; Ferretti, G.; Marchiafava, C.; Moscone, D.; Palleschi, G. 2006: A disposable immunosensor for detection of 17beta -estradiol in non-extracted bovine serum. *Analytica Chimica Acta,* **572**: 11-16.

Waldmann, A. 1999: Monoclonal antibodies to progesterone: Characterization and selection for enzyme immunoassay in bovine milk. *Hybridoma,* **18**: 289-296.

Wathes, D. C.; Ayad, V. J.; McGoff, S. A.; Morgan, K. L. 1989: Effect of active immunization against oxytocin on gonadotropin secretion and the establishment of pregnancy in the ewe. *Journal of Reproduction and Fertility,* **86**: 653-64.

Weishaupt, K. R.; Gomer, C. J.; Dougherty, T. J. 1976: Photoradiation therapy. 3. Identification of singlet oxygen as the cytotoxic agent in photo-inactivation of a murine tumor. *Cancer Research,* **36**: 2326-9.

Wood, R. W. 1902: On a remarkable case of uneven distribution of light in a diffraction grating spectrum. *Philosophical Magazine,* **4**: 396-402.

Wu, Y. Q.; Mitchell, J.; Cook, C.; Main, L. 2002: Evaluation of progesterone-ovalbumin conjugates with different length linkers in enzyme-linked immunosorbant assay and surface plasmon resonance-based immunoassay. *Steroids,* **67**: 565-572.

Yoshida, C.; Nakao, T. 2005: Some Characteristics of Primary and Secondary Oestrous Signs in High-producing Dairy Cows. *Reproduction in Domestic Animals,* **40**: 150-155.

Yoshida, H.; Nakai, S.; Nimbari, F.; Yoshimoto, K.; Nishimura, T. 1989: Synthesis of 11 alpha-hydroxyprogesterone haptens. *Steroids,* **53**: 727-38.

Yuan, J.; Oliver, R.; Li, J.; Lee, J.; Aguilar, M.; Wu, Y. 2007: Sensitivity enhancement of SPR assay of progesterone based on mixed self-assembled monolayers using nanogold particles. *Biosensors and Bioelectronics,* **23**: 144-148.