Rapid On-line Glycogen Measurement and Prediction of Ultimate pH in Slaughter Beef

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ATTESTATION OF AUTHORSHIP

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person (except where explicitly defined in the acknowledgements), nor material which to a substantial extent has been submitted for the award of any other degree or diploma of a university or other institution of higher learning.

Signed: _____ Date: _____

Dominic Lomiwes

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CONFIDENTIAL MATERIAL

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ABSTRACT

The rapid determination of glycogen on indicator muscle immediately after slaughter is advantageous as it permits the prediction of a muscle's ultimate pH (pH_u) and allows the identification of high pH_u meat carcasses by extrapolation. This thesis examines the development of two rapid glycogen determination methods.

The first aim of this thesis was to find a new glucometer to replace the Bayer ESPRIT™ (Bayer) glucometer currently used in the Rapid pH (RpH) method. Roche's Accuchek® Advantage II (Accuchek) and Abbott's Medisense Optium™ (Medisense) glucometers were compared. Accuchek measurements exhibited a positive linear relationship in glucose standards made with water, RpH buffer and glucose spiked meat/buffer slurries ranging from 0 to 500 mg dL^{-1} (r^2 = 0.999, 0.998 and 0.995 respectively). Medisense also exhibited a strong positive relationship for glucose standards made with water and RpH buffer; however, a non-linear trend in spiked meat slurries was observed.

The second aim of this thesis was to explore the calibration of the KES K201 (KES Analysis Inc., NY, USA) near-infrared (NIR) diode array spectrometer to measure glycogen and pH at approximately 45 minutes after slaughter (pH_{45}), and to predict pH_u in pre-rigor *M. longissimus dorsi* (LD) from beef. This first required finding a reference method to calibrate against the NIR instrument. The RpH, Iodine and Bergmeyer methods were compared. Analysis of glycogen in replicate samples of three beef LD muscles at timepoints post-mortem (1, 4, 9 and 20 hours) was conducted. No significant difference in glycogen concentration was found between an enzymatic and an iodine based colorimetric method at each timepoint; however, the Iodine method was more consistent than the Bergmeyer method at all timepoints. Glucose measurements from the RpH method were consistent; however the pattern of glycogen decline at increasing timepoints post-mortem did not correspond with existing published studies.

NIR spectra (538 to 1677 nm) of LD muscles from steers (n = 47), cows (n = 28) and bulls (n = 20) routinely slaughtered in a commercial abattoir were collected on-line approximately 45 minutes after slaughter. Poor results were obtained for Partial Least Squares (PLS) models generated from the mean reflectance spectra of each animal to measure glycogen and pH_{45}, and predict pH_u (r^2 = 0.23, 0.37

and 0.20 respectively). A high mean square error of prediction (MSEP) for glycogen was also obtained (7.75).

Validation of qualitative models generated with Generalised Partial Least Squares regression (GPLS) found that the optimum model was able to correctly categorise only 42% of high pH_u samples with the remaining portion being wrongly classified as normal pH_u meat. When the effect of gender was removed, only 21% of high pH_u carcasses were correctly categorised.

Exploratory analysis of the absorbance spectra of the LD muscles showed that a group composed predominantly of steers had a significantly lower pH_{45} than other existing groups. Further work is recommended for NIR to be successfully utilised on-line to measure glycogen or predict pH_u in pre-rigor carcasses.

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CHAPTER 1

INTRODUCTION

The rapid glycogen determination of pre-rigor muscle of freshly slaughtered beef carcasses is important in the prediction of ultimate pH (pH_u). Many quality attributes are dependent on the meat's pH_u and meat with the most desirable properties generally have 'normal' pH_u values ranging from 5.4 to 5.6. In some meat processing plants, meat with an elevated ultimate pH_u of 5.7 to 6.1 and greater than 6.1 are classed as intermediate pH_u and high pH_u, respectively. Meat with an elevated pH_u is undesirable as a table cut because it is dark in colour, inferior in flavour and highly susceptible to microbial degradation. For this reason, they meat is usually sold at a lower price than normal pH_u meat.

For muscle to achieve normal pH_u, it must contain sufficient concentration of glycogen at the time of slaughter. When the muscle undergoes anaerobic metabolism after slaughter, glycogen is converted to lactic acid via the glycolytic pathway. Lactic acid accumulates in the meat causing the meat pH to fall. If the glycogen concentration is limited, pH decline is arrested at higher values, frequently resulting in DFD meat.

The New Zealand pastoral production system generates a range of meat pH_u values. Approximately 70 percent of bulls, 30 percent of cows and 10 percent of steers yield beef with high pH_u values ($pH_u > 5.8$). Due to this high production of high pH_u beef, most beef produced from bulls and cows is sold as frozen manufacturing commodities at a lower price than beef from steers. In contrast, prime beef (from steers) are sold as higher value chilled products. This approach leads to loss in profitability as a significant amount of cows and bulls will yield meat with a normal pH_u that could be sold as higher value chilled products. Conversely, there are instances where chilled beef designated as 'prime', is actually high pH_u meat, which leads to customer dissatisfaction.

For processors to optimise profitability from their product, a method of rapidly identifying carcasses that will yield high pH_u meat is required before carcasses are hot boned and packed. A significant curvilinear relationship exists between the pH_u of

beef and the glycogen content immediately after slaughter. This means that it is possible to predict the pH_u of muscle if the glycogen content after slaughter is known.

Developments in two on-line methods of rapidly measuring glycogen in pre-rigor beef muscle are examined in this thesis. The recent discontinuation in production of Bayer glucometers used for the existing Rapid pH (RpH) method has seen the need for the calibration of a new glucometer to replace the Bayer model. The efficacy of near-infrared (NIR) spectroscopy as an on-line instrument to rapidly measure glycogen in pre-rigor beef was also investigated. Although previous studies have shown NIR as a spectroscopic tool capable of measuring several meat quality attributes, its applicability as an on-line tool in commercial abattoirs is largely unknown. Thus, this thesis explores the efficacy of NIR spectroscopy as an on-line instrument to rapidly measure glycogen in pre-rigor beef muscle.

Chapter 3 of this thesis details the materials and methods that were used. This is followed by the presentation of the results and discussions in Chapters 4 and 5 where results obtained on finding a replacement glucometer for the RpH method are presented. Finally, the calibration and success of NIR spectroscopy to quantify glycogen and predict the pH_u of pre-rigor beef muscle is discussed.

Conclusions drawn from the results are presented in Chapter 6. Recommendations for further research with regards to the rapid determination of glycogen using NIR spectroscopy are also discussed.

CHAPTER 2

LITERATURE REVIEW

NEW ZEALAND BEEF

The agricultural sector is a significant revenue earner to the New Zealand economy and makes up 6% of the global meat trade ("Meat and Wool New Zealand," 2007a). In the year ended March 2007, over 360,000 tonnes of beef were exported earning over $1.8 billion, which is up 5% on the previous year (MAF, 2007).

Cattle, which are all eventually destined for beef production in one form or another, are predominantly raised in the North Island, accounting for 75 percent of the 4.4 million cattle in New Zealand (Meat and Wool New Zealand, 2007a). Approximately half of the meat produced from cattle is from steers (male castrates) and heifers (virgin females), collectively classified as prime, with cows and bulls making up most of the remaining half (Statistics New Zealand, 2007). Beef cattle come from a variety of breeds but mainly from Angus, Hereford, Angus × Hereford and Friesian lines.

Up to 80% of beef produced in New Zealand is exported. New Zealand's largest beef market is the United States taking about 50 percent of all New Zealand beef exports. Most of this beef is frozen manufacturing grade beef from cows and bulls. North Asia (Japan, South Korea and Taiwan) is the second largest market with 23% of beef produced in 2006 exported to these countries ("Meat and Wool New Zealand," 2007b). Other major international markets are South Asia and the Pacific nations.

New Zealand cattle are overwhelmingly pasture-fed, which is a key factor that is used to differentiate New Zealand beef from many competitors. This is particularly important in the United States where beef cattle are cereals-finished resulting in meat with a high intramuscular fat content (marbling) compared with meat from New Zealand. The difference between the beef produced from both countries is complementary as they are destined for different food purposes. In the United States, locally produced beef are destined as higher value cuts whereas beef imported from

New Zealand are intended for manufacturing purposes. This is the driver for New Zealand's strong export trade with the United States.

The success of New Zealand beef exports is also due to the marketing of New Zealand beef in North Asia. Consistent emphasis is placed on New Zealand beef cattle as being raised in a 'natural' free-range environment and claims that New Zealand beef is a healthier food alternative to grain-finished beef. Emphasis is also placed in New Zealand beef as a safe, disease-free product. This is particularly important in the North Asian market where bovine spongiform encephalopathy (BSE) was detected in American and Canadian beef in 2003. This led to the immediate ban of North American beef exports between 2003 and 2006 leaving New Zealand and Australia as the only countries allowed to export beef to Japan and South Korea in that period (MAF, 2007). In these years, New Zealand's beef market share in Japan quadrupled from 2.1 to 8.5 percent (Meat and Wool New Zealand, 2007b).

Although international prices for New Zealand beef are currently at an all time high with beef production forecast to increase within the next two years (MAF, 2007), there are challenges that face the beef industry. The increase in the proportions and absolute numbers of discerning consumers in key markets has seen increased emphasis placed on safety, quality and convenience on products that must also be competitively priced. Innovative research is critical to meet these demands where new technologies can be applied to cut costs, add value or to ensure that consistent high quality is achieved.

INDUSTRIAL PROCESSES IN THE SLAUGHTER OF CATTLE

In order to understand the different factors affecting meat quality, it is important to have an overview of processes involved in the slaughter of animals and the general handling of the carcass. In New Zealand, strict guidelines dictating the slaughter process and handling of the carcass revolves around animal welfare and hygiene. Although exact slaughter processes vary between abattoirs, the method of slaughter and the subsequent treatment of the carcass are relatively similar.

Pre-slaughter handling and ante-mortem inspection

Animals scheduled for slaughter are held in lairage before slaughter. This allows animals to be easily directed into laneways that lead to the slaughter line. Ideally, animals are kept in unmixed groups to avoid undue stress and injuries that they may acquire from social interaction and fighting with unfamiliar animals. However, in an abattoir situation, this may not always be the case.

Antemortem examinations of animals are conducted prior to slaughter. At this stage, animals are individually observed for signs of transmittable diseases or stress that may produce meat unacceptable for human consumption. After an animal has passed the inspection, it is cleared for slaughter. Animals that exhibit signs of sicknesses or other abnormalities are inspected further.

The slaughter of cattle

Stunning is the first step in the slaughter process. The stunning of an animal is performed to fulfil humane slaughter guidelines that require the animal to be "rendered insensible to pain prior to being slaughtered by bleeding" (NZFSA, 2002). There are many methods used to achieve insensibility. In New Zealand, electrical stunning of cattle is predominantly employed.

Head-only electrical stunning is the most common method of stunning in New Zealand. It works by passing a current through the animal's brain. The current applied results in an epileptic reaction in the animal where all parts of its brain are stimulated. This results in the induced unconsciousness and insensibility of the animal. In a typical stunning system, the current is passed through electrodes behind the ears and on the nose of the restrained animal. The insensible and relatively motionless animal is then presented for slaughter.

Sticking is the generic term used to describe the severing of the vessels supplying blood to the brain. Once sticking has been performed, the animal is exsanguinated quickly, ultimately leading to the animal's death. In New Zealand, two methods of sticking are used. These are thoracic stick and the halal stick.

In a thoracic stick, an incision is made in the chest of the animal towards the heart where the branchiocephalic trunk is severed using a knife. The branchiocephalic trunk gives rise to carotid arteries that supply blood to the brain. Once the stunned animal has been shackled on a hind leg and hoisted, the stick is then

performed on the thoracic inlet between the first and the second rib of the suspended animal. This results in rapid exsanguination.

A halal stick is made by an incision in the neck with a knife severing the carotid arteries and jugular veins, similarly resulting in exsanguination and death (Shragge & Price, 2004). There are several requirements for the halal slaughter of animals. In New Zealand, for an abattoir to be halal certified, the person conducting the halal stick must be Muslim. The name of Allah (God) must be spoken over the animal and stick must be carried out from the front (chest) of the animal and not from the back. It is required that the death of the animal results from the stick and subsequent exsanguination. It is for this reason that electrical stunning is used only to immobilise animals before slaughter. While electrical stunning induces unconsciousness and insensibility to animals, it does not kill the animal, and given a few minutes, the animal can fully recover and live a normal life (Shragge & Price, 2004).

Most cattle in New Zealand are slaughtered according to halal procedures. The halal slaughter of beef cattle is currently an issue in New Zealand. Beef imports from New Zealand to Malaysia were banned in 2005 after inspectors decided that halal procedures were not fully adhered to (NZPA, 2007). By complying with halal procedures, export meat is accessible by both the Muslim and non-Muslim communities since the latter have no religious requirements.

Electrical stimulation

The application of low voltage electrical stimulation to a carcass is commonly applied following exsanguination and death. Low voltage electrical stimulation is achieved by passing a current through a freshly slaughtered carcass where the current is applied through the shackled left hind hoof and passes out through an electrode manually clipped on the nose, head or neck of the carcass (Devine, Hopkins, Hwang, Ferguson, & Richards, 2004). The application of an electric current causes the muscles in the carcass to contract increasing the metabolic breakdown of glycogen and a rapid fall of pH. This has implications in meat quality where the toughness in meat resulting from a phenomenon known as cold shortening is reduced (Hwang, Devine, & Hopkins, 2003). This is further discussed later in this chapter in the **MEAT QUALITY** section.

Carcass dressing

The dressing of a carcass is carried out from an overhead rail system that continues the production line from the point of stunning. Over the past decades, the Zealand meat industry has invested heavily in the mechanisation of the dressing process to replace as many labour units as possible and to increase process efficiency.

After electrical stimulation, the hooves and head are first removed and the carcass is prepared for hide stripping. This is achieved using capstans which pull the hide downward to separate it from the subcutaneous muscles (Belk & Scanga, 2004). Two operators stand on motorised descending platforms at each side of the carcass and assist with hide removal using rotating shears (Longdell, 2000).

The evisceration of the carcass then follows where the abdomen is split open and the viscera (rumen, intestines, liver and spleen) are removed along with other internal organs. Some organs such as the heart and liver undergo inspection to determine any abnormalities that may result in the carcass being condemned (Belk & Scanga, 2004).

Following evisceration, the carcass is halved by sawing it longitudinally from tail to neck along the vertebral column. It is then quartered between the 12th and 13th ribs but cutting only through the vertebrae so that the carcass remains as two sides rather than four pieces. It then progresses to a grading station where the final inspection for any abnormalities is made and a grade assigned based on weight, fatness, gender, and other factors depending on the abattoir's target market. After passing the final inspection, the sides are then either moved to a chiller or immediately hot boned.

Carcass boning

After dressing, the carcass sides are traditionally held in a chiller set between 2 to 4°C for approximately 36 hours before it is boned into commercial cuts (Ockerman & Basu, 2004). This method is known as cold boning because the carcass had reached the chiller temperature and rigor mortis is complete during boning (Waylan & Kastner, 2004). In meat processing plants, cold boning is normally carried out on prime animals destined for export or animals destined for local consumption. Alternatively, whole carcasses are also transported in refrigerated trucks to local meat

retailers where the meat may be aged before it is boned and sold as table cuts by the retailer.

Hot boning is the removal of lean meat and fat from the carcass preceding rigor mortis. Most hot boned beef processed in New Zealand is exported frozen in plastic-lined cartons for manufacturing purposes. In contrast to cold boning, hot boning is carried out while the carcass is still warm, and in New Zealand, this happens immediately after the grading point, typically within 45 minutes after slaughter. High value cuts such as striploins (*M. longissimus dorsi*) and tenderloins (*M. psoas major* and *minor*) are removed, vacuum packed and chilled. The rest of the carcass is boxed and blast frozen. Hot boned meat from cows and bulls are immediately frozen after boning as the enduring market perception is that muscles from these animals will yield meat that is of inferior eating quality.

There are many advantages to hot boning over the conventional cold boning practice. Hot boning is estimated to save meat processors up to 50 percent of refrigeration costs and space. Other advantages of hot boning are labour savings, shorter processing times and lower transport costs of the product (Ockerman & Basu, 2004).

Although hot boning brings many advantages, concerns regarding its effect on meat quality have been raised. These concerns include the higher microbial susceptibility of hot boned meat (Taylor, 1995). As hot boning is conducted at higher temperatures than the conventional cold boning method, there is a higher risk of bacterial growth on the meat. Although there may be conflicting views on this matter, research has found that hot boned meat are microbiologically equivalent to cold boned meat (Bell, Harrison, Moorhead, & Jones, 1998; Waylan & Kastner, 2004) as long as prescribed hygiene practices are followed. Additional concerns with the hot boning method are the retention of muscle shape and the increased incidence of shortening/toughening of the meat that can occur because, unlike the cold boning method, the muscles are not restrained by the skeleton until rigor mortis is reached.

THE METABOLIC CONVERSION OF MUSCLE TO MEAT

Immediately after slaughter, the transport of nutrients and oxygen to within the body of the animal is stopped due to the massive loss of blood. This leads to changes

in the metabolic processes within the animal, where aerobic metabolism stops and anaerobic metabolism takes over. This has implications to the biochemistry and structure of the muscle as it enters the rigor mortis state to become meat as we know it.

The structure of muscles

Meat is heterogeneous and is macroscopically composed of contractile tissue (true muscle), fat, and connective tissue. Skeletal muscle generally represents a bulk (35 to 65 percent) of the carcass weight of slaughtered animals. Meat also contains some smooth muscle as a component of blood vessels (Hendrick, Aberle, Forrest, Judge, & Merkel, 1994). Due to people's concept of meat and muscle, the two terms are sometimes used interchangeably. In this thesis, the term meat is used to describe muscle that is in rigor.

Skeletal muscle is made up of structural units called muscle fibres (cells) which are bound together into muscle bundles and are in turn collectively grouped to make the muscle. Muscle fibres make up 75 to 92 percent of the total muscle volume, with connective tissues, blood vessels and extracellular fluid accounting for the remaining volume (Hendrick et al., 1994).

Skeletal muscle fibres from mammals are long, threadlike cells with tapered ends (Figure 1). Although they may reach lengths of up to several centimetres long, these unbranched cells usually do not extend the length of the muscle. Muscle fibres contain all the organelles normally found in living cells within their sarcoplasm (cytoplasm) and are bound by a collagen-stabilised membrane known as a sarcolemma. Sarcolemmas have elastic properties, which allows them to endure considerable distortion during muscle contraction, relaxation and stretching (Hendrick et al., 1994).

A breakdown of skeletal muscle and its components are shown in Figure 2. Muscle fibres are unique in that within the sarcoplasm, there is a regular arrangement of cylindrical myofibrils lying side by side and oriented longitudinally along the entire length of the cell. Each myofibril is made up of smaller components known as myofilaments, dominantly comprising of so-called thick and thin filaments. Thick filaments are composed of the multichained protein myosin. Thick filaments are organised longitudinally in exact alignment parallel to each other across the myofibril. Thin filaments, which are made up of actin proteins are also aligned parallel to each

other across the myofibril and are arranged between two sections of thick filaments. This alternating arrangement of the myofilaments shows up as transverse bands along the skeletal muscle when observed microscopically. It is for this reason why skeletal muscle is also referred to as striated muscle (Hendrick et al., 1994).

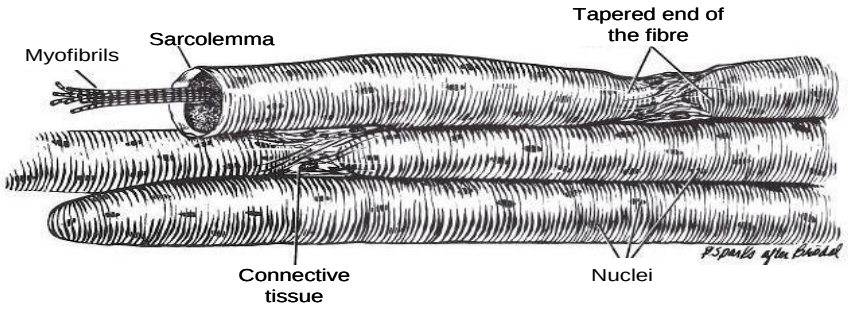

Figure 1: A diagram of a skeletal muscle fibre (Hendrick et al., 1994). They are unbranched, arranged longitudinally and bound by sarcolemmas. The fibres do not extend the length of the cell and have tapered ends.

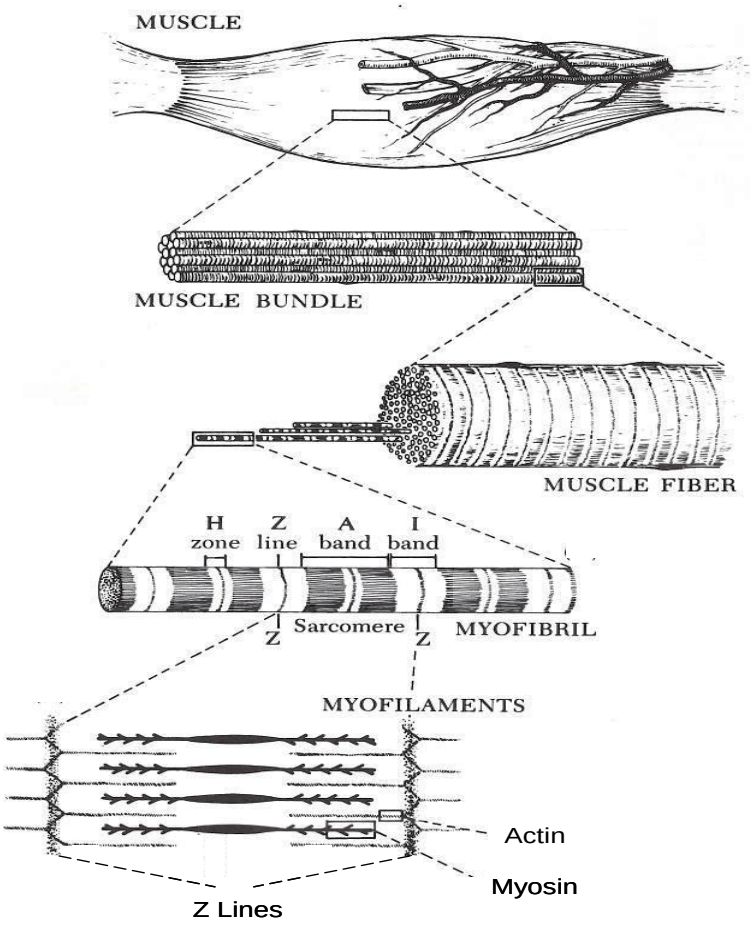

Figure 2: Diagram of a skeletal muscle and its constituents (Hendrick et al., 1994). Myofibrils are predominantly composed of myosin (thick filaments) and actin (thin filaments).

Muscle metabolism in living animals

Muscles cells are developed to convert chemical energy into mechanical energy. Myofilaments are important as they are involved in the contraction and relaxation of the muscle in a mechanism known as the sliding filament hypothesis (Huxley, 1969). During muscle contraction, the thick and thin filaments slide over one another and become linked by cross bridges formed between actin and myosin proteins (Figure 3). When they are in this state, their combined configuration is referred to as actomyosin (Warriss, 2000).

Energy is required to achieve the work of muscle contraction. The energy is dominantly derived from the nucleotide adenosine triphosphate (ATP) produced by enzyme systems in the sarcoplasm and mitochondria of the muscle fibres[1]. Energy production from ATP needed to fuel muscle contraction is catalysed by a calcium ATPase where ATP is hydrolysed to adenosine diphosphate (ADP) and inorganic phosphate. The enzyme system (ATPase) that regulates this reaction is located in the head of the myosin molecule, whose activity is triggered by the release of calcium ions (Ca^{2+}) in the sarcoplasm[2]. The hydrolysis of ATP and the resulting contraction of the muscle due to the cross bridges formed between actin and myosin proteins convert chemical energy into mechanical energy (Hendrick et al., 1994). ATP is also involved in actomyosin dissociation resulting in muscle relaxation (Davies, 2004).

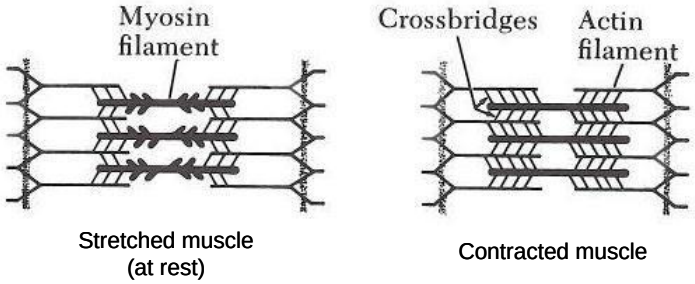

Figure 3: Diagrammatic representation of a sarcomere at various stages of contraction (Hendrick et al., 1994).

In the muscle of living animals, fuel for the production of ATP is mainly sourced from carbohydrates in the blood (mainly glucose), free fatty acids, or glycogen. These substrates undergo aerobic metabolism. In unstressed animals,

[1] Creatine phosphate provides some energy.
[2] In this sense myosin is an enzyme, but it is usually referred to as a structural protein of muscle.

glucose is metabolised for ATP production and surplus energy is stored as glycogen and creatine phosphate in the muscle cells. The production of ATP from glucose sequentially involves glycolysis, oxidative decarboxylation and oxidative phosphorylation. On the whole, the production of ATP is a complex process but only a brief outline is needed for the scope of this thesis.

Glucose in the blood initially undergoes glycolysis where it is broken down to give two molecules of pyruvate[3] (Figure 4). During glycolysis, two ATP molecules are consumed and four ATP molecules are generated from adenosine diphosphate (ADP) and phosphate. Thus a net yield of two ATP molecules and four hydrogen atoms are gained for each glucose molecule converted to pyruvate.

The two pyruvate molecules formed from the glycolysis of glucose then undergoes decarboxylation. A CO_2 molecule is initially lost from pyruvate resulting in the formation of acetyl coenzyme A (acetyl-CoA). Decarboxylation occurs in a cycle known as Krebs or tricarboxylic acid (TCA) cycle. During this cycle, pyruvate loses carbon as CO_2 and in the process generate hydrogen atoms carried as nicotine adenine dinucleotide (NADH) or flavin adenine dinucleotide ($FADH_2$). Ten hydrogen atoms are generated from each pyruvic acid. Thus a net yield of 20 hydrogen atoms is gained from the decarboxylation of two pyruvate molecules.

The final phase of ATP production from the metabolism of carbohydrate in muscle is oxidative phosphorylation. Up to this point, a net yield of 24 hydrogen atoms has been generated from the glycolysis of glucose and oxidative decarboxylation of the pyruvate molecules in the TCA cycle. Oxidative phosphorylation occurs in the cytochrome system where a pair of hydrogens enters the system as NADH. For each two hydrogen, three molecules of ATP are generated. Thus, 36 ATP molecules are generated from the metabolism of one glucose molecule in addition to the ATP generated from glycolysis (Warriss, 2000).

Muscle metabolism post-mortem

Muscles do not immediately stop functioning following the death of an animal as metabolic enzymes controlling glycolysis and ATP production are still active (Hamm, 1977). However, the activities of these metabolic enzymes are variously lost within a few hours or days post-mortem due to the cessation of the circulatory system

[3] Or more accurately, a mix of pyruvic acid and pyruvate depending on the pH in muscle cells.

which transports oxygen and glucose to the muscle. In the absence of oxygen, pyruvate can no longer be metabolised via the aerobic TCA cycle, thus it is anaerobically metabolised to lactic acid. Although much less ATP is produced from the anaerobic metabolism of glucose than by the complete process to CO_2 and water, it is enough to retain muscle extensibility for some hours. Generation of ATP is an attempt to maintain the ATP concentrations to preserve muscle homeostasis (Hendrick et al., 1994).

One significant post-mortem change in muscle due to anaerobic metabolism is the lowering in the pH of the muscle. During anaerobic metabolism, muscles preferentially utilise glycogen over free glucose remaining in the muscle. This is perhaps because glucose has to come in the muscle via the blood stream which is no longer functioning. Glycogen, however, is in situ. Another proposed reason for the preferential utilisation of glycogen over glucose for anaerobic metabolism is that the phosphorylation of glycogen-derived glucose does not require ATP as does hexokinase-catalysed phosphorylation of glucose. Hence, glycogen derived glucose is more energy efficient. The generation of ATP through the anaerobic metabolism of glycogen results in lactic acid as the terminal metabolite (Figure 4). In live animals, any excess production of lactic acid due to temporary oxygen deprivation is transported via the circulatory system away from the muscle (Hendrick et al., 1994). However, in carcasses where the circulatory system has been terminated, lactic acid necessarily accumulates in the muscle. Glycolysis usually ceases before all glycogen has been used up. This may be explained by the inactivation of glycolytic enzyme systems due to the low pH that develops within the muscle. In a well-fed, unstressed animal the pH fall is typically from 7.2 to an ultimate pH of 5.5 (Warriss, 2000).

ATP is essential in keeping muscle in a relaxed state by preventing the formation of actomyosin (Warriss, 2000). Muscle shows extensible properties while ATP is still abundant in the carcass. However, as the pH decreases and the metabolism of glycogen is halted, ATP concentrations finally fall below a threshold required to maintain relaxation in muscles (Greaser, 2001). When this occurs, actin and myosin combine to form permanent cross bridges resulting in rigor mortis where the muscle tends to shorten and, as the name rigor suggests, extensibility is lost (Marsh & Carse, 1974). The anaerobic depletion of glycogen and ATP are observed within a few hours post-mortem. The kinetics of the fall in glycogen concentration, decrease in pH and increase in lactate are illustrated in Figure 5.

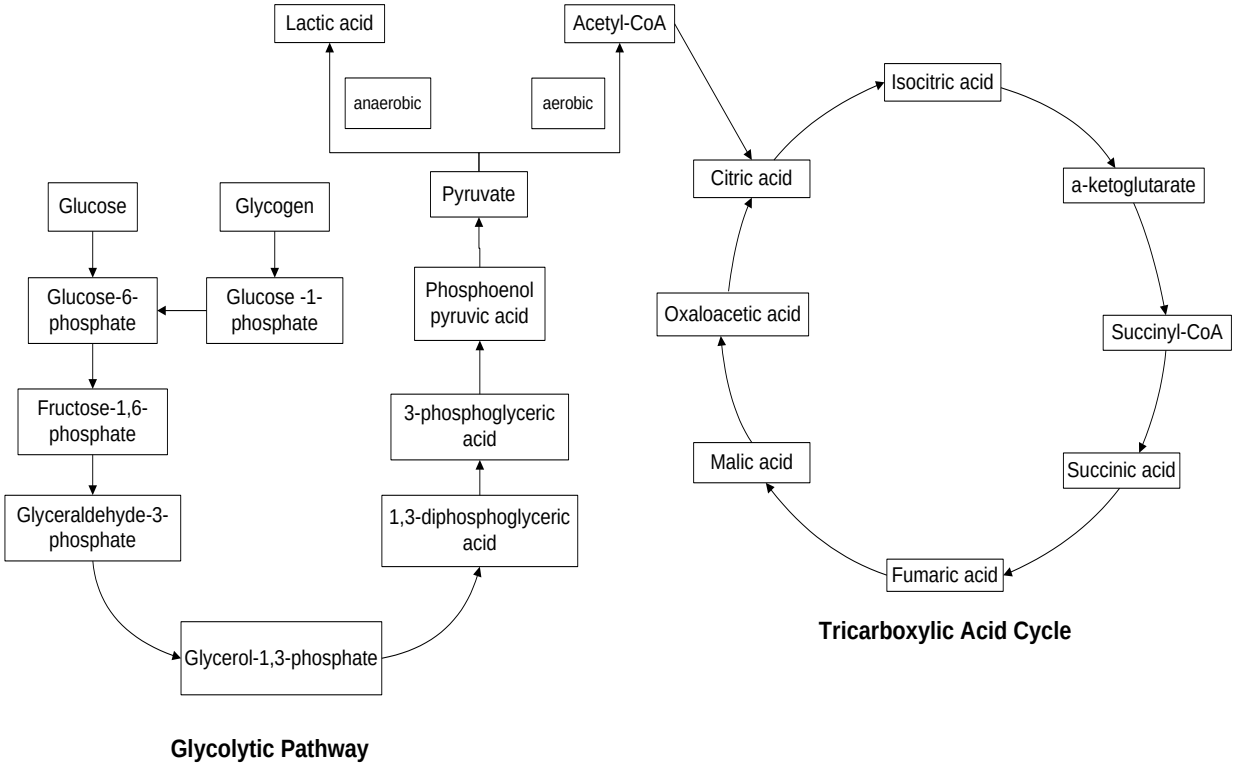

Figure 4: A schematic diagram of the glycolytic pathway and tricarboxylic acid cycle (TCA). During aerobic metabolism, pyruvic acid is converted to acetyl-CoA which enters the TCA cycle. However, in the absence of oxygen, pyruvic acid is converted to lactic acid as the terminal metabolite.

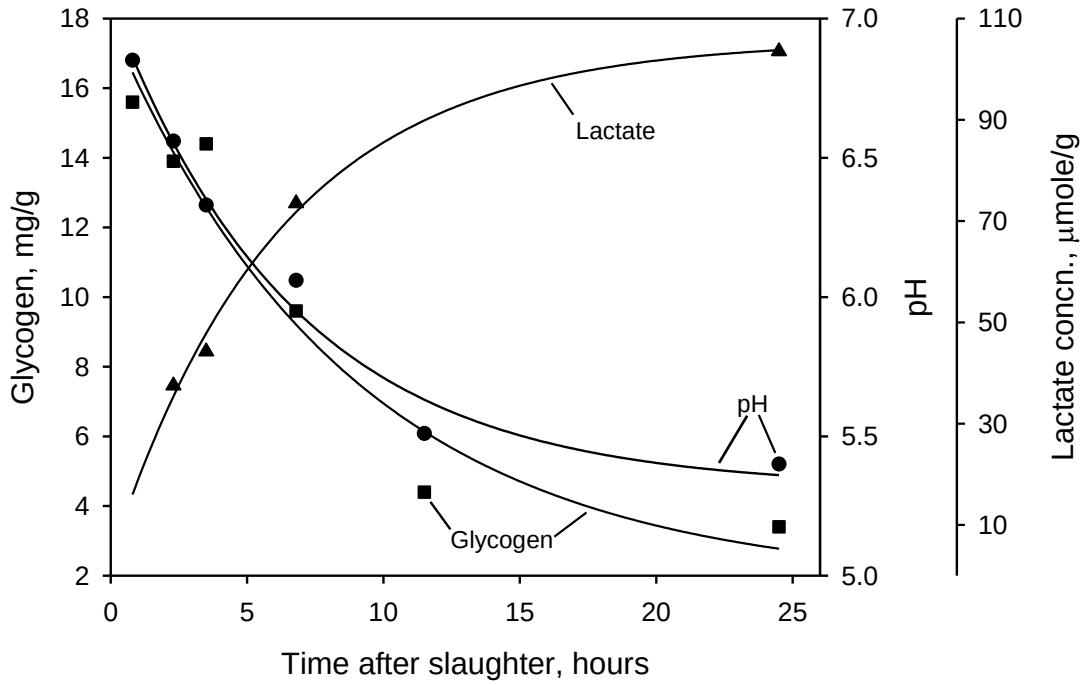

Figure 5: Kinetics of glycogen loss, pH fall and lactate accumulation in *longissimus lumborum* muscle from an unstimulated bovine carcass (Young, West, Hart, & Otterdijk, 2004).

Changes in muscle structure during ageing

During the onset of rigor and continuing after rigor as meat, muscle proteins are selectively and progressively hydrolysed by endogenous enzymes. This process is manifest as the softening of the rigor rigidity and tenderisation of the meat as perceived after cooking and during consumption. The tenderisation process is also known as ageing. Depending on the temperature and other factors, it can take several weeks for a muscle to reach maximum tenderness. At the core of tenderisation is the weakening of the myofibrillar structure due to hydrolysis of certain structural proteins (see below). However, it has also been suggested that the breakdown of intramuscular connective tissue also plays a minimal role in the tenderisation of meat durng ageing (Warriss, 2000).

Tenderisation during the ageing of meat is due to the activity of proteolytic enzymes within the meat. While the actomyosin structure remains intact during ageing, other myofibrillar proteins are degraded. Ageing is mainly observed in the degradation of the muscle structure as the proteins associated with the Z disks and other myofibrillar structures become extensively degraded with increasing storage time. These proteins include desmin (Young, Graafhuis, & Davey, 1981), titin (Locker, 1987) and connectin (Maruyama et al., 1977). Z disks keep the ultrastructure of meat intact by keeping thin filaments and, indirectly the thick filaments, in their organised longitudinal arrangement. During ageing, the degradation of the Z disk and its associated proteins lead to fragmentation of the myofibrils resulting in meat tenderness. The proteolytic activities of the calpain and cathepsin enzyme systems are known to be primarily involved in this process. Although the mechanism of tenderisation is not well understood, factors such as temperature, preslaughter conditions and electrical stimulation are known to affect the rate of ageing (Devine, 2004; Hendrick et al., 1994).

MEAT QUALITY

With regards to meat production, quality is difficult to simply define due to numerous factors that collectively affect meat quality. Functional quality refers to the attributes in meat that affect its appearance and palatability. Functional quality is

addressed in this section as consumers usually refer to functional quality attributes when discussing meat quality.

The three dominant attributes by which consumers judge meat quality are appearance, texture and flavour (Faustman & Cassens, 1990), all of which are sensory properties. The conformance of these attributes to consumer's expectations is important and deviating from this will affect the product's marketability. In this section, meat colour, tenderness and flavour are addressed. Their importance to meat quality and factors affecting these attributes are discussed in this section.

Meat colour

The colour of lean meat is critical to the consumer's purchase decision as it is often the only criterion by which a consumer can judge meat quality (Warriss, 2000). In raw lean beef, a bright cherry red colour is most ideal as consumers perceive this as an indicator of a fresh, good quality meat. Meat colour is also linked to other meat quality attributes. Tests conducted on a sensory panel have found that lean colour is significantly related to the panel's tenderness and flavour intensity scores (Viljoen, Kock, & Webb, 2002).

Myoglobin is the protein largely responsible for the pigmentation of meat (Figure 6). Meat colour is determined by the proportions of the three forms myoglobin present in the meat (Tang et al., 2005). Myoglobin is a molecule with a protein portion (globin) and a non-protein portion known as a haeme ring. Within the haeme ring is an iron atom. The oxidation state of this atom governs the colour of meat (Hendrick et al., 1994).

The development of colour in meat is known as blooming. In uncut meat, which is unexposed to the oxygen in air, myoglobin exists in its reduced ferrous state (Fe^{2+}) with no bound oxygen Meat in this state is purple-red. This form of myoglobin is known as deoxymyoglobin. However, when the meat is exposed to air, through size reduction by cutting for example, deoxymyoglobin reacts with oxygen to form a pigment called oxymyoglobin. This red pigment is responsible for the desirable bright colour in meat.

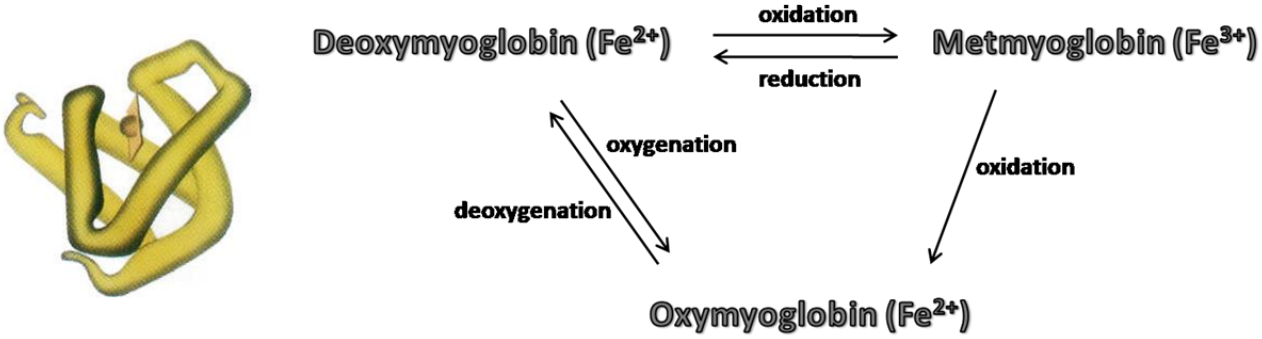

Figure 6: The myoglobin molecule (left) consists of a helical polypeptide chain and a haem group within the folded chain (Garret & Grisham, 2005). The different forms of the myoglobin molecule are shown (right). The colour of meat is regulated by the oxidation state of iron in the haem group within the molecule.

For chemical reasons beyond the scope of this review, at low but not zero concentrations of oxygen, oxymyoglobin tends to undergo oxidation (where the Fe^{2+} in the haem is oxidised to Fe^{3+}) to form the undesirable brown pigment metmyoglobin in meat. This problem is particularly prominent in vacuum-packed meat where low concentrations of air can permeate through the packaging and accelerate metmyoglobin formation (Hendrick et al., 1994). Metmyoglobin can be enzymatically reduced back to oxymyoglobin. However, once meat has undergone conditioning, residual enzymatic activity within the meat has declined and reducing equivalents are lost, metmyoglobin reduction does not occur and the meat remains discoloured (O'Keeffe & Hood, 1982).

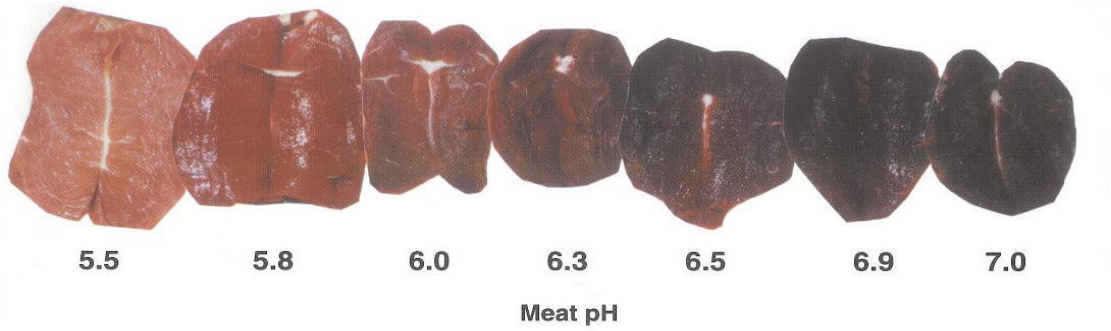

Figure 7: The colour of meat at various pH levels (MIRINZ Food Technology and Research, 1999). With the upper control threshold for the ultimate pH at 5.8, meat with an ultimate pH equal or greater than this is classified as dry, firm and dark (DFD).

The effect of ultimate pH on the colour of meat is widely known (Figure 7). Meat with an ultimate pH ranging from 5.4 to 5.6 has the ideal bright cherry red appearance. Meat exceeding the ultimate pH of 5.8 are categorised as dark, firm, dry (DFD) meat, referring to the meat's qualities. Consumers find the dark colour of DFD meat unattractive and beef less flavourful than normal pH beef (Dransfield, 1981).

There are two possible reasons for the dark colour in high pH meat. It has been suggested that the high water holding capacity of DFD meat results in a tighter meat structure which decreases the rate of oxygen diffusion into the muscle and consequently the formation of oxymyoglobin (Young & West, 2001). The second proposed reason for the dark appearance in DFD meat relates to mitochondrial and respiratory enzyme activity (O'Keeffe & Hood, 1982) confirmed by Zhu and Brewer. The mitochondrial oxygen consumption in meat has been suggested to enhance the conversion of oxymyoglobin to deoxymyoglobin (Tang et al., 2005). The enhanced mitochondrial activity in DFD meat will boost oxygen consumption which maintains myoglobin as deoxymyoglobin rather than oxymyoglobin (Faustman & Cassens, 1990).

Meat tenderness

Although the purchase decision of raw meat is primarily affected by colour, the likeability of meat is markedly affected by the tenderness of the cooked product. Tenderness is also linked to other meat attributes. Only when the tenderness of cooked meat is acceptable that judgements of flavour and juiciness can be made (Dumont, 1981). Meat tenderness is not simple to define and is more than just a measure of biting effort required. The perception of tenderness involves ease of fragmentation, mealiness, texture and the adhesion of muscle fibres during mastication (Hendrick et al., 1994).

The tenderness of meat is not always consistent or acceptable following cooking. This is due to many intrinsic properties of meat that determine tenderness. These include meat ultimate pH, the occurrence of cold shortening and the effect of connective tissues in meat (Purchas, 2004).

A significant relationship exists between tenderness and the ultimate pH, but there are contradicting results regarding the trend of this relationship. One study

demonstrated a linear relationship with minimum tenderness at pH 5.5, the lowest pH attained (Silva, Patarata, & Martins, 1999), whereas others cited a curvilinear relationship between ultimate pH and tenderness with minimum tenderness observed in DFD meat between the intermediate pH values of 5.8 to 6.2 (Devine, 1994; Jeremiah, Tong, & Gibson, 1991). It has been suggested that between pH 5.8 and 6.2 the calpain and cathepsin enzyme systems are not at their optimum activities resulting in the decrease in proteolysis and consequently tenderness. The shortening of muscle fibres at the intermediate pH range has also been associated with the decrease in tenderness (Purchas & Aungsupakorn, 1993).

A decrease in meat cooked tenderness is observed in a condition known as cold shortening which occurs when a muscle is cooled below 10°C before the onset of rigor (Warriss, 2000). This is of particularly relevant to hot boned muscle where muscle cuts are cooled rapidly before reaching rigor. The low temperature is believed to stimulate a massive release of calcium ions within the muscle fibre (Greaser, 2001; Young & Gregory, 2001). Muscle contraction is induced by the increase in the calcium ATPase activity of myosin. This results in so-called cold shortened meat where muscle contraction is not followed by relaxation. It does not tenderise during subsequent storage (Warriss, 2000).

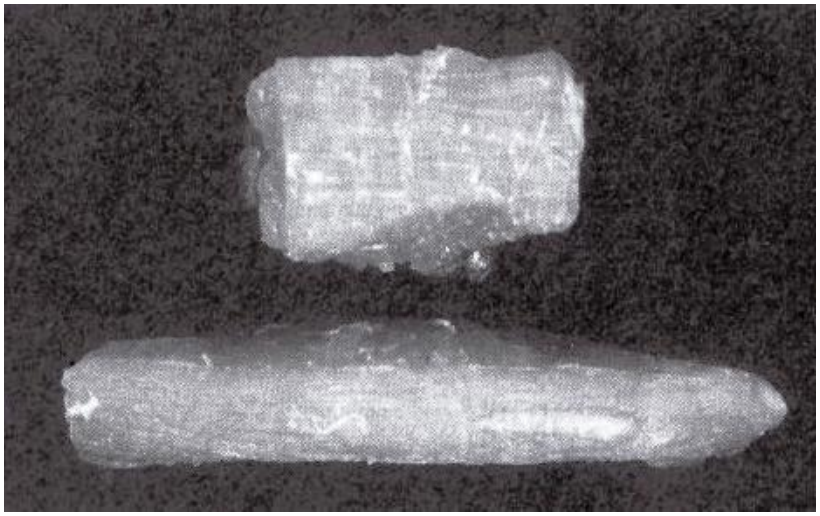

Figure 8: An example of muscle shortening (Hendrick et al., 1994). A freshly excised muscle (bottom) is compared to an identical muscle that has been frozen pre-rigor and subsequently thawed.

The abundance of connective tissue surrounding the muscle fibres, bundles and the entire muscle is an important source of variation in the tenderness of meat

(Purchas, 2004). Although connective tissues are only a minor component of meat, they have structural, protective and mechanical functions. The proteins collagen and elastin are of particular interest. Collagen is a protein composed of three polypeptide chains that form a triple helix. These triple helices align in a staggered and highly ordered manner with intermolecular cross-linkages between the polypeptide chains of the helix.

These cross linkages increase and stabilise the molecule as the animal ages, yielding a decrease in collagen solubility in older animals. The increase in collagen cross-linking and stability in meat from older animals retards gelatinisation during cooking resulting in a chewy texture to meat (Purchas, 2004). Elastin is less prevalent than collagen in muscle. It has elastic properties and is extremely insoluble which may contribute to meat toughness (Hendrick et al., 1994).

Meat flavour

Flavour is mainly a combination of two sensory responses, taste and smell. The smell (here called aroma) profile is mainly of interest when discussing meat flavour. This is because the volatile elements of cooked meat are what is predominantly perceived as meat flavour (Pegg & Shahidi, 2004). Aroma is perceived as the detection of volatile substances by olfactory receptors in passages at the back of the nose (Hendrick et al., 1994; Miller, 2004; Warriss, 2000).

The flavour of meat arises from the interaction of a host of compounds during cooking. The chemical composition of raw meat is what ultimately gives rise to the flavour in cooked meat (Pegg & Shahidi, 2004). Raw meat is a heterogeneous matrix including proteins, fats, vitamins, sugars and nucleotides, which are the flavour precursors in cooked meat. The interactions between these components and their degradation products during cooking are responsible for the flavour profile in meat (Oddy, Harper, Greenwood, & McDonagh, 2001).

Part of the flavour profile of cooked meat can be attributed to compounds generated by the Maillard reaction. Many of the 900-odd volatile components that have been identified in cooked meat are the result of the Maillard reaction (Miller, 2004). The Maillard reaction begins when a carbonyl group from a reducing sugar reacts with an amino group (NH_2) of an amino acid or peptide (Warriss, 2000). The reaction is accelerated by heat during cooking. After this initial reaction, the so-called Amadori compounds degrade to compounds such as furfurals, furanones and

dicarbonyls. These further react with compounds such as amino acids, aldehydes and ammonia to generate a host of aroma compounds. Thiamine and sulphur-containing amino acids have been identified as major contributors to meat flavour compounds mainly because their Maillard reaction products contain sulphur. Such compounds have very low odour thresholds (Belitz, Grosch, & Schieberle, 2004).

Another important contributor to cooked meat flavour is oxidative degradation of fats in meat during cooking. Phospholipids and triacylglycerols are particularly significant contributors in the generation of meat aroma. Flavour derived from fat is due largely to a plethora of carbonyl compounds (aldehydes and ketones) generated from the oxidation of unsaturated fatty acids and the volatilisation of fat soluble compounds during cooking (Pegg & Shahidi, 2004). Whereas these fat oxidation products are partly responsible for meat flavour, they are also responsible for unpleasant aromas when generated in excess (Oddy et al., 2001). This can occur when meat is stored, chilled or frozen, for long periods but exposed in some way to air.

The ultimate pH of meat has been found to contribute to the variation of flavour in meat. For beef, the flavour of normal pH meat is favoured over DFD meat (Warriss, 2000). It was proposed that the lower concentrations of sugars in DFD meat consequently reduces the Maillard reaction and leads to poorer flavour (Warriss, 2000). Equally though the Maillard reaction is strongly affected by pH such that one pH will lead to one profile of aroma compounds and another pH to a markedly different profile (Martins, Jongen, & Boekel, 2001).

Pre-slaughter factors affecting meat quality

It is clear then that variations in meat colour, tenderness and flavour are affected by the ultimate pH of meat. At the root of pH is the glycogen concentration (more commonly called ‘content’) in muscle at the moment of slaughter, as discussed in the earlier section **Muscle metabolisn post-mortem**. Stress experienced by the animal prior to slaughter is the foremost contributor high pH meat. This can be caused by poor lairage conditions, cattle management and handling of the animal prior to slaughter (Scanga, Belk, Tatum, Grandin, & Smith, 1998). Several studies have also shown that weather conditions can affect glycogen content and thus ultimate pH (Scanga et al., 1998). The effects of stress on meat quality are discussed in detail in the following section.

Many meat quality attributes are also affected by the animal's diet. For instance, meat from animals fed on pasture generally has a more intense flavour than meat from corn-finished cattle (Warriss, 2000). Studies have found that supplementing a corn-diet with antioxidants such as selenium (an antioxidant precursor) and vitamin E increases colour stability in raw meat (Oddy et al., 2001; Wood et al., 2003). Antioxidants also improve the shelf life of meat by combating fat oxidation (Geay, Bauchart, Hocquette, & Culioli, 2001). The management of nutrition before slaughter is critical to maintaining the concentration of stored muscle glycogen.

Age and gender also affect meat quality attributes. The effect of age is seen in the tenderness of meat, where a decrease in tenderness is observed in older animals due to prevalence and stability of collagen cross-links as discussed earlier. Gender effects on meat quality is evident where higher incidences of DFD meat from bulls observed (Brown, Bevis, & Warriss, 1990; Graafhuis & Devine, 1994).

Post-mortem factors affecting meat quality

Low voltage electrical stimulation of carcasses immediately after exsanguination is applied to accelerate the glycolytic processes in muscle by causing intense muscle contractions. Induced muscle contractions promote the depletion of ATP and glycogen leading to the early onset of rigor. This is particularly advantageous in eliminating cold shortening by ensuring that muscle has attained rigor before it is chilled below 10°C. Along with the prevention of cold shortening, low voltage stimulation has also been shown to increase tenderness and improve the appearance of meat (Warriss, 2000; White, O'Sullivan, Troy, & O'Neill, 2006). The reasons behind this are not completely understood.

The chilling regime of the carcass during conditioning and ageing is important with regards to the tenderness, appearance, eating quality and shelf life. Studies have addressed the effect of post-mortem chilling regimes of muscle on the tenderness of meat. Researchers have recommended electrical stimulation followed by slow chilling (10°C for 24 hours, then 2°C until 48 hours) or delayed chilling (16°C for 24 hours, then chill down to 2°C) of carcasses as the best chilling regimes to obtain meat with optimum tenderness (Hwang et al., 2003; Ockerman & Basu, 2004; White et al., 2006). Slow chilling avoids cold shortening by preventing muscles from cooling below 10°C before reaching rigor. It is important to note although the chilling regime

of 10°C or higher favour tenderness, the growth of some pathogenic and psychrophilic spoilage bacteria at these temperatures occurs (Hendrick et al., 1994). Therefore, hygienic considerations also need to be made when discussing chilling regimes.

A meat's ultimate pH impacts on its colour and cooked flavour and tenderness. For a muscle to attain optimal meat quality, it needs to reach ultimate pH levels between 5.2 and 5.6. As established earlier in this thesis, the ultimate pH that a muscle will reach is dependent on glycogen concentrations in muscle at the time of slaughter. Thus the glycogen content in muscle early post-mortem is an indicator of future meat quality.

GLYCOGEN

Glycogen is a polymer of glucose units. Its three dimensional structure comprises of glucose units forming a helical chain that is linked by α-1,4 acetal bonds, with α-1,6 bonds present where branches occur in the chain. Structurally glycogen is very similar to amylopectin in plants, but it is much larger (up to 50,000 glucose units) and more branched (Warriss, 2000). Glycogen functions as an energy store and is readily available as a source of glucose in the form of glucose-1-phosphate. In animals, dietary carbohydrates that are not immediately needed are converted to glycogen and stored in the muscle and the liver. Glycogen is initially degraded to glucose-1-phospate moieties before it is metabolised to yield ATP.

In situations where animals have been fasted and carbohydrate concentrations in the blood are low, free fatty acids are metabolised from the fat depots of the body. However, when the breakdown rates of carbohydrates and free fatty acids are not sufficient to keep up with the demands of contracting muscles, glycogen is utilised. This is typically a result of an animal going through long periods with no food (fasting) or intense physical activity. Glycogen is also immediately metabolised in response to an external stressor such as fear. Stress releases adrenaline into the bloodstream which immediately triggers the rapid metabolism of glycogen to energy for contraction if, for example, the animal needs avoid predation (Warriss, 2000). Thus physiological and psychological stresses lead to the depletion of glycogen stores in the muscle.

The importance of glycogen for meat quality

Anaerobic metabolism in muscle results in the accumulation of lactic acid and therefore a drop in muscle pH. The pH value that the muscle finally attains is referred to as the ultimate pH. Muscles with adequate glycogen reserves in muscle will yield normal pH meat, while muscles with depleted glycogen stores will yield high pH, DFD meat. As noted in previous sections DFD meat is inferior in colour, tenderness, and flavour and is more prone to microbial contamination. However, due to the high water holding capacity of DFD meat, it is useful for manufacturing purposes.

A New Zealand survey conducted in 1993 with 540 'prime' animals (steers and heifers) and 770 bulls found that approximately 80% of slaughter bulls and 20% of primes had an ultimate pH exceeding 5.7, which the researchers chose as the upper limit of quality LD meat (Graafhuis & Devine, 1994). These results conform with a more recent survey of approximately 13,700 animals (9,255 primes and 4,463 bulls) conducted in 1999 which found that 62% of slaughter bulls 19% of prime had an ultimate pH exceeding 5.7 (Young, Thomson, Merhtens, & Loeffen, 2004).

The incidence of high pH beef has been attributed to the pre-slaughter handling of animals. Stresses incurred during transport and at lairage collectively contribute to the depletion of muscle glycogen. To reduce this, the mixing of unfamiliar animals must be avoided as this will result in stress brought about by aggressive behaviours as the animals try to establish a social hierarchy within the new group (McVeigh & Tarrant, 1982; Warriss, Kestin, Brown, & Wilkins, 1984).

Environmental factors in which the animals have been reared has also been linked to the depletion of muscle glycogen where a significant number of animals yield DFD meat during periods of extreme weather conditions (Scanga et al., 1998; Young, Thomson et al., 2004). The glycogen content of animals in winter has also been found to be significantly lower than in temperate climates. This seasonal effect may be due to the quality and availability of pasture and the effect of the weather on the animals' metabolism (Brown et al., 1990; Knee, Cummins, Walker, & Warner, 2004).

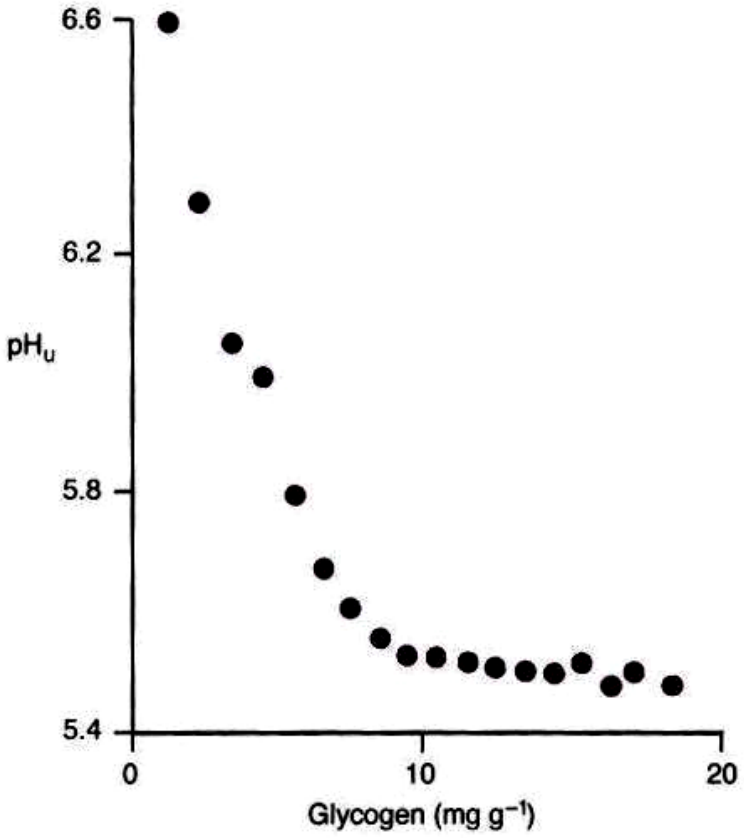

Figure 9: The relationship of ultimate pH (pH_u) to the concentration of glycogen present in muscle at death (Warriss, 2000).

Cattle weight and age are also potential factors affecting the ultimate pH in meat. In New Zealand beef, it was observed that the lower the mean carcass weight of an animal at a given age, the higher the ultimate pH (Graafhuis & Devine, 1994). Restated by other researchers, with increasing cattle weight, the incidence of DFD meat decreases (Kreikemeier, Unruh, & Eck, 1998).

Gender effects on meat quality is evident in bulls where the yield of DFD meat from bulls is higher than those from primes and cows (Brown et al., 1990; Graafhuis & Devine, 1994). Social interactions between bulls while in lairage explains the elevated pH of bulls (Graafhuis & Devine, 1994). The effect of breed on glycogen levels has also been considered, however, some findings show that differences between breeds and meat quality are not significant and that the inclination of a particular breed to yield DFD meat is most likely a result of stress rather than breed (Graafhuis & Devine, 1994; Purchas & Aungsupakorn, 1993; Sanz, Verde, Sáez, & Sañudo, 1996).

The importance of determining muscle glycogen content early in the slaughter process

It is important to note that although high pH DFD meat is of inferior quality to normal pH meat, the meat is not discarded. The high water binding activity of DFD meat makes them very suitable for manufactured meat products such as emulsion sausages of all forms. Adding DFD meat to the formulations of these meat products will lower fluid losses, with additional cohesion and fat emulsifying properties. DFD meat has also been used in the frozen ready-to-eat dishes, where undesirable colour of raw DFD meat is not an issue as the product has already been cooked.

In New Zealand high pH, DFD meat affects the industry in two distinct ways. First, meat from most cows and bulls is generally frozen and sold as manufacturing grade beef based on the assumption and market perception that the meat is DFD (or that the meat from older cows is intrinsically tougher due to collagen-related toughness). The price of manufacturing beef is usually lower than that of prime. This is disadvantageous as the economic potential from these carcasses is not fully realised. A significant quantity of bull and cow beef is normal pH meat which could be sold as higher-priced chilled meat. Second, there are instances where high value cuts from prime animals sold as table cuts is in fact DFD meat and selling these cuts will lead to lower prices due to consumer dissatisfaction. In Australia, the economic losses due to the reduction in the value of DFD carcasses is estimated to be worth AUD 30 million per year (Gardner, McIntyre, Tudor, & Pethick, 2001).

Although steps can be taken to minimise the occurrence of DFD condition (mob separation, stress reduction etc.), it is commercially important to be able to isolate DFD meat from normal pH meat. A statistically significant curvilinear relationship exists between ultimate pH (pH_u) and the glycogen content of muscle immediately after slaughter (Figure 9). A study conducted on bovine, porcine and ovine muscles found a negative curvilinear relationship between glycolytic potential in pre-rigor muscle and pH_u (Pryzbylski, Venin, & Monin, 1994). The most important implication of this curvilinear relationship is that it would be possible to predict the ultimate pH of a muscle if the glycogen content were known immediately post-mortem. This would be a huge advantage to processors who can then decide whether a carcass is to be chilled and sold as table cuts or frozen and packaged as manufacture grade meat.

METHODS OF MUSCLE GLYCOGEN DETERMINATION

In a commercial hot boning environment where the turnover of carcasses is rapid and where meat is boned out and packed immediately after the carcass weigh station, there is a need to measure glycogen sometime between the moment of slaughter and that station. The pH cannot be measured directly in a hot boning environment as it takes 24 hours or so for muscles to reach their ultimate pH – long after the meat has been packaged and chilled. Ultimately, an ideal method is one that is rapid, simple, cheap and applicable to the abattoir environment.

There are numerous methods available to measure glycogen. The progressive development of several of these methods has made it possible to determine glycogen content in tissue samples in the laboratory environment relatively quickly and accurately.

Isolation of glycogen from muscle

As glycogen cannot be measured directly by any known means, existing methods aim to initially isolate glycogen from the sample matrix then measure it indirectly by colorimetric or enzymatic methods. It is critical that all glycogen is extracted from the muscle tissue and recovered for subsequent assays. There are a number of approaches to isolating glycogen from muscle tissue. These include alkaline and acid extraction. Several published articles debating the merits and faults of these methods have been published.

Alkaline extraction

Alkaline extraction of glycogen is based on the classical method of glycogen determination known as Pflüger's method. In this method, glycogen is extracted from the tissue sample by hydrolysis in concentrated potassium hydroxide (KOH) (Sahyun, 1931). Two volumes of 95% alcohol are then added to the hydrolysate and further heated. The solution is then cooled allowing extracted glycogen to precipitate from the solution (Good, Kramer, & Somogyi, 1933).

The glycogen residue is then collected by filtration and the alkali-alcohol supernatant is discarded. This is followed by the hydrolysis of glycogen to glucose by heating in concentrated sulphuric followed by neutralisation with an alkali. Glycogen

from the hydrolysate is indirectly quantified by measuring glucose. As glycogen is a polymer of glucose, the amount of glucose in the hydrolysate is directly proportional to the glycogen content in the sample. Once the glucose content from the hydrolysate is known, this value is back calculated to the glycogen content of the sample.

Glucose is quantitatively determined using a colorimetric method described by Folin and Wu (1920). Glucose from the sample is oxidised by a weakly alkaline copper tartrate solution. A phenol reagent is then added to the glucose-copper solution where it develops a blue colour due to the formation of cuprous oxide. The intensity of the blue colour that develops is directly proportional to the amount of glucose in the sample.

One concern about Pflüger's extraction method is that the precipitation of glycogen from a strong alkali solution can result in partial losses of glycogen during the precipitation and filtration of glycogen from the solution (Keppler & Decker, 1974; Sahyun, 1931). However, Sahyun (Sahyun, 1931, 1933) demonstrated that the use of activated charcoal as a medium for glycogen adsorption during glycogen precipitation coupled with centrifugation to ensure complete precipitation and recovery of glycogen. Nonetheless, this method is completely impractical to apply in an abattoir environment because it is complex and time consuming.

Acid extraction

Using acid is another approach to extract glycogen from muscle. This involves the dispersion of meat in acid solutions typically between 0.03 and 2 mol L^{-1}. This procedure extracts glycogen from the muscle leaving it suspended in the liquid phase. Glycogen is then either precipitated with alcohol then analysed as described in *Alkaline extraction* or the supernatant is immediately assayed for glycogen content.

Acid extraction is less favoured to Pflüger's method because it lacks specificity, where reducing substances not related to glycogen are assumed to be glucose. This led in the overestimation of glycogen in tissue samples (Good et al., 1933). However, developments in methods of glycogen measurement have eliminated errors associated with acid extraction making it a viable method for glycogen extraction. A study comparing several methods of glycogen extraction found no significant difference in the recovery of glycogen from muscle using Pflüger's method and acid extraction at room temperature (Fabiansson & Reuterswärd, 1984).

The literature variously recommends different acids. Although hydrochloric acid (HCl) (Chan & Exton, 1976; Fabiansson & Reuterswärd, 1984; Passonneau & Lauderdale, 1974) and perchloric acid (Dreiling, Brown, Casale, & Kelly, 1987; Keppler & Decker, 1974) are more commonly used, sulphuric acid (H_2SO_4) (J. A. Johnson, Nash, & Fusaro, 1963) and trichloroacetic acid (TCA) (Roe & Dailey, 1966) have also been used with mixed success. While there is no standardised acid used for the acid extraction method, studies have found that some acids have advantages over others. For instance, perchloric acid was found to yield higher recoveries of glycogen from muscle as well as precipitating protein bound hexose residues which interfered with subsequent glycogen assays (Roe & Dailey, 1966).

It is evident that glycogen determination through alkaline and acid extraction is both time consuming and complex. These methods also use strong acidic and alkaline solutions which are hazardous. Applying these methods to commercial abattoir is impractical and unsafe. Enzymatically hydrolysing glycogen from tissue is a method of extracting glycogen that is simple, safe and rapid.

Hydrolysis of extracted glycogen

Once glycogen has been isolated from the muscle sample, the next step is to hydrolyse the glycogen into glucose monomers. As glycogen is a polymer of glucose, the glycogen concentration of a sample can be indirectly quantified by hydrolysing glycogen into glucose monomers and determining the amount of glucose derived from glycogen. The most common approaches of glycogen hydrolysis are by acid or enzymatic hydrolysis. Although there is no significant difference in the results obtained from either methods (Passonneau & Lauderdale, 1974), enzymatic hydrolysis is favoured as it is less hazardous and no loss of glycogen is incurred during hydrolysis (Fabiansson & Reuterswärd, 1984; Keppler & Decker, 1974).

Acid hydrolysis of glycogen typically involves the heating sample in concentrated or diluted acid in a boiling water bath for up to two hours. While HCl was used by some studies (D. J. Bell & Young, 1934; Keppler & Decker, 1974; Passonneau & Lauderdale, 1974; Wagtendonk, Simonsen, & Hackett, 1945), H_2SO_4 is also used as an alternative as chlorides that result from using HCl may interfere with subsequent colorimetric glucose measurement methods (Sahyun, 1931).

Enzymatic hydrolysis employs amyloglucosidase (Chan & Exton, 1976; Dreiling et al., 1987; Keppler & Decker, 1974; Passonneau & Lauderdale, 1974; Young, West et al., 2004), because it is able to hydrolyse both the α-D-(1–4) and the α-D-(1–6) linkages of glycogen. Enzyme hydrolysis is performed by incubating the glycogen extract with the buffered enzyme solution for several hours at between 37 and 55°C. Incubation is usually ended by the addition of a concentrated acid to lower the pH and terminate any enzyme activity.

Methods of glycogen/glucose measurement

Once glycogen has been hydrolysed, the resulting glucose is quantified using colorimetric or enzymatic assays. Traditional glycogen determination methods employed colorimetric assays to quantify glucose. However, variation in pH due to the presence of sodium bicarbonate and loss of colour due to the reoxidation of copper have been shown to give inconsistent results (Folin & Wu, 1920). Due to inconsistencies experienced with colorimetric assays, enzymatic assays are generally favoured.

Colorimetric assay

Colorimetric methods involve the addition of an alkaline copper reagent to an aliquot of the hydrolysed glycogen. Alkaline copper reagents essentially contain sodium bicarbonate and crystalline copper sulphate. Published literatures cite these components in varying concentrations along with the addition of other components (Folin & Wu, 1920; Somogyi, 1926). The solution is then heated in a boiling water bath where glucose is oxidised by the reducing sugar, in this case glucose, and cuprous oxide is formed. A solution containing sodium molybdate is then added and the resulting blue colour is measured.

Enzymatic assay

A conventional enzyme assay for glycogen determination developed by Keppler and Decker (1974) describes a method where glucose is enzymatically phosphorylated into glucose-6-phosphate. Glucose-6-phosphate is then oxidised in the presence of nicotinamide adenine dinucleotide phosphate ($NADP^+$) by the enzyme glucose-6-phosphate dehydrogenase. This reaction also results in the reduction of $NADP^+$ into

NADPH which is determined at 340 nm. The quantity of glucose liberated from glycogen is proportional to the change in absorbance at this wavelength.

Enzymatic assays are favoured over colorimetric assays as they are more consistent with a coefficient of variation reported at 2.5% (Keppler & Decker, 1974) and are not affected by problems associated with chemical colorimetric assays. Chemical producers have also translated this method into a commercial assay kit, which makes this method convenient and rapid.

Other methods of measuring glycogen

Other methods of determining glycogen that exist take a different approach to the colorimetric and enzymatic assays detailed above. The iodine method directly measures extracted glycogen without hydrolysis, while the Rapid pH (RpH) method immediately measures glycogen as glucose after enzymatic hydrolysis.

Iodine method

The iodine method was developed as a rapid colorimetric method of measuring glycogen at very low levels. It is based on the characteristic amber-brown colour complex formed when iodine is added to glycogen. The colour intensity is dependent on the degree of helicity, branching and chain length of the glycogen polymer (Dreiling et al., 1987; Krisman, 1962). In their development of the iodine method, van Wagtendonk et al. (1946) dissolved glycogen in water. Lugol's reagent (a solution containing iodine and potassium iodide) was then added and the resulting amber-brown solution read with a colorimeter. However, a review of this method found that the colour formed by glycogen with iodine lacked stability, specificity and sensitivity especially at varying temperatures (Morris, 1946).

In her review of the iodine method, Krisman (1962) proposed the analysis of glucose samples and standards in saturated calcium chloride as a means of stabilising the glycogen colour complex. This amendment to the original method was shown to stabilise the colour at varying temperatures. The modified method also is also specific to glycogen and eliminated the overestimation of glycogen due to the interference of other polysaccharides experienced with the original method. A study conducted on bovine muscle samples has shown the iodine method to be faster and more consistent than the enzyme assay (Dreiling et al., 1987).

Rapid pH method

The RpH method was developed as a means predicting a carcass' ultimate pH by indirectly determining the glycogen content in pre-rigor *longissimus dorsi* (LD) muscle immediately after slaughter. Due to the simplicity of the method, it is currently the only glycogen measurement method that is applicable to commercial abattoirs. In the RpH method, pre-rigor LD muscle is hydrolysed by amyloglucosidase in acetate buffer and the meat slurry is incubated at 55°C for five minutes. The liberated glucose in the meat slurry is then measured using a diabetic glucometer (Young, Thomson et al., 2004; Young, West et al., 2004).

Although the RpH method has been successfully applied to the industry, collective financial costs associated with running this method is a perceived disadvantage. On the whole, the materials cost averages at $1.50 per carcass tested. The RpH method is also labour intensive and is best suited for slower processing lines where one labour unit is sufficient to operate the procedure (Young, Thomson et al., 2004). However, up to three labour units have been used in abattoirs where the processing line speed is much faster, adding to the costs of running the method.

NEAR-INFRARED SPECTROSCOPY

Near-infrared (NIR) had been largely ignored since its discovery in 1800 as spectroscopists could not find any additional information in the NIR spectral region that the much recognised mid-infrared region already provided (Pasquini, 2003). The acceptance of NIR as a serious analytical tool was initiated by research conducted by one Dr Karl Norris in the US Department of Agriculture in the 1960s. Since then, NIR has expanded beyond the agricultural industry and its application has diversified into many fields including pharmaceuticals, food processing and remote imaging spectroscopy (Barton, 2002).

Principles of NIR

NIR spectroscopy is a spectroscopic technique that employs photon (light) energy corresponding to this wavelength range (Pasquini, 2003). NIR occupies the

high energy side of the infrared spectrum which ranges between 750 and 2500 nm. NIR spectroscopy aims to obtain qualitative and/or quantitative information from interactions between NIR electromagnetic waves with the constituents of a given sample.

In respect of models used to describe to infrared spectroscopy, molecular bonds are commonly likened to springs where atoms of a molecule vibrate due to the oscillating action of the bonds. Vibrations are classified as stretching or bending, and these are further classified into more specific groups. For example, water has three principle vibrations; symmetric stretch, asymmetric stretch and scissoring of O–H bonds (Barton, 2002).

Spectra from the infrared region arise from vibrations associated with C–H, O–H and N–H bonds. At ambient temperature, atoms in a molecule generally remain in configurations where the kinetic energy of bond vibration is lowest (Pasquini, 2003). When NIR light is absorbed by molecular bonds, the energy is transferred into mechanical energy. This results in the excitation of the chemical bonds in the molecule and the promotion of the bonds into a higher vibrational energy level. However, the NIR frequencies required for bonds to reach a higher vibrational state is dependent upon the configuration of the vibration of the bonds. This means that when an organic molecule is exposed to a given NIR wavelength range, some frequencies will be absorbed while others will not. Other frequencies may also be partially absorbed. The NIR spectrum arises from this complex association between NIR frequencies and vibrational configurations of organic molecules in a substance.

NIR instrumentation

NIR spectroscopy is favoured above many analytical methods as it is rapid and non-invasive. Test samples require little or no preparation (Pasquini, 2003). NIR spectroscopy normally works by passing a beam of NIR light through a sample. Tungsten lamps are most commonly employed lights. NIR light interacts with the molecular bonds in the sample as discussed above and some light is absorbed. Transmitted light reaches a wavelength selector, which selects a particular wavelength to be quantified. The selected wavelengths of NIR light strike the detector which measures the extent to which the sample has absorbed that particular wavelength. The amount of NIR light absorbed is proportional in some way to the concentration of the compound of interest (e.g. glycogen) in the sample (Pasquini, 2003).

As a piece of meat is nominally opaque, obtaining an NIR absorbance spectrum by passing light through it would not be feasible unless a very powerful light source were used (and this would heat the sample). With a low energy source, all NIR wavelengths would be obscured by the sample. Diffuse reflectance measurement of NIR wavelengths is a better approach (Pasquini, 2003). In diffuse reflectance measurement, NIR light is beamed into a sample where light is scattered and some is absorbed. Unabsorbed light is reflected back to the surface of the sample where a detector measures the reflectance of selected wavelengths.

NIR spectrophotometers can be categorised according to the detector installed in the instrument. A diode-array detector (Figure 10) was the detector present in the NIR spectrometer used in this thesis. Once light enters a diode array detector, it is reflected into a grating, which fractionates light into individual wavelengths. These wavelengths then reach the diode array where the sensors simultaneously measure the signal of the wavelength range of interest. Diode arrays are obviously favoured over traditional monochromators which measure only one wavelength at a time.

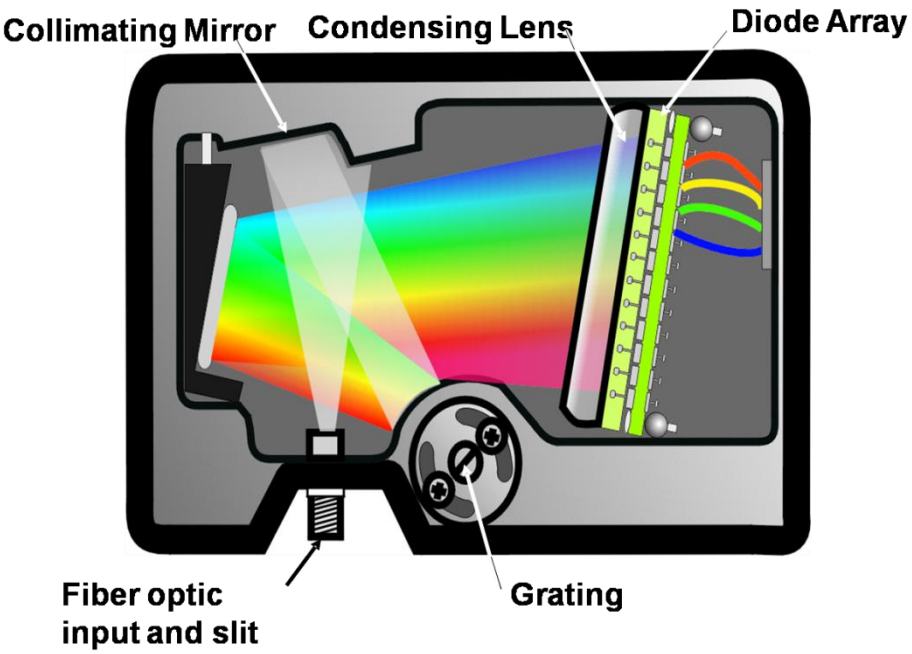

Figure 10: A diagram of a diode-array detector (courtesy of Ocean Optics). NIR light from the sample enters through an optical fibre, and is fractionated into individual NIR wavelengths by the grating; their signals are simultaneously measured.

NIR calibration and chemometrics

The complexity of NIR reflectance spectra of meat is due to the presence of several components in meat. A successful approach to determining given components in meat will require multivariate analysis based on the measurements of multiple wavelengths where the whole NIR spectrum and not just individual wavelengths is considered (Small, 2006). In order to extract information from the spectra and correlate it to the property of interest in the sample, chemometrics is used. Chemometrics is a discipline which uses mathematical and statistical techniques to uncover relevant information from analytical data such as the NIR spectral data (Pasquini, 2003). By partnering NIR spectroscopy with chemometrics, robust determinative models can be achieved. The most common chemometric techniques used in NIR spectroscopy are Multiple Linear Regression (MLR), Principle Components Analysis (PCA) and Partial Least Squares Regression (PLS). All these techniques assume some linear relationship exists between the spectral data and the concentration of the component of interest.

Applications of NIR spectroscopy in the meat industry

Extensive research has been conducted on the capability of NIR spectroscopy as an analytical tool to measure several meat quality attributes. Published research on the efficacy of NIR to quantify tenderness in post-rigor beef meat has shown promising results with the r^2 of predictive models ranging from 0.55 to 0.67[4] (Byrne, Downey, Troy, & Buckley, 1998; Liu et al., 2003; Park, Chen, Hruschka, Shackelford, & Koohmaraie, 1998). When qualitative models were fitted to categorise meat into tenderness groups, even better models were obtained with up to 96% of meat correctly classified (Liu et al., 2003; Park et al., 1998). Good predictive NIR models on other attributes such as colour, ultimate pH, and drip loss have also been reported in both beef (Andrés et al., 2007; Liu et al., 2003) and pork (Andersen, Borggaard, Rasmussen, & Houmøller, 1999; Hoving-Bolink et al., 2005; Savenije, Geesink, Palen, & Hemke, 2006).

Although NIR has shown to be a promising analytical tool in the meat industry, most of the published research in this area was conducted on post-rigor meat where

[4] r^2 values are the square of a linear correlation coefficient, and show what fraction of the prediction, 0 to 1, is explained by the fitted model.

NIR scans were collected in a controlled laboratory environment. The capability of NIR to predict the ultimate outcome of meat quality attributes from scans collected on-line from pre-rigor carcasses is largely unknown. On-line validation of NIR spectroscopy is necessary for the potential of this method to be realised in the meat industry. A recent study aimed to predict the cooked tenderness of aged beef from NIR scans collected on-line. A low error rate (3.7%) was found when classification models were fitted to categorise the muscles into their predicted tenderness groups (Rust et al., 2008).

To date, no research has been published regarding the on-line quantitative measurement of glycogen or the prediction of pH_u using NIR spectroscopy. The successful on-line calibration of NIR spectroscopy to quantify glycogen or predict pH_u of pre-rigor muscle would capitalise on all the advantages of NIR as a quantitative technique, while avoiding the disadvantages associated with the Rapid pH method (RpH).

RESEARCH AIMS AND OBJECTIVES

Glucometer calibrations for the RpH method

The RpH method has been successfully used for several years in three hot boning processing plants in New Zealand. The Bayer glucometer, a domestic diabetic's meter, is used in this method where the glucose readings are used to predict the ultimate pH of the muscles in the carcass. Unfortunately, the Bayer glucometers, which had been specifically calibrated for the RpH method are no longer manufactured, and the corresponding sensor disks used to detect glucose are slowly being phased out. This is a problem because meat processors using the RpH method are heavily dependent on it as a quality control measure to detect carcasses that will yield high ultimate pH in potential table cuts. Thus arose the need to find a replacement for the Bayer glucometer.

The first aim of this thesis is to find a glucometer to replace Bayer in the RpH method. Two glucometers were tested as prospective replacements of the Bayer glucometer. These were Roche Accuchek® Advantage II (Accuchek) and Medisense Optium™ (Medisense). Both meters are commercially available at relatively similar retail prices. Each glucometer's response to a range of glucose standards prepared in

water, RpH buffer and spiked meat slurries has been evaluated. Finally, the glucometers were tested in a commercial abattoir where measurements were taken on pre-rigor samples. The results obtained were then used to determine whether one or both of the new meters could be used to replace the existing Bayer ESPRIT® meter.

Rapid determination of glycogen in slaughter beef using NIR spectroscopy

Looking beyond the wet chemistry method that is RpH, NIR spectroscopy may be a better alternative to RpH for the following reasons: NIR spectroscopy is non-destructive in the sense that a sample of meat is not required to be excised from the carcass; it is less time consuming and can be used on-line on whole carcass muscle; it requires only one labour unit. The second aim of this thesis was thus to determine the efficacy of NIR spectroscopy as an on-line instrument to measure glycogen and so predict ultimate pH.

To achieve this aim first involved finding a reference glycogen method to calibrate the NIR instrument for glycogen measurement. Three glycogen methods were compared. First was the Iodine method, which is based on the method originally developed by Krisman (1962). This method is currently used in the AgResearch MIRINZ laboratory. The second method was the enzymatic method (Keppler & Decker, 1974) referred to by some as the 'gold standard' for glycogen measurement. Third was the RpH method (Young, West et al., 2004) which is in current commercial use in some New Zealand abattoirs. Each of the methods was used to determine glycogen concentration in replicated LD muscles with varying glycogen concentrations. The results were then statistically analysed and the method with the best repeatability was used for NIR calibration.

The final aim of this thesis was the calibration of a commercially available NIR instrument to measure pre-rigor glycogen on-line in a commercial abattoir where hot boning of beef carcasses is applied. NIR measurements were carried out on cut LD muscle at the thirteenth rib. The temperature and pH of the LD muscle were recorded and small samples of the muscle were frozen immediately in liquid nitrogen for subsequent glycogen and ultimate pH analysis. The reference method chosen from the previous aim was used to determine the glycogen content of the replicate LD muscles. All measured glycogen and ultimate pH values were then used for calibration against the NIR spectra. To obtain the best possible calibration between the measured ultimate pH and glycogen values and the NIR spectra, samples were

collected from a broad range of animal categories (bulls, cows and prime) and from animals in varying physical condition as routinely presented in commercial abattoirs.

The next chapter describes in detail the materials and methods used to achieve the aims of this thesis. The methods used to find a replacement for the Bayer glucometer is firstly described, followed by the materials and methods used to find a reference glycogen method. Finally, the methods and chemometric tools used to calibrate the NIR instrument for glycogen and ultimate pH measurements are described.

CHAPTER 3

MATERIALS AND METHODS

CALIBRATION OF ROCHE'S ACCUCHEK® ADVANTAGE II AND ABBOTT'S MEDISENSE OPTIUM™ FOR RAPID pH MEASUREMENT

Chemicals, reagents and equipment

The designated Rapid pH (RpH) buffer (Young, West et al., 2004)) was prepared. Amyloglucosidase reagent was prepared from *Aspergillus* (Enzidase® L300) supplied by Zymus International Limited, Auckland. The enzyme was diluted with RpH buffer to give a final enzyme activity of six glucose activity units mL^{-1}. Analytical grade D-glucose purchased from LabServ™ was used as the chemical standard.

The Bayer ESPRIT™ glucometers and their corresponding test sensors were provided by a commercial abattoir currently using the RpH method in their on-line process to predict meat ultimate pH. Roche Accuchek® Advantage II and Medisense Optium™ glucometers and their corresponding test sensors were purchased from a local pharmacy.

For experiments carried out in the MIRINZ laboratory, an Ika®-Labortechnik (Staufen, Germany) Utra-Turrax T25 was the homogeniser used in this project. The drive was fitted with a S25 dispersing tool 18 mm in diameter. The homogeniser was fixed to a retort stand with a clamp.

Glucometer responses to glucose standards made with water and RpH buffer

Two stock glucose standards of 1000 mg dL^{-1} glucose were prepared. One was prepared with milli-Q water and the other with RpH buffer. A series of glucose standards ranging from 50 mg dL^{-1} to 500 mg dL^{-1} was then prepared by dispensing aliquots of the stock water glucose standard into 100 mL volumetric flasks and making it up to volume with milli-Q water. A second series of glucose standards was similarly prepared using the RpH buffer glucose stock and made up to volume with

RpH buffer. The standards were left to stand overnight at room temperature to allow the equilibration of α and β-glucose isomers in solution.

One hundred microlitres of each standard (in water and RpH buffer) were pipetted onto a square piece of Biolab Parafilm and then sampled with a glucometer. Single readings for all glucometers were obtained for the both series of standards.

Glucometer response to spiked meat slurries

The method used in this section and the following section is based on the technology developed and described by Young et al. (2004). Post-rigor rump steak (*M. gluteus medius,* GM) purchased from a local butcher was used in this experiment. A mass of 1.5 ±0.05 g of post-rigor meat was weighed accurately into 30 mL polyethylene centrifuge tubes. Seven millilitres of each of the previously prepared RpH glucose standards were then pipetted into separate centrifuge tubes followed by 0.5 millilitres of amyloglucosidase reagent 6 units mL^{-1}. The meat was then dispersed with an Ultra-Turrax for 20 seconds at 20,000 rpm. This gave a series of meat slurries with approximately equal meat fragment concentrations and varying glucose concentrations. The spiked meat slurries were then incubated in a water bath at 55°C for 5 minutes. One hundred microlitres of each of the spiked meat slurries were then pipetted onto a square piece of parafilm. The droplet was then sampled with a glucometer. All spiked meat slurries were measured in triplicate with each of the three glucometers.

Abattoir practice

The calibration trial took place in a commercial abattoir that slaughters cows, bulls and prime (steers and heifers) for both domestic and international markets. The cattle are slaughtered according to New Zealand animal welfare standards (NZFSA, 2002). Animals were immobilised using captive bolt stunning followed by exsanguination with a thoracic stick. After low voltage electrical stimulation applied through the shackled hind leg to a nose clip, the hide was removed. At this point (approximately 10 minutes after slaughter) GM muscle is collected from the right side of the carcass for RpH analysis. The carcass then goes through normal commercial abattoir dressing and grading processes after which it is hot-boned and the meat packed.

On-line RpH measurement of beef samples

Forty five beef carcasses were sampled and analysed using the RpH method. Readings were taken with all three glucometers. Pre-rigor GM muscle was sampled on-line approximately 10 minutes after slaughter using the designated biopsy tool. The biopsy tool is useful as it consistently extracts between 1 and 2 g of meat (Young, West et al., 2004). Sample collection was carried out by piercing GM muscle with the biopsy tool. The muscle sample was immediately placed in a flat bottom plastic tube and weighed. RpH buffer (7 mL) warmed to 55°C was dispensed into the tube followed by 0.5 mL of amyloglucosidase reagent kept at room temperature. This was followed by dispersing the sample with the abattoir's Utra-Turrax at 20,000 rpm for 20 seconds, then placing the tube in a temperature controlled aluminium heating block set at 55°C and incubating for five minutes. After incubation, 100 µL of the clear fluid from the meat slurry was pipetted onto Parafilm. The fluid was then sampled with a glucometer. The fluid was then sampled with the three glucometers.

Data analysis for calibration of two new meters against the Bayer glucometer

All three glucometers have a different dynamic range, with readings outside these limits giving error reading of LO or HI. The respective dynamic ranges for Bayer, Accuchek and Medisense glucometers are 10 to 600 mg dL^{-1}, 11 to 600 mg dL^{-1} and 20 to 500 mg dL^{-1}. For the purpose of calibrating a glucometer against the Bayer glucometer, measurements that returned a LO or HI glucose readings were discarded as the glucose concentration in these samples could not be known.

The data collected from the abattoir for the Accuchek and Medisense were plotted separately against the Bayer data as scatterplots. The correlation coefficients and resulting regression equation between the new glucometers versus the Bayer were calculated using the mathematical functions available in SigmaPlot version 9.01. The regression equation and correlation coefficient were then used to determine the capability of the new meters as a replacement for the Bayer glucometer in the RpH method.

COMPARISON OF THREE METHODS FOR GLYCOGEN DETERMINATION

Chemicals, reagents and equipment

All chemicals and reagents were analytical grade. Potassium iodide, calcium chloride dihydrate, 70% (w/v) perchloric acid, magnesium sulphate and potassium hydroxide were purchased from BDH Laboratory Supplies (Poole, England). The Sigma Chemical Company (St. Louis, MO, USA), supplied sodium acetate trihydrate (102364Q), β-nicotinamide-adenine dinucleotide phosphate disodium salt (β-NADP) (N-0505), glycogen Type IX from bovine liver (G-0885), adenosine triphosphate disodium salt (ATP) from equine muscle, glucose-6-phosphate dehydrogenase (G6P-DH) from baker's yeast (G-4134), hexokinase Type VI from baker's yeast (H-5375), and amyloglucosidase from *Aspergillus niger* (A-7420). Resublimed iodine was purchased from Hopkin and Williams Ltd. (England), potassium hydrogen carbonate was purchased from M&B Australia Pty Ltd., 96% (w/v) glacial acetic acid was sourced from J.T. Baker Chemical Company (Phillipsburg, NJ, USA) and triethanolamine hydrochloride was purchased from ICN Biomedicals Inc. (Ohio, USA).

An Ultra-Turrax T25 with the previously-described fittings was the disperser used in this section. Samples were centrifuged using a Sorvall® (Newtown, PA, USA) RC-5C+ centrifuge fitted with a Number 5 rotor. Absorbance readings were primarily measured with a Spectronic Unicam (Cambridge, England) UV300 UV-Visible spectrophotometer. All samples and chemicals were weighed on a Mettler Toledo (Switzerland) AB54 analytical balance.

Sample collection and storage

M. longissimus dorsi (LD) from the right side of the carcass from four cows were collected less than one hour post-mortem from the AgResearch abattoir, Hamilton, and immediately transported to the MIRINZ site where they were kept in a 5°C chiller. The cows were slaughtered according to approximately the same methods described in **Abattoir practice**. Portions of each muscle were sub-sampled at different time points (1, 4, 9 and 20 hours post-mortem). Subsampling was done by transversely slicing a steak from the muscle and placing a lean portion of this into an

aluminium cap. The samples were then frozen by immersion in liquid nitrogen and stored in a -80°C freezer for subsequent glycogen extraction.

Perchloric acid extraction of glycogen from meat

The frozen samples were crushed in a vacuum bag with a pestle and approximately 1 g of each sample was weighed into a 30 mL polyethylene centrifuge tubes. Care was taken to ensure that the samples did not thaw by keeping them in liquid nitrogen. Nine millilitres of ice cold 0.6 mol L^{-1} perchloric acid was immediately dispensed and the sample dispersed at 20,000 rpm for 20 seconds. The homogenate was held on ice for 30 minutes followed by centrifugation at 10,000 rpm (11,953 gravities) for 15 minutes. The supernatant was poured into a 10 mL centrifuge tubes, capped, and stored in the 5°C chiller for the following enzymatic and iodine glycogen determinations.

Iodine method for glycogen determination

This method is based on the method developed by Krisman (1962). A 15 mg mL^{-1} glycogen stock standard was diluted 1:9 in perchloric acid (0.6 mol L^{-1}). Aliquots of the stock standard were pipetted and made up to 400 µL with 0.6 mol L^{-1} perchloric acid to prepare glycogen standards ranging from 0 to 0.2 mg mL^{-1}. Standards were made up to 3 mL with Krisman's iodine reagent (iodine/potassium iodide in saturated calcium chloride), vortexed and allowed to stand for 10 minutes, transferred into a plastic cuvette with a 1 cm pathlength, and their absorbances measured at 460 nm. The spectrophotometer was zeroed using distilled water.

Glycogen measurements of the samples were carried out by adding 2.6 mL of iodine reagent into 400 µL of the glycogen extracts in a glass test tubes. After vortexing, the samples were allowed to stand for 10 minutes, transferred to cuvettes, and their absorbances measured at 460 nm. The glycogen contents of the samples were calculated from the standard curve obtained from the absorbances of the glycogen standards and expressed as mg g^{-1} of muscle.

Enzymatic method for glycogen determination

This method is based on the method developed by Keppler and Decker (1974). Aliquots (200 µL) of perchloric glycogen extract were pipetted into 10 mL centrifuge tubes and neutralised with 100 µL of 1 mol L^{-1} potassium carbonate and 10 mg mL^{-1}

amyloglucosidase and capped. After vortexing, the assays were incubated in a water bath at set at 40°C. Glycogen hydrolysis was terminated with 1 mL perchloric acid (0.6 mol L^{-1}) after two hours.

Fifty microlitres of the hydrolysed sample assays were then pipetted into 1 cm plastic cuvettes followed by 1 mL of ATP/NADP/G6P-DH solution as defined by Keppler and Decker (1974). After mixing, the absorbances of the samples were measured and recorded once the absorbance had stabilised at 340 nm (zeroed against air). Fifty microlitres of hexokinase was then dispensed into each cuvette and mixed thoroughly. The absorbance was then measured at 340 nm and recorded once it had stabilised.

The glycogen contents of the samples were calculated using a given constant used for glycogen measurements taken at 340 nm. The change in absorbances, dilutions and samples weights were also taken into account. Glycogen content was expressed as mg g^{-1} of muscle.

RpH method for glycogen determination

A series of spiked meat standards at varying glucose concentrations were prepared as described in **Glucometer response to spiked meat slurries**. Glucose measurements of these meat slurries were taken with a Medisense Optium Xceed™ glucometer and a standard curve was obtained.

Approximately 1.5 g of crushed meat samples were weighed into 30 mL polyethylene centrifuge tubes. Care was taken to ensure that the samples did not thaw by submerging them in liquid nitrogen. The previously described RpH buffer (7 mL) and 0.5 mL amyloglucosidase reagent were then dispensed into each tube. The samples were then dispersed at 20,000 rpm for 20 seconds followed by incubation for five minutes in a 55°C water bath. Glucose measurement was carried out with a Medisense glucometer. All glucometer readings were corrected for liquid associated with meat (assumed to be 78% w/v) and normalised to a glucose value of mg dL^{-1} per gram of muscle.

Glycogen concentrations for all samples were calculated using the normalised standard curve obtained from spiked meat standards. Glycogen content for each sample was expressed as mg glycogen g^{-1} of meat.

Data analysis

Calculated glycogen data for the three samples at four timepoints from each method were plotted in scatterplots. In addition to this, one-way analysis of variance was conducted to determine whether significant differences ($p \leq 0.05$) existed between the glycogen means and values obtained from the three glycogen methods. Statistical analysis was conducted using mathematical functions available in SPSS version 15.0 (SPSS Inc., USA).

NIR CALIBRATION FOR THE QUANTITATIVE DETERMINATION OF GLYCOGEN IN PRE-RIGOR MUSCLE

Chemicals, reagents and equipment

Chemicals and reagents and equipment used for the iodine determination of glycogen were as described in the section **Iodine method for glycogen determination** and purchased from the same source. Ultimate pH measurements were taken with a Testo® 230 meter (Testo, Germany) with attached temperature and pH probes. NIR scans were taken with a KES model K201 diode array spectrometer (KES Analysis Inc., NY, USA) fitted with a 16 mm fibre optic reflectance probe.

NIR scanning of pre-rigor samples

NIR scans, pH and temperature measurements, and samples for glycogen determination were collected approximately 45 minutes post-mortem from the left LD muscles of steers (n = 47), bulls (n = 28) and cows (n = 20) that were routinely slaughtered. To do this the NIR instrument was positioned beside the carcass grading station. Using a reflectance probe, the NIR spectra of muscle samples were collected between the wavelengths of 538 to 1677 nm on a freshly cut surface of the LD muscle (between the 11th and 12th rib) just before carcass grading (Figure 11). The detector had 152 diodes, each programmed to measure the reflectance of the muscle at an average of 9 nm intervals. Twenty scans were collected at different locations of a transverse section of the LD muscle and the spectra obtained were saved into the computer database for further analysis.

Collection and storage of muscle samples

The LD muscle was removed from the bone once the carcass had reached the hot boning area. After the pre-rigor pH (pH_{45}) and temperature at that time of the striploin had been measured with the Testo® meter, a transverse section of LD was taken, sliced into small cubes and placed into a labelled aluminium cap before freezing in liquid nitrogen. The samples were transported to the MIRINZ site and stored in a -80°C freezer for subsequent glycogen and ultimate pH (pH_u) measurements.

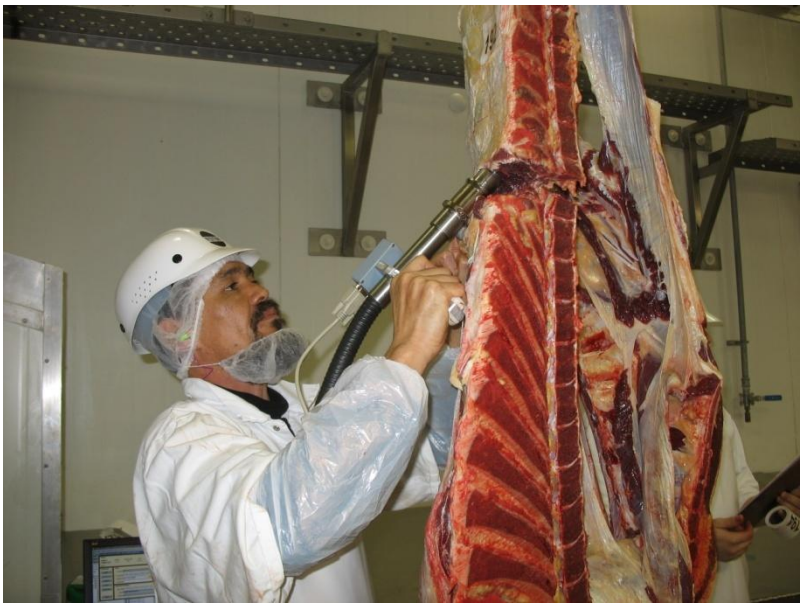

Figure 11: On-line collection of NIR spectra from a beef carcass. Spectra were collected using a fibre optic reflectance probe on a freshly cut surface of the LD muscle. (Photograph courtesy of AgResearch)

Glycogen and ultimate pH measurements of NIR scanned beef samples

The glycogen content of the beef samples from this trial were measured using the Iodine method.

The ultimate pH measurement method used was based on the MIRINZ protocol for measuring ultimate pH (MIRINZ). Approximately one gram of frozen beef muscle crushed under liquid nitrogen was weighed into a 30 mL polyethylene centrifuge tube. After allowing the sample to thaw to room temperature (approximately two hours), 8 mL of deionised water was dispensed into the centrifuge

tube followed by dispersion of the meat for 20 seconds at 20,000 rpm with the Utra-Turrax. The ultimate pH of the meat slurry was then measured with the calibrated pH meter.

Principal Component Analysis of NIR spectra to find a representative spectrum for each animal

From the 20 scans collected from each animal, the 10 most similar spectra that were most representative of the muscle were selected using Principal Component Analysis (PCA). This was achieved by removing one spectrum from the data set and averaging the remaining 19 spectra. The distance between the removed spectrum and the mean spectrum of the 19 spectra were then calculated using PCA. This step was repeated until PCAs for all 20 spectra was completed.

The 10 spectra most representative of the muscle scanned were defined as the spectra with the least calculated distances from the mean spectrum. Once these were identified by PCA, they were averaged to give a mean spectrum that was most representative of the muscle. The mean spectrum from each animal was then used to develop the following quantitative and qualitative models for the measurement of glycogen and pH_{45} and prediction pH_u in the beef samples.

Development of calibration models to measure glycogen and pH_{45} and predict pH_u in pre-rigor *M. longissimus dorsi*

Partial least squares (PLS) models using leave-one-out cross validation were fitted to predict the glycogen concentration, pH_{45} and pH_u. Numerous calibration models were fitted with increasing latent variables (LVs) for these three parameters. Up to 20 LVs were used. Two PLS models were generated for each of the three parameters (glycogen, pH_{45} and pH_u); the first was generated using the mean absorbance spectra, and the second using the mean reflectance spectra.

Validation of each model was conducted using leave-one-out cross validation. This was achieved by removing one animal's (average) spectrum from the data set. The remaining 94 spectra were then used to construct the PLS model. Finally, the model was used to measure the missing animal's glycogen concentration and pH_{45} and predict the animal's pH_u. The error of prediction was subsequently calculated. This procedure was repeated for every (average) spectrum in the data set for each model that was fitted. As a result, 1900 trials were required (95 samples $\times$ 20 LVs)

for each parameter. The mean square error of prediction (MSEP) was calculated from the differences between predicted and actual value for each model. The coefficient of determination (r^2) of predicted values was calculated from the regression plot of measured values versus predicted values obtained from the model.

PLS models were generated using the statistical package PLS version 2.0.1 available in software R version 6.2.0 (The R Foundation of Statistical Computing, www.r-project.com).

Development of classification models to categorise meat into two classes of ultimate pH

Generalised Partial Least Squares (GPLS) models were developed to estimate the probability that an animal would attain a normal pH_u (< 5.7). From this, animals were then categorised into two groups: animals that would attain a normal pH_u (< 5.7) and animals that would attain a higher pH_u (> 5.7). Two models were developed, the first where gender was taken into account, and the second where the gender effect was removed.

GPLS models were generated by dividing the data into calibration and validation sets. The former is used to produce a model to estimate the probability of a muscle becoming a normal pH_u meat. The independent validation set was then fitted into the model and the model was evaluated through the rate of correct classification versus incorrect classification.

The gender-into-account model first involved dividing the range of pH_u data from the 95 animals into four groups, with each group comprising 25 percent (quartiles) of the range of pH_u values. The ranges were pH_u 5.20 to 5.39, 5.40 to 5.50, 5.51 to 5.77 and 5.78 to 5.85. Then, 75 percent of the bulls, cows and steers from each quartile were allocated randomly to form the calibration data set. This procedure ensured that the range the calibration data at each quartile was equally represented by bulls, cows and steers. The calibration data set was then divided further into two classes: samples with a $pH_u > 5.7$ and $pH_u < 5.7$.

GPLS was then used to generate a linear model (calibration model) to estimate the probability that an animal would attain a pH_u less than 5.7. The remaining validation data containing 25 percent of the data set from bulls, cows and steers were then fitted to the calibration model. The number of latent variables used to fit the model was progressively increased from 1 to 15, with 20 calibration models generated

for each latent variable. As a result, 300 (15 latent variables × 20 models) models were fitted.

For the second model, the gender effect was reduced by splitting spectra in three subsets (bulls, cows and steers). The average spectra of each subset was calculated and then subtracted from spectra in the corresponding subset. Seventy-five percent of the data set in each quartile was randomly allocated to produce the model and the remaining 25 percent was used for validation. Calibration models were fitted in exactly the same manner as the first model where 20 models were generated with increasing latent variables resulting in 300 models fitted.

The mean reflectance spectra from each animal were used to generate both GPLS models. Models were generated using the statistical package GPLS version 1.3.1 available in software R version 6.2.0 (The R Foundation of Statistical Computing, www.r-project.com).

Exploratory analysis of NIR spectra to evaluate factors that give differences between animal classes

Exploratory analysis of the mean absorbance spectra from each animal was attempted to identify natural groupings within the data. This was conducted by calculating and comparing the distances between pairs of samples in a data set.

The spectral differences in between pairs of animals were calculated using Hierarchical Cluster Analysis (HCA). This was achieved by determining the Euclidean distances between the mean spectra from each animal over a multi-dimensional plot that can have any number of dimensions. The 9 nm intervals at each diode were treated as the variables.

When the distance between two animals is small, this implies that their spectra are relatively similar. Once the distances between animals have been determined with HCA, spectra were designated into groups, where naturally similar spectra (with small distances between them) were allocated into the same groups. HCA results are presented in the form of a dendrogram to facilitate the recognition of groups within the data and their similarity to other groups.

HCA analysis was conducted using the statistical package Stats version 2.6.0 available in the software R version 6.2.0 (The R Foundation of Statistical Computing, www.r-project.com).

CHAPTER 4

RESULTS AND DISCUSSION – PART I

CALIBRATION OF ROCHE ACCUCHEK® ADVANTAGE II AND MEDISENSE OPTIUM™ GLUCOMETERS FOR RAPID pH MEASUREMENT

Glucometer responses to glucose standards in water and RpH buffer

The likelihood of new glucometers as prospective replacements for the Bayer in the RpH method were evaluated by testing their response in a series of glucose standards made with water and RpH buffer. As these glucometers are designed to measure glucose in human blood, this preliminary check was conducted to determine whether they would be able to quantitatively determine a range of glucose concentrations in media other than blood.

Each of the glucometers was able to detect increasing glucose concentrations in water. Figure 12A shows that for each glucometer, a strong positive correlation exists between the standard glucose concentrations and corresponding glucometer readings. No values were obtained for zero glucose concentration because the glucometers return an error message at very low glucose concentrations. No results were recorded for the Bayer glucometer between 400 and 500 mg dL^{-1} because these glucose concentrations exceeded the upper limit of the dynamic range of that glucometer.

Both the Bayer and Accuchek plots were best described by a linear function, while a non-linear function best described the Medisense plots. The Medisense plot showed a curved trend which tended to plateau at higher glucose concentrations.

The three glucometers each returned different values at most glucose concentrations. Accuchek was the most accurate glucometer, with readings closest to the actual glucose concentration. The Bayer glucometer tended to overestimate the glucose concentration of most standards and the Medisense glucometer exhibited a non-linear trend which overestimated at lower glucose concentrations and underestimated at higher glucose concentrations.

Although each glucometer responded differently to the glucose standards, the coefficient of determination (r^2) of the fitted regression line for each glucometer suggested that all three glucometers would be able to accurately predict glucose concentration in water, using a suitable calibration equation. Moreover, the precision was very high (all the more remarkable because only single readings were taken). For the Bayer and Accuchek glucometers greater than 99.9% of the variation in standard glucose concentrations could be explained by the variation in the glucometer values. The equivalent value for Medisense was 97.8%.

The RpH method uses acetate buffer adjusted to pH 5.0 rather than water, so determining the effects this buffer on the glucometer readings was critically important.

At glucose concentrations equivalent to those shown in Figure 12A, higher readings were returned in RpH buffer than in water (Figure 12B). Whether this effect is due to the sodium ions, acetate, acetic acid, or hydrogen ions is not known, nor is it important in this study. But whatever the cause, the pattern of responses in RpH buffer was similar to that in water. The Accuchek glucometer returned values closest to the actual glucose concentration. The Bayer glucometer overestimated values of glucose and its limited dynamic range could not measure glucose concentrations above 400 mg dL^{-1}. A non-linear trend was again observed with the Medisense glucometer. As with water, nearly all the variation in standard glucose concentrations in RpH buffer can be explained by the variation in the glucometer values: Bayer 99.3%; Accuchek 99.8%; Medisense 99.7%.

Glucometer responses to meat slurries spiked with glucose

The RpH method involves the determination of glucose (originating from glycogen) in pre-rigor muscle dispersed in RpH buffer, trivially called meat slurries. The performance of the three glucometers was tested in meat slurries made from post-rigor meat spiked with glucose in the concentration range expected for glucose from glycogen in pre-rigor muscle. (As described in Chapter 1, meat as we know it is post-rigor muscle, where the glycogen has been largely metabolised to lactate. Residual glycogen concentrations are low. Thus, addition of glucose to meat slurries simulates pre-rigor meat treated with an amylolytic enzyme.)

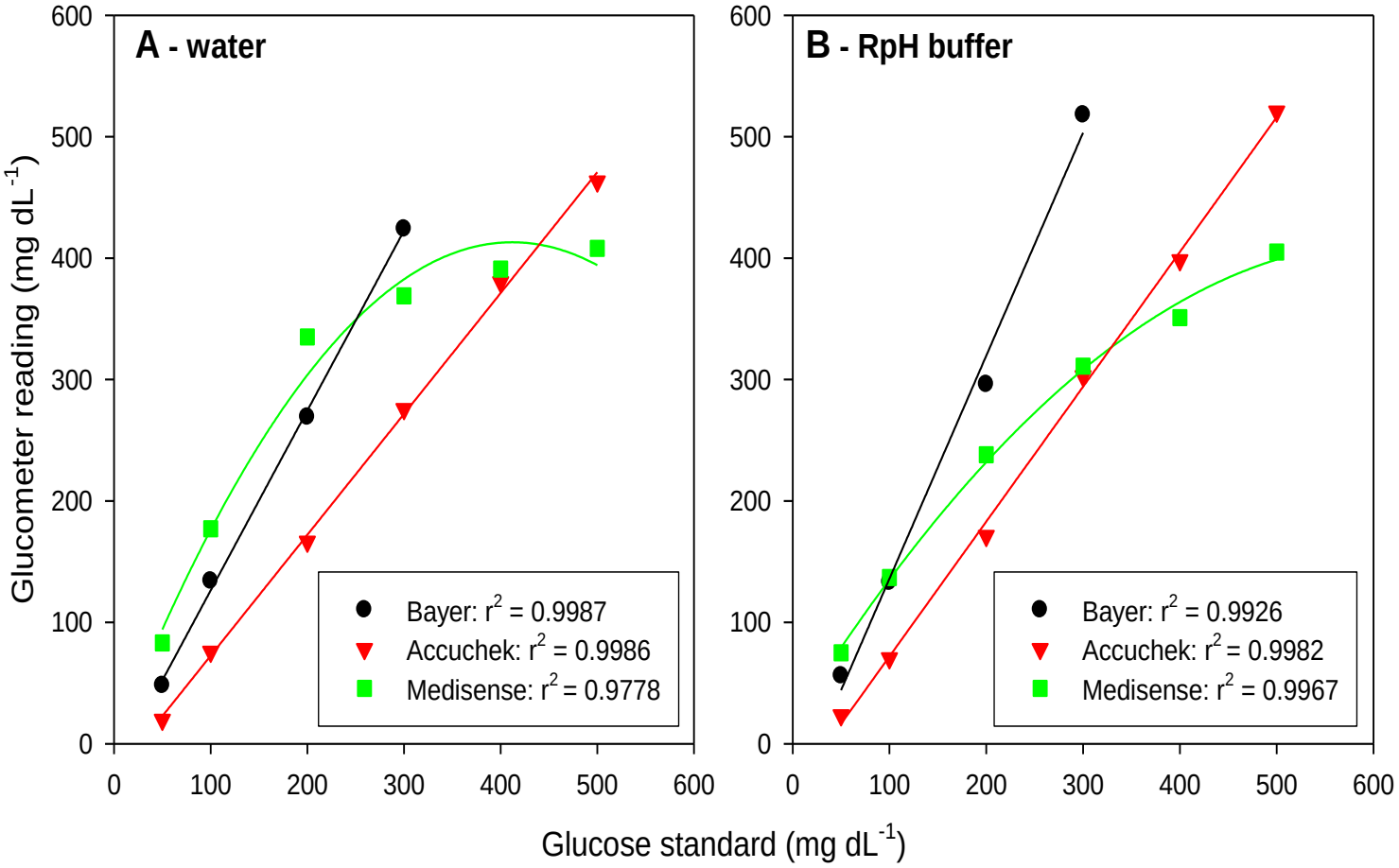

Figure 12: Glucometer readings of a range of glucose concentrations in water (A) and Rapid pH (RpH) buffer (B). Bayer and Accuchek gave higher readings for standards made in RpH buffer than in water. Medisense exhibited a curved trend in both solvents.

The RpH method – described in detail in Chapter 3 – was simulated such that post-rigor meat samples (1.5 g ± 0.05 g) were dispersed in 7.0 mL of glucose standards made up with RpH buffer ranging from 0 to 500 mg dL^{-1} and 0.5 mL of amyloglucosidase solution. Triplicate readings were taken at each standard concentration to check the accuracy and precision of the glucometers in this chemically messy environment. The responses of the three glucometers were very similar to the responses previously observed in water and RpH buffer. The Bayer and Accuchek glucometers showed a positive linear correlation and a non-linear trend was once again observed for the Medisense glucometer (Figure 13).

Unlike the situation with glucose in water and in RpH buffer, two of the meters, Bayer and Medisense, returned values at zero glucose addition (Figure 13). During glycolysis, monomeric glucose is liberated at α-1-6 branch points in glycogen, and accumulates in meat to a small extent. Moreover, the 'industrial grade' of amyloglucoside used in the RpH method contains some residual glucose (O.A. Young, personal communication). These sources were presumably responsible for the positive values at zero-added glucose. In the case of Accuchek, an error message was still returned, showing that the 'endogenous' glucose concentration was still below the detection limit for that meter. At the high end of the concentration scale, the Bayer glucometer was unable to determine glucose at 500 mg dL^{-1}.

Inspection of the data points in Figure 13Figure 13 shows that in each slurry the Medisense glucometer readings were the most variable. In other words this meter was the least precise. However, any of the three glucometers could perform the task adequately, all other factors being equal. Nearly all the variation in standard glucose concentrations in meat slurries can be explained by the variation in the glucometer values as follows: Bayer 99.7%; Accuchek 99.5%; Medisense 99.1%. This suggests that both Accuchek and Medisense may be viable replacements for Bayer glucometers for the RpH method.

Comparison of three glucometers in on-line RpH measurement of beef carcasses in a commercial abattoir

To help ensure that a wide range of glycogen concentrations would be encountered, the pre-rigor muscle samples were taken from steers, cows and bulls. Forty-five carcasses were processed at a commercial abattoir and the normal RpH procedure applied on the pre-rigor *gluteus medius* samples. From each of the 45

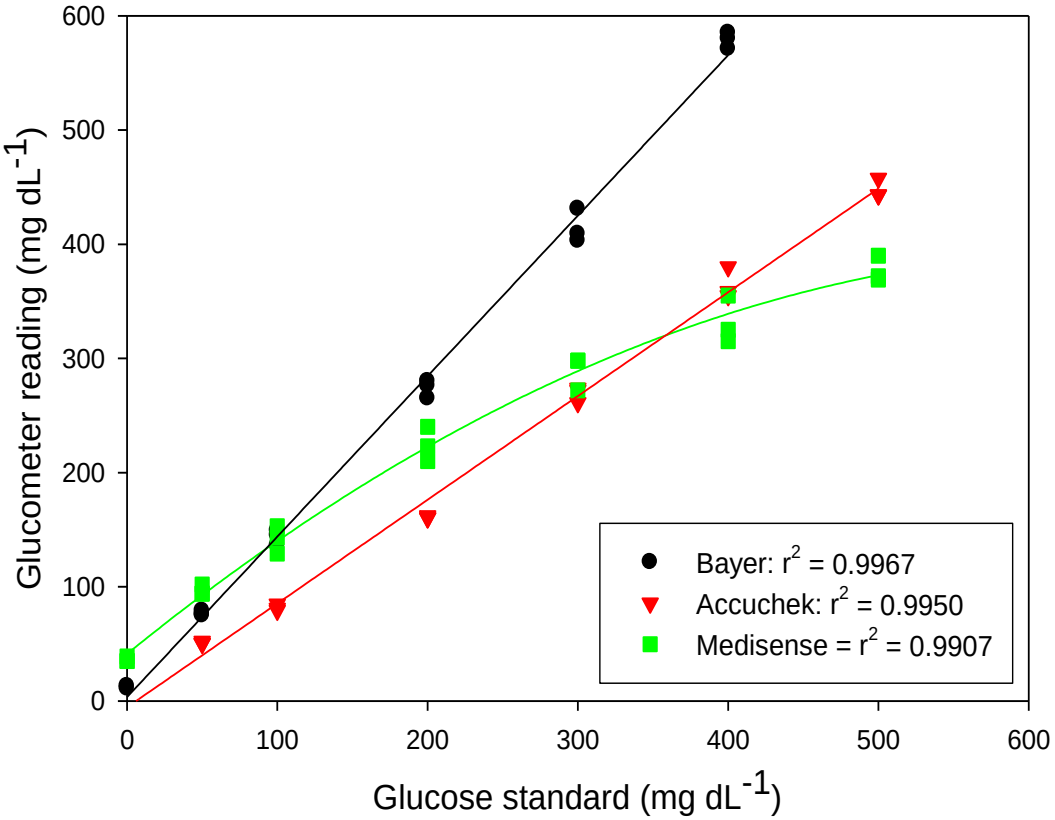

Figure 13: Glucometer readings from spiked postrigor meat slurries in RpH buffer and amyloglucosidase. Triplicate readings were taken.

slurries, a single glucose reading was taken with each of the three glucometers. The 135 readings were individually normalised to 1.0 g of muscle to compensate for the variation in the mass of muscle taken from each of the 45 animals. Subsequently the three normalised values for each animal were averaged, yielding 45 abscissa values. The individual glucometer readings (3 by 45) were the plotted against the 45 means (Figure 14). This was conducted to compare individual glucometer performances.

Response patterns observed in simpler mixtures (Figures 12 and 13) were also evident in Figure 14. Bayer and Accuchek gave linear responses while Medisense again gave a non-linear response (Figure 14). The Accuchek response to increasing glycogen concentration had the steepest slope, and up to 200 mg dL^{-1}, the Medisense and Bayer readings were closely similar.

When individual-animal, normalised glucometer readings from the Accuchek and the Medisense glucometers were plotted against the equivalent Bayer data on the abscissa; two very different graphs (Figures 15, 16) were obtained as might be expected from close inspection of Figure 14.

A strong positive linear correlation was observed (r^2 = 0.95) between Bayer and Accuchek readings (Figure 13).

Although a quadratic response was observed when the Bayer glucometer readings were plotted against equivalent Medisense data (Figure 16A), this response could be linearised by taking the square root of the Bayer data and replotting (Figure 16B). There was a strong linear correlation for which r^2 = 0.89.

Collectively the results in Figure 14, 15 and 16 indicate that either the Accuchek or Medisense glucometer could be a viable replacement for the Bayer glucometer in the RpH method. The next section discusses the choice of replacement meter.

Choice of a replacement meter for the Bayer glucometer

The Bayer glucometer had been calibrated for its routine operation of the RpH method in commercial abattoirs. However, the decline and inevitable discontinuation of the Bayer glucometer and its corresponding test strips saw the need for a replacement. At the time this project started, there were two commercially available glucometers in the New Zealand market – Roche's Accuchek® Advantage II and Abbotts's Medisense Optium™.

The three glucometers have the same operating principle, based on glucose determination by amperometry. When a sample is applied to the sample test strip, glucose from the sample reacts with glucose oxidase incorporated in the test strip. The products of this reaction generate an electrical current that is measured and is expressed as glucose concentration by some embedded mathematical function. All the glucometers have a specified dynamic range beyond which they either return inaccurate results or error messages.

Studies comparing the accuracy and precision of glucometers with their intended medium, human blood (Hawkins, 2005; Johnson & Baker, 2001), have found small differences between several commercial glucometers. The non-linearity of the Medisense glucometer reported here was also reported by Hawkins (2005), where the glucometers tended to over-report at low glucose concentrations and under-report at high glucose concentrations. Johnson and Baker (2001) reported that the Accuchek glucometer tended to under-report glucose at both low and high concentrations, with a bias of -1.9%. However, the fact that they are approved for medical use throughout the world strongly indicates their accuracy and precision are high enough to be of positive benefit to diabetics.

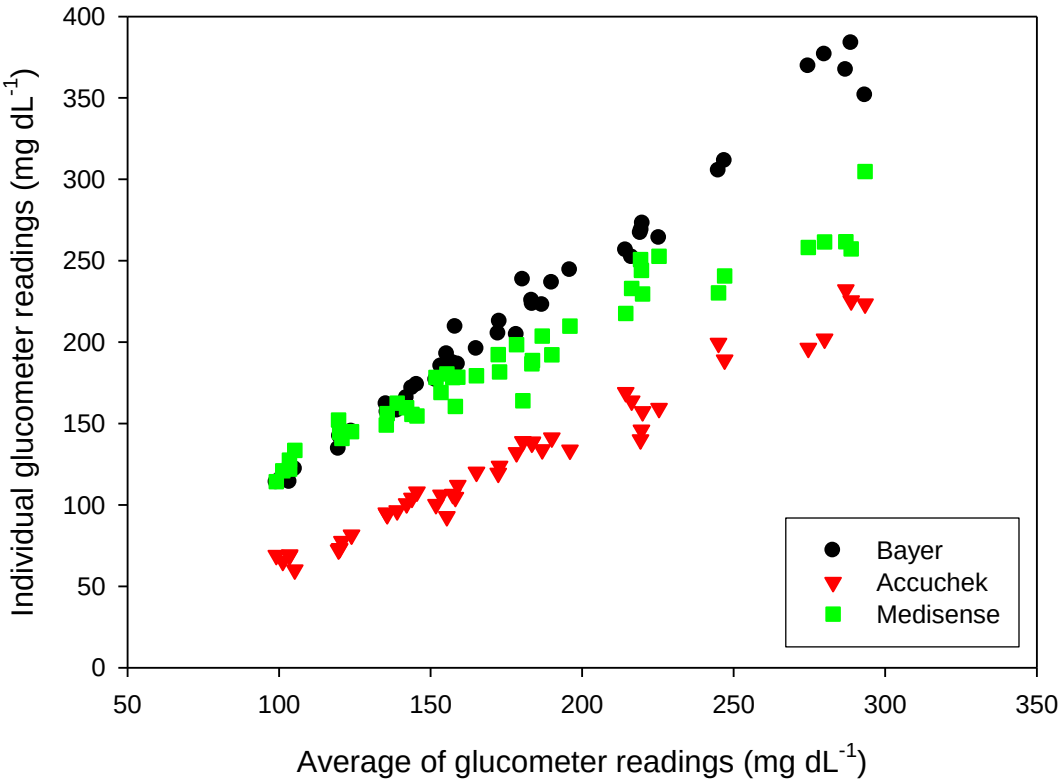

Figure 14: Plots of three glucometer readings from individual animals (ordinate axis) as a function of the mean of the three glucometer readings from each animal muscle slurry (abscissa). All values have been normalised to 1.0 g.

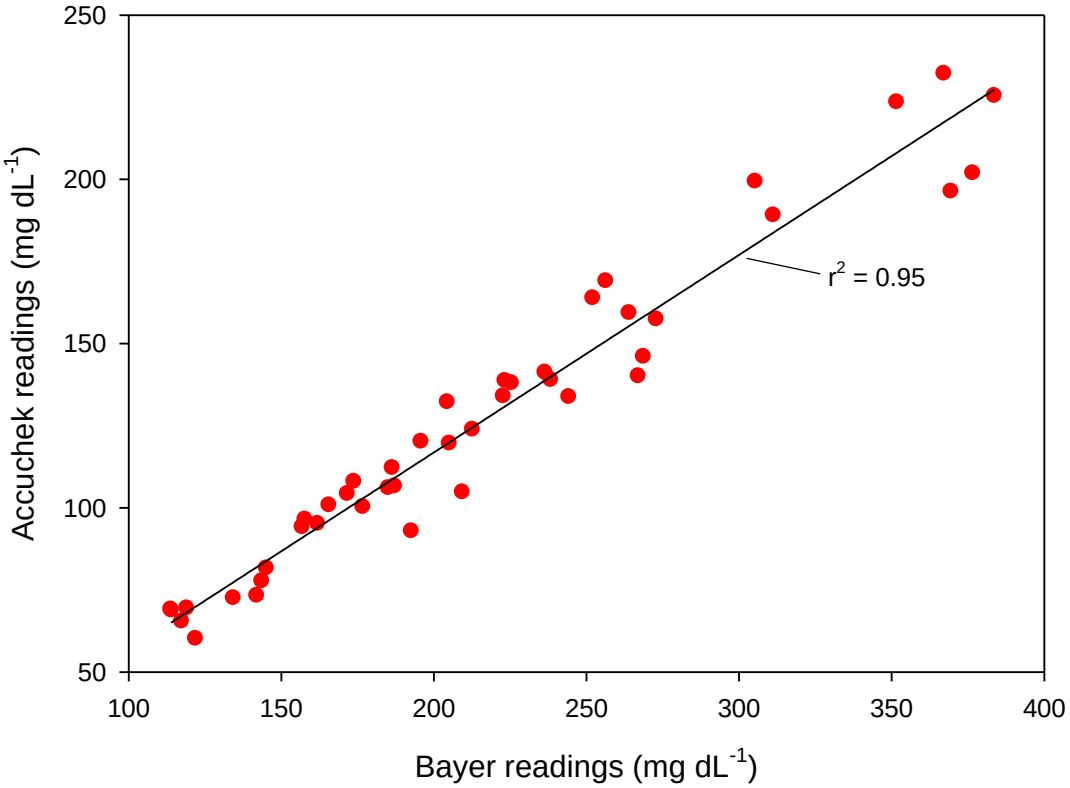

Figure 15: Accuchek glucometer readings as a function of Bayer glucometer readings for each of the 45 muscle slurries.

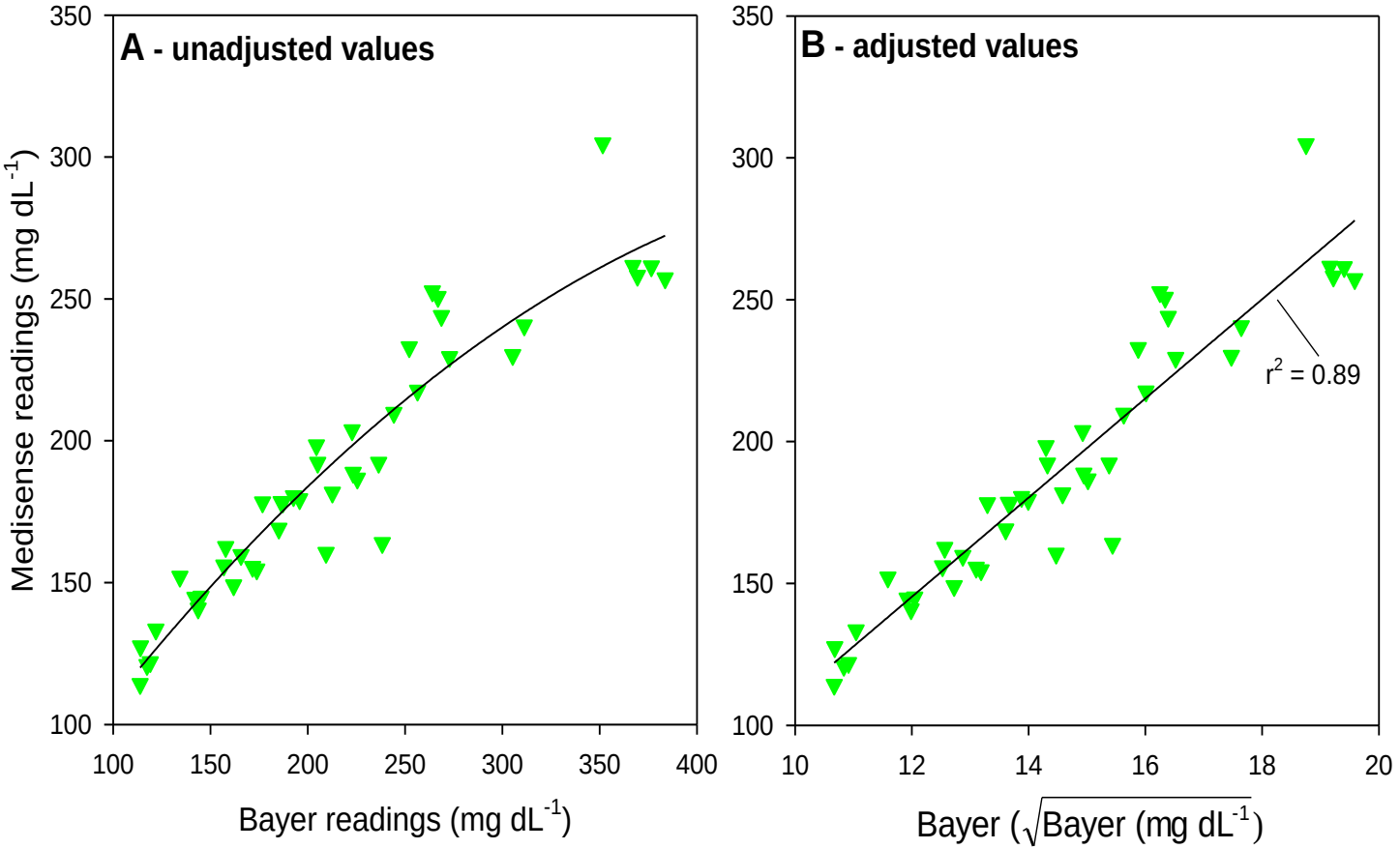

Figure 16: Medisense glucometer readings as a function of Bayer glucometer readings for each of 45 muscle slurries. A quadratic curve described the relationship well (A). This curve could be linearised by taking the square root of the Bayer glucometer data (B).

Inspection of Figures 12, 13 and 14 shows that the three meters do not usually return accurate values of glucose concentration. Moreover each glucometer behaves differently in slope and in offset from the origin (e.g. the constant in a linear equation). Thus they behave differently when the glucose is in a matrix other than blood. Other researchers have obtained parallel results to those reported here (Gardner, Moreland, & Thomson, 2004; Young, West, Hart, & Otterdijk, 2004). There are several possible reasons for why the responses in blood and RpH buffer could be different. The glucometer sensors are sensitive to liquid viscosity, and it is likely that the viscosity of the liquid phase of slurry that enters the minute sensor cavity would be lower than that of blood. The RpH buffer contains high concentrations of acetate, while the tonicity of blood is that of normal saline. Moreover, the pH values of blood and RpH buffer are very different, about 7.0 and 5.0 respectively. The temperature within the full sensor chamber is likely to be slightly different for the two liquids having originated at 37°C (blood) and 55°C (meat slurries).

Although the responses of glucometers to glucose in RpH buffer and blood are different, the dynamic responses in RpH buffer and their precisions show that either the Accuchek or the Medisense glucometer could contend to replace the Bayer glucometer. Arguably, the linearity or otherwise of glucometer responses is unimportant because in the practical application of the RpH technology it is only necessary to determine if the Bayer glucose reading (normalised to 1 g of muscle) is greater or less than 130 mg dL^{-1}. Provided the slope of the response curve is approximately linear around that value, nothing else is important from a commercial perspective. However, the interests of meat science will be better served with a glucometer that is linear over a wide dynamic range. There was strong linear relationship between the Bayer and Accuchek readings (Figure 15). The Medisense values were closer to those of the Bayer glucometer, especially at lower glucose concentrations (Figures 12 and 14), but were non-linear. Either glucometer could be commercially useful, but the linearity of Accuchek glucometer would make it the meter of choice. But there are other factors to consider. These are ease of use in an abattoir environment and price.

Bayer glucometers were easy to use in the abattoir because the test strips are arranged in a sealed carousel disc. One disc was enough for 10 readings (10 animals) before the disc had to be replaced. Readings take approximately 30 seconds.

The Accuchek glucometer take 20 seconds to return a reading. Test strips are held in batches of 50 in a cylindrical plastic container, and each test strip has to be individually inserted into the glucometer for each reading. This consumes time but is unavoidable. The Medisense glucometer takes only 10 seconds to return a reading, but test strips are individually wrapped and valuable time would be lost unwrapping each test strip prior to individual insertion. The 10 second response time of the Medisense glucometer might seem to be an advantage but in an abattoir environment that is not the case. The incubation time of the sensor is not dead time because other activities can be and are done in that window of opportunity.

Comparison of glucometer prices found that Accuchek glucometers are more expensive than Medisense glucometers. When purchased directly from the suppliers, the glucometer starter kits retail for $34.95 and $22.17, respectively. However, given that a meter will last for many months, the difference in price is not important. In contrast, the price of the test strips is important because each test is a consumable cost. As quoted by the suppliers in early 2008, Accuchek strips cost $22.50 for a pack of 50 strips, and Medisense strips cost $22.00. But these quoted values may be misleading because a better price could probably be negotiated when buying in the bulk that a beef processing company would consume in a year. (In this respect the suppliers, Roche and Abbott, are unaware of the comparative data in this thesis and as such are in a weak bargaining position.) The price of the test strips is very important because consumables savings mount up in the long run.

Subject to test strip price, Accuchek appears to be the best replacement for the Bayer glucometer. Gardner et al. (2004) found that Accuchek was able to qualitatively predict high ultimate pH carcasses with 80% accuracy. Although the aims of that study were rather different aims of those the present research, that study indicates that the Accuchek glucometer would be suitable. It also has a higher upper dynamic range limit than the current Bayer glucometer, which as discussed earlier is useful from a meat science perspective. At the low concentration end of the dynamic range the Bayer and Medisense meters are slightly more sensitive than the Accuchek. This is shown in Figure 13 where an error message was returned by Accuchek at zero added glucose, but not by the other two meters. However this does not detract from Accuchek's desirability. The normalised quality threshold value used commercially is 130 mg dL^{-1} with the Bayer glucometer (Young, Thomson et al., 2004). Inspection of Figure 15 shows that the equivalent Accuchek result would be approximately

70 mg dL^{-1}. Accuchek's lower detection limit lies somewhere between 0 and 50 mg spiked glucose dL^{-1} as displayed by that meter (Figure 13)

In respect of the lower limit of detection, only five samples were identified as high pH carcasses with Bayer glucose readings below 130 mg dL^{-1}. It was hoped that sampling different animal classes (steers, cows and bulls) would capture the whole spectrum of glucose values observed in beef in New Zealand. This was unfortunate but nothing can be done about this. With very little data below the quality threshold value of 130 mg dL^{-1} (Bayer) in Figure 15, one cannot be certain that data below this point would still follow the linear correlation between Bayer and Accuchek data (Figure 15). However, the curves in Figure 12, 13 and 14 strongly support the notion that linearity continues to low concentrations. With this assumption, the equivalent Accuchek threshold value was calculated by extrapolating the regression line of existing between Bayer and Accuchek (Figure 15). The new cut-off value for pre-rigor muscle using Accuchek glucometers is now set at 74.8 mg dL^{-1}. This is the equivalent Accuchek reading for the old Bayer threshold.

For the RpH method to be useful to meat processors, proper training of staff members who carry out the RpH method is essential. Several visits to commercial abattoirs that employ the RpH method revealed significant inconsistencies such as sampling of the wrong muscle, overheating of the meat slurry and the use of a faulty homogeniser that did not completely disperse the meat sample. These and other errors give unreliable results rendering the RpH method less useful as a quality control tool. However, as pointed out by Young et al. (2004), most of the possible errors in technique yield lower than actual glucose values. In this respect, the method tends to 'fail safe' in that low pH animals are more likely to be classed as high pH animals than the other way around. However, that can adversely affect profit. At all times the abattoir should aim for an accurate classification.

Although the RpH method is a relatively simple method of rapidly measuring glycogen in pre-rigor muscle, the inherent cost of applying the method is a perceived disadvantage. The current trend of glucometer manufacturers replacing old glucometer models with newer ones (as experienced in this thesis) is also an issue and recalibrating the RpH method with new glucometer models is costly and inconvenient to meat processors. Even with the costs taken into account, the RpH method it is still a worthwhile investment for the meat processors that use it. However, it would be good to find a method that reduces the cost of the method.

It has been proposed that near infra-red (NIR) spectroscopy could be a cheaper and more robust method for rapidly measuring glycogen in pre-rigor meat. Although the initial cost of purchasing a NIR spectrophotometer would be high, NIR instruments are more cost effective in the long run as they are longer lasting and can be specifically designed for industrial use. For NIR to be successfully applied on-line to measure glycogen, calibration and validation steps are required. This firstly involves finding a reliable reference glycogen determination method. Secondly, developing predictive models to quantify glycogen in pre-rigor muscle collected on-line. These are the objectives of the following chapter.

CHAPTER 5

RESULTS AND DISCUSSION – PART II

COMPARISON OF THREE GLYCOGEN DETERMINATION METHODS

With the high collective costs of running the Rapid pH (RpH) method and the inconvenience experienced by meat processors in calibrating the RpH method with new glucometer models, it has been proposed that that near infra-red (NIR) spectroscopy is ultimately a better method of rapidly measuring glycogen in pre-rigor meat. The overall aim of this chapter is to develop predictive models to quantify glycogen in *M. logissimus dorsi* (LD) muscle from NIR spectra collected on-line.

Developing models to measure glycogen first requires a reference glycogen determination method that is reliable and precise. The first part of this chapter describes work to find an accurate and consistent reference method from three popular glycogen determination methods. Once a reference glycogen method has been found, the final objective is to develop calibration models to measure glycogen concentration and pH at approximately 45 minutes post-mortem (pH_{45}) and to predict ultimate pH (pH_u) of pre-rigor LD muscles from NIR spectra collected on-line.

Determination of glycogen in *M. longissimus dorsi*

M. longissimus dorsi (LD) from three cows were collected less than one hour after slaughter and stored in a 5°C chiller. Portions from each muscle were sub-sampled at 1, 4, 9 and 20 hours post-mortem. The glycogen content of the LD samples at each timepoint was then assayed using the Iodine, Bergmeyer and RpH methods, all described in Chapter 3. The glucose content (originally glycogen) of the LD samples analysed using the RpH method was calculated in mole terms using the equation of the standard curve obtained from spiked meat slurries. For reasons discussed later, the meter used for this technique was the Medisense glucometer. The Bergmeyer method is similarly an enzymatic method that also expresses glycogen content as glucose units in mole terms. Glycogen values from the Bergmeyer method were converted into glycogen content in mass terms using the multiplication factor of

0.0162 (Keppler & Decker, 1974). The Iodine method expresses glycogen content directly as glycogen determined by colorimetry based on iodine's reaction with glycogen. Six replicates were conducted for each LD sample at each time point for each method. Finally glycogen values were expressed as mg g^{-1} fresh weight of muscle.

Figure 17 shows that the loss of glycogen by glycolysis during the onset of rigor was detected by all methods. A major decrease in glycogen concentration was observed within the first nine hours post-mortem, but less so when measured by the RpH method. Between nine and 20 hours post-mortem, the changes in glycogen concentration as measured by each method were essentially unchanging, such that at 20 hours the measured concentration by RpH was about three times higher than for the other two methods.

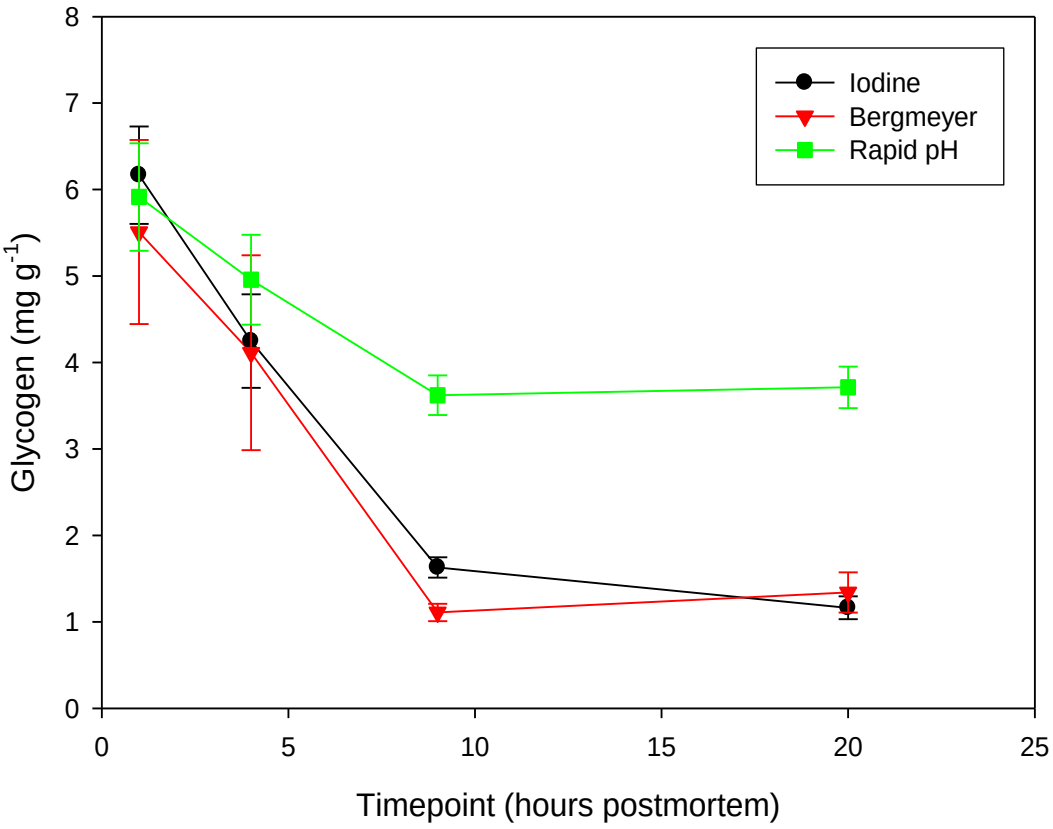

Figure 17: Mean glycogen values of *longissimus dorsi* muscle samples and their corresponding standard deviations at different time points post-mortem. Three glycogen determination methods were compared.

The fact that the Iodine and Bergmeyer methods gave similar results, and were consistent with the known pattern of glycogen depletion after slaughter (Dreiling et al., 1987; Young, West et al., 2004) suggests that these two methods gave more believable results. It also implies that one of these is most likely to be the reference glycogen determination method. However, accuracy is arguably less important than precision in a reference method, so percent variances were calculated at each time point (Table 1).

Table 1: Percent variances of glycogen concentration in *longissimus dorsi* muscle samples at different timepoints post-mortem as measured by three methods

Timepoint (hours postmortem)	Iodine	Bergmeyer	Rapid pH
1	9.2	19.3	10.5
4	12.7	27.3	10.5
9	7.2	9	6.3
20	11.4	17.4	6.5
Mean percent variance ± SD	10.1 ±	18.3 ±	8.5 ±

There was no obvious trend in percent variance with time. The Bergmeyer method yielded the most variable results, with little difference between the Iodine and RpH methods. At this point the Iodine method appeared to be the method of choice.

Final choice of glycogen reference method for NIR calibration

It is important to reiterate that a new Medisense glucometer (Medisense Optium Xceed™) was used in this section to determine glucose values through the RpH method. Although Accuchek was originally suggested to replace Bayer (see previous section), the manufacturers of Accuchek glucometers announced the replacement of the recommended model with a newer model just weeks after the glucometer work in the previous section was completed. Preliminary assessments of the new Accuchek model found that it was not able to measure glucose in meat slurries at the pH of the RpH buffer. Coincidentally during this period, a new Medisense model became commercially available. Although it exhibited the non-linear response in meat slurries as observed in the older model, it was the only commercial glucometer

available at the time that was able to measure glucose in RpH buffer. It was therefore the only viable replacement for the Accuchek glucometer recommended in the previous section.

The purpose of comparing the three glycogen measurement methods was to determine which method would be able to measure a range of glycogen values most consistently, and so become the reference glycogen method for the NIR calibration work (next section). Because glycogen in the muscles of freshly slaughtered carcasses can be variable depending on the psychological and physiological state of the animal before and at the time of slaughter, a method that could consistently quantify glycogen at over a wide range of values was required.

Previous research on glycogen methods support the findings reported in this section. The depletion of glycogen as monitored by the Iodine and Bergmeyer methods at increasing timepoints follows the trend reported by Dreiling *et al.* (1987) and Young *et al.* (2004). The glycogen content measured at both 1 hour and 24 hours post-mortem for the Iodine and Bergmeyer methods also agree with results published by Fabiansson and Reuterswärd (1984) and Dreiling *et al* (1987). However, some studies (Immonen, Kauffman, Schaefer, & Puolanne, 2000; Lahucky et al., 1998; Young, Thomson et al., 2004) have reported significantly higher levels of glycogen one hour post-slaughter. This difference may be due to the analysis of a different muscle, animal variation, variability in sample collection and preparation, or the particular glycogen method used.

Results from the RpH method show that the glycogen results stabilised at approximately 3.5 mg g^{-1} at 10 hours post-mortem. These results do not agree with previous research that have found glycogen content in muscle from unstressed animals approximately 24 hours post-mortem to be in the within 1 and 3 mg g^{-1} (Dreiling et al., 1987; Fabiansson & Reuterswärd, 1984; Lahucky et al., 1998). This suggests that the RpH method may not give accurate results at low levels of glycogen. These high readings may be due to errors introduced by the new Medisense glucometer. The behaviour of this glucometer at low glycogen concentrations in meat slurry needs to be further evaluated. Because of this uncertainty due to the high readings at low levels of glycogen obtained, the RpH method was not chosen for the calibration of the NIR instrument in the next section.

After assessing glycogen determination methods in post-mortem beef muscles, Fabiansson and Reuterswärd (1984) suggested that enzymatic hydrolysis of glycogen

followed by measurement of glucose by the Bergmeyer method as the best way of measuring glycogen. However, a later study comparing the reliability of the Iodine and Bergmeyer methods to measure glycogen in meat samples (Dreiling et al., 1987) found that the Iodine method gave more consistent results than the Bergmeyer method. The results reported in this section agree with this study. Although the mean glycogen measurements for both methods in each timepoint were statistically similar ($p > 0.05$), the mean percent variance for the Iodine method was numerically lower than for the Bergmeyer method (Table 1), but this difference was not statistically significant ($p = 0.085$).

Although there was no statistical significance in the consistency of the Iodine and Bergmeyer methods, the Iodine method protocol is simpler and less time consuming than the enzymatic Bergmeyer method. Because of its simplicity and higher precision, the Iodine method was chosen over the Bergmeyer and RpH methods as the reference glycogen method to calibrate the NIR instrument for glycogen determination in pre-rigor muscle.

The following section of this chapter details work to determine the efficacy of NIR spectroscopy as an on-line method of rapidly measuring glycogen in a commercial abattoir. The results of this objective are presented in the following section where the NIR spectra were collected on-line and their glycogen content subsequently determined using the Iodine method.

NEAR-INFRARED SPECTROSCOPY

Near-infrared scanning of muscles and selection of scans for data analysis

M. longissimus dorsi from 95 animals were scanned. In an effort to ensure that a wide range of range of glycogen, pH at approximately 45 minutes post-mortem (pH_{45}) and ultimate pH (pH_u) values were represented in the data set, spectra from three animal classes (steers, bulls and cows) were collected. Twenty near-infrared (NIR) scans between 538 and 1677 nm were recorded for each animal from a freshly cut surface of the *logissimus dorsi* (LD) muscle between the 11^{th} and 12^{th} rib using the reflectance probe described in Chapter 2.

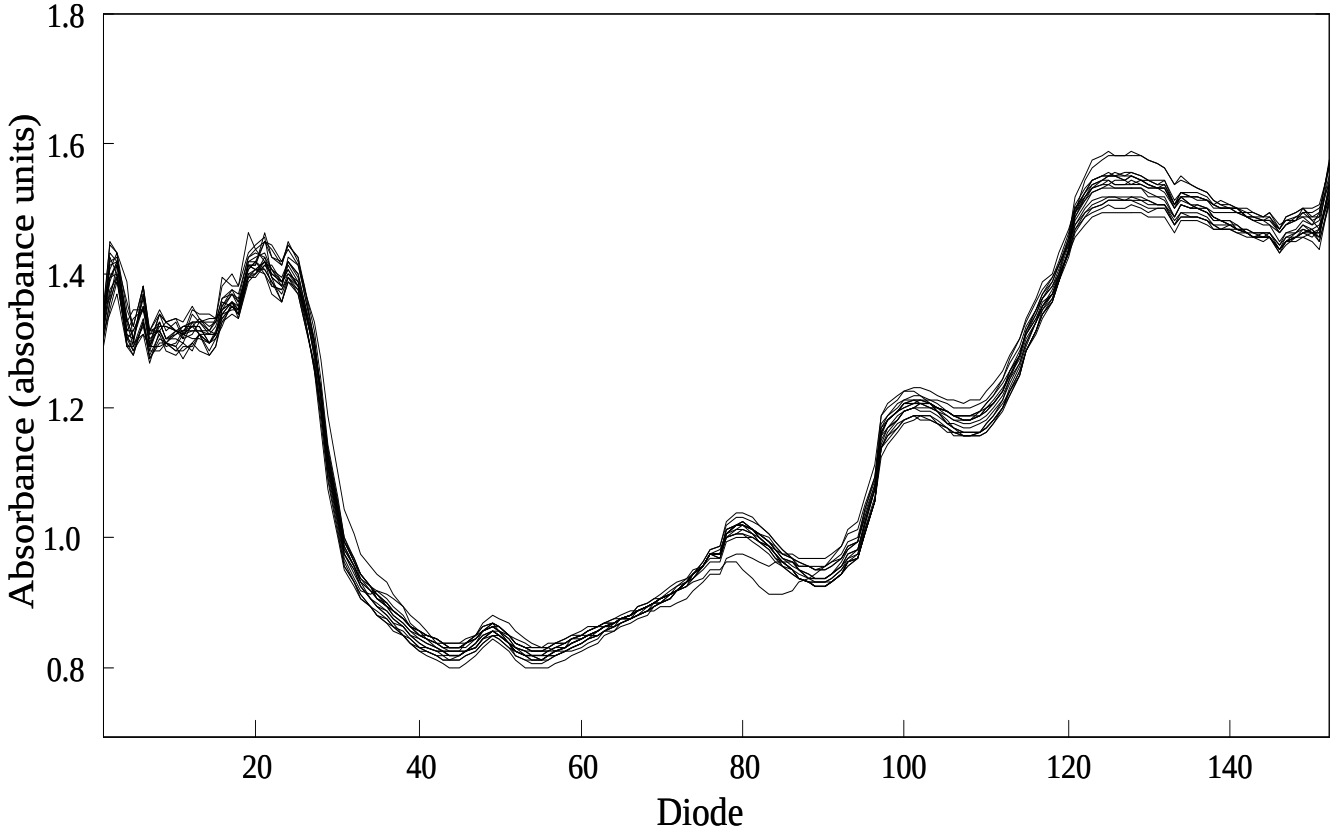

Figure 18: A typical plot showing the 20 near-infrared spectra collected from prerigor *M. longissimus dorsi* muscle of one animal. The KES detector contained 152 diodes which covered the spectral range of 538 nm to 1677 nm.

Figure 18 shows there is variation in the spectra at different locations of the same LD muscle, in this case, from a single animal. This is almost certainly due to the heterogeneous nature of muscle where components such as collagen, fat and connective tissue are more or less represented in given sections of the muscle.

Due to the variability of the NIR spectra collected from each animal, it is important to identify spectra that are most representative of the muscle. As discussed in Chapter 3, this was achieved by applying Principal Component Analysis (PCA) to identify the 10 most similar spectra from the 20 collected from each animal. Figure 19A shows the 10 spectra that were selected using PCA. The mean of these spectra were averaged to give a representative spectrum for the animal (Figure 19B). The 95 mean spectra from each animal were used for the subsequent development of models to quantify glycogen in pre-rigor muscle, measure pH_{45} and predict the pH_u and of prerigor LD muscle.

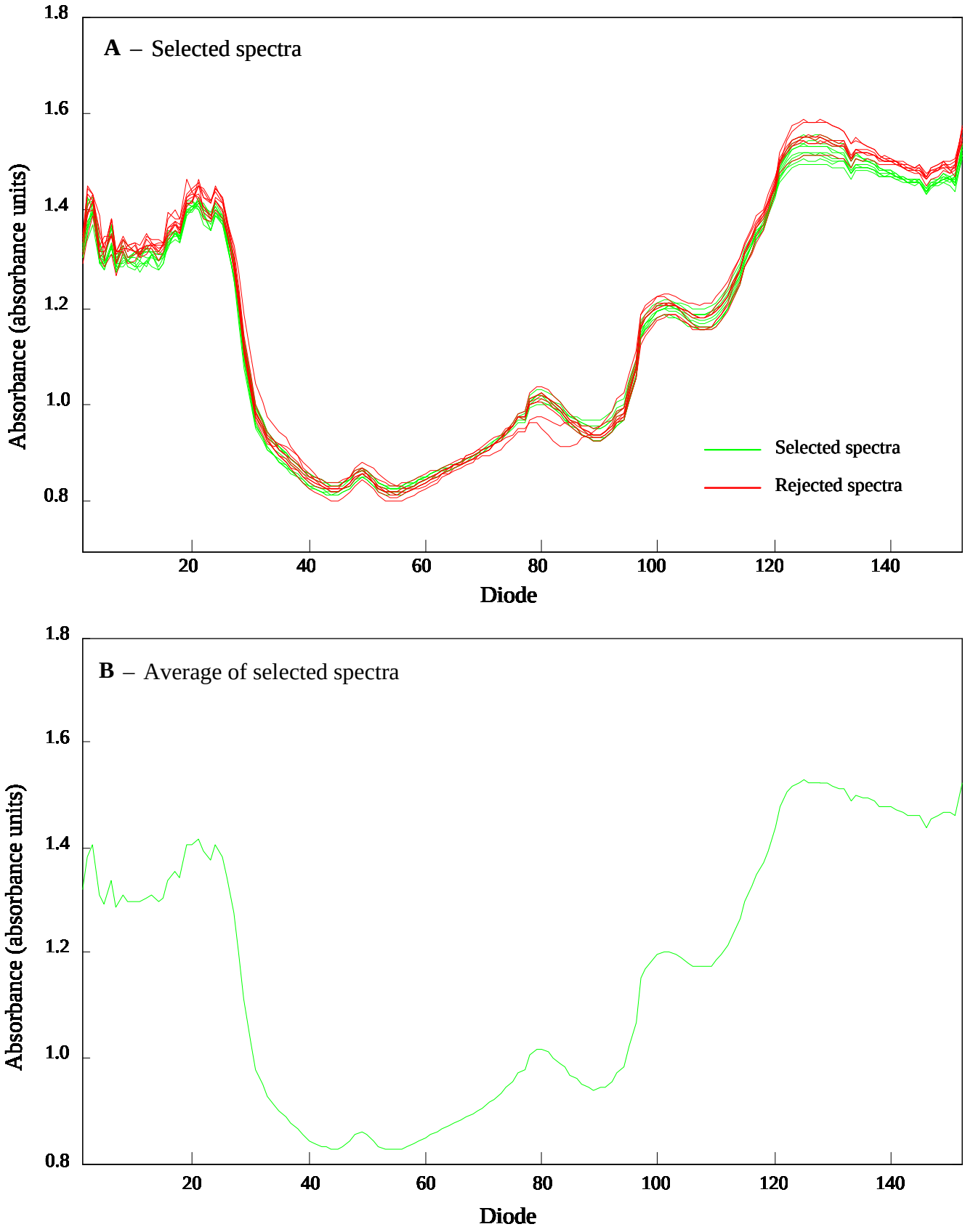

Figure 19: Principal Component Analysis of near-infrared (NIR) spectra showing the 10 selected spectra (A). The selected spectra were then averaged to give a representative NIR spectrum for the animal.

Development of calibration models to measure the glycogen concentration and pH_{45} and predict pH_u in pre-rigor muscle

Any capability of NIR to quantify glycogen in pre-rigor muscle is based on the assumption that glycogen affects the scattering and absorption of light in the NIR region. This would be due to the ability of glycogen, a polymer of glucose, to absorb or reflect light at particular NIR wavelengths characteristic to the molecule. As light is beamed into a muscle, it is scattered, and either absorbed or reflected back into the probe where it reaches the detector to produce a signal. The reflectance spectrum is proportional in some way to the concentration of glycogen in the muscle. At the same time, the reflectance spectrum can be used to calculate the NIR light absorbed by the muscle because the spectral characteristics of the light generated by the probe are completely known.

Partial least squares (PLS) regression is a statistical technique that assumes a linear relationship exists between the spectral data and the parameter of interest. In this thesis, PLS was used to develop linear models that predict glycogen concentrations, pH_{45} and pH_u in prerigor LD muscle. Successful predictive models for these parameters rely upon the NIR instrument being able to detect spectral variation as reference measurements changes from sample to sample.

For models generated using PLS regression, a linear model is applied to identify if any significant covariation between predicted and measured values exist. This is achieved by dividing the data into two sets – one set for calibration the other set for validation. The calibration set is used to generate the model while the validation set evaluates whether the model can be applied to new samples independent from those used to construct the model. As outlined in Chapter 3, the capability of the PLS models that were developed was tested with leave-one-out cross validation.

PLS models are assumed to be directly influenced by a number of latent variables (LVs). In contrast to measured variables (glycogen, pH_u and pH_{45}), latent variables are not directly observed. Rather, they are underlying variables which are assumed to be directly related observed variables and NIR spectra. The number of LVs influencing a model us usually not known, and the aim of PLS is to estimate the number of LVs that give the optimum predictive model.

The significance of a model is verified mainly by their Mean Square Error of Prediction (MSEP) and coefficient of determination (r^2) values. MSEP is an indicator of how accurate the model is at predicting the values of independent samples

compared to their reference measurements. The significance of a model is also indicated by the covariation between the predicted values and measured values. If significant covariation in a model exists, a high r^2 value is attained.

The generated models were verified by plotting 'predicted' against 'measured' values. In an accurate model where significant covariation exists, the points in the plot will form a linear trend and plot over a 45° line. Hence optimum predictive models are identified by the number of latent variables that have the maximum r^2 and the least MSEP from leave-one-out cross validation predictions.

Predictive models for glycogen measurement were determined from both the absorbance and reflectance spectra to determine which spectral data generated better models. Table 2 shows that models generated from reflectance spectra gave better results for glycogen, pH_u, and pH_{45} and so the reflectance spectra was used to generate PLS models for their prediction in pre-rigor muscle.

Table 2: Summary of results for PLS models fitted to predict glycogen, pHu and pH45 in pre-rigor LD muscle by leave-out-one cross validation. Models generated from reflectance spectra gave slightly better models

	Spectra (538 to 1677 nm)					
	Prediction from absorbance			Prediction from reflectance		
LV[a]	11	6	10	13	6	0.13
r^{2}[b]	0.22	0.18	0.31	0.23	0.20	0.37
MSEP[c]	7.75	0.13	0.04	7.75	0.13	0.04

[a] LV: Number of latent variables in fitted model
[b] r^2: coefficient of determination
[c] MSEP: Mean square error of prediction for cross validation predictions

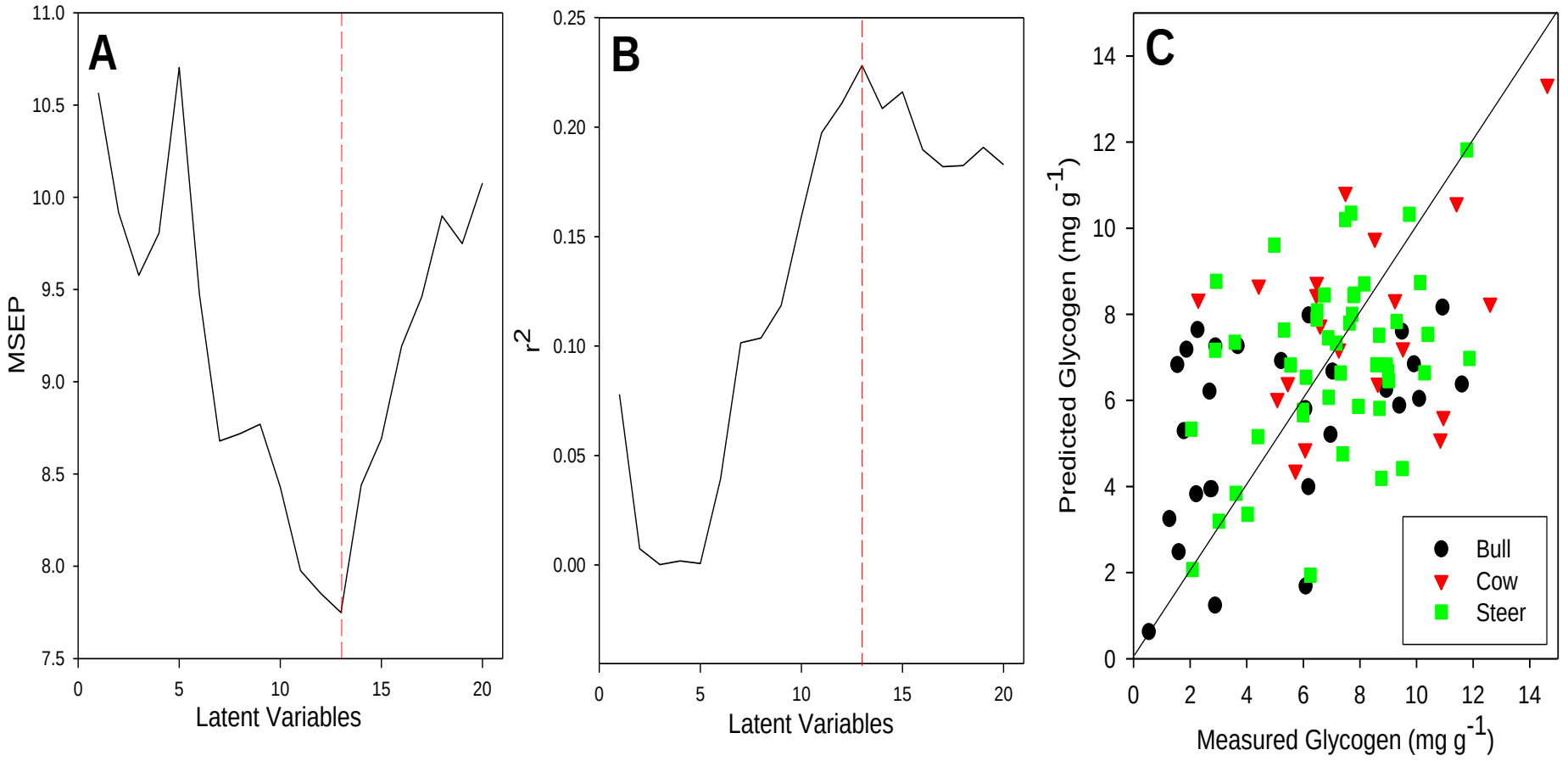

Figure 20: Mean Square Error of Prediction (MSEP) (A) and coefficient of determination (r^2) (B) values of Partial Least Squares (PLS) models of the reflectance spectra (538 to 1677 nm) for the prediction of glycogen in pre-rigor muscle. Predictions of leave-one-out cross validation are presented in terms of measured and predicted glycogen values. Thirteen latent variables were used to generate the optimum PLS model.

The PLS calibration models for glycogen determination were poor. The optimal calibration model from the reflectance spectra had 13 latent variables which gave an r^2 of 0.23 and a large MSEP of 7.75 mg g^{-1} (Figure 20). The r^2 value of 0.23 indicates that the best attainable calibration model for glycogen can explain only 23 percent of the variation in the predicted glycogen values. The optimal calibration model from the absorbance spectra was statistically very similar to the reflectance spectra with a lower r^2 of 0.22.

The best calibration model fitted to predict pH_u from reflectance spectra was also very poor. Cross validation predictions yielded a very low r^2 of 0.20 with an MSEP of 0.13. The regression plot (Figure 21C) shows that the pH_u of most steers and cows fall below pH 5.7 with a predicted pH_u ranging from approximately pH 5.2 to 6.0. It also shows that the distribution of the pH_u of bulls was different from that of steers and cows. Although the measured pH_u of the bulls ranged between pH 5.5 and 6.8, the predicted pH_u of most bulls was within the narrow pH range of 5.7 to 6.2. This suggests that a separate calibration model may need to be fitted for bulls to predict pH_u. Inspection of Figure 20C shows no obvious differences between the three gender classes that could be exploited in developing gender-specific calibration models for glycogen prediction. However, the regression plot of predicted pH_u from the reflectance spectra (Figure 21C) show that the bulls, cows and steers each follow a different trend which suggests that a different model may be required for each animal class.

PLS models were also developed to predict pH_{45}. Validation was performed to find the model with the optimum number of latent variables that produced the maximum r^2 and the lowest MSEP (Figure 22). Results from the reflectance spectra show that the best attainable calibration model was still poor (r^2 = 0.37). Although the MSEP of the model after cross validation was very low (0.039), only 37 percent of the variation in the pH_{45} of muscle could be explained by the calibration model.

The regression plot of muscle at pH_{45} (Figure 22C) show that data tend to be clustered according to animal class. Most bulls had a measured and predicted pH_{45} value above 6.5, while almost all steers had measured and predicted pH_{45} values ranging from 5.9 to 6.6. Again this suggests that separate models might need to be fitted to the different animal classes.

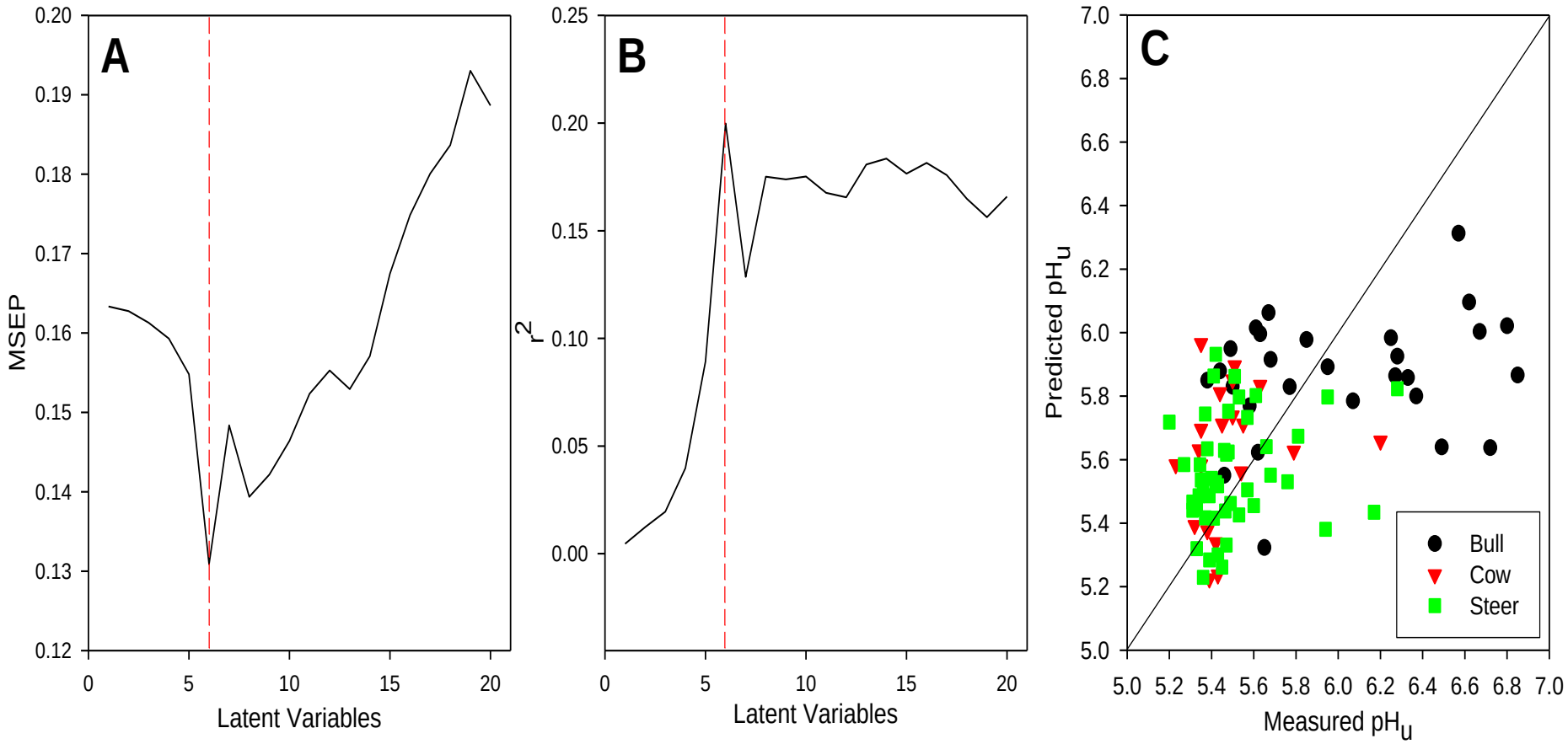

Figure 21: Mean Square Error of Prediction (MSEP) (A) and coefficient of determination (r^2) (B) values of Partial Least Squares (PLS) models of the reflectance spectra (538 to 1677 nm) for the prediction of ultimate pH (pH_u) in pre-rigor muscle. Predictions of leave-one-out cross validation are presented in terms of measured and predicted pH_u values. Six latent variables were used to generate the optimum PLS model. Samples for pH_u measurements were frozen in liquid nitrogen and the pH_u measured a month later.

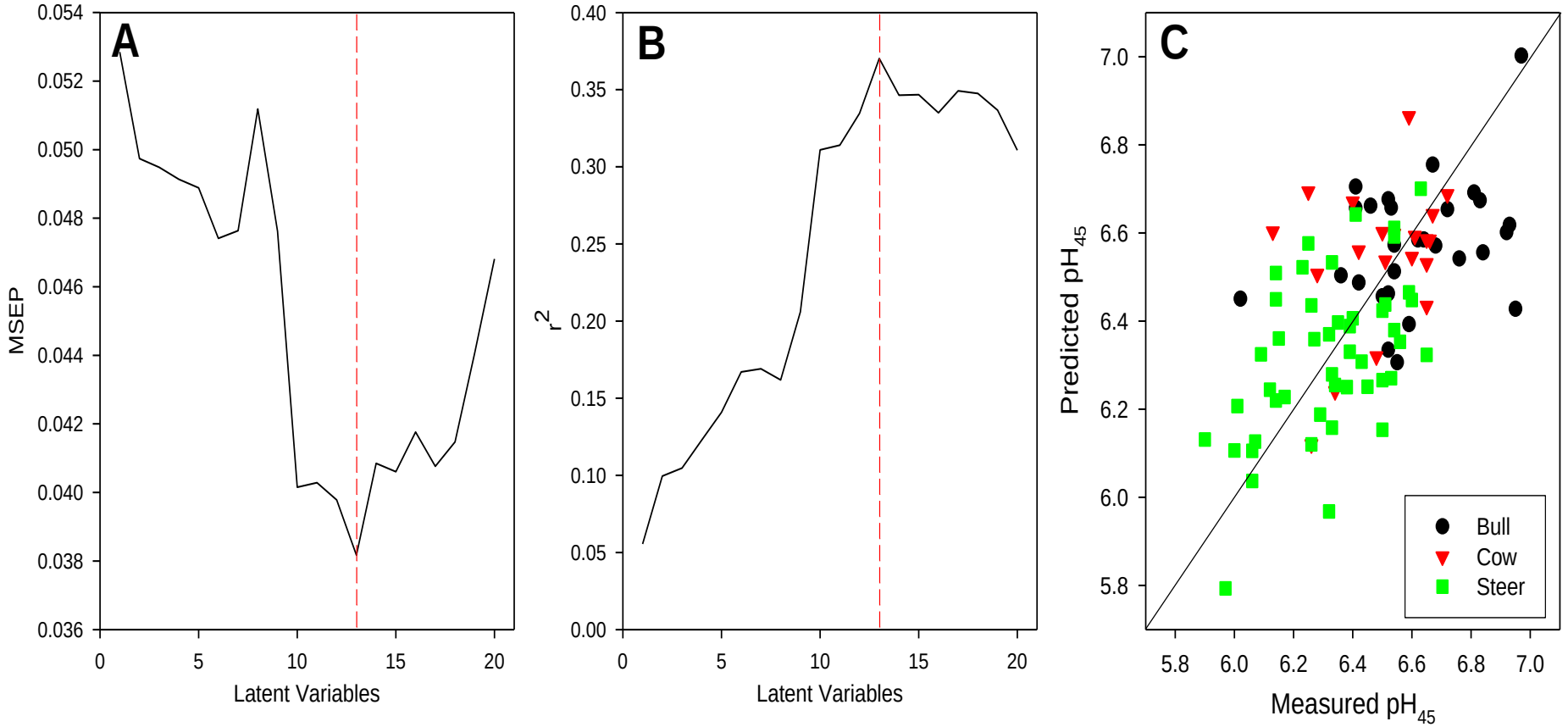

Figure 22: Mean Square Error of Prediction (MSEP) (A) and coefficient of determination (r^2) (B) values of Partial Least Squares (PLS) models of the reflectance spectra (538 to 1677 nm) for the prediction of pH_{45} in pre-rigor muscle. Predictions of leave-one-out cross validation are presented in terms of measured and predicted pH at approximately 45 minutes post-mortem (pH_{45}) values. Thirteen latent variables were used to generate the optimum PLS model (C). pH_{45} was measured in the abattoir immediately after near-infrared scanning.

Development of classification models to categorise meat into two classes of pH_u

As presented in the previous section, PLS models generated to predict muscle glycogen were very poor. Determining the glycogen concentration of muscle is ultimately not useful for meat processors. They only want to know whether a carcass will be above the acceptable threshold of pH_u. Thus the development of models to classify meat into two pH_u categories was attempted in this section.

As discussed in the earlier, Generalised partial least squares (GPLS) was used to develop classification models to categorise beef carcasses into their predicted pH_u. GPLS is similar to PLS modelling. However, GPLS algorithms are specifically for constructing classification models and are different from that of PLS.

GPLS classification models were achieved by dividing the reflectance data set into two sets one for calibration and the other for validation. Seventy-five percent of the data set (72 animals) was randomly chosen to produce calibration models. The remaining 24 animals were used as an independent data set for validation.

Two models were developed, the first where gender was taken into account, and the second where the gender effect was removed.

As described in Chapter 2, the gender-into-account model first involved dividing the range of pH_u data into four groups (quartiles), with 75 percent of the data from each quartile allocated randomly to form the calibration data set. The calibration data set was used to fit a linear model to estimate the probability of a sample to have a pH_u greater than 5.7. The independent validation set was then fitted to the model to test the model's accuracy at classifying the pH_u of prerigor muscle. Three hundred (15 LVs $\times$ 20 models) models were fitted. Twenty models were attempted at each LV as models generated beyond this were not significantly different from the twentieth model.

For the second model, the gender effect was reduced by splitting spectra in three subsets (bulls, cows and steers). The average spectra of each subset was calculated and then subtracted from spectra in the corresponding subset. Calibration and validation of was carried out as for the gender-into-account model.

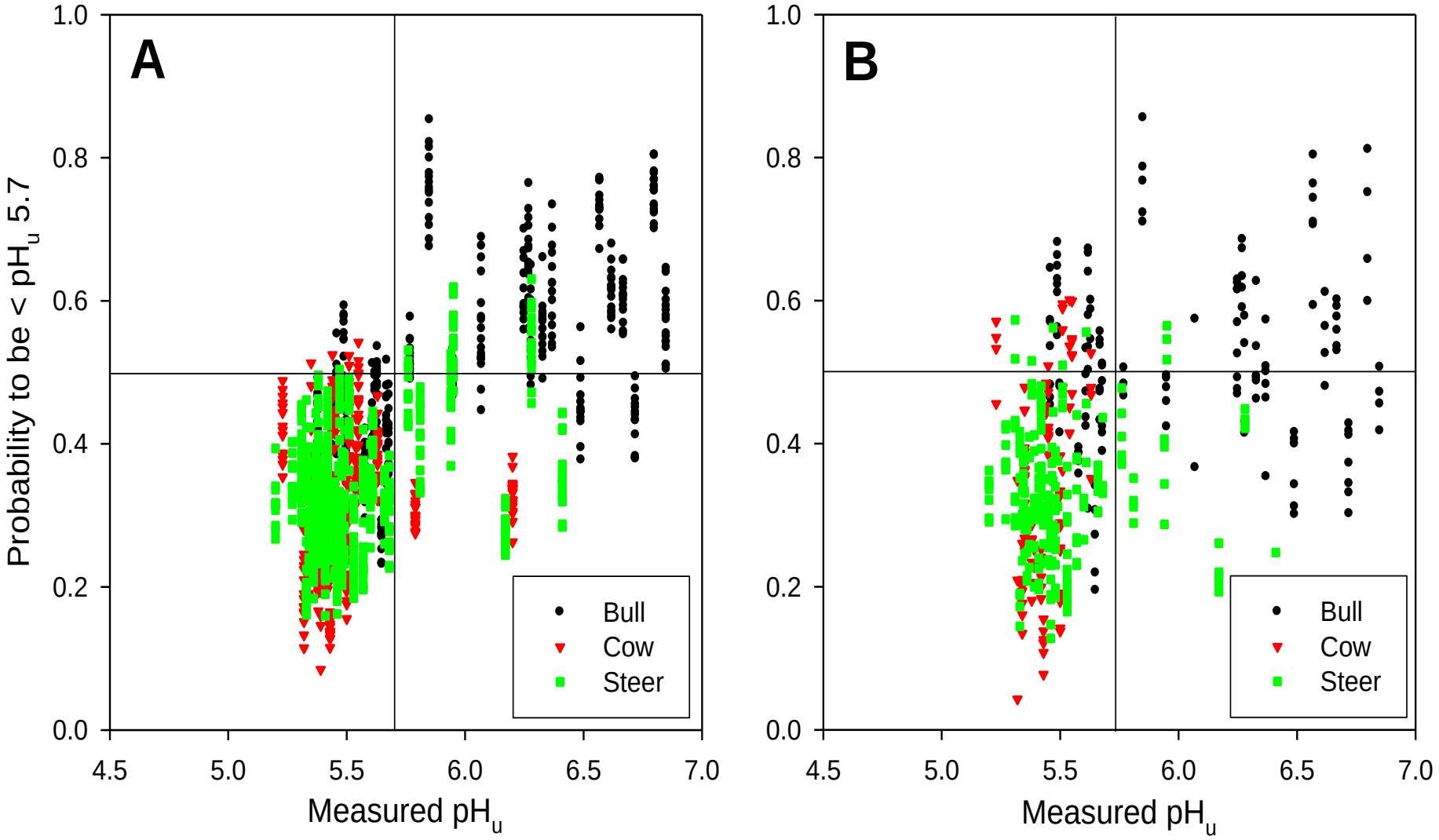

Figure 23: Calibration model (A) and validation (B) of classification models with gender effect taken into account. Classification was based on the probability of samples reaching an ultimate pH (pH_u) lower than 5.7. The reflectance spectra were used with nine latent variables giving the optimum model.

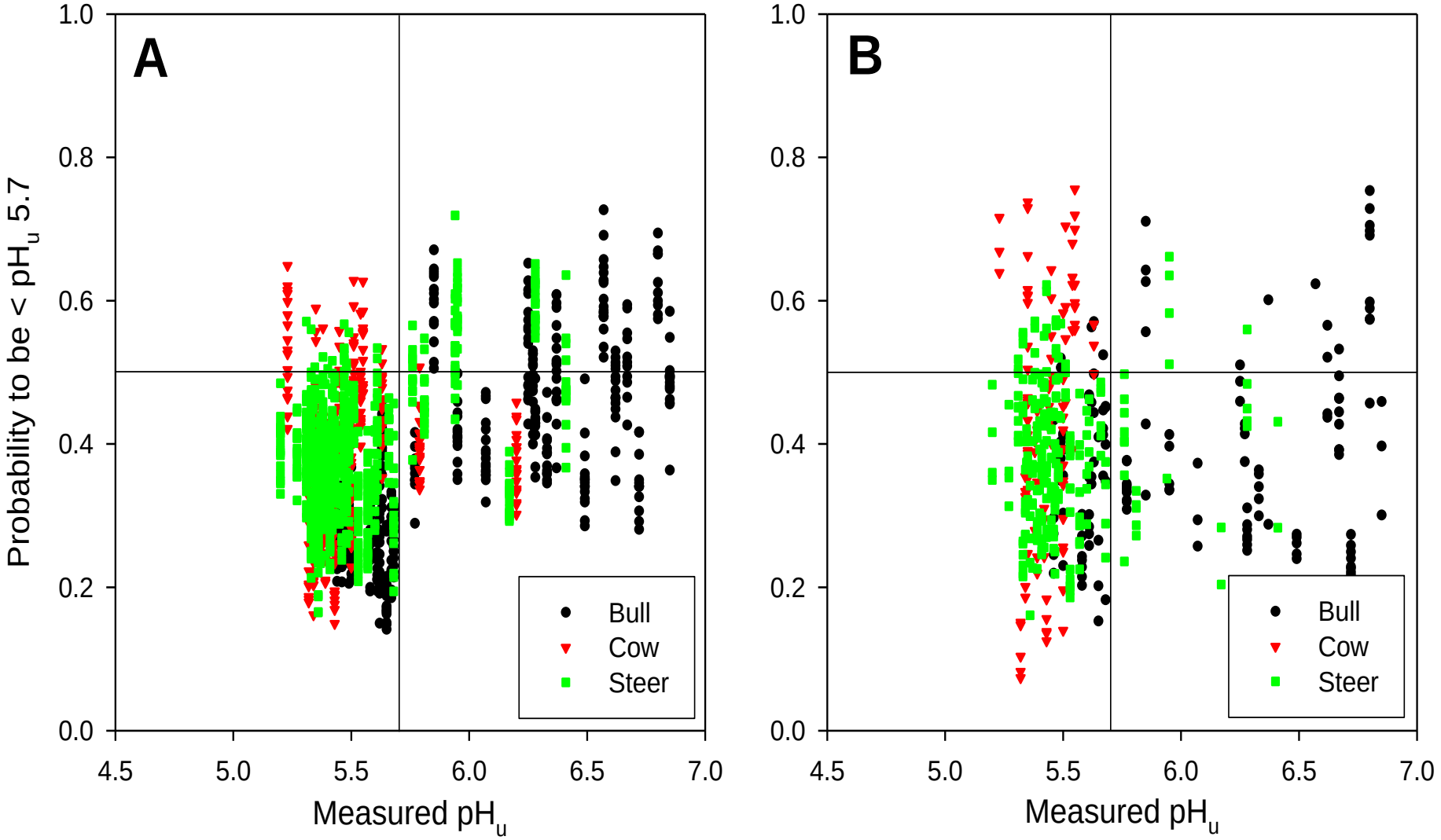

Figure 24: Calibration model (A) and validation (B) of classification models after ignoring the gender effect. Classification was based on the probability of samples reaching an ultimate pH (pH_u) lower than 5.7. The reflectance spectra were used with nine latent variables giving the optimum model.

Table 3: Percentage of correct and incorrect classification of calibration and validation data for GPLS classification models with the inclusion and exclusion of gender effects. The reflectance data was used with nine LVs giving the optimum model

	Gender effect included				Gender effect excluded			
	Calibration		Validation		Calibration		Validation	
	Low pH	High pH	Low pH	High pH	Low pH	High pH	Low pH	High pH
Correct classification (%)	98	61	83	42	94	39	85	21
Incorrect classification (%)	2	39	17	58	6	61	15	79

Bull data from the calibration and validation GPLS models exhibited a different trend from steers and cows (Figure 23). Most of the data with measured and predicted pH_u values above 5.7 were predominantly from bulls. This confirms findings from the previous predictive PLS models for glycogen, pH_{45} and pH_u and suggests that since the trend and pH_u profile of bulls are different, they may require a separate GPLS model to predict their pH_u.

Table 3 shows that the classification models yielded poor results. For the first model, where gender was taken into account, most of the calibration data with normal pH_u were fitted well by the model: 98% of the calibration data was correctly classified for all models fitted which suggests that the GPLS calibration model is good at predicting muscle that will reach a normal pH_u correctly. However, a significant proportion of the calibration data (39%) with a high pH_u did not correctly fit the model. When the independent data (validation) was fitted into the calibration models, poorer results were obtained. For normal pH samples, 83 percent of the validation samples were correctly classified as normal pH_u. However, at high pH, only 42% of the validation samples were correctly classified.

When the effect of gender was removed (Figure 24), an even a poorer classification model was generated. Most of the calibration data fitted correctly into the model at normal pH_u, but only 39% of the calibration model fitted correctly at high pH_u. The poor calibration model resulted in most of the validation data being classified wrongly at the high pH_u range where only 21% of high pH_u animals were classified correctly.

Exploratory analysis of NIR spectra to evaluate factors that give differences between genders

Attempts to develop models that quantify glycogen in prerigor muscle or predict pH_u of muscle from NIR spectra collected on-line have been unsuccessful thus far. In order to explain why such poor models were attained, and in an attempt to extract additional information from the spectra, exploratory analysis of the NIR spectra was conducted.

The method for exploratory analysis of the NIR spectra using Hierarchical Cluster Analysis (HCA) was described in Chapter 2. Briefly, HCA is a technique which primarily aims to present data in a manner which emphasises natural

groupings. The distances between the spectra on a two, three or multi-dimensional plot were calculated where naturally similar spectra are separated by small distances. Once the distances between all spectra have been calculated, HCA results are presented in the form of a dendrogram to facilitate the recognition of groups within the data (Figure 25).

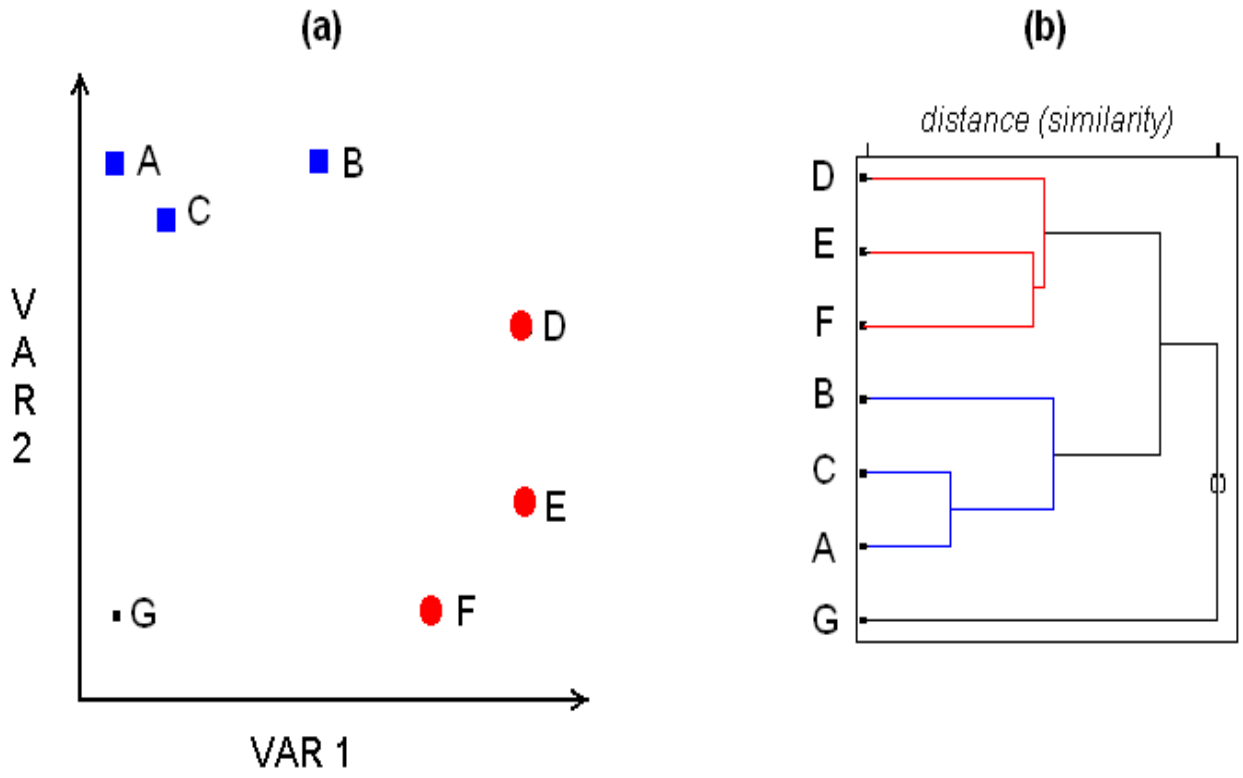

Figure 25: Illustration of the exploratory analysis of a small data set (seven samples described by two variables). Three groups are identified and are presented accordingly in a dendrogram (b). Group 1 (Sample G) is characterised having the lowest values for both variables. Group 2 (Samples A, C and B) is characterised by having the highest values of variable 2 (VAR 2) and low values in variable 1 (VAR 1). Group 3 (Samples D, E and F) is characterised by samples with high values in VAR 1 and low values in VAR 2.

In this thesis, HCA was conducted on the mean absorbance spectra to identify groups of animals with naturally similar NIR spectra. The absorbance spectra were chosen for HCA analysis because they were easier to manipulate and gave slightly better results than the reflectance spectra. The first objective in HCA was to determine a numerical value for the similarity between the mean absorbance spectra

from each animal. As mentioned in Chapter 2, this was achieved by calculating the Euclidean distances between pairs of samples. Euclidean distance is simply the geometric distance between two data points in a space with any number of dimensions. Once the distances between spectra were determined, spectra were then divided into groups where the spectra in each group were naturally similar.

Exploratory analysis of the absorbance spectra from the beef samples show that groups with naturally similar NIR spectra do exist. This is illustrated in the dendrogram (Figure 26). Five distinct groups of animals were identified. Most of the data can be distributed among Groups 1, 2 and 3. Although all animal classes are present in all groups, it is evident that they are each more represented in certain groups. The majority of Group 1 comprises bulls. Groups 2 and 3 are predominantly comprised of steers; however, Group Three has a significant proportion of bulls and cows. As only a few animals were classed into Groups 4 and 5, and both groups have approximately the same proportion of animals found in Groups 2 and 3, these groups may not bear the same statistical significance as Groups 1, 2 and 3.

After groups with naturally similar spectra were identified (Figure 26), the comparison of glycogen, pH_u, pH_{45}, and live weight values of the animals within those groups was conducted, as illustrated in the boxplots (Figure 27).

The results show that all groups have similar glycogen concentration with approximate means between 6 and 8 mg g^{-1}. Although Group 1 has a greater range of pH_u values, the pH_u average for all groups was approximately pH 5.5. Most groups also showed similar pH at pH_{45}. An interesting finding was the significantly lower pH_{45} of animals in Group 2. The reason why this group, which is predominantly comprised of steers, has a lower pH_{45} is unknown. However, the ability of NIR to detect a difference in this group when the glycogen content and pH_u of all groups were very much the same is interesting.

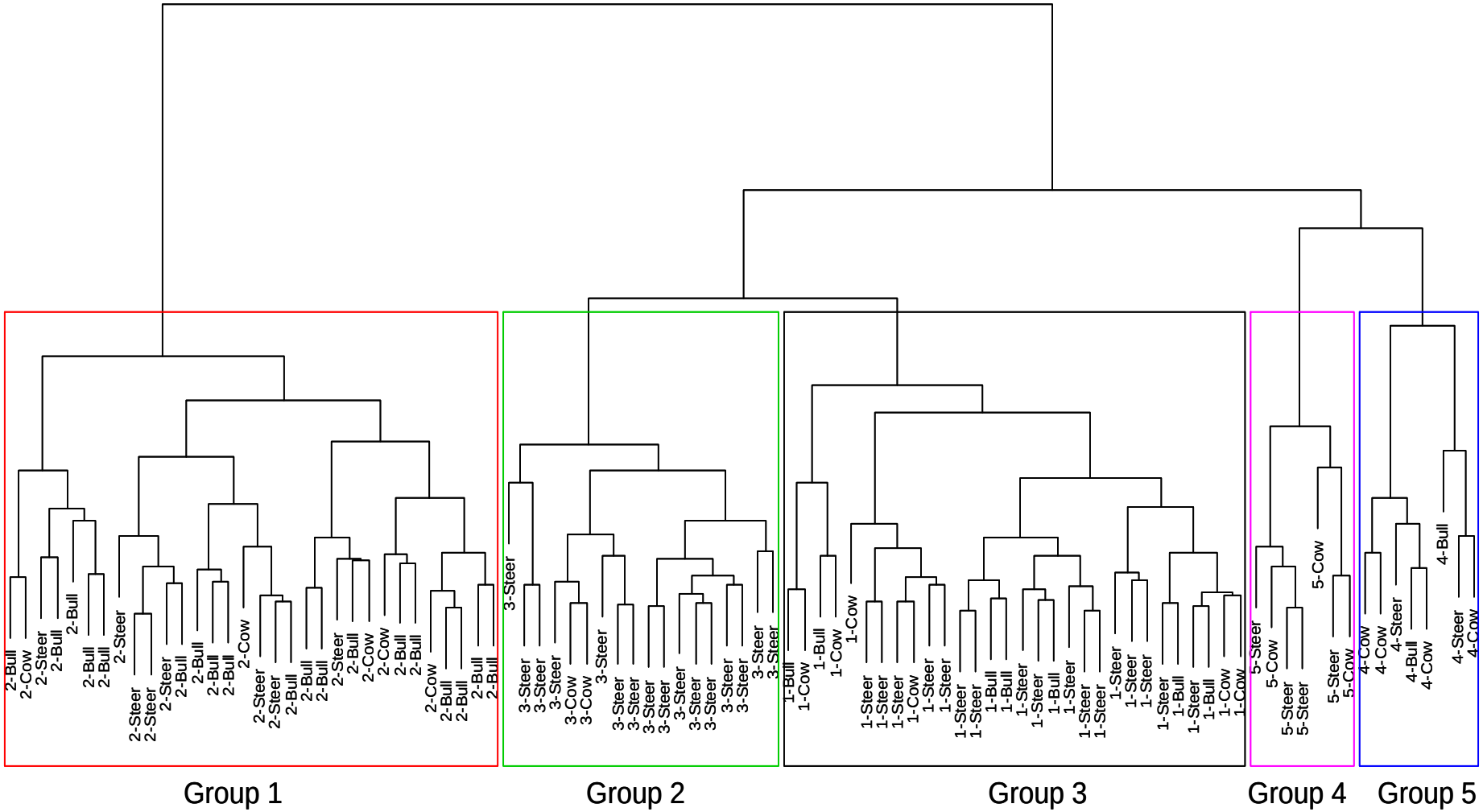

Figure 26: Hierarchical Cluster Analysis of the absorbance spectra (538 to 1677 nm) of pre-rigor LD muscle from 96 animals. The most similar groups are arranged closer together. Thus Group 2 is the most similar group to Group 1, whereas Group 5 is the most dissimilar to Group 1.

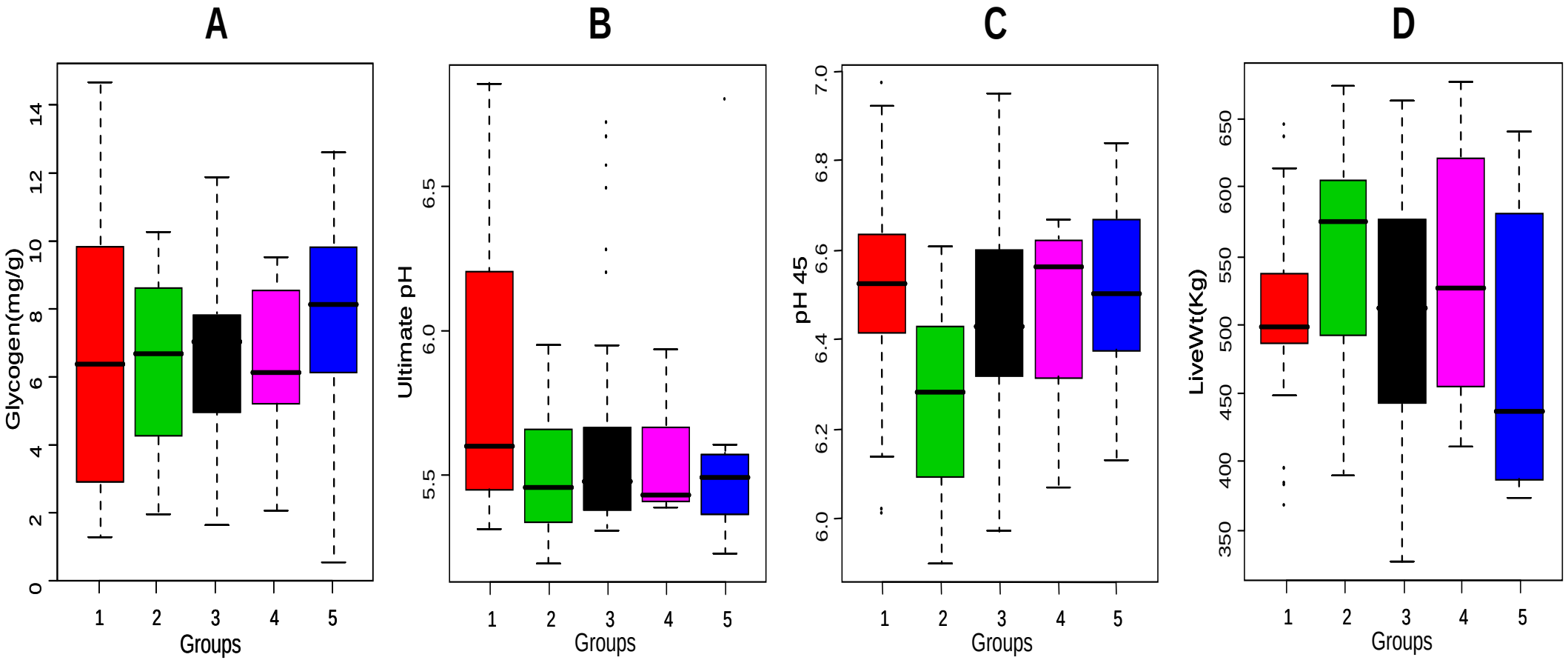

Figure 27: Means of reference values for similar groups were been identified after Hierarchical Cluster Analysis of the absorbance spectra of 96 animals. Plot A represents glycogen concentration, Plot B ultimate pH, Plot C pH at approximately 45 minutes post-mortem (pH_{45}), and Plot D liveweight.

Discussion

Some commercial abattoirs in New Zealand employ the current Rapid pH (RpH) method as a discriminatory tool used to identify carcasses that will produce high pH meat. Currently, the cut-off point for normal pH in some meat processing plants is pH 5.7. Carcasses that are predicted to have an ultimate pH (pH_u) greater than 5.7 are separated and sold as manufacturing grade beef. For a number of technical and economic reasons discussed in Chapter 1, it was decided to explore the potential for a relatively non-invasive method of predicting pH_u, based on NIR spectroscopy to replace the RpH method.

A capability of NIR to predict glycogen and the pH_u of pre-rigor muscle assumes that variation in the glycogen concentration, or chemical composition somehow related to pH in a muscle, can be reproducibly detected by NIR spectroscopy. If this assumption were true, then the spectra collected from each animal could be used to produce models that could ultimately predict glycogen and pH_u from a NIR scan taken immediately after slaughter. More formally, a successful model using PLS and GPLS depends upon the covariation between laboratory measurements of glycogen and pH_u and their corresponding NIR spectra that were collected on-line.

Two statistical models were developed in this thesis to predict pH_u in pre-rigor beef. These include Partial Least Squares (PLS) and generalised Partial Least Squares (GPLS), the principles of which were described in Chapter 2 and summarised earlier in this chapter.

Studies addressing the potential of NIR as a non-destructive method of measuring pH have reported strong positive correlations between the measured pH and predicted pH from statistical models generated from NIR specta (Andersen et al., 1999; Andrés et al., 2007). However at present, there are no reports of the ability of NIR to predict the pH_u of a muscle from spectra collected on-line. Thus, this thesis addressed the efficacy of a commercially available NIR instrument to quantify glycogen and to predict the pH_u of pre-rigor *longissimus dorsi* (LD) muscle. NIR spectra of pre-rigor LD muscles were collected on-line in a commercial abattoir approximately 45 minutes after slaughter.

Poor calibration models were generated using PLS to predict pre-rigor glycogen, pH_{45}, and pH_u with a high prediction error observed for the glycogen model. A different model may be needed for each animal class as their trends were distinctly

different. The low r^2 observed for steers, bulls and cows for the three attributes measured after cross validation suggest PLS regression may not be the best approach to generating a calibration model.

Andersen et al. (1999) and Andrés et al. (2007) have both explored NIR as a non-invasive method of measuring pH one day after slaughter in pork and beef respectively. Their calibration models that were generated using PLS had very good prediction and little error with respective r^2 of 0.80 and 0.97, and standard error of predictions of 0.08 and 0.10 pH units.

Results from this thesis are not consistent with these findings. PLS models generated from pH_{45} measurements showed a very weak r^2 of 0.37. Even though the measurements of pH and collection of NIR spectra was not carried out at the exact timepoint (5 to 10 minutes difference), the capability of NIR to measure pH_{45} and predict pH_u was still very poor.

It is important to consider that the approaches to NIR pH measurement in this project and the published studies are different. Published studies primarily focussed on investigating the capability of NIR to measure meat pH in a controlled laboratory environment after pH_u has been attained. In contrast, the capability of NIR to measure the pH_{45} of pre-rigor muscle in a realistic industrial situation was investigated in this thesis. The superior PLS calibration models reported by Andersen et al. (1999) and Andrés et al. (2007) are possibly not unexpected because factors that may contribute to spectral variation can be minimised in a controlled laboratory environment. However, this is not the case in an industrial environment where variations in temperature, muscle surface and NIR spectra collection are not easily controlled.

Also, it must be reiterated that the commercial environment driving the two approaches is different. In a hot-boning environment, there is no opportunity to measure pH_u, because the dissected meat is packed, chilled and even frozen many hours before pH_u has been attained. The window of opportunity to predict pH_u is between the minutes leading up to slaughter and some point after pelting and before the hot-boned meat is packed, certainly no more than one hour. In a conventional cold boning-environment the NIR scans can be taken at the time pH_u would otherwise be measured. Thus the favourable results of Andersen et al. (1999) and Andrés et al. (2007) are perhaps not surprising.

The failure of PLS calibration models to predict the glycogen content and pH_u in pre-rigor beef muscle demanded another approach to spectral data analysis. As measurement of glycogen in pre-rigor muscle as an indirect method of predicting the pH_u, classification models were attempted to find the probability of a muscle reaching a pH_u greater than 5.7. Classification models were developed using GPLS. These models have less complexity than the PLS calibration models and function by simply classifying samples into two pH_u categories – high pH_u (> 5.7) or normal pH_u (≤ 5.7). The performances of these qualitative models, which included and excluded gender effects, were still poor with a significant proportion of samples being classified into the incorrect pH_u category. Poorer models were obtained when the effects of gender were removed. The incorrect classification of a significant proportion of animals suggests that NIR is not able differentiate high pH_u from normal pH_u muscle.

Observations from the PLS and GPLS models showed that the distribution of glycogen, pH_{45} and pH_u in steers, bulls and cows varied considerably. Hierarchical Cluster Analysis (HCA) of the NIR absorbance spectra was performed in an attempt to identify which variables contributed to the differences observed between the animal classes. The aim of the HCA was to categorise samples with naturally similar spectra into groups. Five groups with similar spectra were identified. The glycogen values and the pH_u between groups were relatively similar. However for pH_{45}, a group predominantly consisting of steers was found to have a significantly lower pH. Further work would be required explain the physicochemical reasons for this difference.

Collecting NIR spectra from whole LD muscles to quantify glycogen was the favoured approach as it was efficient, simple and would only require one labour unit in its commercial execution. However, attempts to predict glycogen in whole pre-rigor muscles or to classify them into two pH_u groups with NIR spectroscopy have been unsuccessful.

An alternative approach to quantify glycogen using NIR is now suggested. The current RpH method operates by enzymatically hydrolysing glycogen into glucose in pre-rigor meat then quantifying glucose using a diabetic glucometer. It is proposed that NIR could be a replacement for diabetic glucometers in the RpH method.

To determine whether NIR is capable of quantifying glucose in meat slurries, preliminary tests of NIR response in increasing complex sample matrices would be required. This could be achieved by initially evaluating whether NIR is able to detect

increasing glucose concentrations in water, RpH buffer and spiked meat slurries. In this respect, other research (Giangiacomo, 2006; Marquardt, Arnold, & Small, 1993) have found that NIR can detect increasing concentrations of glucose in water and protein matrices. However, the capability of NIR to detect glucose in meat slurries needs to be investigated. If confirmed, NIR calibration for the RpH method could be carried out by preparing amyloglucosidase-treated meat slurries from muscle collected on-line using the RpH method and scanning these slurries with the NIR probe. Predictive models could then be generated from the spectra. The known glycogen content and pH_u of the calibration samples could then be correlated with the collected spectra to determine whether NIR could replace glucometers in the RpH method.

One drawback in using NIR in the RpH method is that it would not be available to measure any other meat quality attributes. The main interest for NIR spectroscopy in industry is that by using this technique it would be possible to simultaneously measure several parameters from a single scan. In the meat industry, NIR has the potential and capability to simultaneously measure attributes including connective tissue, fat content and protein content. The dispersion and heating of muscle in buffer during the RpH method would change the composition of the muscle. This would make it infeasible to simultaneously measure other attributes in the muscle in that timepoint. However, theoretically another NIR instrument could be used on-line in the slaughter plant for prediction of other valuable quality attributes.

If the above wet chemistry proposal were to work, or if future NIR work could develop useful calibrations, NIR equipment would replace glucometers, or the RpH method in its entirety. Replacement of glucometers with the NIR in the existing RpH method would probably lower costs. NIR instruments are robust and can be designed for industrial applications. Although the capital cost of NIR is substantial, in the long run they are cheap to run. Glucometers by contrast are cheap (less than $60 for a Medisense glucometer and a packet of 50 test strips), but usually last for a maximum of two weeks with continuous use, and frequent replacement of these instruments and the consumables cost of test strips is an on-going expense. NIR instruments are also less time consuming and give instantaneous results once the spectra have been collected. Moreover, a minimum of two labour units is required to operate the existing RpH method. Less work is involved with NIR however applied and it is

likely that only labour unit would be required. Thus the incentive to apply NIR to pH issues in meat remains strong.

CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS

CONCLUSIONS

Results reported in this thesis found that Accuchek should be the glucometer of choice to replace the superseded Bayer glucometer in the RpH method. Trials with glucose standards in water and RpH buffer showed that Accuchek responded well in these media with a positive linear correlation. In contrast, Medisense returned a non-linear response with readings plateauing at higher glucose concentrations. Similar observations were made when the glucometers were tested on glucose-spiked meat/buffer slurries, with more variation observed with the Medisense glucometers when replicate readings were made.

The Iodine method was found to be the optimum reference method to calibrate the NIR instrument for glycogen measurement. When the Iodine, Bergmeyer and RpH methods were compared, results obtained from the Iodine and Bergmeyer methods were comparable with published results. However, the Iodine method was ultimately the reference method of choice as it was less variable than the Bergmeyer method and its protocol was simpler and less time consuming to employ.

Using NIR, attempts to quantitatively predict the ultimate pH (pH_u) of pre-rigor *longissimus dorsi* (LD) or to qualitatively discriminate animals into a high pH or a normal pH category were both unsuccessful. Predictive models that were developed using NIR spectra to quantitatively measure glycogen and predict pH_u yielded very poor results with a high prediction error for glycogen predictions. Classification models that were developed to identify high pH_u meat also yielded poor results with a majority of high pH_u meat predicted as normal pH_u meat. These results suggest that NIR was not able to differentiate muscles that are high pH_u from normal pH muscles.

HCA analysis of the NIR data found that animals fell naturally into five groups on the basis of spectra similarity. Although these groups had similar glycogen and pH_u values, a group predominantly composed of steers had lower pH_{45} values compared with the other groups. The reason for this is unknown.

RECOMMENDATIONS FOR FURTHER RESEARCH

Although NIR predictive models did not yield good results, PLS and GPLS models consistently found different trends between animal classes with the majority of bulls having lower glycogen and higher pH_u and pH_{45} values than for cows and steers. It is recommended that separate predictive models be generated for bulls, cows and steers. Although having an NIR predictive model that encompassed all animal classes would be more useful, results from this thesis suggest that predictive models for individual animal classes may be more robust and accurate in the measurement of glycogen or prediction of muscle pH_u.

It is also recommended that the possibility of replacing glucometers with NIR spectroscopy in the existing RpH method be investigated. The current replacement of older glucometer models has resulted in the inconvenience and added costs of recalibrating a new glucometer for the RpH method. Previous studies have confirmed that NIR is able to detect increasing glucose concentrations in water and protein standards. Successfully implementing NIR technology into the RpH method would be advantageous as it is more rapid than glucometer determinations, more robust and less costly in terms of consumables. However, capital costs are much higher, so net present value calculations would need to be done before decisions were made.

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