Effect of slaughter age between 5 and 14 months on the quality of sheep meat with a specific focus on collagen concentration and solubility

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Abstract

In New Zealand, pasture-raised lamb is considered the highest quality sheep meat product while hogget is a downgraded product considered to be of poorer quality. The objectives of this study were to investigate the difference in meat quality between sheep slaughtered at 5, 8 and 14 months-of-age. This study also aimed to develop an assay to measure collagen concentration and solubility and to compare shear force to collagen concentration and solubility for meat from sheep slaughtered at 5, 8 and 14 months. Sixty Romney crossbreed rams born in the spring of 2015 were allocated to one of three slaughter age groups: 5-group (n=20), 8-months group (n=20) and 14-months group (n=20). The ram lambs were grazed together on perennial ryegrass-based pasture. At slaughter, the loin (*Longissimus lumborum*) from the left side of the carcass was excised, chilled for 24 hours and then frozen. Shear force, pH, colour, driploss, myofibrillar fragmentation index, sarcomere length, soluble collagen, insoluble collagen and total collagen were measured on the loin. *Longissimus lumborum* muscle from the 5-months group was darker (P=0.045) with higher pH (P<0.001) than 8 and 14-months groups. *Longissimus lumborum* muscle from the 14-months group was redder (P<0.001), yellower (P<0.001) with higher intramuscular fat (0.003), shorter sarcomere length (P<0.001), lower collagen concentration (P=0.020) and lower soluble collagen in percentage (P=0.007) and in g/100g fresh weight (P=0.008) than 5 and 8-months groups. The peak shear force was lower at 14 months, intermediate at 5 months and greater at 8 months (P<0.001). *Longissimus lumborum* muscle from the 8-months group had greater drip loss (P<0.01) than 5-months group and 14-months group. The results suggest that slaughtering sheep at 14 months could have benefits on eating quality due to an increase in intramuscular fat.

Keywords*:* lamb; slaughter; age; meat; quality

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Chapter 1. Literature Review

1.1 Introduction

New Zealand contributes approximately 47% of the total world sheep meat trade and 95% of sheep meat produced in New Zealand is exported (Morris, 2009). The increase in global population is likely to increase demand for sources of protein including meat (Godfray et al., 2010). The New Zealand governmental goal to increase the return from primary industry exports drives the sheep industry towards increasing meat production (Morris, 2013). However, due to a limited land base for primary production, it is unlikely New Zealand will produce large quantities of sheep meat. For New Zealand the focus is likely to be on high quality and a high value meat product to cater to the demands of discerning and affluent consumers to maximise the value of sheep meat exported from New Zealand (Joo et al., 2013). To achieve a consistent high value of meat and meat product, it is important to consider meat quality of sheep throughout the meat production chain.

1.2. The quality of meat from sheep

Meat quality of sheep is highly associated with the consumer acceptance of sheep meat at point of purchase or after eating the cooked sheep meat based on previous eating experience. Most important sheep meat quality attributes include appearance, palatability, nutritive value, safety and wholesomeness characteristics (Joo et al., 2013; Przybylski & Hopkins, 2015; Purchas, 1989). Appearance attributes include lean meat and fat colour (Becker, 2000) that contribute to the visual attributes of meat during carcass grading and that influence consumer purchasing decisions (Grunert et al., 2004; Hopkins, 1996) and can also give a cue on the safety and wholesomeness characteristics of sheep meat. Consumers' expectation of quality fresh meat at purchasing is based on their experience of appearance characteristics and for sheep meat can include a preference for bright red colour of lean meat (Kropf, 1993; Pethick et al., 2005), firm texture, less drip and white or creamy fat colour (Purchas et al., 1989). Deviation from desirable conditions such as yellow fat (Boom & Sheath, 1997), discoloration, soft texture, large amount of drip and excessive visible fat can negatively affect the

consumers purchasing decision and reduce the value of the meat products (Boom & Sheath, 1997; Joo et al., 2013).

Meat palatability traits are those characteristics that become apparent when meat is being eaten and this can influence the consumer's decision to repurchase. Consumers may buy visually attractive meat without return to purchase from the same retailer following disappointing palatability attributes mainly flavour, juiciness and tenderness (Joo et al., 2013). Palatability attributes are assessed subjectively by using a sensory panel or consumer survey and objective testing using warner Bratzler shear force device and consumers are willing to pay for the meat if palatability attribute is guaranteed (Mintert et al., 2000). A small incidence of unacceptable meat quality attributes at point of purchase or eating experience can change consumer preferences to another food product in this product quality oriented market (Bermingham et al., 2008; Pethick et al., 2006).

Appearance and palatability of lamb meat is predominantly determined by intrinsic factors including breed, sex, specific genes associated with callipyge gene, weight at slaughter, age at slaughter and nutrition (Hopkins & Mortimer, 2014; Hopkins et al., 2007; Johnson et al., 2015; Johnson et al., 2005; San et al., 1998). Meat quality can also be altered by post slaughter factors including chilling, extent of proteolysis during post-mortem ageing and activity of proteases (calpain and cathepsins) that are involved in post-mortem meat tenderization, freezing, methods and temperatures of cooking (Ablikim et al., 2016) and the extent of rigor affecting sarcomere length (Maltin et al., 2003). Moreover, muscle location within the sheep body and alteration of myofibrillar protein structure (Field et al., 1996) can also alter meat quality attributes.

Among intrinsic factors, sheep slaughter age can alter meat colour by increasing the concentration of myoglobin pigment in muscle leading to increased meat redness and darkness (Gardner et al., 2007; Hopkins et al., 2007; Warner et al., 2007). Meat tenderness, juiciness and flavour changes with the increase of intramuscular fat content as the animal ages (Thompson et al., 2005b). Meat tenderness is also affected by changes in connective tissue particularly soluble and insoluble collagen that occur as a consequence of sheep slaughter age (Young et al., 1993; Young & Braggins, 1993).

In New Zealand, sheep carcasses are age classified based on dentition. Lamb is for young sheep 0 to 12 months or with no permanent incisor teeth in wear. Hogget is assigned to a carcass from a sheep having two permanent incisor teeth in wear, normally aged between 12 and 24 months. Mutton is the term used to define carcass from sheep with more than two permanent incisor teeth in a wear and normally aged above 24 months of age (Young et al., 2005). Carcasses classified as hogget and mutton are downgraded in their value due to perceived meat quality issues (Barwick & Thwaites, 1980; Young & Lim, 2001). However, studies in Australia have shown the acceptability of meat from hogget and implies the penalty placed on hoggets carcasses is not valid (Wiese et al., 2005).

1.2.1 Effect of sheep slaughter age on colour of lean meat

Protein pigments myoglobin and haemoglobin as well as cytochrome C in meat are the main determinants of the colour of red meat (Mancini & Hunt, 2005). Of all determinants myoglobin is believed to play the major role in determination of red meat colour (Mancini & Hunt, 2005; Pethick et al., 2005; Suman & Joseph, 2013). Concentration of these pigments differs with muscle fibre type. A higher concentration of myoglobin is found in aerobic muscle fibre type compared to anaerobic fibre type (Pethick et al., 2005). Muscle fibre type can define the concentration of pigments present in the muscle fibre however; meat colour on display is also affected by the extent of oxygenation and oxidation.

On display, meat colour can appear red, brown or purple depending on the relationship between myoglobin and oxygen. Before the muscle is exposed to air (oxygen) it is characterised by purplish- red or purplish-pink colour (Mancini & Hunt, 2005). But after cutting the meat, the exposed myoglobin reacts with oxygen from air to form either oxymyoglobin (bright cherry red colour) or metmyoglobin (brown colour) depending on the concentration of oxygen. Exposure to higher concentrations of oxygen in meat favours oxygenation reaction that produces oxymyoglobin which is a normal bright cherry red colour of meat. Oxygenation of myoglobin occurs rapidly as soon as meat is exposed to air on cutting in which a bright cherry red colour can be observed on the surface of meat within 30 to 45 minutes (Mancini & Hunt, 2005) a process termed as

"blooming". However, on exposure to low concentrations of oxygen the surface of the meat develops a brown colour met-myoglobin.

A Chroma meter can be used to objectively measure colour of meat by measuring light reflectance. A colour space such as L*, a*, b* where lightness (L*), redness (a*) and yellowness (b*) can be used to quantify colour (Hopkins, 1996; Khliji et al., 2010). There is a strong correlation between myoglobin content and colour parameters such as lightness (L*) and redness (a*). This means that increase in concentration of myoglobin leads to decrease in L* and an increase in a* which corresponds to a more intensely red coloured meat.

The concentration of myoglobin is known to increase with animal age leading to an increased intensity of red colour (Table 1; Gardner et al., 2007; Hopkins et al., 2007; Warner et al., 2007). Hogget and mutton meat has an elevated myoglobin concentration which gives a darker red colour compared to the meat from lamb (Hopkins & Mortimer, 2014; Hopkins et al., 2007; Jacob et al., 2007; Pethick et al., 2005). This is evident in Table 1 that shows as slaughter age increased from 8 to 22 months-of-age there is an increase in myoglobin concentration and this is associated with decreased lightness (L*) and increased redness (a*) of *Longissimus lumborum* which in turn lead to decline in meat colour acceptance (Pethick et al., 2005; Hopkins et al., 2007).

Correlations between objective and subjective colour measurement indicate that lamb meat with a* value of at least 9.5 and L* value of 34 to 35 is considered the most acceptable colour for consumers (Hopkins, 1996). However, a decline in meat lightness and colour acceptability as slaughter age advances is evident for sheep slaughtered beyond 12 months-of-age (Hopkins, 1996). Sheep slaughtered beyond 12 months had a darker colour compared to those slaughtered below 12 months-of-age indicating the need to consider slaughter age to satisfy the demand of the discerning consumers (Table 1). Moreover, Hopkins et al. (2005) found meat from suckler lambs (lambs slaughtered before weaning off the mother) to have a lighter colour than meat from weaned lamb and hoggets.

Table 1: Effect of slaughter age on colour and tenderness of meat from different muscles of sheep.

Reference	Age (Months)	Colour	Myoglobin concentration	Shear force (kgF)
Jacob et al. (2007) [4,5,6]	5	L* 33, a* 21.3		
	12	L* 30.9, a*22.3		
Purchas et al. (2002)[1]	5	L* 38.2		7.45
	11	L* 36.8		11.16
Bouton et al. (1978)[1,2,3,4]	2-3			2.82
	18 -10			3.32
	12-19			3.74
	18-24			4.24
	23-36			4.34
	28-48			4.50
	72-92			4.72
Jeremiah et al. (1997)[5]	3-6	L*31.31, a* 6.15		
	6-9	L* 30.81, a* 6.41		
	9-12	L*30.81, a*6.22		
	12-15	L* 30.04, a*6.84		
Hopkins et al. (2007)[5]	4	L* 38, a* 18.1		
	8	L* 36.7, a* 18.6		
	14	L* 35.2, a* 19.4		
	22	L* 32.7, a* 20.5		
Gardner et al. (2007)[5]	4		5.58 (mg/g muscle)	
	8		8.50 (mg/g muscle)	
	14		8.78 (mg/g muscle)	
	22		10.01 (mg/g muscle)	
Young et al. (1993)[1]	0			3.8
	1			5.8
	2			6.1
	9			7.2
	12			6.8
Pethick et al. (2005)[5]	9	L* 39	20.1 (g/100g protein)	

20	L* 35.3	26.2 (g/100g protein)
33	L* 36.4	24.8 (g/100g protein)
45	L* 37.1	27.4 (g/100g protein)
57	L* 37.7	24.6 (g/100g protein)
69	L* 36.6	27.1 (g/100g protein)

[1]*Semimembranosus* muscle (SM), [2]*Biceps femoris* (BF), [3]*Semitendinosus* (ST) ,[4]*Gluteus medius* (GM), [5] *Longissimus thoracis et lumborum* (LL), [6]*Rectus femoris* (RF).

The age of the animal also affects muscle fibre type. As the age of sheep increases muscle fibres become more aerobic and therefore have a higher myoglobin concentration that leads to an increased red colour. However, the extent varies depending on breed and nutrition (Picard et al., 2002; Warner et al., 2007). In Australia suckler lamb slaughtered at 5 months had lower met-myoglobin concentration of 5 g/100 protein compared to 5.7 g/100g in carryover lamb slaughtered at 12 months (Jacob et al., 2007).

A two-fold increase in myoglobin concentration associated with increased oxidative metabolism was reported in the *semitendinosus*, *rectus abdominis* and *Longissimus* muscles as the slaughter age of lambs progressed from 4 to 22 months and over half of this increase occurred between 4 and 8 months of age (Gardner et al., 2007; Warner et al., 2007). Sheep slaughtered at 8-14 months of age found to be a critical transition age in meat colour based on L* value and meat from suckler lamb was more acceptable to consumers compared to 14 months of age (Hopkins et al., 2007).

1.2.2 Effect of sheep slaughter age on colour of fat

Fat colour is one of the attribute which is taken in to account by consumers during carcass grading and at a point of purchase. New Zealand has reported approximately 1 in 1000 lamb carcasses being rejected for export due to unacceptably elevated yellow fat colour (Kirton et al., 1975). Yellowness of fat can be due to presence of fat soluble pigments called carotenoids that are deposited in the fat as a consequence of the animal consuming a green, forage diet. Alternatively yellow fat can occur due to illness creating jaundice and the retention of bile pigments in the fat as a result of liver damage or dysfunction such as that occurs in facial eczema (Kirton et al., 1975). Carcasses with yellow fat colour may be considered fit for human consumption however, consumers prefer a white fat colour. Yellow fat is considered unacceptable due to perceptions of

ill-health in the animal from which the meat was sourced and creating a sense that the meat is unfit to eat (Boom & Sheath, 1997). Consumers unaccustomed to a more yellow fat colour from animals fed on forages may hesitate to purchase meat (Boom & Sheath, 1997). Fat carotenoid concentration is reported to increase as slaughter age increases. Boom & Sheath (1997) and Jeremiah et al. (1997) reported a decline in fat whiteness in heavier weight lambs (> 67.7kg) compared to lighter weight.

1.2.3 Effect of sheep slaughter age on meat flavour

Consumer perception of sheep meat eating quality is highly influenced by flavour followed by tenderness and juiciness (Pethick, 2006). Taste and aroma are the two components of meat flavour. Flavour of meat is developed from a complex series of heat induced reactions between the non-volatile lean portion and fat portion as well as oxidative degradation of fats in meat upon cooking (Pethick, 2006; Pethick et al., 2005).

Meaty flavour, pastoral flavour and foreign flavour are the three different meat flavour that can be perceived by consumers when eating cooked lamb meat (Schreurs et al., 2008). Meaty flavour is the normal species specific flavour. Pastoral flavour is the flavour which occurs in meat from sheep grazed on pasture. Pastoral flavour is normally considered acceptable to consumers who have been used to eat meat from sheep grazed on pasture however it might be undesirable to consumers who have been used to eat meat from grain finished sheep (Watkins et al., 2013). Foreign meat flavour is undesirable flavour that is considered unacceptable and maybe a consequence of taints or oxidative degradation of the meat.

At least 1000 flavour compounds have been reported to contribute to the flavour of meat and some are species-specific. Lipid derived volatile compounds are responsible for variation in flavour within animal species (Pethick, 2006). Methyl-branched-chain fatty acids (BCFA) such as 4-methyloctanoic and 4-methylnonanoic acids, 3- methyl indole (skatole) and products of oxidation of linolenic fatty acid and its derivatives are the known compounds responsible for development of meat flavour in sheep. However, methyl-branched-chain fatty acids (BCFA) such as 4-methyloctanoic and 4-methylnonanoic acids are known to be responsible for undesirable sheep meat flavour. Several studies have reported the increase in undesirable flavour of sheep meat with

age and this has been associated with an increase in methyl-branched-chain fatty acids (BCFA) compounds such as 4-methyloctanoic and 4-methylnonanoic acids compounds (Young et al., 1997). Young et al. (1994) reported stronger odour and flavour in meat from mature sheep compared to young sheep. Moreover, Jeremiah et al. (1998) found lambs slaughtered at 6-9 months of age to have more acceptable flavour as assessed by consumers compared to meat from sheep that were 12-15 months of age. Moreover mutton has been reported to produce meat with undesirable flavour to consumers compared to lamb (Pethick et al., 2005). This is due to the fact that flavour compounds associated with intense flavour in older sheep appear to reside in fat so the increase in fat content of older animal may exacerbate flavours. Pethick et al. (2005) concluded that the effect of undesirable flavour in meat from older sheep can be mitigated via turnover of fat and undesirable flavour in meat from older lamb can be minimized by avoiding serving the product with subcutaneous and intramuscular fat.

1.2.4 Effect of sheep slaughter age on meat juiciness

Meat juiciness is associated with the ability of the meat to hold onto moisture and release it during eating. Tenderness, flavour, intramuscular fat, water holding capacity and overall liking relates positively to juiciness (Jeremiah et al., 1997; Pethick et al., 2005). Meat with higher juiciness is considered more tender and more flavoursome than meat with low juiciness. Intramuscular fat has a lubricating effect and with cooking the melted intramuscular fat assists in reducing the force required to chew the meat (Blumer, 1963; Wood, 1985). In a study by Hopkins et al. (2006) increased intramuscular fat was associated with improved tenderness and eating scores for lamb and intramuscular fat concentration between 15-20 % is associated with peak juiciness score (Thompson, 2004).

Juiciness tends to decrease as the animal slaughter age increases which in turn negatively affects other eating qualities such as tenderness and flavour (Pethick et al., 2005). Pethick et al. (2005) reported a decline in juiciness score by 7.6 points on a 0 (very tough) to 100 (very tender) score for tenderness, juiciness, flavour liking and overall liking of *Longissimus lumborum* muscle from sheep slaughtered at average of 44.5 month of age compared to sheep slaughtered at 20 months of age when assessed by Australian untrained consumers. There was no decline in *Longissimus lumborum*

juiciness in sheep slaughtered below 20 months of age (Pethick et al., 2005). A decline in juiciness is associated with reduced water holding capacity as slaughter age and weight increases. This is evident in sheep slaughtered at 12 to 15 months-of-age with live weight greater than 68.2kg which had greater percentage of expressible juice compared to those slaughtered at age of 3 to 12 months-of-age (Jeremiah et al., 1997). Meat with higher expressible juice loses more water upon cooking which in turn reduce tenderness, juiciness and flavour.

1.2.5 Effect of sheep slaughter age on meat tenderness

Sheep meat consumers consider tenderness as one of the most important palatability characteristics (Cho et al., 2010). Objective assessment of tenderness uses instruments such as the Warner Bratzler shear force (Shackelford et al., 1999) or the MIRINZ tenderometer which measures compression forces (Chrystall & West, 1987). Instrumental meat tenderness assessments are significantly correlated to the subjective assessments made by a trained sensory panel (Young & Braggins, 1993) indicating that either objective or subjective measurements can effectively assess meat tenderness.

There is a decrease in meat tenderness as the slaughter age of the sheep advances (Bouton et al., 1978; Jeremiah et al., 1998; Pethick et al., 2005; Purchas et al., 2002; Warner et al., 2007). Sensory panel assessment of meat tenderness by Thompson et al. (2005b) comparing weaner lamb (6 months old ewe lambs) and mutton (48 month old ewes) found that the largest proportion of variation in meat tenderness score was due to animal age when considering the effects of animal age, muscle type, ageing period, hanging method and electrical stimulation. Also, the best eating quality score was evident for meat from suckler lamb (3-5 months of age) compared to weaned lamb (9-12 months-of-age) and hogget (20 months-of-age) (Hopkins et al., 2005). Moreover, meat from lamb (8.5 months of age) and hoggets (20 months of age) scored on a single sheet using four 100 mm line scale marked with 20mm gradients as developed by Thompson et al. (2005a) reported higher score for tenderness, juiciness, flavour liking and overall liking than meat from older sheep (68.5 months of age) (Pethick et al., 2005).

There is a positive correlation between slaughter age and shear force values measured on the *semimembranosus* from sheep ranging in age from 2.5 to 22 months (Jeremiah et al.,1971). Also tenderness and intramuscular fat of sheep correlate positively (Hopkins et al., 2006) and intramuscular fat tend to increase as slaughter age advances (Thompson et al., 2005b). Therefore, the reduced tenderness in old sheep despite of the different cooking condition, core shape and size in the two studies is associated with an increase in shear force values as the slaughter age of the sheep increases (Table 1) and it can also be associated with the change in connective tissue component (Ablikim et al., 2016; Dutson et al., 1976; Purchas et al., 1989; Weston et al., 2002; Young & Braggins, 1993) and intramuscular fat level. The study by Thompson et al. (2005b) did not specify age but reported higher intramuscular fat (7.58%) in mutton compared to lamb (3.56%). However, the relationship between intramuscular fat level and connective tissue properties is not clear in New Zealand pasture-raised sheep.

Distribution, density, and properties of connective tissue play an important role in determining meat tenderness (Ablikim et al., 2016; Dutson et al., 1976; Purchas et al., 1989b; Weston et al., 2002; Young & Braggins, 1993). The contribution of connective tissue to meat tenderness is dependent on collagen concentration (Dransfield, 1977; Light et al., 1985), collagen solubility and extent of its cross-linking (Ablikim et al., 2016; McCormick, 1994; Young & Braggins, 1993). Collagen is one of the fibrous protein molecules that form the structural component of connective tissue of muscle. Collagen solubility is the ability of collagen to transform from its fibrous protein state found in living animals into soluble protein gelatin when heat is applied. Collagen cross- linking is an intrinsic property of collagen which is formed by condensation of its unique protein lysine or hydroxylysine residues and their aldehyde (McCormick, 1994) to form electron microscopically visible collagen fibrils.

1.2.6 Collagen in meat

Muscle structure is composed of individual muscle fibres which is surrounded by a connective tissue sheath called *endomysium* (Purslow, 2005). Individual muscle fibres are assembled in bundles by connective tissue called *perimysium* and the connective tissue surrounding the muscle is called *epimysium* (Pearson, 2012). These three layers of connective tissues are all interconnected and also connect to ligament and bone

(Figure 1). *Endomysium*, *perimysium* and *epimysium* form the three collagen depots in muscle to assist with muscle structure, give muscle its strength and flexibility as well as a means of transmitting and absorbing force generated by muscle contraction (Harper, 1999; McCormick, 1994; Purslow, 2002; Purslow, 2005).

Collagen is a large elongated protein which forms into strong fibrils of muscle connective tissue (Figure 1). These strong fibrils are linked covalently to form collagen fiber that increases the strength of connective tissue. Collagen forms between 1-15 % of muscle connective tissue depending on species (Purslow, 2005). At least 19 different types of collagen have been identified (Bateman et al., 1996). Of all types fibrous collagen type I and III are the most abundant collagen types of skeletal muscle (Bateman et al., 1996; Lepetit, 2007; Light & Champion, 1984; McCormick, 1994).

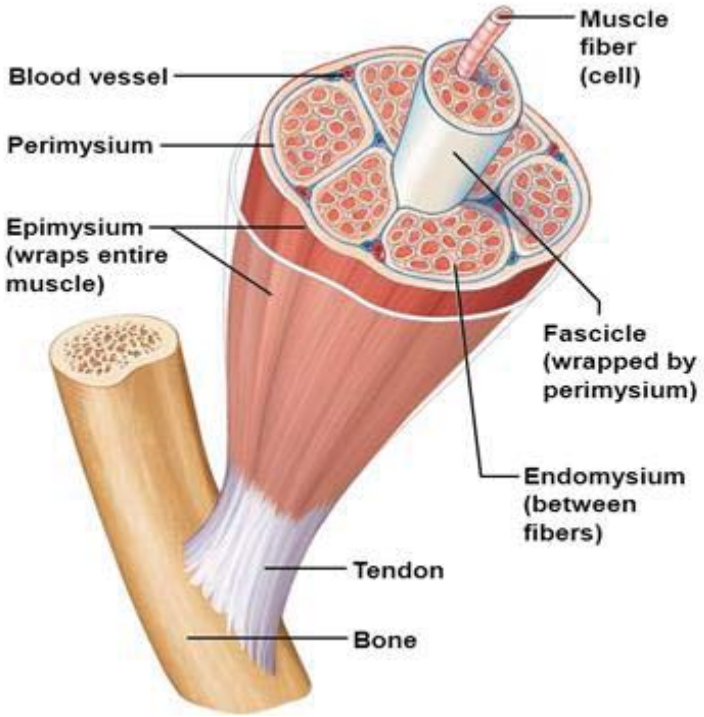

Figure 1. Structure of skeletal muscle showing three connective tissues associated with the muscle structure (Pearson, 2012).

1.2.6.1 Measurement of collagen concentration and solubility

Quantification of collagen is of particular importance when assessing meat tenderness associated with connective tissue component. The complexity of the collagen structure due to its covalent cross linking makes the collagen molecules insoluble and difficult to

extract (McCormick, 1994). Hydroxyproline is the specific amino acid in collagen therefore total, soluble and insoluble collagen in meat is estimated from analysed hydroxyproline (Ablikim et al., 2016).

Different methods that determine hydroxyproline concentration in meat samples has been successfully established and include colorimetric method (Bergman & Loxley, 1963), near infrared reflectance spectroscopy (Oh & Groβklaus, 1995), electrokinetic chromatography (Dugan et al., 2000; Vazquez-Ortiz et al., 2004), ultrasonic spectra-analysis (Park et al., 1994), magnetic resonance imaging (Bonny et al., 2001), quantitative carbon-13 Fourier transform nuclear magnetic resonance spectrometry (Jozefowicz et al., 1977) and Auto fluorescence quantification (Egelandsdal et al., 2005). Conventional methods such as high liquid chromatography (Vazquez-Ortiz et al., 2004) and mass spectrometry (Colgrave et al., 2008) have been used to quantify hydroxyproline concentration in meat samples but they are limited due to a long time required to dry the samples after hydrolysis.

Colorimetric assay is the most predominant method used to measure the concentration of hydroxyproline in acid hydrolyzed, neutralized and diluted sample from animal muscle (Ablikim et al., 2016; Archile-Contreras et al., 2010; Bergman & Loxley, 1963; Edwards & O'Brien, 1980). Standard solutions containing 0, 0.6, 1.2, 1.8 and 2.4 µg hydroxyproline per mL is prepared (AOAC 990.26, 1993). Modification of hydroxyproline residue to pyrrole is completed by timed oxidation of hydrolysate and standard solutions with chloramine T. to a pyrrole. The formed pyrrole is stained with 4-dimethylaminobenzaldehyde colour reagent and heated at 60 ^{o}C during which red chromophore developed. The intensity of red colour depends on the amount of pyrrole present. The absorbance is then read (AOAC 990.26, 1993; Archile-Contreras et al., 2010; Bergman & Loxley, 1963; Edwards & O'Brien, 1980). A standard curve is produced from the standard solutions enabling the hydroxyproline of the sample to be calculated based on its absorbance and dilution factors. A standard curve is produced for each series of measurements (AOAC 990.26, 1993).

Skin from vertebrate which contains a high amount of collagen has been shown to contain from 10-14 g hydroxyproline/100 g protein (Neuman & Logan, 1950) therefore collagen is assumed to weigh 7.25 times the measured hydroxyproline (Palka, 1999). Elastin is reported to contain small quantities (1-3 g/100 g protein) of hydroxyproline and can be considered as the source of error when determining collagen by analysis of hydroxyproline (Neuman & Logan, 1950). However, elastin is estimated from hydroxyproline of the insoluble fraction after collagen extraction (Neuman & Logan, 1950) due to its resistance to solubilisation when meat is heated.

Collagen solubility is predominantly determined by homogenisation of sample with 8 mL of one-quarter-strength Ringer's solution (Hill, 1966) or distilled water (Ablikim et al., 2016; Archile-Contreras et al., 2010) and heated using a water bath for a set temperature and time. The sample is then cooled down and then centrifuged (Ablikim et al., 2016; Archile-Contreras et al., 2010). Sediments and supernatants are collected separately and then acid (Ablikim et al., 2016; Archile-Contreras et al., 2010) or alkali (Hofman et al., 2011; Wierbicki & Deatherage, 1954) hydrolyzed to determine the percentage of soluble (supernatant) and insoluble collagen (sediment). Collagen of each portion is calculated from the hydroxyproline concentration in each supernatant and sediment hydrolysate. The conversion factor of 7.25 for sediment and 7.52 for supernatant is used to convert hydroxyproline to collagen (Crouse et al., 1986) however Archile -Contreras et al. (2010) used the conversion factor of 7.25 for both supernatant and sediment.

To minimize time required for one set of experiments total collagen could be calculated by adding soluble collagen to insoluble collagen but there is a need to validate whether the addition of the two separate assays can give the same value as the measured total collagen. Archile -Contreras et al. (2010) used the two separate assays to determine total collagen and heat soluble collagen in which case the insoluble collagen could be determined by calculation.

The pH of muscle post rigor is 5.5 and 1/4 ringer's solution at pH of 5.6 is recommended during determination of collagen solubility (Latorre et al., 2016). However, Ablikim et

al. (2016) and Hill (1966) used 1/4 ringer's solution at pH of 7.4 without any explanation of the choice of pH. Moreover, distilled water (Archile-Contreras et al., 2010; Kristensen et al., 2002) and 0.9% NaCl (Christensen et al., 2011) has been accurately used to determine soluble and insoluble collagen in meat samples.

1.2.6.2 Collagen concentration and meat tenderness

Within animal species collagen concentration in the muscle depends on a range of factors including the muscle location (Cross et al., 1972), age of animal (Young & Braggins, 1993), extent of activity, animal species and animal breed (Crouse et al., 1985; Purslow, 2005). Muscles in different parts of the body differ in collagen concentration (Ablikim et al., 2016; Young et al., 1993). Muscles with frequent activation such as leg or shoulder including rectus *femoris* or *semimembranosus*, *semitendinosus* or *supraspinatus* have quite high collagen concentration compared to muscles that a less frequently activated such as the *Longissimus lumborum* of the back (Table 2; Starkey et al., 2016; Tschirhart-Hoelscher et al., 2006).

Table 2. Studies investigating collagen concentration and collagen solubility in the *Longissimus lumborum* or several muscles of sheep.

Reference	Age (Months)	Treatment	Collagen concentration g/100g	Collagen Solubility (%)
Maiorano et al. (1993)[1]	6	Ram	1.851*	12.21
	6	Wether	1.676*	9.67
	6	Wether + Zeranol	1.525*	15.34
Field et al. (1996)	8	Normal lamb	1.68*	
	8	Callipyge lamb	1.47*	
Starkey et al. (2015)[2]		1-day aging	0.96*	0.15***
		7 days aging	0.97*	0.20***
		14 days aging	1.06*	0.20***
Tschirhart-Hoelscher et al. (2006)	*3-8*	*Adductor*	0.32*	
	3-8	*Gluteobiceps-distal*	0.50*	
	3-8	*Gluteobiceps proximal*	0.56*	
	3-8	*Gluteus medius*	0.61*	
	3-8	*Infraspinatus*	0.9*	
	3-8	*Latissimus dorsi*	0.5*	
	3-8	*Longissimus lumborum*	0.26*	
	3-8	*Longissimus thoracis*	0.29*	
	3-8	*Psoas major*	0.45*	
	3-8	*Pectoralis profundus*	0.5*	
	3-8	*Retus femoris*	0.43*	
	3-8	*Semimembranosus*	0.35*	
	3-8	*Semitendinosus*	0.37*	
	3-8	*Supraspinatus*	0.55*	
	3-8	*Serratus ventralis*	0.41*	
	3-8	*Triceps brachii*	0.50*	
	3-8	*Vastus lateralis*	0.39*	
Ablikim et al. (2016)[3]	7- 9 (8)	*Supraspinatus*	0.36*	26.21
	7- 9 (8)	*Gluteus*	0.49*	50.73
	7- 9 (8)	*Longissimus dorsi*	1.70*	22.03
Veiseth et al. (2004)	*2*	Age at slaughter	0.388**	
	4	Age at slaughter	0.372**	

	6	Age at slaughter	0.386**	
	8	Age at slaughter	0.369**	
	10	Age at slaughter	0.362*	
Camacho et al. (2016)[1]	*2*	Canarian breed	0.311*	84.90
	3	Canarian breed	0.318*	76.99
	5	Canarian breed	0.284*	74.17
	2	Canarian hair breed	0.326*	86.31
	3	Canarian hair breed	0.332*	78.22
	5	Canarian hair breed	0.335*	77.81
Hopkins et al. (2013)	*7 & 8*	*Longissimus lumborum*	0.22*	
	7 & 8	*Semimembranosus*	0.45*	

*fresh weight basis
**g of collagen/100g of cooked muscle at 71^{o}C
*** Soluble collagen in g/100g
[1] Collagen solubility samples were heated in a water bath for 63 min at 77°C
[2] Collagen solubility samples were heated in a water bath for 2 h at 80 °C.
[3] Collagen solubility samples were heated in a water bath for 60 min at 77°C.

Moreover, in the hind leg the *vastus lateralis* muscle has a higher collagen concentration compared to *semimembranosus and semitendinosus* (Table 2 and Table 3; Starkey et al., 2016). Within the same age range *Longissimus lumborum* had the lowest collagen concentration compared to other 17 muscles studied (Table 2; Tschirhart -Hoelscher et al., 2006) which is attributed to less activity associated with *Longissimus* muscle due to its position in animal body compared to leg muscles (Aalhus et al., 1991). Collagen concentration of sheep *Longissimus lumborum* muscles ranges from 0.2 - 2 g/100 g whole tissue basis and this depend on age, breed and sex (Table 2).

Collagen concentration tends to decline with an increase in slaughter age (Table 2; Camacho et al., 2016; Veiseth et al., 2004). With advanced age, weight increase due to increase in muscle fibre size (Cross et al., 1972) leading to decline in collagen concentration. Young et al. (1993) observed higher collagen concentration in the *semimembranosus* and *gluteus* muscle of lambs at birth which then declined as the animal grows up to 1 month of age then remains almost static up to one year of age. Also, in all four muscles studied by Cross et al. (1972) higher collagen concentration

was observed in lamb slaughter at 2 months -of -age compared to 20 months of age (Table 3).

Table 3. **Collagen concentration and collagen solubility of *ovine* different muscle and different age (Cross et al., 1972).**

	Collagen (g/100g of whole tissue)				Soluble collagen (%)				Tenderness rating[1]			
Average age (Months)	**SM**	**ST**	**BF**	**VL**	**SM**	**ST**	**BF**	**VL**	**SM**	**ST**	**BF**	**VL**
2	1.19	1.36	1.49	1.61	17.8	14.9	22.2	16.6	3.6	5.7	5.3	6.2
4	0.92	1.07	1.21	1.57	8.0	6.4	14.1	9.8	3.8	6.9	6.3	6.4
6	0.98	0.78	1.45	2.05	6.6	8.4	11.2	9.2	4.9	6.4	5.7	5.5
8	1.15	1.44	1.49	1.98	6.7	7.9	10.1	7.5	3.7	6.1	3.9	4.8
10	1.42	1.16	1.53	1.63	6.9	7.7	12.6	6.7	3.6	6.2	5.4	5.8
12	1.08	1.05	1.06	1.68	6.1	5.1	5.3	4.2	3.9	7.4	6.1	5.4
14	0.73	1.00	1.23	2.05	4.2	6.3	5.6	3.4	4.2	6.3	5.4	4.5
16	0.90	0.94	1.71	2.23	4.9	8.3	13.4	7.1	3.4	6.1	5.3	5.0
18	0.90	0.77	1.14	1.76	4.8	5.7	5.4	4.2	2.8	6.1	5.1	4.6
20	1.19	0.92	1.37	1.45	3.6	4.6	4.4	3.9	2.1	5.3	3.0	3.8

SM = *Semimembranosus*, ST = *Semitendinosus*, BF = *Biceps femoris*, VL = *Vastus lateralis*

[1] Means based on a nine-point hedonic scale (9 = like extremely; 1 = dislike extremely)

Collagen is required for muscle structure (Figure 1) but in meat it is associated with creating toughness of meat during eating. Therefore, it would be expected that when there is less collagen in the muscle the meat will be perceived to be more tender and have lower shear force values and *vice versa* (Dransfield, 1977; Light et al., 1985). However, more often it is observed that a lower concentration of collagen can result in higher shear force values and tougher meat as the effect of age and muscle. This is clearly shown in (Figure 2) as sheep slaughtered at 1, 2, 3 and 4 months had low *semimembranosus* collagen concentration but higher shear force values unlike newborn lambs which had higher collagen concentration but low shear force values (Young et al., 1993). Cross et al. (1972) did not consider shear force but sensory results suggested that tenderness rating declined with increase in slaughter age (Table 3).

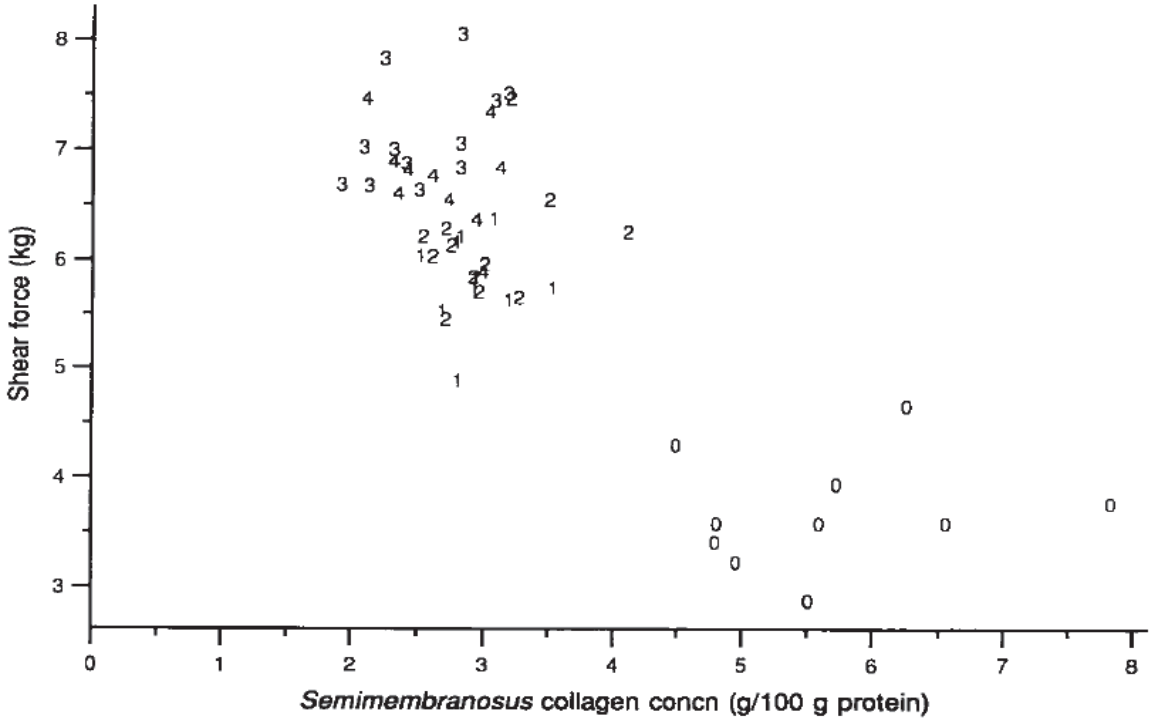

Figure 2. Shear force as a function of collagen concentration. Data are plotted by age i.e., 0 means new born and 1,2,3 and 4 months-of- age (Young et al., 1993).

1.2.6.2 Collagen solubility and meat tenderness

Collagen solubility is considered as a potentially major determinant of meat tenderness (Purchas et al., 1989; Weston et al., 2002; Young & Braggins, 1993) and it is determined by the level of cross-linking between collagen molecules (Light et al., 1985; Weston et al., 2002). The higher the level of cross-linking between collagen molecules the lower the level of solubilisation and the tougher the meat will be (Light et al., 1985; Weston et al., 2002). Intramolecular (cross-linking within the collagen molecule) and intermolecular (cross-linking between the collagen molecules) are two types of collagen cross-links. Intermolecular head to tail cross-links are normally present in a newly formed collagen fibrils to form an infinite polymer (Figure 3a). Collagen has low rate of metabolic turnover which leads to permanent formation of cross-linkages (Purslow et al., 2012). Increase in intermolecular collagen cross-links connect two or three collagen molecules together which increases the tensile strength of collagen leading to reduced collagen solubility and subsequent decline in meat tenderness (McCormick, 1994).

Solubilization of collagen cross links depends on temperature and time of cooking. Collagen is solubilized to gelatine when meat is heated to internal temperature ranges

between 60 to 80°C for 1 hour (Light et al., 1985; Palka & Daun, 1999; Young & Braggins, 1993). However, Burson & Hunt (1986) reported greater percent of solubilized collagen when meat was heated at 90°C for 140 minutes than at 70°C for 70 minutes. Moreover, Hill (1966) suggested the increase in sensitivity of hydroxyproline assay if the heating temperature could be raised above 77°C.

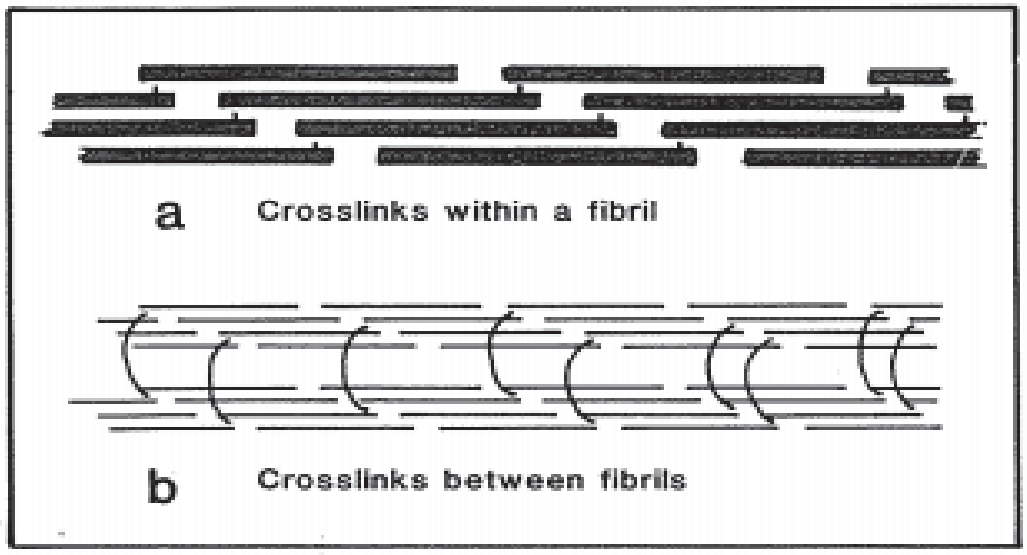

Figure 3 (a). Collagen cross-link structure in young animal with collagen molecule linked head to tail to form an infinite polymer. b. Showing collagen cross-link in older animal as it passes between the fibrils. Adopted from Purchas et al. (1989).

The increase in collagen cross-linking and in turn increase in stability of collagen upon heating depend on muscle activity and age of the animal (Hill, 1966; Purchas et al., 1989). Between muscles collagen solubility in the *gluteus* muscle is higher ranging from 13.5 to 70.5 % compared to *semimembranosus* which ranges from 4.1 to 16.9 % (Young & Braggins, 1993). Also at a given age, collagen solubility for the *ovine biceps femoris* muscle is higher than that of *semimembranosus* (Table 2; Cross et al., 1972). Within similar age range, *Longissimus lumborum* is found to have higher collagen solubility than *semimembranosus and Biseps femoris (*Table 3; Starkey et al., 2016).

Collagen has low metabolic turn over (Purslow et al., 2012) therefore, a decrease in collagen solubility is associated with presence of mature collagen cross-link (non-reducible cross-link) in advanced animal slaughter age (McCormick, 1994; Young & Braggins, 1993). Presence of heat stable collagen cross-links limits the solubility of collagen in meat from mature sheep, even at higher temperature (Light et al., 1985).

Berge et al. (2003) reported a decline in collagen solubility as lamb slaughtered advances from 1 month of age to 12 months of age (Figure 5). There is also a decline in collagen solubility as lamb slaughter age increases from 2 to 5 months- of-age (Table 2; Camacho et al., 2016). Also, a decline in collagen solubility from around 50% down to 12% was evident in lamb slaughtered at 0 compared to 12 months of age (Young et al., 1993). Santos-Silva et al. (2002) reported no effect of the degree of maturity at slaughter on collagen solubility in lamb meat however, lambs were slaughtered at an average age of 53 days and it is likely that the young age and also small age difference between the treatments was unable to allow the variation in collagen solubility to be expressed.

Young & Braggins (1993) assessed the eating quality of *semimembranosus* from sheep aged 4 to 60 months and found that collagen solubility declines as animals get older and correlates with an increase in shear force. Berge et al. (2003) reported a decline in collagen solubility of *Longissimus et lumborum* muscle of lamb as slaughter age advances from 0 to 14 months (Figure 5). Bouton et al. (1978) reported a clear increase in shear force as age increased in combined data for 4 muscles of sheep aged from 2-96 months (Figure 6).

Meat with higher collagen solubility requires less shear force compared to meat with low collagen solubility (Table 4). Moreover, lamb slaughtered at 0 months had a higher collagen solubility and subsequent low shear force value whereas meat from lambs slaughtered at 3 and 4 months-of-age had less collagen solubility and greater shear force (Figure 4; Young et al., 1993). This relationship is consistent with the reported higher tenderness rating of meat from sheep slaughtered when they were younger than 3 months compared to those slaughtered at 19-21 months of age or older (Cross et al., 1972).

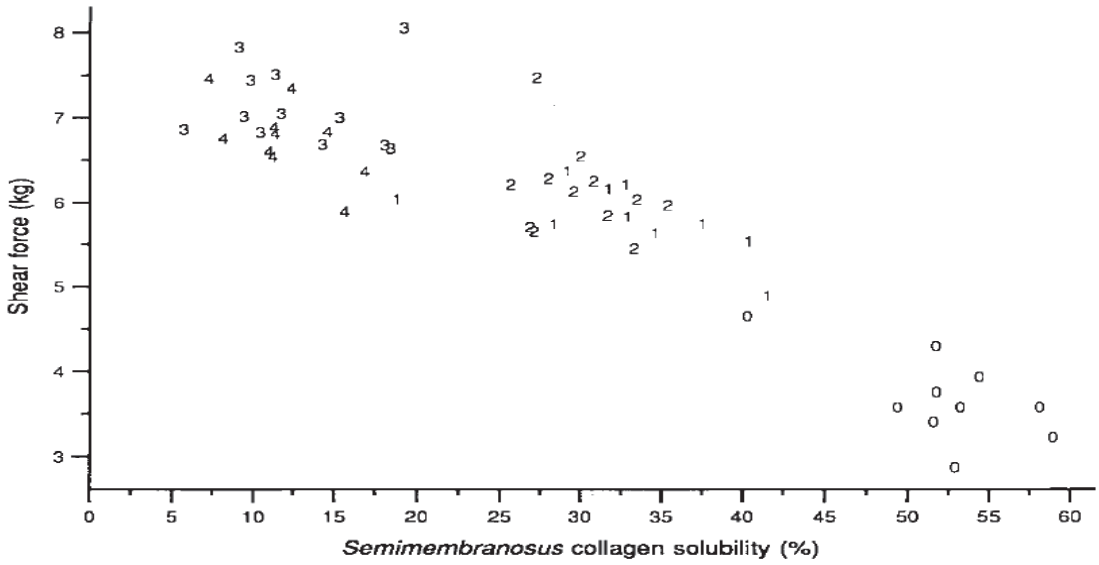

Figure 4. Shear force as a function of collagen solubility. Data are plotted by age (o means new born and 1, 2, 3 and 4 months- of- age). Lower shear force values are associated with meat that is more tender (Young et al., 1993).

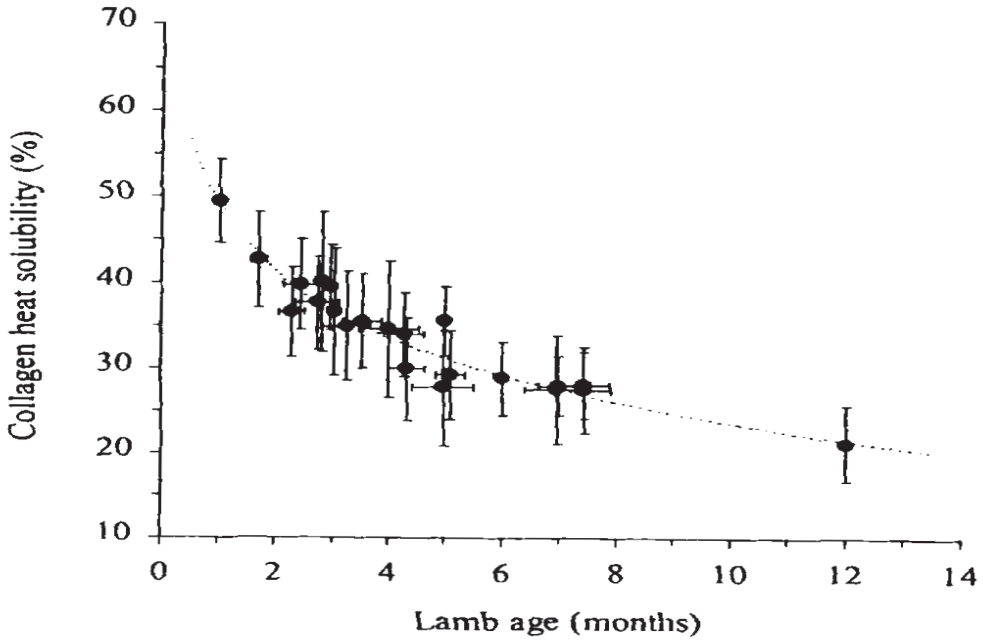

Figure 5: Decline in collagen solubility of sheep *Longissimus* muscle with age. Adopted from Berge et al. (2003).

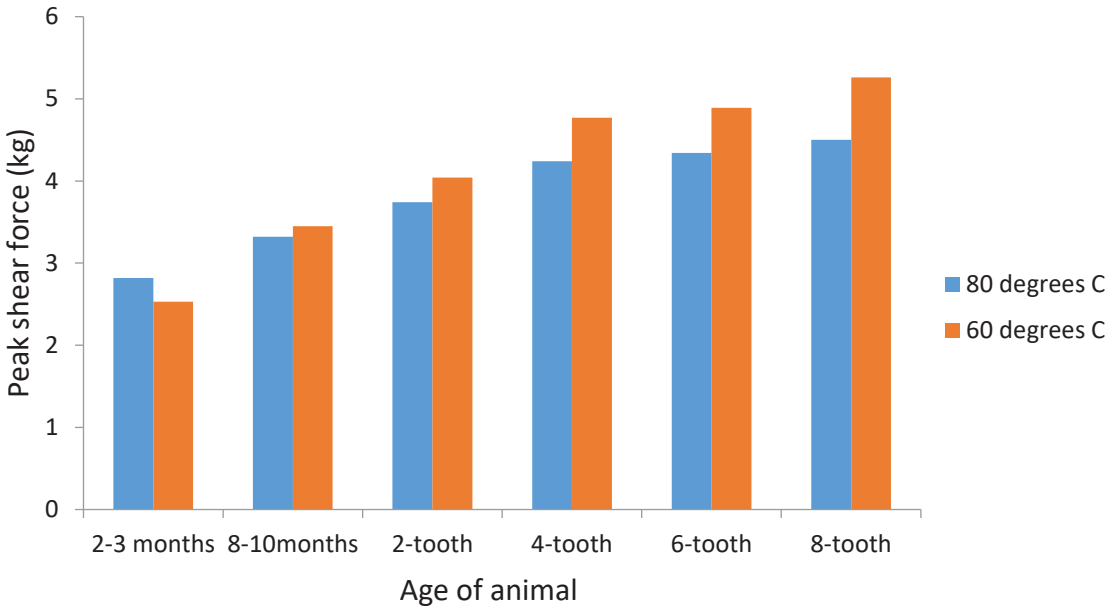

Figure 6. Shear force for muscle samples cooked at 60 or 80 °C for 90 min from weaners 2–3 months, lambs 8–10 months, 2 tooth (12-19months), 4 tooth (18-24 months), 6 tooth (23-36 months) and 8 tooth (28-48 months). (Adapted from (Bouton et al., 1978) also presented in (Hopkins & Mortimer, 2014).

A previous study in Australia has reported higher score in meat from 8.5 months old lambs and 20 months hogget which was scored by consumers using a procedure described by Thompson et al. (2005a) compared to 68.5 months old sheep and suggested that to ensure acceptable eating quality sheep should be marketed at below 20 months of age (Pethick et al., 2005). However, in New Zealand there is insufficient literature to understand the effect of changes in meat quality and specifically collagen solubility between younger and older lambs.

Table 4. Age at slaughter, collagen concentration, collagen solubility, tenderness rating (9 = like extremely; 1 = dislike extremely) and shear force in sheep *semimembranosus* muscles.

Reference	Age at slaughter (months)	Collagen concentration (g/100g whole tissue)	Collagen solubility (%)	Tenderness rating	Shear force (kgF)
Young et al. (1993)	0	1.25	49.5		3.8
	1	0.58	31.7		5.8
	2	0.61	28.4		6.1
	9	0.52	14.8		7.2
	12	0.59	12.2		6.8
Young & Braggins (1993)	4 to 60	0.36 - 0.78	4.1- 16.9		
Cross et al. (1972)	<3	0.48	17.8	3.6	
	3-5	0.37	8.0	3.8	
	5-7	0.39	6.6	4.5	
	7-9	0.46	6.7	3.7	
	9-11	0.57	6.9	3.6	
	11-13	0.431	6.1	3.9	
	13-15	0.291		4.2	4.2
	15-17	0.361		4.9	3.4
	17-19	0.354		4.8	2.8
	19-21	0.476		3.6	2.1

1.2.6.3 Effect of collagen concentration and solubility on meat tenderness

Collagen solubility is reported to account for at least 30 and 50% of the variation in tenderness and classified as the best predictor of meat toughness (Light et al., 1985). Oddy et al. (2001) considered that up to 90% of variation in meat tenderness to be explained by collagen solubility. Both collagen concentration and collagen solubility are higher for *semimbranosus* muscle and *Longissimus* muscle in new born lambs which then decline as as animal gets older leading to greater shear force (Figure 2, 4 and 5; Berge et al., 2003; Young et al., 1993). Although collagen concentration and collagen solubility in bovine muscle has been reported to have an additive effect on meat tenderness (McCormick, 1999), across each individual study in sheep there is less change in collagen concentration with an increase in slaughter age but collagen solubility declines at a great rate as the slaughter age increases (Table 2 and 4). Young et al. (1993) found insignificant effect of *semimembranosus* collagen concentration on shear force value and suggests that collagen solubility is a better predictor of meat

tenderness than collagen concentration. A study on beef muscles reported collagen solubility as a greater contributor of connective tissue related toughness compared to collagen concentration (Cross et al., 1973). Similarly, shear force data of lamb suggest collagen solubility to be more important determinant of meat eating quality than collagen concentration (Young & Braggins, 1993).

1.3 Research Objectives

The literature concerning meat quality of lamb and sheep meat from animals slaughtered at different ages has focused on comparisons between mature sheep and young lambs. There has been little consideration of young lambs at weaning (approximately 3-5 months of age) in comparison to older lambs (7-9 months of age) and hoggets (12-15 months of age). In the meat industry, the perception is that meat quality problems present themselves in hoggets rather than lambs, hence the downgraded and lower carcass price for sheep carcasses classified as hogget.

A study from Australia suggest that the collagen solubility in lamb meat could be a strong driver of tenderness (Hopkins & Mortimer, 2014). Recent focus in New Zealand for improving tenderness of lamb has been on intramuscular fat and the selection of breeding animals for partitioning of fat for deposition within the muscle (Johnson et al., 2015; Johnson et al., 2016). Given the research from Australia indicating the importance of collagen for the tenderness of lamb it suggests that New Zealand research in the eating quality of lamb should not just focus on intramuscular fat but also place equal emphasis on collagen and the solubility of collagen in the meat. Studying collagen concentration and collagen solubility in lamb meat requires the development of an assay that accurately estimates collagen concentration and solubility. Previous studies suggest methods that utilise acid hydrolysis, buffered chloramine T oxidant solution and Erhlich's reagent to create a colour change proportional to the hydroxyproline in the sample are the most reliable.

The age at which collagen solubility becomes a predominant problem in meat tenderness is not well understood for New Zealand pasture-raised lamb. In particular, there is a lack of understanding of the change in collagen solubility for young lambs (approximately 3-5 months of age) in comparison to older lambs (7-9 months of age)

and hence, the level of management required to finish lambs as young as possible to maximise the eating quality. In relation to this, it is not known if there is substantial change in collagen solubility between 9 and 14 months of age when the lamb cuts it's teeth and the hogget carcass classifications then applies. Having this understanding of the solubility of collagen as the slaughter age of the lamb increases would allow the slaughter age at which the best quality meat is produced to be identified for discerning markets that are likely to pay a premium. Also, the extent to which the presence of two permanent teeth in a wear when they are higher than adjacent teeth really reflects a change in meat quality could be identified.

1.3.1 Research objectives and hypothesis

1) To use objective measurements to understand the differences in meat quality between sheep slaughtered at 5, 8 and 14 months of age.
2) To develop an assay to measure collagen concentration and solubility.
3) To compare shear force as a measurement of tenderness to the collagen concentration and solubility of meat from sheep slaughtered at 5, 8 and 14 months-of-age.

The experimental work of this thesis was designed to provide the opportunity to develop an assay to measure collagen concentration and solubility in lamb meat and investigate whether lamb compared to hogget has differing meat quality and establish if there is an association with collagen concentration or collagen solubility. The experiment also aims to elucidate the point in the animals' growth and development where the effect of decrease in collagen solubility becomes a tenderness problem. It is hypothesised that the decline in meat quality of older lambs and carcasses classified as hogget is due to a decrease in collagen solubility.

Chapter 2. Materials and Methods for Objective Meat Quality Test

2.1 Animal management and slaughter

Sixty (60) Romney ram lambs born in late August and early September (spring) of 2015 were used in this experiment. The lambs were raised in one group on perennial ryegrass-based pastures following standard commercial farming operations at Massey University's Keebles farm (latitude 41° 10'S, longitude 175° 36'E) 5km south of Palmerston North, New Zealand. The lambs were allocated to slaughter-age groups of 5, 8 and 14 months of age with 20 lambs in each group. Lambs were balanced across the slaughter age groups for birth weight. Slaughter occurred on 20th of January, 26th April and 3rd November 2016 when the average age of the sheep in each group was 5.1 (4.5-5.2), 8.2 (7.7-8.5), and 14.5 (14.1-14.8) months, respectively.

All lambs were weighed at birth, at docking on the 29th September, 2015, 2nd November, 2015, 3rd December, 2015, 15th January, 2016 and 19th January, 2016. For the remaining animals after the 20th of January and 26th of April slaughter dates the final live weight was recorded on farm prior to each slaughter group being transported to the abattoir.

The sheep were slaughtered and processed at the Alliance Group plant in Dannevirke, (latitude 40° 12'S, longitude 176° 44'E) 58 km east of Palmerston North, New Zealand following standard commercial procedures.

2.2 Carcass measurement and meat sampling

Each carcass was given an identification number which was linked to the electronic ID of each sheep to allow tracing of individual sheep information. Hot carcass weight was measured and the GR (tissue depth 11 cm from the midline on the 12th rib) and yield in the leg, loin and shoulder were estimated using the Alliance Group VIAscan® system. After the carcasses were chilled at 4°C for 24 hours the bone-in, short loin (*Longissimus lumborum)* was collected from each carcass, vacuum-packed with the carcass identification tag and transferred to Massey University Food Pilot Plant. The loin samples were stored at -20°C for three weeks.

2.3 Preparation and partitioning of the loin sample

Samples were removed from the freezer and thawed at 1^{o}C for 24 hours. The vacuum-packed bone-in loin sample was weighed and then the packaging removed. Both the packaging and meat sample was blotted dry with a paper towel and then weighed so that the thaw loss could be calculated. The *Longissimus lumborum* muscle was excised from the bone to obtain the boned-out loin. A 2.5 cm segment from the caudal end of the loin was cut and used for the measurement of pH, colour, myofibrillar fragmentation index, expressed juice and then sarcomere length. Moving from the caudal to cranial, three segments that were 2.5 cm wide were then cut and placed in the plastic bag for cooking and the determination of shear force. A further 3 cm wide segment was cut for assessing drip loss and the most cranial segment that remained was vacuum packed and frozen at -20^{o}C as a reserve (Figure 7).

Any raw loin tissue that remained after the meat quality analysis was trimmed of fat and visual connective tissue (epimysium), finely minced (Kenwood MG450, 3 mm hole-plate), vacuum-packed and frozen for the subsequent assessment of fat (intramuscular fat) using a Soxhelet ether extraction method (AOAC 911.36) and collagen concentration and solubility (Figure 7).

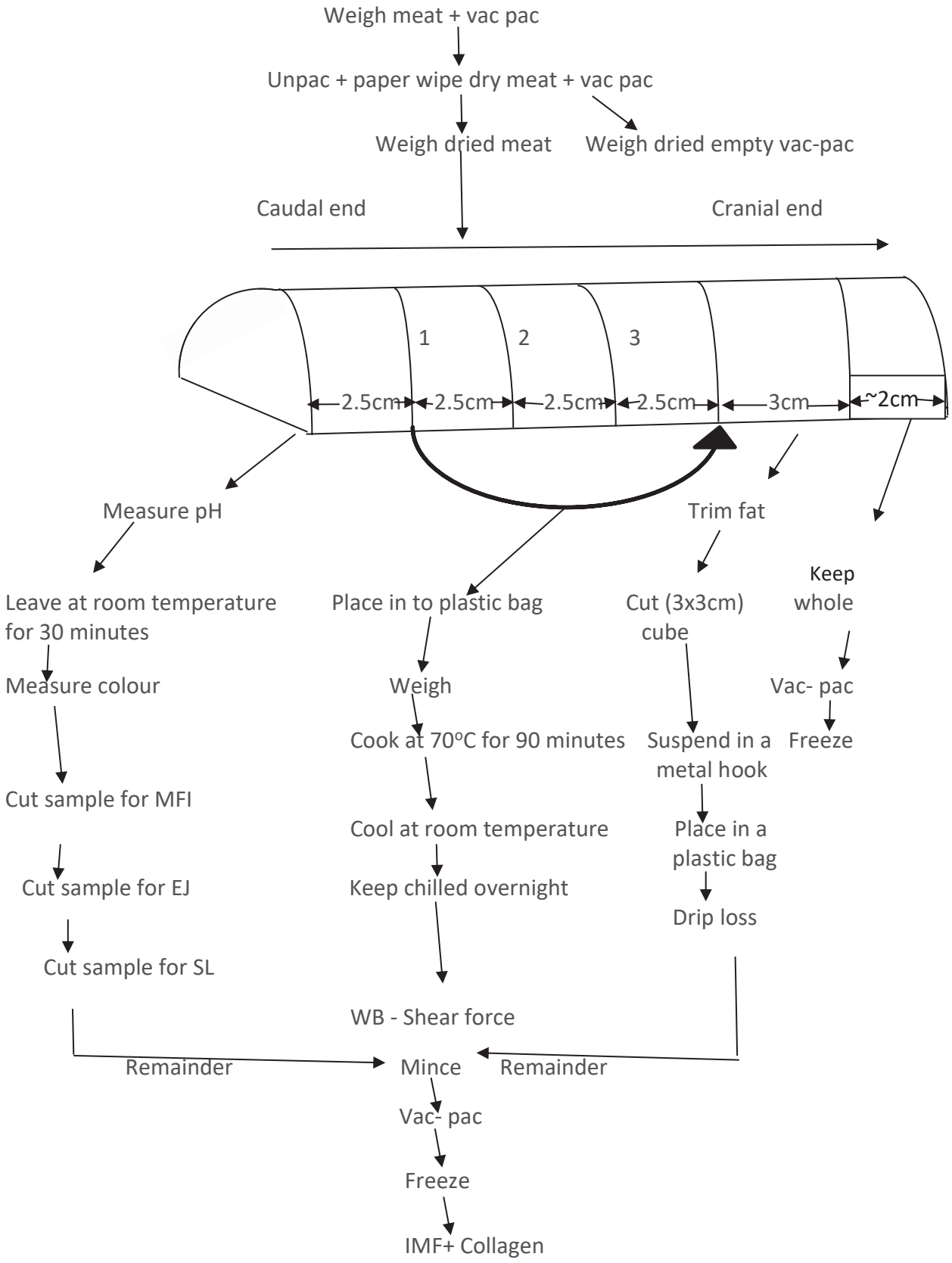

Figure 7. A schematic diagram showing the partitioning of lamb *Longissimus lumborum* muscle for meat quality analysis. MFI= Myofibrillar fragmentation index, EJ= Express juice, SL = Sarcomere length, IMF = Intramuscular fat.

2.4 Objective meat quality tests

The ultimate pH was measured at three points from medial to distal across a transverse, internal cut of the loin with a pH spear (Eutech Instruments, Singapore). The pH spear was calibrated to pH 4.01, 7.00 and 10.01 standard buffers. The three values were averaged to obtain the pH value for the loin sample.

The lean meat colour was also measured on the transverse cut surface after 30 min exposure to air (blooming). The colour was measured using the Minolta Colour Meter calibrated to a standard white tile supplied by the manufacturer (CR-200, Konica Minolta Photo Imaging Inc., Mahwah, NJ, USA). The CIE *L** (lightness), *a** (redness) and *b** (yellowness) values were measured (Illuminant D65, 8 mm aperture, 0° standard observer) at three points from medial to distal across the loin and the three values averaged to obtain the *L*,* a** and b** values for the loin sample.

Water holding capacity (WHC) was assessed as thaw loss, expressed juice and drip loss of the raw meat sample and cooking loss from the samples for shear force. Expressed juice was measured by filter-paper-press method (Purchas & Aungsupakorn, 1993). Thaw loss (water loss from thawing of frozen meat) was measured using the weight of thawed frozen meat in a vacuum pack, weight of unpacked paper dried meat and paper dried vacuum pack and labels separately using the following equation.

$$\text{Thaw loss (\%)} = \frac{[(\text{Whole weight} - \text{packaging weight}) - (\text{Paper dried meat weight})]}{(\text{Whole weight} - \text{packaging weight})} \; X \; 100$$

Approximately 0.5 g of fresh meat cube with sides of about 6 mm was placed on Whatman No. 1 filter paper and pressed between two Perspex plates for 2 min using a 10 kg weight. The wetted area from expressed juice was measured using a planimeter (Placom KP-90N, Tokyo, Japan) and the expressed juice value expressed as the total wetted area per unit weight of sample (cm^2/g).

Driploss by suspension was measured using a cube of raw meat with 3 cm sides that was cut from the 3cm steak. The 3cm cube was weighed then suspended on a metal hook in a plastic bag at 1°C. After 24 and 48 hours the suspended cube was blotted dry using tissue paper and reweighed. Drip loss was calculated as the original weight minus

the weight at 24 or 48 h and the value was expressed as a percentage of the original weight.

The three 2.5 cm steak were placed into a plastic bag and suspended in a water bath at 70°C for 90 min for cooking and then chilled at 1°C for 24 hours. Cooking loss was calculated as the difference in weight of the three steak before and after cooking and expressed as a percentage of the weight before cooking.

The force required to shear through a 13 mm × 13 mm cross-section of the cooked sample (from the 2.5 cm steak) at right angles to the fibre axis was performed using a shear force device with a square blade (50 mm blade with a 25 mm horizontal cutting section and blade thickness of 1.016 mm, crosshead speed of 230 mm/min; G-R Electric Manufacturing, Manhattan, KS, USA). Parameters recorded were: work done, initial yield and peak force. Work done is an average of the 436 values used to produce a force-by-time curve. Initial yield represents a change in shear force and appears as an inflexion in the force-by-time curve. Peak force is the maximum recorded force (Purchas & Aungsupakorn, 1993). Six replicates were measured for each cooked sample and results are expressed in kilograms of force (kgF).

Sarcomere length was measured by laser diffraction. A small slither of muscle of approximately 8-10 mm along the direction of fibres and 1 x 1 mm in cross section was prepared using scalpel blades. The samples were then transferred onto a microscope slide and teased out and compressed between two microscope slides after adding 2-3 drops of distilled water. A helium-neon gas laser (632.8 nm, Melles Griot, Carlsbad, CA, USA) was passed through the sample to create a diffraction pattern on a screen set 10 cm from the sample. The distance between the first order diffraction bands in 12 replicate per sample was measured in millimetres and the averages were recorded. Sarcomere length was calculated using the following equation.

$$\text{Sarcomere length (µm)} = 0.6328 * \left[\sqrt{\left(\left(\frac{x}{10*2}\right)^2 + 100\right)}\right] \Big/ \left(\frac{x}{10*2}\right)$$

Where; x = Distance (mm) between first order diffraction bands.

Myofibrillar fragmentation index was measured by assessing the muscle fragments that passed through 231 µm mesh by filtration following a standard homogenization procedure (Purchas et al., 1997). Approximately 5 g of the loin in which visible connective tissue has been removed was roughly diced into cubes with edges of about 3 mm and added into 50 mL of 0.85% NaCl. Anti-foam solution (5 drops) was added into the tube to prevent foam formation while homogenizing. The mixture was homogenised using Ultra-Turrax, 18 mm diameter shaft at one-third speed for one minute. The homogenized mixture was then rapidly poured into pre-weighed 231 µm mesh filters. The filters were allowed to drip for 4 hours and then dried at 30^{o}C for 40 hours. Myofibrillar fragmentation index was calculated as: one hundred percent minus the percentage weight of the dried sample retained on the filter compared to the sample weight. Myofibrillar fragmentation index normally range from 78% when no fragments passed through the filter up to 100% when all fragments pass through (Purchas et al., 1997).

2.5 Soluble, insoluble and total collagen determination

For the analysis of collagen, all chemicals were procured from Sigma-Aldrich, New Zealand Ltd and the buffer solution, chloramine T oxidant solution, Erhlich's colour reagent (4-dimethylamino benzaldehyde), NaOH (6M), HCL (6M) and trans-4-Hydroxy-L-proline standard solution were prepared according to AOAC 990.26, (1993), Bergman & Loxley (1963) and Archile-Contreras et al. (2010). Total, soluble and insoluble collagen was determined as hydroxyproline which is the amino acid that form large proportion of collagen molecule according to methods of (Ablikim et al., 2016; Archile-Contreras et al., 2010; Bergman & Loxley, 1963; Colgrave et al., 2008; Edwards & O'Brien, 1980). Full detail of the method is provided in Chapter 3 which describes the development of the collagen assay in the Massey University laboratory.

Chapter 3. Collagen Assay Development

3.1 Introduction

Quantification of soluble, insoluble and total collagen in meat is of particular important when assessing tenderness associated with connective tissue component. Collagen is the fibrous protein molecule that forms the structural component of connective tissue sheath (Purslow, 2005). The complexity of the collagen structure due to its covalent cross linking make the collagen molecules difficult to extract (McCormick, 1994). Therefore, collagen in muscle is predominantly estimated from determined hydroxyproline which is an exclusive amino acid for the triple helix structure of collagen (Ablikim et al., 2016; Archile-Contreras et al., 2010; Bergman & Loxley, 1963; Colgrave et al., 2008; Edwards & O'Brien, 1980).

Collagen concentration can be estimated from hydroxyproline determined from tissue following acid hydrolysis, neutralization, dilution of hydrolysates and oxidation of hydroxyproline to pyrrole using chloramine T oxidant solution. The pyrrole is converted to a relatively specific chromophore with Erhlich's reagent (Kivirikko et al., 1967) and heated to develop red chromophore colour. The intensity of colour is proportional to the pyrrole present. The absorbance is then read (Archile-Contreras et al., 2010; Bergman & Loxley, 1963; Edwards & O'Brien, 1980) and total hydroxyproline concentration of the sample is calculated from the hydroxyproline standard curve linear equation produced from standard solution (AOAC 990.26, 1993). Collagen is reported to contain 13.8 % hydroxyproline (AOAC 990.26, 1993) thus, the factor for the conversion of hydroxyproline to its equivalent collagen in muscle is assumed to be 7.25 (Ablikim et al., 2016; Archile-Contreras et al., 2010; Palka, 1999, 2003).

Alternatively, total collagen concentration in meat can be calculated by the summation of soluble collagen and insoluble collagen (Archile-Contreras et al., 2010). Soluble and insoluble collagen is estimated from hydroxyproline determined from tissue after heating step followed by centrifugation to separate soluble and insoluble fractions (Archile-Contreras et al., 2010). To obtain hydroxyproline concentration and in turn collagen concentration in each soluble and insoluble fractions then undergo acid

hydrolysis, neutralization, dilution, oxidation and staining similar procedures as for total collagen concentration procedures above.

The aim of this section of work was to test and to develop an assay to measure collagen concentration and collagen solubility. The initial methodology was established from reviewing the literature to identify the most common and reliable methods used in other studies.

3.2. Methods in Literature

As stated in introduction above determination of total hydroxyproline for total collagen concentration in meat is initiated by freeze drying, grinding followed by acid hydrolysis of meat sample (Ablikim et al., 2016; Archile-Contreras et al., 2010; Bergman & Loxley, 1963; Palka, 1999, 2003; Zhao et al., 2015). Also determination of soluble and insoluble collagen involves the heating and centrifuging of the freeze dried and grinded meat sample followed by acid hydrolysis of supernatant and sediment separately (Archile-Contreras et al., 2010). The main limitation of acid hydrolysis is the long time required to complete hydrolysis that is 24 h at 110^{o}C (Archile-Contreras et al., 2010; Palka, 1999, 2003). Alkali hydrolysis requires a shorter time than acid hydrolysis, however, hydroxyproline recovery is slightly greater in acid hydrolysed muscle compared to alkali hydrolysed muscle (Hofman et al., 2011; Wierbicki & Deatherage, 1954). Neutralization of acid hydrolysate predominantly uses sodium hydroxide and dilution of hydrolysates can be completed after neutralization (Palka, 1999, 2003) or in part of hydrolysate after neutralization (Hofman et al., 2011).

Buffered chloramine T solution is predominantly used to oxidize hydroxyproline to pyrrole (Edwards & O'Brien, 1980; Hofman et al., 2011; Stegemann & Stalder, 1967). However, non-buffered chloramine T solution with distilled water as solvent can be used without affecting the accuracy of the assay (Edwards & O'Brien, 1980). Staining of pyrrole is achieved by adding Erhlich's reagent that contain p-Dimethylamino-benzaldehyde, 70% perchloric acid and 2-propanol (AOAC 990.26, 1993; Stegemann & Stalder, 1967).

In order to separate soluble and insoluble collagen the sample is heated. Different authors have used different conditions for the heating step with variations of temperature from 75 to 90^{o}C for 60 to 80 minutes (Hill, 1966). Maximum collagen solubility is reported in muscle heated to internal temperature of 80^{o}C (Palka, 2003) as a result of gelatinization of collagen fibres in the perimysium (Palka & Daun, 1999). Hill (1966) also suggested the increase in sensitivity and accuracy of determination of hydroxyproline upon raising the temperature of solubilisation to a value above 77^{o}C. Following the heating a separation of the soluble and insoluble collagen step is required and then the hydroxyproline can be determined on each fraction.

Heating for colour development is predominantly achieved at 60°C water bath (Fisher Scientific Isotemp 210) for 15-30 minutes (Archile-Contreras et al., 2010; Bergman & Loxley, 1963; Edwards & O'Brien, 1980; Hofman et al., 2011; Palka, 1999). Following cooling in an ice water the absorbance can be accurately read at 550 nm (Edwards & O'Brien, 1980) 558nm (AOAC 990.26, 1993; Bergman & Loxley, 1963; Palka, 1999), 570 nm (Archile-Contreras et al., 2010) by using colour spectrophotometer.

3.3 Outline of method to be tested and developed

3.3.1 Preparation of hydroxyproline standard solution and standard curve

Hydroxyproline standard curve was determined using a modification of AOAC 990.26 (1993) procedures. Hydroxyproline was dissolved in water to a concentration of 600 µg/L as a stock solution. This stock solution was then diluted to create an intermediate solution with a concentration of 6 µg/mL. Intermediate solution was further diluted to create the working solution with a concentration of 0.6, 1.2, 1.8 and 2.4 µg/mL. Each concentration of working solution was oxidized to pyrrole with chloramine T solution and stained with Erhlich's reagent to convert the pyrrole to relatively specific chromophore. To develop the colour the samples were incubated in the water bath at 60^{o}C for 20 minutes during which red chromophore developed and then cooled in an ice water for 5 minutes and absorbance of each solution versus distilled water was measured at 558 nm with spectrophotometer and the values were recorded. A standard curve was produced and linear equation was fitted.

3.3.2 Determination of total collagen concentration

Approximately 0.5 g of freeze dried and powdered meat samples was hydrolysed with 20 mL 6 M HCl in an oven at 110°C for 24 hours (Zhao et al., 2015). The hydrolysates were then neutralized with 6M NaOH to a pH of 6 and diluted to a concentration that was within the range of the hydroxyproline standard solutions. Hydroxyproline concentration of the diluted samples was determined using AOAC 990.26, (1993) and the modifications of Archile-Contreras et al. (2010, Bergman & Loxley, (1963) Edwards & O'Brien, (1980) and Zhao et al. (2015) as follows. Hydroxyproline in hydrolysates and working standard solution was oxidised to pyrrole with chloramine T solution and then converted to relatively specific chromophore with Erhlich's reagent solution. The samples and standard solution were heated in the water bath at 60^{o}C for 20 minutes during which red chromophore developed and cooled in an ice water for 5 minutes then the absorbance was read at 558 nm. The standard curve of hydroxyproline standard solution absorbance versus hydroxyproline concentration was produced. The hydroxyproline of the sample was calculated from the linear equation developed from the standard curve and multiplied by 7.25 as a factor to get total collagen concentration (Palka, 1999).

3.3.3 Determination of heat soluble and insoluble collagen in lamb meat

Exactly 10 mL of distilled water was added into a Kimax tube containing 0.5 g of freeze dried powdered meat sample and then heated at 80°C for 75 minutes, with stirring every 15 minutes including at 75 minutes (Archile-Contreras et al., 2010). The homogenates were centrifuged at 4000 rpm (3270*g*; Heraeus Multifuge 1S-R centrifuge, Thermo Fisher Scientific, Germany) for 10 minutes and each supernatant and sediment was collected and hydrolysed separately in an oven at 110^{o}C for 24 hours. The supernatant constitutes the soluble collagen and sediment constitutes the insoluble collagen. Both supernatant and sediment were neutralized with 6 M NaOH to pH about 6.0 (Latorre et al., 2016) and 1 mL of each supernatant and sediment was diluted to 50 mL. The hydroxyproline concentration of each supernatant and sediment was determined using the modifications of methodology described above for total collagen (Archile-Contreras et al., 2010; Bergman & Loxley, 1963; Edwards & O'Brien, 1980). Soluble collagen in supernatant and insoluble collagen in sediment was calculated by

assuming that collagen weighed 7.25 times the measured hydroxyproline value and expressed as g of collagen per 100 g of fresh weight (Palka, 1999).

3.3.4 Preparation of buffer solution

Buffer solution of pH value 6.0 was prepared by dissolving 30 g of citric acid monohydrate ($C_6H_8O_7.H_2O$), 15 g NaOH and 54.3 g of sodium acetate (CH3COONa. $3H_2O$) in about 500 mL of water. The solution was transferred to 1 litre volumetric flask and 290 mL of 1-propanol was added. The pH was checked with pH meter and adjusted with 6 M hydrochloric acid. The solution was made up to 1 litre with water and stored at 5^oC.

3.3.5 Preparation of oxidant solution

The oxidant solution was prepared by dissolving 1.41 g of Chloramine T (the sodium salt of p – toluene sulfon-chloramide) in 100 mL of buffer solution. Due to its lack of stability it was prepared immediately before use (Edwards & O'Brien, 1980).

3.3.6 Preparation of colour reagent solution (Erhlich's reagent)

The colour reagent was prepared by dissolving 2.5 g of p-Dimethylamino-benzaldehyde in 6.5 mL perchloric acid 70% (S.G. 1.54) followed by addition of 16.6 mL of 2- propanol with stirring. The colour reagent solution is stable for 1 hour; therefore it was prepared immediately before use (Edwards & O'Brien, 1980).

3.3.7 List of equipment and chemical

The equipment and chemicals used are listed in Table 5 and 6 respectively.

Table 5. List of equipment and use

Equipment	Use
Bottles	To store buffer solution and stock solution
Volumetric flasks	Measuring volume during dilutions
Pyrex test tubes	Heating samples in the water bath
Kimax tubes	Heating samples in the oven and water bath
Pipette	Transferring volume of sample and reagents
Beaker	Aid the transferring of samples and reagent
Spatula	Take the reagent from containers when weighing
Weighing scale	Weighing samples and reagents
pH meter	To check the pH of hydrolysates and buffer solution
Water bath at 80^{o}C	To heat the sample for soluble and insoluble collagen determination
Water bath 60 ^{o}C	Heat the samples and standard for colour development
Glass cells (cuvette)	To take the sample for absorbance reading
Spectrophotometer	To read the absorbance at 558nm
Centrifuge and tubes	To separate soluble from insoluble part of sample

Table 6. List of chemicals and use

Chemical	Use
Citric acid monohydrate ($C_6H_8O_7.H_2O$)	Preparation of buffer solution
15 g NaOH	Preparation of buffer solution
Sodium acetate (CH3COONa.3H_2O)	Preparation of buffer solution
1-propanol	Preparation of buffer solution
6M hydrochloric acid (HCL)	Sample hydrolysis
6M sodium hydroxide (NaOH)	Hydrolysate neutralization
Hydroxyproline	To prepare standard solution
Buffer solution	Preparation of oxidant solution
Chloramine T	Preparation of oxidant solution
4-dimethylaminobenzaldehyde (Erhlich's reagent)	Preparation of colour reagent
Perchloric acid (70% w/w)	Preparation of colour reagent
2-propanol	Preparation of colour reagent

3.4 Considerations in the method development

3.4.1 pH of hydrolysate

Accuracy of determination of hydroxyproline requires the pH of hydrolysate of 5.6-6.5 (Latorre et al., 2016). Archile -Contreras et al. (2010) did neutralization by adding equal volume of 6 M sodium hydroxide (NaOH). During the first trial 20 mL of NaOH was added but the resulting pH was as high as 12.5 indicating an excess of NaOH. The experiment was repeated and the volume of hydrolysate was measured prior to the neutralization

step. It was found that the volume of hydrolysate was less than the initial volume before hydrolysis and varied with sample. The volume of NaOH equal to the volume of hydrolysate was measured and added drop wise to attain the pH of 6. The volume of hydrolysate and the volume of NaOH were recorded to account for the first sample dilution factor when calculating hydroxyproline of sample from the equation generated from hydroxyproline standard curve.

3.4.2 Standard curve and sample dilution

The absorbance values for hydroxyproline standard solution ranges from 0.1 to 0.7 (Table 7). The absorbance value was plotted against hydroxyproline to produce the hydroxyproline standard curve (Figure 8). The curve showed linear relationship between absorbance and hydroxyproline concentration. The curve was fitted with R-squared value of 0.9957 and the linear equation; Y= 4.5448x - 0.6084. Since from the standard curve Y - axis represent hydroxyproline concentration and X - axis absorbance values therefore, substituting Y with hydroxyproline and X with absorbance it gives the following equation that was used to estimate the amount of hydroxyproline in meat sample. Hydroxyproline = 4.5448 (Absorbance) - 0.6084.

Table 7. Absorbance values for standards

Hydroxyproline conc. (µg)	**Absorbance unit (558 nm)**		
	Rep1	Rep2	**Mean**
0	0.141	0.125	0.133
0.6	0.268	0.257	0.263
1.2	0.423	0.411	0.417
1.8	0.514	0.508	0.511
2.4	0.663	0.669	0.666

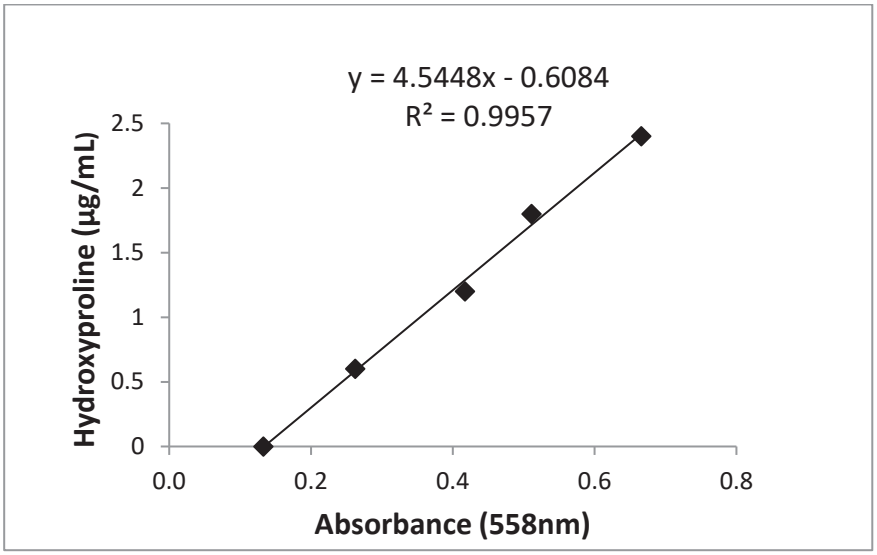

Figure 8. Hydroxyproline standard curve for estimating total hydroxyproline in meat sample

To accurately calculate hydroxyproline from the produced standard curve linear equation, the sample absorbance values need to be within the range of the absorbance values of the standard solutions. Therefore, the sample hydrolysates need to be diluted to attain the absorbance value which is within the range of produced standard solution absorbance values. Hydrolysates was filtered and diluted by a factors of 10, 25 and 50 (Archile-Contreras et al., 2010) but absorbance values (Table 8) were greater than 0.7 the highest absorbance from the produced standard curve (Table 7).

Table 8. Absorbance of Spring 165 green sample using 10, 25 and 50 dilution factor

Dilution factor	Absorbance (558nm)
10	2.44
25	1.45
50	1.30

To check if the standard curve would still remain linear at higher hydroxyproline concentrations, a standard curve was produced with hydroxyproline concentrations of 0, 1.2, 2.4, 4.8 and 9.6 µg/mL (Table 9).

Table 9: The absorbance value of higher hydroxyproline concentration

Hydroxyproline conc. (µg/mL)	Absorbance unit (558 nm)		
	Replicate1	Replicate2	Average
0	0.269	0.284	0.28
1.2	0.559	0.594	0.58
2.4	0.811	0.900	0.86
4.8	1.215	1.185	1.20
9.6	1.452	1.461	1.46

The standard curve with high concentrations of hydroxyproline is given in Figure 9. The curve is non-linear at higher concentration which is reflected by the R-squared value of 0.8935 that is lower than the previous standard curve. This is in agreement with Wierbicki & Deatherage (1954) who found a slight reduction in the expected colour development at higher concentrations of hydroxyproline. Therefore, rather than using a wider range standard curve it is better to use higher dilutions of the sample.

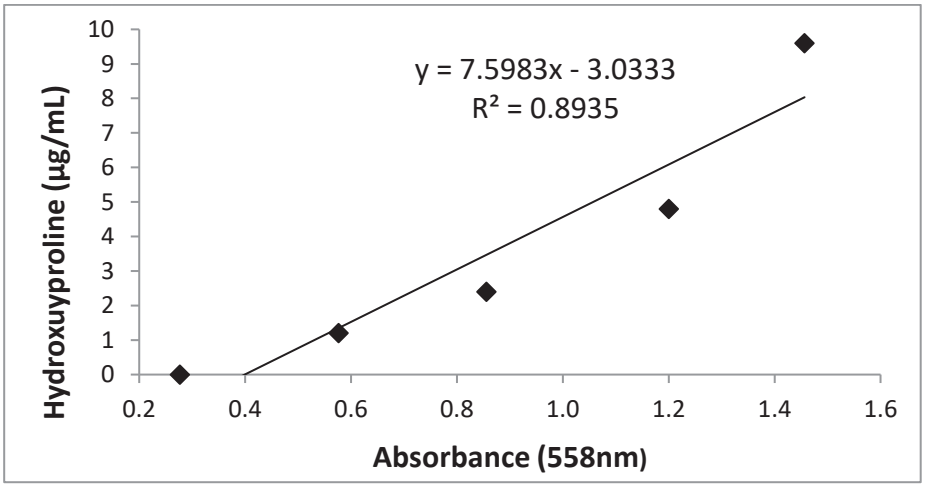

Figure 9. Hydroxyproline standard curve of higher hydroxyproline concentration.

1 mL of the sample was further diluted to 75 and 100 mL. Hundred (1 in 100 mL) dilution produced a mean absorbance unit of 0.648 (Table 10) which lies in the range of the standard curve (Figure 8). This confirmed that a dilution factor of 100 is required, but this will depend on the actual concentration in other samples.

Table 10: Absorbances of Spring 165 green sample using 50 and 100 dilution factor

Dilution factor	Absorbance (558nm)
75	0.703
100	0.648

For soluble and insoluble collagen determination 1 mL of each supernatant and sediment hydrolysate was diluted at 25 and 50 mL factors with RO water. Both 25 mL and 50 mL dilution produced the absorbance value within the range of standard curve. However, the hydroxyproline recovery was higher in 50 mL dilution compared to 25 mL dilution. The calculated total collagen at 50 mL dilution factor was 1.642 g/100g fresh weight which was greater than 1.274 g/100g fresh weight at 25 mL dilution factor. The total collagen at 50 mL dilution factor was similar to total collagen of the same sample analysed using the total collagen assay above confirming a dilution factor of 50 is required for both sediments and supernatants, but this will depend on the actual concentration in other samples.

3.4.3 Colour stability

Leaving the sample for more than 3 hours before reading the absorbance the red chromophore colour starts to break down leading to decline in absorbance values (Edwards & O'Brien, 1980). Therefore, it was important to know the stability of sample colour that will help to estimate the number of tubes in one set of experiment to minimize the error associated with the decline in colour with time. To confirm sample colour stability with time one sample was prepared and left in the spectrophotometer for 50 minutes and the absorbance was taken every two minutes. The colour was almost stable showing a decline of about 0.03 up to 50 minutes (Figure 10) confirming that the reading of absorbance within 50 minutes does not affect the produced red chromophore.

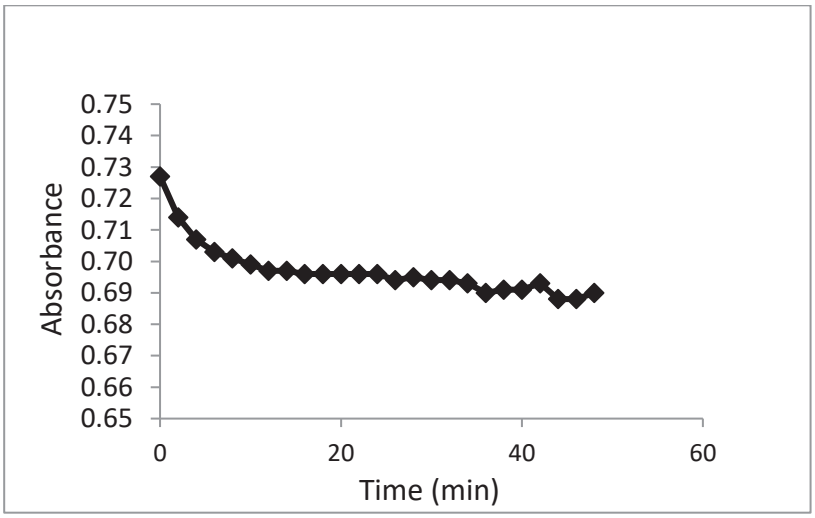

Figure 10. Change in absorbance with time

3.5 Outcomes of initial tests

3.5.1 Total collagen concentration of lamb meat samples – assessing the methodology on samples

A hydroxyproline standard curve was produced in each series of measurements. The sample used was *Longissimus lumborum* from lambs (Spring 165 green Table 10) at around 6 months-of-age. Hydroxyproline concentration was calculated from the standard curve equation produced (Figure 11). Average hydroxyproline and collagen concentration of meat sample was 0.906 g/100 gDM / 0.2265 g/100g in fresh weight and 1.642 g/100 g in fresh weight basis respectively.

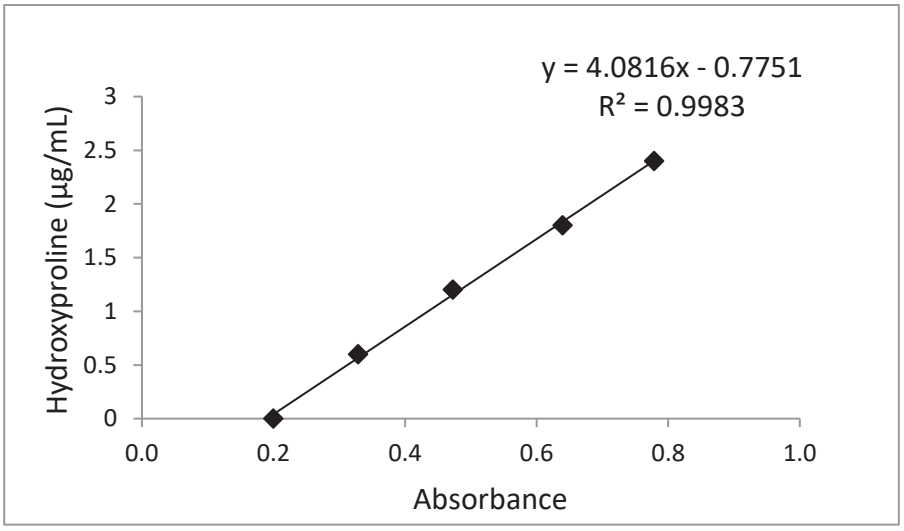

Figure 11. Hydroxyproline standard curve used to estimate total collagen in meat sample

The collagen concentration of the same sample was reanalysed using a fresh standard curve (Figure 12) and the mean hydroxyproline and collagen concentration was 0.866 and 1.569 g/100 g in fresh weight basis respectively demonstrating repeatability within 5%. Maiorano et al. (1993) work on *Longissimus lumborum* muscle of 6 months old sheep reported collagen concentration of 1.851 g/100g and 1.676 g/100g fresh weight in ram and withers respectively. Moreover, Starkey et al. (2016) measured collagen concentration for *Longissimus lumborum* muscle of lamb and hogget and reported total collagen values ranges from 0.69 to 2.09 g/100 g fresh weight. Since the values obtained are within the range of values reported then, the method can be used to determine total collagen concentration in meat samples.

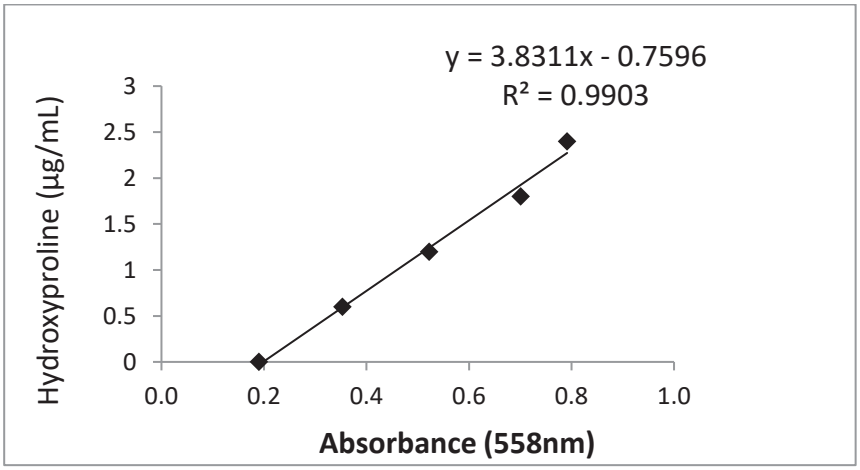

Figure 12. Hydroxyproline standard curve to determine total collagen of Spring 165 green meat sample when the experiment was repeated.

3.5.2 Soluble and insoluble collagen of lamb meat sample

The same *Longissimus lumborum* muscle sample (Spring 165 green Table 10) was used to determine soluble and insoluble collagen. Soluble collagen and insoluble collagen was 0.94 and 0.70 g/100 g fresh weight respectively and total collagen was 1.642 g/100 g fresh weight. The total collagen value is consistent with the total collagen value produced in section 3.5.1 above for the same sample.

The same sample was repeated and produced soluble and insoluble collagen of 1.061 and 0.389 g/100g fresh weight respectively and total collagen concentration of 1.451 g/100g fresh weight basis (FWB). The insoluble collagen was low and soluble collagen was higher than the first analysed values above. This indicates that the day of analysis can affect the obtained values therefore; analysis of sample in replicates can minimize the error.

To confirm the suitability of this method a replicate analysis of soluble and insoluble collagen of Spring 165 green and other 5 lamb meat samples all aged about 6 months was performed (Table 11) and the respective hydroxyproline standard curve was produced (Figure 13).

Table 11: Average soluble, insoluble and total collagen of 6 lamb meat samples utilised to assess the collagen assay that was developed.

Meat sample ID	Soluble collagen (g/100g FWB) (% in brackets)			CV	Insoluble collagen (g/100g FWB)			CV (%)	Total collagen (g/100g FWB)
	Rep 1	Rep 2	Average		Rep 1	Rep 2	Average		
Spring 165 green	0.74	0.97	0.86 (58)	19	0.70	0.57	0.63	15	1.49
Spring 39 red	0.64	0.91	0.78 (57)	24	0.51	0.64	0.58	16	1.35
Spring 128 blue	0.81	0.52	0.66 (54)	31	0.55	0.60	0.57	6	1.24
Autumn 132 green	1.47	1.40	1.43 (62)	3	0.66	1.07	0.87	33	2.30
Autumn 6 red	1.32	0.72	1.02 (60)	42	0.73	0.65	0.69	8	1.71
Autumn 154 blue	0.98	0.91	0.94 (55)	5	0.73	0.84	0.78	10	1.73

Rep = Replicates, FWB = Fresh weight basis, CV = Coefficient of variation

The replicate analysis of soluble and insoluble collagen of Spring 165 green (Table 11) produce the values that are in agreement with the single analysis of soluble and insoluble collagen values with variations between replicates. Therefore, accurate measurement of hydroxyproline in meat sample requires replicate values to account for variability between replicates. Studies have reported soluble collagen values between 22% (Ablikim et al., 2016) and 74 % (Camacho et al., 2016) in *Longissimus lumborum* of lamb aged 5 and 8 month respectively which agrees with the percentage of soluble collagen values in the bracket (Table 11). Moreover, the total collagen of 1.5 g/100 g fresh weight is in agreement with the repeated total collagen value obtained using total collagen assay in section 3.5.1.

The values obtained for Spring 165 green and other five samples are between the collagen solubility values reported in the literature confirming that the method can be used to determine soluble, insoluble and total collagen in meat sample with reliability.

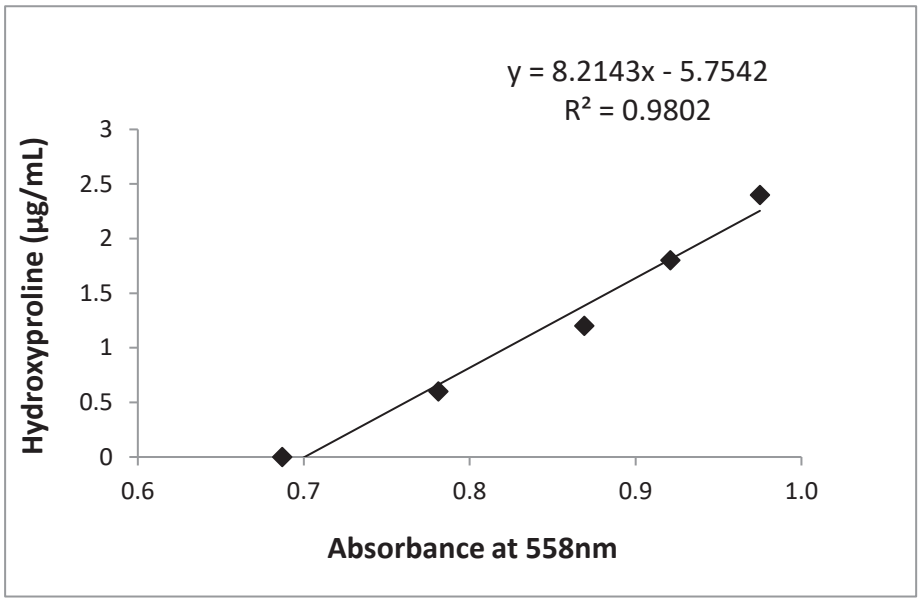

Figure 13. Hydroxyproline standard curve of the 6 lamb meat samples

3.5.3 Conclusion

The total collagen value obtained when determining soluble and insoluble collagen are in agreement with the value obtained from total collagen procedure and are within the range of values reported in the previous studies on this muscle. Also, the soluble collagen value obtained is in agreement with the range of values of the same muscle reported in other studies. Therefore, it can be confirmed that the established and modified method that determine soluble and insoluble collagen as detailed final method utilised in section 3.7 is repeatable and reliable and can be used to determine soluble, insoluble and total collagen concentration in other meat samples provided that the value obtained are the average of replicate measurements.

3.6 Further considerations

The established method successfully determined soluble, insoluble and total collagen in meat sample but the time required to complete one set of experiment is long which is partly due to 24 hours being required to complete acid hydrolysis. Further improvements of the method to reduce the hydrolysis time could minimize the time required to complete one batch of samples.

Neutralization of supernatant and sediments with 6M NaOH is also a time consuming process. Further development of the method by establishing a hydrolysis method that can reduce evaporative losses and establish the volume of NaOH that will increase the

pH of hydrolysates to about 6 could minimize without having to take into account of the volume of each sample could reduce the time required for each batch of samples. This should also take into consideration the sensitivity of the results to slight variations in pH by running the method with a single sample which has the pH after hydrolysis adjusted to values at and around pH 6.

The method can be further developed by the use of ionic strength solution at pH 5.6 to homogenize the samples before heating at $80^{O}C$ for 75 minutes. Ringer's solution, NaCl and KCl have been used to homogenized the meat samples (Latorre et al., 2016) and Ringers solution has been suggested by Hill (1966) to be more effective in weakening the intermolecular forces of collagen than distilled water.

There is a need to consider the effect of cooking time and temperature on hydroxyproline recovery and finding the ideal cooking temperature and time and establish if cooking time and temperature alters the results. Also, there is a need to consider the minimization of variability since the coefficient of variation is disturbingly high and highly variable (Table 11).

3.7 Final method utilised

3.7.1 Preparation of buffer solution

Buffer solution of pH value 6.0 was prepared by dissolving 15 g of citric acid monohydrate ($C_6H_8O_7.H_2O$), 7.5 g NaOH and 27.15 g of sodium acetate ($CH3COONa.3H_2O$) in about 250 mL of water. 145 mLs of 1-propanol was added and the pH was checked with pH meter and adjusted with 6M HCL. The solution was transferred to 500 mL volumetric flask and made up to volume with water and stored at $5^{o}C$.

3.7.2 Preparation of oxidant solution

The oxidant solution was prepared by dissolving 0.564 g of Chloramine T (the sodium salt of p – toluene sulfon-chloramide) in 40 mL of buffer solution. Due to its lack of stability it was prepared immediately before use (Edwards & O'Brien, 1980).

3.7.3 Preparation of colour reagent solution (Ehrlich's reagent).

The colour reagent was prepared by dissolving 5 g of p-Dimethylamino-benzaldehyde in 13 mL perchloric acid 70% (S.G. 1.54) followed by addition of 33.2 mL of 2- propanol with stirring. The colour reagent solution is stable for 1 hour (Edwards & O'Brien, 1980) therefore, it was prepared immediately before use.

3.7.4 Preparation of standard solution

Standard stock solution was prepared by dissolving 0.06 g of hydroxyproline (trans-4-Hydroxy-L-proline) in a beaker containing about 50 mL water. The solution was poured into 100 mL volumetric flask and diluted to volume with water. In every set of determination 2 mL of stock solution was then diluted to 200 mL with RO water. Working solution that contain 0.6, 1.2, 1.8 and 2.4 µg hydroxyproline per mL respectively was prepared by pipetting 5, 10, 15 and 20 mL portions of intermediate solution into 50 mL volumetric flasks and each diluted to volume with RO water. Exactly 2 mL of each concentration was pipetted into Pyrex test tube followed by oxidation and addition of Ehrlich's colour reagent in every set of determination of soluble and insoluble collagen in meat sample (see section 3.7.5).

3.7.5 The method for determination of heat soluble, insoluble and total collagen in lamb meat

Briefly, a subsample of the minced raw meat from the loin was freeze-dried. The sample was weighed before and after freeze drying so that the collagen concentration could be expressed on a whole tissue weight basis. The freeze-dried sample was broken up with mortar and pestle and 0.5 g replicates weighed into a 20 mL Kimax test tube. Ten (10) mL of distilled water was added and the sample was heated at 80°C for 75 minutes, with stirring every 15 minutes including at 75 minute (Archile-Contreras et al., 2010). The homogenates were centrifuged at 4000 rpm (3270*g*; Heraeus Multifuge 1S-R centrifuge, Thermo Fisher Scientific, Germany) for 10 minutes and supernatant collected. The sediment was washed with 5 mL of RO water and centrifuging was performed again at 4000rpm (3270*g*) for 10 minutes. The supernatant and sediments were collected into separate Kimax tubes. Pooled supernatants and sediments was hydrolysed separately with 20 mL of 6M HCl for 24 hours in an oven at 110°C (Zhao et al., 2015) and neutralized with 6 M NaOH to pH about 6.0 (Latorre et al., 2016). The volume of supernatant and

sediment hydrolysate and NaOH was recorded. Then 1 mL of each supernatant and sediment hydrolysate was diluted to 20 mL with RO water.

Hydroxyproline concentration of each supernatant and sediment was determined using the modification of (Archile-Contreras et al., 2010; Bergman & Loxley, 1963; Edwards & O'Brien, 1980; Zhao et al., 2015) as follows: 2 mL of each supernatant and sediment hydrolysates was pipetted into Pyrex test tube. 2 mL of working standard solution was also pipetted into test tubes and 2 mL of water was pipetted into test tube as a blank. Only the volume of oxidant solution required was prepared at this stage due to its instability. One (1 mL) of buffered chloramine T oxidant reagent was added into each tube and tubes were shaken and let stand for 25 minutes at room temperature. During this reaction period the colour reagent was prepared.

After the oxidation reaction period, 1 mL of colour reagent was added and mixed thoroughly and each tube was capped with aluminium foil. Immediately the tubes were placed in 60^{o}C water bath for exactly 20 minutes during which the red chromophore developed.

The tubes were cooled in the plastic beaker containing ice water for 5 minutes. The content of the tube was poured into 4 mL glass cells (Cuvette). The absorbance of each tube contents was read immediately at 558 nm in a spectrophotometer (Genesys 10 Bio, Thermo Electron Corporation (UV-160A, UV visible recording spectrophotometer, Shimadzu, Japan) against distilled water as reference sample. The standard curve was produced by plotting the scatter plot of hydroxyproline (µg/mL) against absorbance of the standard solution. Hydroxyproline concentration of the supernatant and sediment was estimated from the linear equation of the standard curve and the values were recorded. Soluble collagen concentration (supernatant) and insoluble collagen (sediment) was calculated by assuming that collagen weighed 7.25 times the measured hydroxyproline value and expressed as g of collagen per 100 g of fresh weight (Palka, 1999). Total collagen in g per 100 g of fresh weight was calculated from the sum of soluble (supernatant) and insoluble (sediment) collagen of respective meat sample.

Chapter 4. Meat Quality of Sheep Slaughtered at 5, 8 and 14 Months of Age

4.1 Introduction

New Zealand contributes approximately 47% of the total world sheep meat exports and 95% of sheep meat produced is exported (Morris, 2009). The increase in global population is likely to increase the demand for sources of protein including meat (Godfray et al., 2010). Due to a limited production capacity determined by land area, it is likely the focus for New Zealand will be top-quality and high value meat products.

In New Zealand, lamb is considered the highest quality sheep meat products while hogget is a downgraded product considered to be of poorer quality (Wiese et al., 2005; Young & Lim, 2001). Studies examining the meat quality of lamb and sheep meat from animals slaughtered at different ages have reported an increase in redness, a decline in lightness and an increase in shear force in mutton compared to lamb meat (Bouton et al., 1978; Gardner et al., 2007; Hopkins et al., 2007; Jeremiah et al., 1998; Pethick et al., 2005; Warner et al., 2007). The shear force of lamb meat was observed to increase as slaughter age increased from 0 to 12 months of age (Young et al., 1993). However, sensory studies in Australia have shown the acceptability of meat from animals classified as hogget which may mean the penalty placed on hogget carcasses in New Zealand is not validated (Wiese et al., 2005).

The aim of this study was to use objective measurements of meat quality to investigate the differences in meat quality between sheep slaughtered at 5, 8 and 14 months of age and hence, elucidate the possibility of extending lamb meat classification to include older animals than currently classified.

4.2 Materials and methods

Onfarm sheep management, slaughtering procedure, carcass measurement and meat quality analysis of the samples for each slaughter group was undertaken following method described by (Schreurs et al., 2013) as per the method detailed in Chapter 2.

Any raw loin tissue that remained after the meat quality analysis was trimmed of fat and visual connective tissue (visual epimysium), finely minced (Kenwood MG450, 3 mm

hole-plate), vacuum-packed and frozen for the subsequent assessment of intramuscular fat using Soxhelet ether extraction method (AOAC 911.36) and collagen assay as per chapter 3.7 on final method of measurement of soluble and insoluble collagen in meat sample.

The liveweight, slaughter age, carcass weight, GR, leg yield, loin yield, shoulder yield, lean meat yield, intramuscular fat, soluble collagen, insoluble collagen, total collagen and meat quality measurements were analysed using general linear models (PROC GLM, SAS, v. 9.4) with slaughter age treatment as the fixed effect. The model with peak force, initial yield, work done, intramuscular fat, insoluble collagen, soluble collagen and total collagen was fitted to establish their relationship on affecting meat shear force. Forward selection stepwise regression analysis for peak force was performed to establish the best predictor of shear force amoung intramuscular fat, soluble collagen, insoluble collagen, express juice, myofibrillar fragmentation index and sarcomere length. Hydroxyproline standard curve was produced for every day of determination using excel scatter plot to assertain the hydroxyproline concentration of meat samples.

4.3 Results

4.3.1 Sheep live weights and carcass characteristics

Actual slaughter age was 5.1 ± 0.05 months, 8.2 ± 0.05 months and 14.5 ± 0.05 months ($P<0.001$). In the 14.5 group 6 out of 20 sheep were classified as mutton (having more than two permanent incisors in a wear) according to New Zealand sheep carcass classification. Weight at 29th September 2015 (docking) ($P=0.002$), 2nd November 2015, 3rd December 2015, 15th January 2016 and at 19th January 2016 ($P<0.001$) were heavier for lamb slaughtered at 5 months but similar in lambs at 8 and 14 months (Figure 14). Final live weight was greater at 14 months, lower at 5 months and intermediate at 8 months ($P<0.001$; Table 12; Figure 14). Carcass weight ($P<0.001$), dressing out percentage ($P=0.002$), GR soft tissue depth ($P<0.001$) and intramuscular fat ($P=0.003$) were greater in the sheep slaughtered at 14 months of age but similar in lambs at 5 and 8 months of age (Table 12). Leg yield ($P<0.001$), loin yield ($P<0.01$) and total lean meat yield ($P=0.018$) was lower in 14month old sheep but similar in the lambs slaughtered at 5 and 8 months of age (Table 12). Shoulder yield was not affected by slaughter age ($P=0.18$; Table 12).

Table 12: Final on-farm live weight, actual slaughter age, carcass weight, dressing out percentage, GR soft tissue depth, leg yield, loin yield, shoulder yield, lean meat yield and intramuscular fat for 5, 8 and 14 months old Romney rams. Values are least square means ± standard error of the mean.

	Age at slaughter (months)			P- value
	5	8	14	
Birth weight (kg)[1]	6.19 ± 0.25	5.94 ± 0.25	6.04 ± 0.25	0.779
Final live weight (kg)[1]	42.1 ± 1.0[c]	45.0 ±1.0[b]	61.6 ± 1.0[a]	**<0.001**
Actual slaughter age (months)[1]	5.1 ± 0.05[c]	8.2 ± 0.05[b]	14.5 ± 0.05[a]	**<0.001**
Carcass weight (kg)[1]	16.8 ± 0.6[b]	18.2 ± 0.6[b]	28.3 ± 0.6[a]	**<0.001**
Dressing out (%)[1]	40.0 ± 1.3 [b]	40.7 ± 1.3[b]	46.2 ± 1.3[a]	**0.002**
GR (mm)[2]	4.01 ± 0.76[b]	5.64 ± 0.80[b]	11.3 ± 0.80[a]	**<0.001**
Leg Yield (%)[2]	22.7 ± 0.8[a]	22.3 ± 0.8[a]	18.4 ± 0.8[b]	**<0.001**
Loin Yield (%)[2]	14.9 ± 0.6[a]	15.0 ± 0.6[a]	12.5 ± 0.6[b]	**0.005**
Shoulder Yield (%)[2]	16.7 ± 0.2	17.1 ± 0.2	17.3 ± 0.2	0.180
Total lean meat yield (%)[2]	54.3 ± 0.8[a]	54.4 ± 0.8[a]	51.6 ± 0.8[b]	**0.018**
Intramuscular fat (% whole muscle)[3]	2.5 ± 0.3[b]	3.0 ± 0.3[b]	3.8 ± 0.3[a]	**0.003**

[a,b,c] Within rows, values without superscript letters or with common superscripts letter are not significantly different at the P<0.05 level.

[1]20 Lambs per treatment.

[2] Sample size (n) for both 8 and 14 months was 18 per treatment. GR and lean meat yield were measured using the VIAscan® system at the Alliance meat processors in Dannevirke, New Zealand.

[3] 10 samples per treatment.

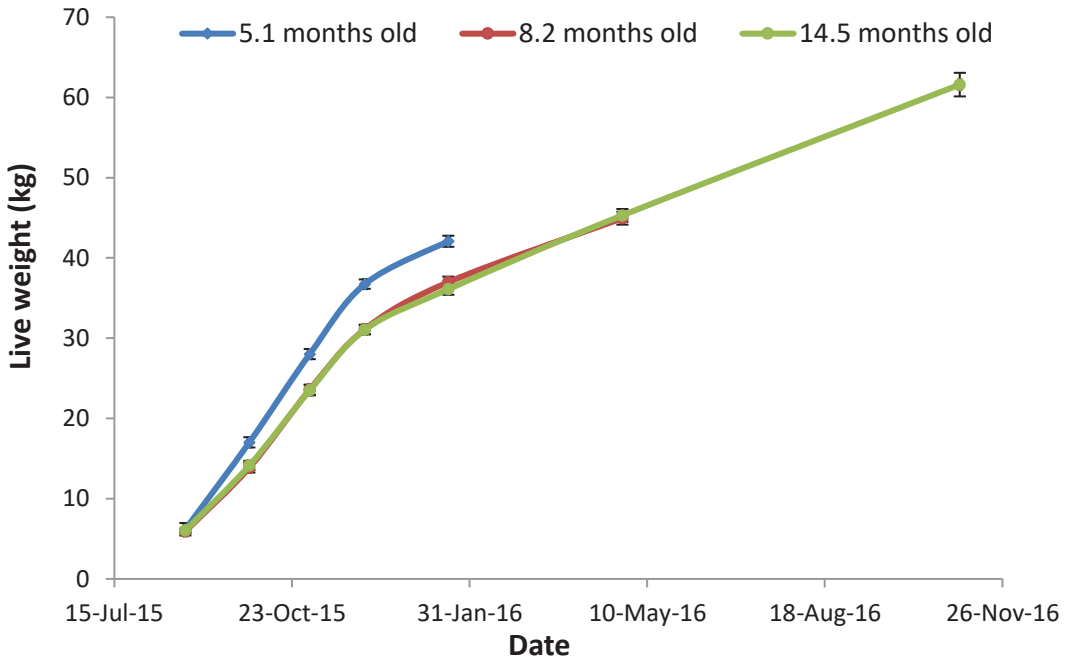

Figure 14: On-farm live weight for actual slaughter age 5.1, 8.2 and 14.5 months old Romney rams. Points are least squares means with standard error bars.

4.3.2 Objective meat quality characteristics

The pH was higher in the lamb slaughtered at 5 months old but the same for the sheep slaughtered at 8 and 14 months of age ($P<0.001$; Table 13). Lambs slaughtered at 5 months of age had meat that was darker than 8 and 14 months ($P=0.045$; Table 13). The meat from the sheep slaughtered at 14 months of age was redder and yellower compared to the lambs slaughtered at 5 and 8 months of age ($P<0.001$).

Drip loss after 24 and 48 hours was greater in lamb slaughtered at 8 compared to 5 and 14 months of age ($P=0.006$; Table 13). Thaw loss was greater at 14 months compared to 5 and 8 months of age ($P<0.001$; Table 13). Expressed juice ($P=0.101$) and cooking loss ($P=0.181$) were not affected by slaughter age (Table 13).

Shear force was lower in meat from 14 months old sheep and greater in meat from lambs slaughtered at 8 months old ($P<0.001$; Table 13). Total work done was greater in meat from sheep slaughtered at 8 months compared to 5 and 14 months of age ($P=0.016$). Sarcomere length was shorter in sheep slaughtered at 14 months of age ($P<0.001$; Table 13). Initial yield ($P=0.132$) and myofibrillar fragmentation index ($P=0.255$) was not affected by slaughter age (Table 13).

4.3.3 Total collagen, soluble collagen and insoluble collagen

The meat from sheep slaughtered at 14 months old had lower total collagen compared to meat from lamb slaughtered at 5 and 8 months ($P=0.020$; Table 13). Soluble collagen was greater in lamb slaughtered at 5 months compared to lamb slaughtered at 8 months and sheep slaughtered at 14 month old ($P=0.008$; Table 13). Insoluble collagen was not affected by slaughter age ($P=0.118$; Table 13).

Table 13. Meat quality attributes of *Longissimus lumborum* of Romney ram slaughtered at 5, 8 and 14 months old assessed using objective measures on twenty samples per age treatment with each sample coming from a different animal. Values are the least square mean ± standard error of the mean.

Meat quality attribute	Age at slaughter (month)			P-value
	5	8	14	
pH	5.54 ± 0.02[a]	5.42 ± 0.02[b]	5.45 ± 0.02[b]	**<0.001**
Meat colour				
L* (Lightness)[1]	37.76 ± 0.45[b]	39.09 ± 0.45[a]	39.24 ± 0.45[a]	**0.045**
a* (Redness)[1]	13.19 ± 0.22[b]	13.34 ± 0.22[b]	15.07 ± 0.22[a]	**<0.001**
b* (Yellowness)[1]	3.59 ± 0.15[b]	3.33 ± 0.15[b]	4.53 ± 0.15[a]	**<0.001**
Water holding capacity				
Drip loss after 24h (%)[1]	3.6 ± 0.4[b]	5.7 ± 0.4[a]	4.6 ± 0.4[ab]	**0.006**
Drip loss after 48h (%)[1]	5.2 ± 0.5[b]	8.0 ± 0.5[a]	6.0 ± 0.5[b]	**0.001**
Expressed juice (cm^2/g)[1]	36.7 ± 0.8	35.1 ± 0.8	34.2 ± 0.8	0.101
Thaw loss (%)[1]	0.34 ± 0.04[b]	0.41 ± 0.04[b]	0.63 ± 0.04[a]	**<0.001**
Cooking loss (%)[1]	30.8 ± 0.5	30.8 ± 0.5	29.7 ± 0.5	0.181
Tenderness				
Shear force (kgF)[1]	6.87 ± 0.35[b]	7.98 ± 0.35[a]	5.72 ± 0.35[c]	**<0.001**
Total work (kgF)[1]	1.96 ± 0.09[b]	2.25 ± 0.09[a]	1.88 ± 0.09[b]	**0.016**
Initial Yield (kgF)[1]	2.95 ± 0.13	3.10 ± 0.13	2.72 ± 0.13	0.132
Myofibrillar fragmentation index (%)[1]	95.6 ± 1.0	93.7 ± 1.0	96.0 ± 1.0	0.255
Sarcomere length (μm)[1]	1.84 ± 0.01[a]	1.87 ± 0.01[a]	1.69 ± 0.01[b]	**<0.001**
Total collagen (g/100g fresh wt)[2]	0.96 ± 0.09[a]	0.87 ± 0.09[a]	0.62 ± 0.09[b]	**0.020**
Soluble collagen (g/100g fresh wt)[2]	0.42 ± 0.05[a]	0.31 ± 0.05[ab]	0.19 ± 0.05[b]	**0.008**
Soluble collagen (%)[2]	43.1 ± 4.3[a]	32.2 ± 4.3[ab]	30.6 ± 4.3[b]	**0.007**
Insoluble collagen (g/100g fresh wt)[2]	0.54 ± 0.05	0.56 ± 0.05	0.42 ± 0.05	0.118

[abc] Within rows, values without superscript letters or with common superscripts letter are not significantly different at the P<0.05 level.

[1]20 samples per treatment

[2]15 samples per treatment

4.3.4 Interrelationships

The Pearson correlations showed that peak shear force was not correlated with insoluble collagen, soluble collagen or intramuscular fat (Table 14).

Table 14: Pearson correlation coefficients and P-values of peak force (kgF), initial yield (kgF), work done (kgF), intramuscular fat (%), insoluble collagen (g/100g fresh weight), soluble collagen (g/100g fresh weight) and total collagen (g/100g fresh weight) of Romney rams slaughtered at 5, 8 and 14 months old.

	Peak[1] force	Initial[1] yield	Work[1] done	IMF[2]	Insoluble collagen[3]	Soluble collagen[3]	Total collagen[3]
Peak force	1	0.29	0.87	-0.08	0.02	0.05	0.04
P-value		**0.04**	**<0.001**	0.67	0.91	0.75	0.79
Initial yield	0.29	1	0.14	-0.09	0.15	0.21	0.21
P-value	**0.04**		0.34	0.61	0.35	0.18	0.19
Work done	0.87	0.14	1	-0.03	-0.09	-0.09	-0.10
P-value	**<0.001**	0.34		0.89	0.55	0.56	0.51
IMF	-0.08	-0.09	-0.03	1	-0.20	-0.14	-0.19
P-value	0.67	0.61	0.89		0.38	0.54	0.40
Insoluble collagen	0.02	0.15	-0.09	-0.20	1	0.53	0.86
P-value	0.91	0.35	0.55	0.38		**<0.001**	**<0.001**
Soluble collagen	0.05	0.21	-0.09	-0.14	0.53	1	0.88
P-value	0.75	0.18	0.56	0.54	**<0.001**		**<0.001**
Total collagen	0.04	0.21	-0.10	-0.19	0.86	0.88	1
P-value	0.79	0.19	0.51	0.40	**<0.001**	**<0.001**	

[1] 20 Samples per treatment
[2] 10 Samples per treatment
[3] 14 Samples per treatment

The forward selection stepwise regression of myofibrillar fragmentation index, soluble collagen, sarcomere length, intramuscular fat, expressed juice and insoluble collagen on peak shear force showed that the combination of these variables explained a maximum of 19% of the variation in shear force values. Soluble collagen explained 5% and total collagen explaining 9% of the variation in peak shear force values however, none of the variables were considered to be significant in explaining variation in shear force values (Table 15).

Table 15. Step wise regression using forward selection to consider the influence of soluble collagen (g/100g fresh weight), myofibrillar fragmentation index (%), insoluble collagen (g/100g fresh weight), sarcomere length (µm), express juice (cm^2/g) and intramuscular fat (% whole muscle) on peak shear force (kgF).

Step	Variable	Partial R^2 (%)	Model R^2 (%)	Pr > F
1	Soluble collagen	5.1	5.1	0.33
2	Intramuscular fat	2.2	7.2	0.52
3	Expressed juice	2.8	10.1	0.48
4	Total collagen	9.0	19.1	0.20
5	Sarcomere length	0.1	19.2	0.94

4.4 Discussion

This study evaluated the quality of sheep meat when the slaughter age was 5, 8 and 14 months of age. The objective was to understand if the current lamb classification could be extended to older animals which are currently classified as hogget once permanent incisors are in wear at 12-14 months of age.

The study did not intend to look at growth path but the lambs in the different treatments had different growth path although they were with similar birth weight (Figure 14). This is a potentially confounding aspect of the study however, it does reflect farm practice where faster grown lambs are sent for slaughter at a younger age and slower grown lambs will be older. The heavier final live weight as the slaughter age progressed was expected and contributed to higher carcass weights in the older sheep. Given the large differences in age it was not possible to control for carcass weight at slaughter and this could be further confounding the results. Further research on growth pattern of the sheep in relation to meat quality would help to understand the confounding effects as studies with sheep have shown changes in meat quality with carcass size (Santos-Silva et al., 2002).

Lean meat yield was lower for 14-month-old sheep compared to the lambs slaughtered at 5 and 8 months of age. Lean meat yield will decline as the proportion of fat in the carcass increases (Semts et al., 1982) so, the increased carcass fat in 14 month old sheep, as evidenced by the greater GR and intramuscular fat, is likely to be the reason for the lower lean meat yields.

Lamb meat colour assists the consumer's decision to purchase displayed meat basing on the experienced bright red colour of fresh meat (Pethick et al., 2005). Meat colour from young animals is lighter than that from mature animals or animals that have been growing for long periods of time (Hopkins et al., 2007; Jacob et al., 2007) so, it was surprising that the lambs slaughtered at 5 months old tended to have darker meat compared to the sheep slaughtered at 8 and 14 months of age. However, the lightness values differ by no more than 2 units and so, this difference is unlikely to be detectable by visual comparison. It is possible that greater fat in the muscle contributed to higher lightness values when measured by chromameter and is also likely to be the reason for the higher yellowness values in the meat from 14-month-old sheep. The higher redness values in the sheep slaughtered at 14 months of age is attributed to the increase in myoglobin concentration that occurs in the muscle as animals get older (Gardner et al., 2007; Hopkins et al., 2007; Warner et al., 2007).

Meat with a lower water holding capacity is associated with unpleasant appearance during packaging and a potential drying of the cooked meat product because moisture has been lost from the meat (Otto et al., 2004). Although the study did not consider subjective meat quality assessment, the higher drip loss in lamb slaughtered at 8 months and higher thaw loss in 14 month could reduce the juiciness of cooked meat. Meat juiciness is partly associated with the ability of the meat to hold onto moisture and release it during eating. Joo et al. (2013) reported a positive relationship between juiciness and intramuscular fat. Therefore, the juiciness of cooked meat upon chewing from sheep slaughtered at 14 months old could be sustained due to lubrication effect of greater level of intramuscular fat (Dryden & Maechello, 1970; Horcada et al., 1998). Moreover, a significant decline in *Longissimus lumborum* juiciness is reported in sheep slaughtered when they are older than 20 months of age (Pethick et al., 2005).

It is known that meat from older sheep is tougher due to the increased level of cross-linking of collagen that reduces solubility of collagen during cooking and making meat more difficult to chew with eating (Light et al., 1985; McCormick, 1994; Weston et al., 2002). Greater collagen concentration which was highly soluble in lamb slaughtered at 5 months old agrees with the values reported in the literature for *Longissimus* muscle and other sheep muscles (Camacho et al., 2016; Veiseth et al., 2004; Young et al., 1993).

Slaughter age did not affect insoluble collagen concentration and the effect of slaughter age appears to be altering the proportion of the collagen that is soluble with cooking.

Although the sarcomere length was shorter in the sheep slaughtered at 14 months of age, it was not having an influence on the shear force which would be expected to be greater with a shorter sarcomere (Wheeler & Koohmaraie, 1999) which is difficult to explain in the current study however, it could be attributed to unknown differences in slaughter conditions.

The lower soluble collagen in 14 months old sheep was expected to increase the shear force value. Young et al. (1993) measured tenderness objectively using shear force in sheep slaughtered at 0, 42, 70, 274 and 365 days and reported the lowest shear force for meat from sheep slaughtered at 0 days of age and increasing shear force value as slaughter age advanced with the major change occurring between day 0 and 42 of age. Unexpectedly, the meat from 14 month old sheep had lower shear force than the meat from 5 and 8 month old lambs. This suggests that other determinants of meat tenderness have reduced the shear force at 14 months old and it is likely that the higher intramuscular fat was having a role. Increased intramuscular fat has been associated with improved tenderness and eating scores for lamb meat (Dryden & Maechello, 1970; Hopkins et al., 2006). Intramuscular fat can improve tenderness and reduce shear force values by creating a dilution of the muscle fibres. Muscle fibres contain the contractile apparatus and collagen tissues that are associated with meat toughness (Young et al., 1993). By having intramuscular fat in the muscle there are less muscle fibres in a unit of area and so the toughening effect of the muscle fibres is reduced (Nishimura et al., 1999). Also, intramuscular fat has a lubricating effect and with cooking the melted intramuscular fat assists in reducing the force required to chew the meat (Blumer, 1963; Wood, 1985).

The greater shear force and greater total work done at 8 months of age could be attributed to less intramuscular fat and less collagen solubility. The lack of correlation of peak force and collagen and intramuscular fat is not surprising given the between animal variation that was observed in the measurements and the relatively small data set. Soluble collagen explaining 5% and total collagen explaining 9% of the variation in

peak shear force signifies that collagen is likely to be an important contributor to tenderness in sheep meat when there is multiple factors that could be of influence. Together, the results from the slaughter age treatments and stepwise regression analysis suggest that shear force values are to some extent explained by the solubility of collagen when sheep do not have substantial intramuscular fat to ameliorate potential tenderness issues associated with collagen.

4.5 Conclusion

Tenderness as measured by Warner Bratzler shear force was greater in meat from sheep that were 14 months old at slaughter than meat from lambs slaughtered at 5 and 8 months of age and this was attributed to greater level of intramuscular fat. This suggests that meat from hogget classified carcasses (2 permanent incisors in wear) should not be downgraded on the basis of tenderness if sufficient intramuscular fat is present. In this study the differences in age were confounded with differences in carcass weight and possibly with growth rates up to the time of slaughter. Further study is needed to consider other eating qualities of sheep meat from older animals because some consumers favour the mild flavour of younger lambs unlike old sheep (Hopkins et al., 2006).

Chapter 5. General Discussion

5.1 Collagen method development

This study developed an assay to measure collagen concentration and solubility. The objective was to be able to measure collagen reliably so that it could be utilised as a measure of meat quality of sheep slaughtered at 5, 8 and 14 months of age. The results were then used to compare shear force to collagen concentration and solubility to understand if the difference in meat tenderness is associated with collagen concentration or collagen solubility. Collagen method worked well and total collagen and soluble collagen values are in agreement with the values reported in the literature (Ablikim et al., 2016; Camacho et al., 2016; Field et al., 1996; Maiorano et al., 1993; Starkey et al., 2016; Tschirhart-Hoelscher et al., 2006; Young et al., 1993; Young & Braggins, 1993).

Standard curves were prepared for each day that the samples from the slaughter age trial were analysed (Appendix 3). Looking at the standard curves suggests that repeatability across days could be improved however; pinpointing the source of the variation is difficult as many factors could be of influence and the variation between animals also can affect the ability to observe repeatability in this study (Appendix 1 and 2). A useful mechanism to understand the methodology variation would be to run a single meat sample through every batch of analyses and compare the results between batches.

The time required to complete one batch of samples is long due to 24 hours required to complete acid hydrolysis. Therefore, further development to reduce the time required for analysis is warranted. Possible considerations could include assessing the effect of length of time for acid hydrolysis on the variability of measurements, acid hydrolysis with an increased heating temperature to 120^{o}C in a pressure vessel for 2-4 hours (Edwards & O'Brien, 1980) or alkali hydrolysis (2M NaOH) (Hofman et al., 2011) .

Neutralization of hydrolysates was achieved by adding NaOH which also took a long time to accurately get the pH of hydrolysate to pH 6. Therefore, further consideration on the effect of pH and neutralization on the accuracy and repeatability of results is justified. The Beckman automatic titrator could also be of consideration for reducing the time required to neutralize the samples (Herring et al., 1967). Moreover, because the dilution factor was 20 it could be useful to consider how sensitive was the analysis to pH and consider whether neutralisation is necessary.

The method developed used RO water to homogenise the sample before heating at 80^{o}C for 75 minutes but there is a suggestion that ionic solution at pH 5.6 could improve the weakening of intermolecular forces of collagen than distilled water (Hill, 1966; Latorre et al., 2016). Therefore, further development of the method should consider the use of NaCl or KCl solution or alternatively, Ringer's solution at pH 5.6 for the cooking step of the methodology.

5.2 Effect of slaughter age on meat quality

This study evaluated the quality of sheep meat when the slaughter age was 5, 8 and 14 months of age. Total collagen was higher in lamb slaughtered at 5 months and the

solubility of the collagen was also higher which is likely to be due to less collagen crosslinking in young animals (Light et al., 1985; McCormick, 1994; Weston et al., 2002). Although the lower shear force values for the 14 month old rams were unexpected they are explained to some extent by the higher intramuscular fat concentration of the meat. Meat from lamb slaughtered at 8 months had a higher shear force partly due to reduced collagen solubility (Young et al., 1993) and low intramuscular fat (Blumer, 1963; Nishimura et al., 1999; Wood, 1985).

5.3 Relationship between shear force and collagen concentration

Collagen concentration was higher in lamb slaughtered at 5 months and 8 months but lower in sheep slaughtered at 14 months old which is inconsistent with studies by Young et al. (1993) which observed higher collagen concentration in the *semimembranosus* and *gluteus* muscle of lambs at birth which then declined as the animal grows up to 1 month of age and then remains almost static up to one year of age. However, there was no significant correlation of shear force with total collagen concentration which is suggesting that the solubility rather than total concentration of collagen may be influencing the shear force results.

5.4 Relationship between shear force and collagen solubility

Collagen solubility is considered as a potentially major determinant of meat tenderness (Purchas et al., 1989; Weston et al., 2002; Young & Braggins, 1993). Meat with lower soluble collagen became more resistance to cutting across the muscle fibre due to increased complexity of muscle fibres as a result of greater collagen cross-link leading to greater shear force (Light et al., 1985; McCormick, 1994; Weston et al., 2002).

The combination of the results looking at the mean values across the treatments, correlations and stepwise regression suggest that variation in tenderness can be substantially explained by collagen solubility however, in situations where collagen solubility is low, the intramuscular fat has a role in compensating and overcoming tenderness issues especially in older sheep. To verify and substantiate this result, the measurement of collagen, intramuscular fat and shear force on a larger set of animals is required along with validation across multiple studies.

5.5. Conclusion

The method developed to measure collagen in meat worked well and the values of total collagen, insoluble collagen and soluble collagen were in agreement with the values reported in the literature. However, the method could undergo further development especially to understand what is creating variation between batches. This information is vital when needing to analyses multiple samples associated with animal replication which requires several days to do the analyses.

The contributory effect of collagen and intramuscular fat to variation in shear force could not be fully elucidated due to the low numbers of animals utilised in this study. However, there is some evidence that collagen solubility is a contributor to tenderness, especially when intramuscular fat is low. The establishment of the collagen assay means that further studies investigating the quality of lamb can include the measurement of collagen concentration and solubility. It is envisioned that the results from the current study will provide the basis of a database collating information on collagen and intramuscular fat in muscle of lambs across many studies occurring at Massey University. This will allow for a detailed analysis of the influence that both collagen and intramuscular fat have on the tenderness of lamb, with greater statistical power.

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Appendix

Appendix 1. Soluble, insoluble and total collagen of 14 samples per treatment in two replicates for 5, 8 and 14 month old Romney rams

Sample ID	Day of analysis	Age	Replicates	Collagen concentration (g/100g fresh weight)				
				Sediment (insoluble)	Average	Supernatant (soluble)	Average	Total collagen
101851	1	5	A	0.55		0.09		
101851	1	5	B	0.00	0.27	0.15	0.12	0.39
101852	1	5	A	0.56		0.00		
101852	1	5	B	0.45	0.50	0.61	0.31	0.81
101853	2	5	A	0.54		1.15		
101853	2	5	B	0.37	0.45	0.18	0.67	1.12
101854	2	5	A	0.74		0.00		
101854	2	5	B	1.01	0.87	0.50	0.25	1.12
101855	3	5	A	0.29		0.67		
101855	3	5	B	0.94	0.62	0.78	0.73	1.34
101856	3	5	A	0.85		0.28		
101856	3	5	B	0.86	0.85	0.83	0.55	1.41
101859	4	5	A	0.42		0.70		
101859	4	5	B	0.41	0.41	0.13	0.41	0.83
101860	4	5	A	0.55		0.36		
101860	4	5	B	0.40	0.48	0.50	0.43	0.91
101861	5	5	A	0.49		0.45		
101861	5	5	B	0.54	0.52	0.22	0.34	0.85
101862	5	5	A	0.48		0.10		
101862	5	5	B	0.62	0.55	0.44	0.27	0.82
101865	6	5	A	0.48		0.28		
101865	6	5	B	0.47	0.48	0.43	0.36	0.83
101866	6	5	A	0.47		0.50		

101866	6	5	B	0.59	0.53	0.48	0.49	1.02
101869	7	5	A	0.56		0.44		
101869	7	5	B	0.43	0.50	0.66	0.55	1.05
101870	7	5	A	0.46		0.29		
101870	7	5	B	0.55	0.50	0.56	0.42	0.93
90007	1	8	A	0.22		0.35		
90007	1	8	B	0.63	0.43	0.00	0.17	0.60
103534	1	8	A	0.32		0.00		
103534	1	8	B	0.00	0.16	0.00	0.00	0.16
103535	2	8	A	0.83		1.00		
103535	2	8	B	0.33	0.58	0.00	0.50	1.08
103536	2	8	A	0.57		0.00		
103536	2	8	B	0.33	0.45	0.00	0.00	0.45
103537	3	8	A	0.84		0.39		
103537	3	8	B	0.94	0.89	0.44	0.41	1.30
103538	3	8	A	0.86		0.59		
103538	3	8	B	0.67	0.76	0.30	0.44	1.21
103541	4	8	A	0.90		0.59		
103541	4	8	B	0.95	0.92	0.70	0.64	1.56
103542	4	8	A	0.80		0.20		
103542	4	8	B	0.84	0.82	0.31	0.25	1.07
103543	5	8	A	0.56		0.09		
103543	5	8	B	0.54	0.55	0.29	0.19	0.74
103544	5	8	A	0.46		0.28		
103544	5	8	B	0.62	0.54	0.23	0.25	0.79
103547	6	8	A	0.34		0.43		
103547	6	8	B	0.33	0.34	0.45	0.44	0.78
103548	6	8	A	0.39		0.29		
103548	6	8	B	0.32	0.36	0.42	0.36	0.71
103551	7	8	A	0.78		0.30		

103551	7	8	B	0.72	0.75	0.33	0.31	1.06
103552	7	8	A	0.10		0.61		
103552	7	8	B	0.57	0.33	0.00	0.31	0.64
102785	1	14	A	0.21		0.00		
102785	1	14	B	0.26	0.24	0.00	0.00	0.24
102786	1	14	A	0.40		0.00		
102786	1	14	B	0.73	0.56	0.21	0.10	0.66
102787	2	14	A	0.42		0.00		
102787	2	14	B	0.31	0.36	0.00	0.00	0.36
102790	2	14	A	0.47		0.00		
102790	2	14	B	0.35	0.41	0.00	0.00	0.41
102791	3	14	A	0.18		0.00		
102791	3	14	B	0.10	0.14	0.00	0.00	0.14
102792	3	14	A	0.34		0.02		
102792	3	14	B	0.14	0.24	0.00	0.01	0.25
102795	4	14	A	0.44		0.39		
102795	4	14	B	0.41	0.42	0.24	0.32	0.74
102796	4	14	A	0.33		0.00		
102796	4	14	B	0.35	0.34	0.08	0.04	0.38
102797	5	14	A	0.42		0.07		
102797	5	14	B	0.34	0.38	0.34	0.20	0.58
102798	5	14	A	0.73		0.51		
102798	5	14	B	0.45	0.59	0.35	0.43	1.02
102801	6	14	A	0.49		0.41		
102801	6	14	B	0.55	0.52	0.79	0.60	1.12
102802	6	14	A	0.38		0.50		
102802	6	14	B	0.64	0.51	0.30	0.40	0.91
102805	7	14	A	0.69		0.27		
102805	7	14	B	0.32	0.50	0.28	0.27	0.78
102811	7	14	A	0.41		0.23		

102811	7	14	B	1.02	0.71	0.43	0.33	1.04

Appendix 2: Ranges, standard deviation (SD) and means of soluble and insoluble collagen of 14 samples per treatment in two replicates for 5, 8 and 14-month-old Romney rams.

	Insoluble (g/100g Fresh weight)			Soluble (g/100g Fresh weight)		
Age (months)	Range	SD	means	range	SD	means
5	0.27-0.87	0.16	0.54	0.12-0.73	0.17	0.42
8	0.16-0.89	0.23	0.56	0-0.64	0.18	0.31
14	0.14-0.71	0.17	0.42	0-0.60	0.20	0.19

cv = Coefficient of variation

sd = standard deviation

Appendix 3. Hydroxyproline standard curves for each day of analysis of samples from the slaughter age experiment.