The Development and Validation of a Probe to Measure Meat Quality Traits

Stephanie M. Fowler
Bachelor of Agriculture
Bachelor of Science (Hons)

A thesis submitted to Charles Sturt University, Wagga Wagga for the Doctor of Philosophy (PhD).

March 2015
# Contents

Figures ........................................................................................................................................... 7

Tables ............................................................................................................................................... 11

Published Papers ............................................................................................................................ 16

Abstract ........................................................................................................................................... 18

List of Abbreviations ....................................................................................................................... 20

1. **General Introduction** .................................................................................................................. 24

2. **Literature Review** ...................................................................................................................... 26

   2.1 Introduction ................................................................................................................................ 26

   2.2 Meat Quality ............................................................................................................................... 27

      2.2.1 Meat Tenderness .................................................................................................................. 27

      2.2.2 Flavour .................................................................................................................................. 28

      2.2.3 Juiciness ............................................................................................................................... 28

   2.3 Biochemical and Biophysical Structure of Meat ......................................................................... 28

      2.3.1 Topographical Muscle Anatomy ........................................................................................ 29

      2.3.2 Muscle Proteins .................................................................................................................... 31

         2.3.2.1. Myofibrillar Proteins ..................................................................................................... 31

         2.3.2.2 Cytoskeletal Proteins ..................................................................................................... 32

      2.3.3 Connective Tissue ................................................................................................................ 32

      2.3.4 Fatty Acids ........................................................................................................................... 33

   2.4 Changes to the Biochemical and Biophysical Properties of Meat .............................................. 36

      2.4.1 Immediate Post Mortem Changes ....................................................................................... 37

      2.4.2 Post Mortem Ageing Period ................................................................................................ 38

   2.5 Traditional Indicators of Meat Quality ....................................................................................... 40

      2.5.1 Shear Force Values .............................................................................................................. 40

      2.5.2 Sarcomere Length ............................................................................................................... 41

      2.5.3 pH Values ............................................................................................................................ 43
2.5.3.1 pH decline ........................................................................................................... 43
2.5.3.2 Ultimate pH value .............................................................................................. 44
2.5.4 Particle Size ......................................................................................................... 46
2.5.5 Histology .............................................................................................................. 46
2.5.6 Colour .................................................................................................................. 47
2.5.7 Water Holding Capacity ....................................................................................... 48
2.6 Technologies to Measure Meat Quality .................................................................... 49
2.7 Raman Spectroscopy .............................................................................................. 54
   2.7.1 Use of Raman Spectroscopy in Previous Meat Studies ...................................... 57
   2.7.2 Assignment of Bands .......................................................................................... 61
      2.7.2.1 Protein ........................................................................................................... 61
      2.7.2.2. Fatty Acids ................................................................................................. 64
2.7 Limitations of Current Literature ............................................................................ 65
2.8 Future Research ...................................................................................................... 66
3. Raman spectroscopy compared against traditional predictors of shear force in m.
   longissimus lumborum ............................................................................................... 67
   3.1 Introduction ............................................................................................................ 67
   3.2 Materials and Methods .......................................................................................... 68
      3.2.1 Samples ............................................................................................................ 68
      3.2.2 Raman Spectroscopy ....................................................................................... 68
      3.2.3 Traditional Indicators of Tenderness ................................................................. 69
      3.2.3.1 24 h Aged LL ............................................................................................ 69
      3.2.3.2 Day 5 Post Mortem Aged LL ....................................................................... 70
      3.2.4 Data Analysis .................................................................................................. 70
   3.3 Results ................................................................................................................... 71
      3.3.1 Raman Spectroscopy ....................................................................................... 71
      3.3.2 Traditional Indicators of Shear force ............................................................... 76
### 3.4 Discussion

- 3.4.1 Prediction of Shear force with Raman Spectroscopy
- 3.4.2 Prediction of Shear force with Traditional Indicators

### 3.5 Conclusion

### 4. Prediction of intact ovine *m. semimembranosus* shear force using Raman Spectroscopy

- 4.1 Introduction
- 4.2 Materials and Methods
  - 4.2.1 Samples
  - 4.2.2 Raman Spectroscopy
  - 4.2.3 Traditional Indicators of Tenderness
  - 4.2.4 Data Analysis
- 4.3 Results
  - 4.3.1 Raman Spectroscopy
  - 4.3.2 Traditional Indicators of Shear Force
- 4.4 Discussion
  - 4.4.1 Prediction of Shear force with Raman Spectroscopy
  - 4.4.2 Traditional Indicators and shear force variation
  - 4.4.3 Raman Band Assignments
- 4.5 Conclusion

### 5. Can confocal Raman microscopy help us understand variation in shear force?

- 5.1 Introduction
- 5.2 Materials and Methods
  - 5.2.1 Samples
  - 5.2.2 Raman Confocal Microscopy
  - 5.2.3 Traditional Indicators of Variation in Tenderness
  - 5.2.4 Statistical Analysis
5.3 Results.................................................................................................................................................. 111
  5.3.1 Traditional Indicators of Variation in Tenderness ............................................................................. 111
  5.3.2 Chemical Imaging ............................................................................................................................. 112
5.4 Discussion ............................................................................................................................................. 115
5.5 Conclusion ............................................................................................................................................. 118

6. Predicting meat quality traits of fresh intact ovine m. semi membranosus using a Raman spectroscopic hand held device .................................................................................................................. 119
  6.1 Introduction .......................................................................................................................................... 119
  6.2 Materials and Methodology .................................................................................................................. 120
    6.2.1 Samples ........................................................................................................................................... 120
    6.2.2 Raman Spectroscopy ....................................................................................................................... 121
    6.2.3 Traditional Indicators ....................................................................................................................... 122
    6.2.4 Data Analysis .................................................................................................................................... 123
  6.3 Results .................................................................................................................................................. 124
    6.3.1 Traditional Indicators of Meat Quality ............................................................................................... 124
    6.3.2 Raman Spectra ................................................................................................................................... 132
  6.4 Discussion ............................................................................................................................................. 135
    6.4.1 Traditional Indicators of Meat Quality ............................................................................................... 135
    6.4.2 Prediction of Tenderness ................................................................................................................... 135
    6.4.2 Prediction of pH values ..................................................................................................................... 137
    6.4.3 Prediction of Purge ........................................................................................................................... 143
    6.4.4 Prediction of L* Value ....................................................................................................................... 146
  6.5 Conclusion ............................................................................................................................................. 149

7. Changes to Raman Spectra and the prediction of eating quality indicators with the freezing and thawing of intact lamb m. semimembranosus ........................................................................................................... 150
  7.1 Introduction .......................................................................................................................................... 150
  7.2 Materials and Methods ......................................................................................................................... 151
    7.2.1 Samples .......................................................................................................................................... 151
7.2.2 Raman Spectroscopy .............................................................. 151
7.2.3 Traditional Indicators ......................................................... 152
7.2.4 Data Analysis ........................................................................ 153
7.3 Results ...................................................................................... 153
7.4 Discussion ................................................................................. 157
7.5 Conclusion ................................................................................ 160
8. Prediction of intramuscular fat content and major fatty acid groups of ovine m. longissimus lumborum using Raman Spectroscopy .................................................. 161
8.1 Introduction ................................................................................ 161
8.2 Materials and Methods ............................................................... 162
  8.2.1 Samples ................................................................................ 162
  8.2.2 Raman Spectroscopy ............................................................. 162
  8.2.3 Intramuscular Fat and Fatty Acid Analysis .............................. 163
  8.2.4 Data Analysis ........................................................................ 164
8.3 Results ...................................................................................... 165
  8.3.1 Intramuscular Fat and Fatty Acid Composition ....................... 165
  8.3.2 Raman Spectroscopy ............................................................. 170
8.4 Discussion ................................................................................. 177
  8.4.1 Intramuscular Fat and Fatty Acid Composition ....................... 177
  8.4.2 Prediction of Fatty Acid Composition ....................................... 179
  8.4.3 Fatty Acid Band Assignments ............................................... 181
8.5 Conclusion ................................................................................ 187
9.  General Discussion/ Conclusion ................................................... 188
10. Implications for Industry .............................................................. 190
11. References .................................................................................. 191
Figures

Figure 2.1. Illustration of the topographical anatomy of a skeletal muscle, showing the arrangement of the connective tissue layers and bundles of myofibres (Scanes, 2003). ...........29

Figure 2.2. Diagram of the organisation of skeletal muscle into myofibres within the sarcolemma, illustrating cross-striation of the myofibrils (Scanes, 2003)...............................30

Figure 2.3. Schematic diagram of the arrangement of thin (red circles) and thick (blue circles) filaments within the myofibril (adapted from Warriss, 2010). ........................................30

Figure 2.4. Illustration of the chemical conformation of a phospholipid showing the hydrophobic tail consisting of two fatty acid groups and the polar head group consisting of the glycerol, phosphate and choline groups (Clayton, 2014).......................................................35

Figure 2.5. Illustration of the chemical composition of a triglycerol showing the hydrophobic tail consisting of three fatty acid groups and the polar head consisting of a glycerol group (Clayton, 2014). ..................................................................................................................36

Figure 2.6. An example Raman spectra showing the peaks and troughs associated with Anti-Stokes and Stokes Raman Scattering..........................................................55

Figure 2.7. Examples of the chemical bond vibrations which are characterised by Raman spectroscopy.................................................................................................55

Figure 2.8. Schematic diagram of a Bench Top Raman Device (adapted from Yang and Ying, 2011). ........................................................................................................59

Figure 2.9. Schematic diagram of a Raman hand held device (adapted from Schmidt et al., 2009). ........................................................................................................60

Figure 2.10. Measurement of meat using a hand held Raman sensor head .................60

Figure 3.1. Hand held Raman spectroscopic device showing the measurement of fresh intact LL with the epimysium removed.........................................................68

Figure 3.2. An example of the unprocessed Raman spectra of lamb muscle, illustrating the main intensity peaks of the aromatic amino acid (tryptophan, tyrosine and phenylalanine) side chains and the peptide backbone signals (α- helix, amide I and III as well as C-H deformations) (Pézolet et al., 1988)................................................................................72

Figure 3.3. Difference in intensity peaks from the loins with the greatest change in shear force on day 1 (black) and day 5 (grey). ......................................................................................74

Figure 3.4. Cross validated prediction of shear force values (N) at 24 h post mortem based on Raman spectra collected 1d post mortem and analysed with 2 latent vectors..........75
Figure 3.5. Cross validated prediction of the difference between 1 and 5 day post mortem LL shear force values (N) based on the difference between Raman spectra taken 1 and 5 days post mortem and analysed with 6 latent vectors.

Figure 3.6. The relationship between LL shear force values (N) and sarcomere length (µm) 24 h PM.

Figure 3.7. Electron microscopy 50 x image of ovine *m. longissimus lumborum* showing partial breaks across the myofibril (indicated by arrows).

Figure 4.1. Direction of the Raman Spectroscopy measurements on the SM, perpendicular to the muscle fibre.

Figure 4.2. Raman Spectra of the toughest SM samples (66-78N) and the tenderest SM samples (29-36N) collected 24 h PM.

Figure 4.3. Average Raman spectra of SM at 24 h (black) and 5 days (grey) PM highlighting the difference in unprocessed spectra over the ageing period.

Figure 4.4. Difference in intensity peak height and location between tough (grey) and tender (black) intact SM.

Figure 4.5. Cross validated prediction of SM shear force values (N) measured 5 days post mortem using Raman spectra taken 24 h post mortem and analysed with 3 latent vectors.

Figure 5.1. An example of a Raman chemical map generated using the total integrated intensity of Raman bands between 500–2100 cm\(^{-1}\) measured over a 40µm area of the myofibril of ovine *m. semimembranosus*.

Figure 5.2. An example of a Raman chemical image for the integrated intensities for the Amide I peak centred at 1330 cm\(^{-1}\) (A), and the chemical image showing the proportions of high (green), moderate (pink) and low (purple) integrated intensity classifications used for image analysis (B).

Figure 5.3. Chemical image of the most tender ovine *m. semimembranosus* (26 N) showing the integrated intensities of wavenumbers between 500–1900 cm\(^{-1}\).

Figure 5.4. Chemical image of the toughest ovine *m. semimembranosus* (74 N) showing the integrated intensities of wavenumbers between 500–1900 cm\(^{-1}\).

Figure 5.5. An example of a background corrected spectra extracted from the Raman map of the most tender (26 N; black) and the toughest (74 N; grey) ovine *m. semimembranosus* highlighting the bands centred at 853 cm\(^{-1}\) and 930 cm\(^{-1}\).

Figure 6.1. Pre-rigor measurement of the *m. semimembranosus* *in-situ* in processing plant conditions.
Figure 6.2. The correlation between particle size (µm) and shear force values (N) of ovine *m. semimembranosus* measured at 5 days post mortem determined by univariate regression analysis ($R^2 = 0.21$) with a 95% confidence interval (dotted line).

Figure 6.3. The cross validated correlation between purge (%) values predicted using Raman spectra collected 25 min post slaughter and observed values measured from ovine *m. semimembranosus* after 4 days ageing.

Figure 6.4 The cross validated correlation between pHu values of ovine *m. semimembranosus* predicted using Raman spectra collected 24 h post mortem and measured at 5 days post mortem.

Figure 6.5 The cross validated correlation between predicted purge values (%) using Raman spectra collected 5 days post mortem and observed values of ovine *m. semimembranosus* measured after 4 days ageing.

Figure 6.6. The correlation between observed pH$_{24}$ and pHu values determined using a step-wise regression model ($R^2 = 0.27$, P <0.001) with a 95% confidence interval (dotted line).

Figure 6.7. The relationship between observed pHu values and purge (%) of ovine *m. semimembranosus* measured at 5 day post mortem ($R^2 = 0.10$) with a 95% confidence interval (dotted line).

Figure 6.8. The average of all Raman spectra of ovine *m. semimembranosus* measured 25 min (black), 24 hours (dotted line) and at 5 days (grey) post mortem.

Figure 6.9 The average first integration (grey) and the average of the total accumulation (black) for all Raman Spectra measured at 25 min post slaughter.

Figure 6.10. The averaged background corrected Raman spectra collected 24 hours post mortem from the ovine *m. semimembranosous* with the 5 highest (5.90 – 6.16; black) and lowest (5.59 – 5.61; grey) pHu values.

Figure 6.11 The average background corrected spectra collected at 24 hours post mortem from the ovine *m. semimembranosus* with the 5 lowest pH$_{24}$ values (5.44 – 5.48; grey) and the pHu values (5.59 – 5.61; black).

Figure 6.12 The averaged and background corrected spectra collected at 24 hours post mortem from the ovine *m. semimembranosus* with the 5 highest (4.65 – 6.42; grey) and lowest purge (1.10 – 1.23; black).

Figure 7.1 The relationship between pHu and pH$_{24}$ values measured on lamb *m. semimembranosus*. 

9
Figure 7.2. Raman spectra from the 5 lamb *m. semimembranosus* with the highest (grey) and lowest (black) pHu values background corrected with the 4 wavenumbers used in the least angle regression prediction model identified.

Figure 8.1 Hand held Raman Spectroscopy sensor head measuring the cranial portion of a fresh intact lamb LL with the epimysium removed, perpendicular to the muscle fibres.

Figure 8.2. Efficiency of extraction (%) of a one-step (black; $R^2 = 0.55$) and two-step (grey; $R^2 = 0.38$) compared with intramuscular fat amounts (g/100g meat) from ovine *m. longissimus lumborum*.

Figure 8.3. The correlation between saturated fatty acids (mg/100g meat) and intramuscular fat (g/100g meat) extracted using a one-step ($R^2 = 0.6$; black) and two-step ($R^2 = 0.91$; grey) methylation and extraction procedures from ovine *m. longissimus lumborum*.

Figure 8.4. The correlation between monounsaturated fatty acids (mg/100g meat) and intramuscular fat (g/100g meat) extracted using a one-step ($R^2 = 0.6$; black) and two-step ($R^2 = 0.92$; grey) methylation and extraction procedures from ovine *m. longissimus lumborum*.

Figure 8.5. The correlation between polyunsaturated fatty acids (mg/100g meat) and intramuscular fat (g/100g meat) extracted using a one-step ($R^2 = 0.20$; black) and two-step ($R^2 = 0.93$; grey) methylation and extraction procedures from ovine *m. longissimus lumborum*.

Figure 8.6 Correlation between Intramuscular fat (g/100g) of lamb *m. longissimus lumborum* and polyunsaturated fatty acids (mg/100g; diamonds; $R^2 = 0.15$), monounsaturated fatty acids (mg/100g; triangles; $R^2 = 0.92$) and saturated fatty acids (mg/100g; circles; $R^2 = 0.91$) extracted using the two-step extraction procedure from ovine *m. longissimus lumborum*.

Figure 8.7. The average intramuscular fat spectra, with no pre-processing, of lamb LL showing the intensity peaks that are indicative of fat.

Figure 8.8 Average spectra of the 5 ovine *m. longissimus lumborum* samples with the highest (black; 432.28 – 477.25 mg/100g meat) and lowest (grey; 235.70 – 255.73 mg/100g meat) PUFA concentrations without pre-processing.

Figure 8.10 PUFA values in *m. longissimus lumborum* (mg/100g meat) predicted using Raman spectra compared to observed values (optimal RMSECV = 46.57, $R^2 = 0.93$, $R^2_{cv} = 0.21$).
Tables
Table 2.1. Common Fatty Acids in Muscle Foods (Christie, 2003) ..................................................35
Table 2. Summary of spectroscopic technologies and their advantages and disadvantages. ............50
Table 3.1. The position and band assignments of the main peaks in intensity in the Raman spectra of muscle tissue (adapted from Beattie et al, 2004; Beattie et al, 2008; Schmidt et al, 2013) ..................................................................................................................73
Table 3.2 Summary of the PLS regression analysis results using Raman Spectroscopy measured at 24 h and 5 days PM to predict shear force values of fresh intact LL measured at 5 days PM. .................................................................75
Table 3.3. Mean, standard deviation (SD) and range of shear force (N), cooking loss (%), sarcomere length (µm), pH24, Particle Size Analysis (µm) and myofibrillar breaks (%) number of full and partial breaks) of lamb m. longissimus lumborum (n = 80) ........................................77
Table 3.4. The RMSEP for models using traditional indicators and Raman spectra to predict shear force values (N) of lamb LL 24 h and 5 days post mortem .........................................................77
Table 4.1 Summary of the chemometric analysis using Raman Spectroscopy to predict 5 day shear force in SM samples ........................................................................................................92
Table 4.2. Mean, standard deviation (SD) and range for Shear Force (N), Cooking Loss (%), ultimate pH (pHu) and sarcomere length (µm) of m. semimembranosus (SM). .........................93
Table 4.3. The RMSEP for models using traditional indicators and/or Raman spectra to predict shear force values (N) of lamb SM at 5 days post mortem ..................................................94
Table 5.1 Co-efficients used for linear discriminate analysis (LDA) to determine whether intensities at 8 wavenumbers can be used to classify m. semimembranosus into tough or tender shear force groups ......................................................................................................110
Table 5.2 Wavenumbers used to fit a 6th order polynomial through unprocessed spectra to remove contributions of background noise and fluorescence ...............................................110
Table 5.3. Summary statistics for carcase traits and indicators of tenderness determined from tough and tender ovine m. semimembranosus .................................................................111
Table 5.4. Summary statistics for the proportion of the Raman chemical images from tender (26 – 35N) and tough (54 – 74 N) ovine m. semimembranosus with high, moderate and low integrated intensities across the 8 wavenumbers important to meat quality assessment ......113
Table 5.5. The percentages and probabilities of correctly classifying ovine m. semimembranosus into tough and tender categories using key Raman spectroscopy vibrations ..............................................................................................................114
Table 6.1. Wavenumbers used to baseline correct raw Raman spectra measured pre-rigor, 24 h and 5 days post mortem. ........................................................................................................124

Table 6.2 Mean, standard deviation (SD) and range for carcase traits and meat quality indicators of the m. semimembranosus. ........................................................................................................125

Table 6.3 Summary chemometrics for the prediction of shear force values using Raman spectra measured 25 mins (pre-rigor), 1 and 5 days post mortem using the first integration (3 s) and the full accumulation (15 s). ........................................................................................................125

Table 6.4 Summary of univariate regression parameters and P values of the variation in shear force values explained by the traditional indicators including pH, sarcomere length, particle size analysis, collagen content, purge and cooking losses. ........................................................................................................126

Table 6.5. Chemometric analysis using Raman spectra measured 25 min post mortem to predict of meat quality indicators of ovine m. semimembranosus. ........................................................................................................127

Table 6.6. Chemometric analysis using Raman spectra measured 24 hours PM to predict indicators of shear force values of ovine m. semimembranosus. ........................................................................................................128

Table 6.7. Chemometric analysis using Raman spectra measured 5 days PM to predict indicators of shear force values of ovine m. semimembranosus. ........................................................................................................129

Table 7.1. Wavenumbers used to baseline correct raw Raman spectra measured 1, 5 and after freezing and thawing at 8 days post mortem. ........................................................................................................153

Table 7.2 Mean, standard deviation (SD) and range for carcase and eating quality indicators measured. ........................................................................................................154

Table 8.1. Summary statistics of lamb m. longissimus lumborum FA composition (mg /100g meat) determined using either a one-step or a two-step extraction methylation procedure. .166

Table 8.2. The wavenumber positions and band assignments of the main peaks in intensity for Raman spectra of adipose tissue (adapted from Olsen et al., (2007); Beattie et al., (2007); Socrates (2001)). ........................................................................................................172

Table 8.3. Summary of chemometric analysis using Raman spectroscopy to predict key fatty acid traits, including polyunsaturated (PUFA), monounsaturated (MUFA), total unsaturated (USFA), saturated (SFA), total FA amount determined by the methylation and extraction (Id. FA), omega-3 to omega-6 ratio and health claimable (DHA, DPA and DPA) FAs, of ovine m. longissimus lumborum determined by a one-step and a two-step FAME extraction methods. ........................................................................................................174

Table 8.4. Coefficient of determination (R^2) values compared with the cross validated coefficient of determination between observed and predicted values (R^2_cv) for key fatty acid
categories determined using the two-step procedure with and without inclusion of intramuscular fat amount (mg/100g) as a model covariate.................................176

Table 8.5. Summary chemometric analysis for the prediction of key fatty acid traits of ovine m. Longissimus thoracis, adjusted* and unadjusted for mean intramuscular fat levels. .......176
"I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person nor material which to a substantial extent has been accepted for the award of any other degree or diploma at Charles Sturt University or any other educational institution, except where due acknowledgment is made in the thesis. Any contribution made to the research by colleagues with whom I have worked at Charles Sturt University or elsewhere during my candidature is fully acknowledged. I agree that this thesis be accessible for the purpose of study and research in accordance with the normal conditions established by the Executive Director, Library Services or nominee, for the care, loan and reproduction of theses."

Signed:

Date: 22/10/2015
Acknowledgements

“If completing a PhD was easy everyone would have one”- R. Doyle

I am incredibly grateful to the numerous people who have contributed to the process of completing this PhD including:

- My two supervisors Dr David Hopkins (NSW DPI) and Prof. Peter Wynn (CSU). Thank you for your seemingly endless patience, countless hours reading, editing and re-reading and for sharing your years of experience and knowledge over the last three years.

- Heinar Schmidt, Rico Scheier and the team at Forschungsstelle für Nahrungsmittelqualität der Universität Bayreuth am Standort Kulmbach (ForN). Thank you for sharing “my little German friend”, your knowledge and experience and of course your gorgeous corner of the world with me.

- Matt Kerr, Tracy Lamb, Kristy Bailes, Dr. Ben Holman and Jordan Horban (NSW DPI). I can’t thank you all enough for your expertise and help in collecting and processing the 1000’s of samples collected during the three years. It would have been impossible for me to complete this project without you all.

- Remy van de Ven (NSW DPI). Thank you for your contributions of statistical analyses for most of the chemometric analysis, your lessons in R and your in-depth feedback on the papers.

- Dr Ed Clayton (NSW DPI) and Dr Eric Ponnampalam and Matt Kerr (DEPI) for your advice and assistance analysing the samples for fatty acids and intramuscular fat.

- Dr Bayden Wood for the use of the Raman microscope at Monash and Gianluca Baldi and Matteo Ottoboni for your assistance in analysing the images.

- My family and friends at CSU, across Australia and across the world. Thanks for being there and sharing in the journey with me. A special thanks to Dr Rebecca Doyle for all your sage advice and to D. Gale and Claire “with an I” Walker for the countless discussions on paperclips, good food and good wine which kept me sane.

- Clint. A special thanks for doing everything you’ve done over the last 10 months which has kept me doing everything I wanted to do.

- “the whiteboard”. Thank you for reminding me of the realities of research and for keeping me motivated and on track during those long months of conducting experiments and writing up over the last three years.
Published Papers


Conference Proceedings


Conference Abstracts


Abstract

Inconsistent eating quality is one of the major problems faced by the sheep meat industries, as the age, weight, sex and fat scores currently used to determine the value of carcases are poorly related to the traits which indicate eating quality. Raman spectroscopy is one technology which has the potential to be used as a tool for carcase assessment as it is rapid, non-destructive and capable of providing information on the protein and lipids present in meats. Therefore, the aim of this research was to determine the potential of a hand held Raman spectroscopic device to predict meat quality traits of lamb for commercial application.

Spectra collected 25 min, 24 h and 5 days post mortem (PM) using a 671nm hand held Raman device were regressed against traditional indicators of meat quality including shear force values, pH values, cooking loss, purge, colour, sarcomere length, particle size, intramuscular fat levels and major fatty acid group concentrations using partial least squares. Furthermore, models to determine relationships between shear force and traditional indicators (likewise pHu and traditional indicators) were fitted using simple linear regression.

Overall, predictions of the major fatty acid groups were the most promising, yielding coefficients of determination of \( R^2 \) of 0.93 – 0.54 and reductions in error of up to 7.8% for the prediction of polyunsaturated (PUFA) and monounsaturated (MUFA) fatty acids as well as saturated fatty acids which had been adjusted for the level of intramuscular fat (IMF). However, there may be some overlap in Raman signals arising from the head groups of phospholipids, which may be causing a reduction in the accuracy when cross validation is used to determine the robustness of the prediction. Hence, further research needs to determine the impact of various lipid conformations on the prediction of major fatty acid groups in fresh intact muscle.

The best prediction of shear force values at 5 days PM was found using spectra collected 24 h \( R^2_{cv} = 0.27 \), however the prediction of shear force was inconsistent over time as further experiments demonstrated no ability to predict shear force values. Therefore, this research indicates that prediction of shear force of fresh intact lamb using Raman spectroscopy does not have the repeatability and robustness required by industry.
Models for the prediction of other meat quality traits suggested that there was also an ability to predict pH\textsubscript{24} and purge using spectra measured pre-rigor ($R^2_{cv} = 0.27$ and 0.32) and pHu, purge and L* values from spectra measured at 24 h post mortem ($R^2_{cv} = 0.22 – 0.59$). As colour, water holding capacity, structure pH decline and pHu are related, it is hypothesised that Raman spectroscopy is able to predict meat quality traits which relate to early PM metabolism. However, since Raman spectra of intact meat are complex, further research is required to determine the impact of spectral overlap from compounds of similar chemical conformation, determine the biochemical processes which contribute to these predictions and validate these findings.
**List of Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP</td>
<td>Adenodiphosphate</td>
</tr>
<tr>
<td>ALA</td>
<td>Alfa Linoleic Acid</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenotriphosphate</td>
</tr>
<tr>
<td>CCD</td>
<td>Charge Coupled Device</td>
</tr>
<tr>
<td>CV</td>
<td>Coefficient of Variation</td>
</tr>
<tr>
<td>DHA</td>
<td>Docosahexaenoic Acid</td>
</tr>
<tr>
<td>DMb</td>
<td>De-oxymyoglobin</td>
</tr>
<tr>
<td>DPA</td>
<td>Docosapentaenoic Acid</td>
</tr>
<tr>
<td>ES</td>
<td>Electrical Stimulation</td>
</tr>
<tr>
<td>EPA</td>
<td>Eicosapentaenoic Acid</td>
</tr>
<tr>
<td>FA</td>
<td>Fatty Acid</td>
</tr>
<tr>
<td>FAME</td>
<td>Fatty Acid Methyl Ester</td>
</tr>
<tr>
<td>FTIR</td>
<td>Fourier Transform Infra-Red</td>
</tr>
<tr>
<td>GC</td>
<td>Gas Chromatography</td>
</tr>
<tr>
<td>GR Tissue Depth</td>
<td>Greville Tissue Depth (depth over the 12\textsuperscript{th} rib 110mm from the backline)</td>
</tr>
<tr>
<td>HCW</td>
<td>Hot Carcase Weight</td>
</tr>
<tr>
<td>IMF</td>
<td>Intramuscular Fat</td>
</tr>
<tr>
<td>IR</td>
<td>Infra-Red</td>
</tr>
<tr>
<td>IV</td>
<td>Iodine Value</td>
</tr>
<tr>
<td>LAR</td>
<td>Least Angle Regression</td>
</tr>
</tbody>
</table>
LCPUFA: Long Chain Poly Unsaturated Fatty Acids
LDA: Linear Discriminate Analysis
LL: *Longissimus Lumborum*
LLpH$_{24}$: pH of *Longissimus Lumborum* measured 24 h post mortem
LOO: Leave One Out
LV: Latent Variables
Mb: Myoglobin
MMb: Met-myoglobin
MFI: Myofibrillar Fragmentation Index
MSC: Multiplicative Scatter Correction
MFI: Myofibrillar Fragmentation Index
MUFA: Mono- Unsaturated Fatty Acids
NIR: Near Infra-Red
NMR: Nuclear Magnetic Reasonance
PCA: Principal Component Analysis
PCr: Phosphocreatine
pH$_{24}$: pH measured 24 h post mortem
pH$_{PR}$: pH measured pre-rigor
pHu: Ultimate pH
PLS: Partial Least Squares
PM: Post mortem
PR: Pre-rigor
PSA  
Particle Size Analysis

PS  
Particle Size

PSE  
Pale Soft and Exudative

PUFA  
Poly Unsaturated Fatty Acids

PSA  
Particle Size Analysis

$R^2$  
Coefficient of determination of the Raman spectra and average model covariate value

$R^2_{cv}$  
Cross validated coefficient of determination of the Raman spectra and average model covariate value

RMSECV  
Root Mean Square Error of Cross Validation

RMSEP  
Root Mean Square Error of Prediction

RMSEP_{cv}  
Cross validated Root Mean Square Error of Prediction

RS  
Raman Spectroscopy

SC  
Soluble Collagen

SF  
Shear Force

SFA  
Saturated Fatty Acids

sHSP  
Small Heat Shock Proteins

SL  
Sarcomere Length

SM  
Semimembranosus

SNR  
Signal to Noise Ratio

TC  
Total Collagen

temp_{20}  
Temperature measured 20 m post mortem

TIFA  
Total Identified Fatty Acid
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>USFA</td>
<td>Unsaturated Fatty Acids</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra Violet</td>
</tr>
</tbody>
</table>
1. General Introduction

The amounts and types of meat products consumed in Australia have dramatically changed from post-World War II to today. One major driver in changes to meat consumption over this period has been increasing affluence, which has led to a greater demand for high quality animal products, including meat (Lang & Heasman, 2004).

Pre-1970’s, lamb and beef were preferred meats (Dixon, 2002), however the consumption of chicken has since continued to increase to 43 kg per capita whilst consumption of beef has remained steady at approximately 30 kg per capita and consumption of lamb has decreased to 9 kg per capita. Recent improvements to prime lamb production, the promotion of lamb through the “trim lamb” and “we love our lamb” marketing campaigns to address this consumption decline have changed lamb and its perception to that of a premium product. Furthermore, Meat Standards Australia has developed a sheep meat eating quality program using untrained consumer panels (Thompson et al., 2005b). Yet, lamb carcases are still mainly assessed using market specifications of weight, age, sex and fat scores. While informative on meat yield, these attributes are variable and may not be indicative of eating quality characteristics, such as tenderness. Consequently, better carcase assessment methods are required by the sheep meat industry to determine whether prime lamb carcases meet the quantitative and qualitative attributes demanded by domestic and export consumers.

Development of digital camera and computer technologies in the last three decades has provided an opportunity to develop carcase assessment tools, such as spectroscopy since optic equipment can take advantage of the improved efficiency and miniaturisation of electronic components (Damez & Clerjon, 2008). Hence, this thesis describes an investigation into the development and validation of one optic technology, a Raman spectroscopic hand held device for the assessment of meat quality of commercial lamb processed in Australia.
Chapter 2 is a review of literature which discusses the biophysical and biochemical characteristics which contribute to meat quality and the indicators used to measure them. The literature review then seeks to identify the suitability and potential of Raman spectroscopy for online measurement and the lack of research on fresh intact lamb using Raman spectroscopy.

Chapters 3 and 4 address these gaps in literature by investigating the potential for Raman spectroscopy to predict shear force values of intact fresh lamb muscles (m. longissimus lumborum and m. semimembranosus) in comparison to the traditional predictors of shear force values.

Chapter 5 explores the potential for Raman microscopy to further understand the variation in Raman spectra and the relationship between the spectra and the biochemical and biophysical characteristics associated with variation in shear force values.

Chapter 6 seeks to clarify the predictions of shear force found in Chapter 4 as well as investigating the potential to use Raman spectroscopy to predict other indicators of meat quality. This includes the hypotheses that taking measurements earlier post mortem prior to rigor mortis and increasing integration times will improve the predictions.

Chapter 7 tests the hypothesis that predictions of shear force values of lamb are improved by freezing and thawing.

Chapter 8 investigates the potential to predict intramuscular fat (IMF) levels and the major fatty acid groups of intact lamb m. longissiumus lumborum.

Chapter 9 summarises the major findings of this research and provides general conclusions before Chapter 10 discusses the implications of these findings for industry and outlines the opportunities for future research.
2. Literature Review

2.1 Introduction

The term ‘quality’ can have many interpretations and definitions to different people within red meat industries. It may be defined as the characteristics or features of a product or its ability to satisfy the specifications and needs of a customer. The quality of a product is now associated with traits that extend far beyond the cost for product weight (Herrero, 2008b) and now may include the legal, technological and socio-ecological consequences of production and supply (Lang & Heasman, 2004). Consequently, there are many objective (the material properties which can be measured) and subjective (the consumer's acceptability) measures of quality.

Studies that have been conducted to objectively measure the consumer acceptability or subjective quality parameters of meat, found that there are three main factors that influence meat palatability: tenderness, flavour and juiciness (Thompson, 2002, Thompson et al., 2008a, Thompson et al., 2008b, Watson et al., 2008, Polkinghorne & Thompson, 2010). Of these studies, Thompson (2002) has demonstrated that tenderness is a main concern in the purchase of beef, however it has a lesser importance in lamb (Thompson et al., 2005b).

It is well established in literature that meat tenderness is determined by the contribution of connective tissue (background toughness) and the myofibrillar structure (Damez & Clerjon, 2008) and the changes that occur to these structures within the carcase post mortem (Clerjon et al., 2011). Many attempts have been made to measure these changes (Damez & Clerjon, 2008) and of these Raman Spectroscopy has been highlighted as having potential uses as it is not affected by varying levels of water content and it is rapid, non-destructive and non-invasive (Beattie et al., 2004b, Beattie et al., 2008, Yang & Ying, 2011).

This critical review aims to identify the factors which relate to meat quality, critically assess the literature on measuring meat quality and determine the potential to develop new assessment technologies that can be applied in a commercial setting to measure the meat quality of intact muscle.
2.2 Meat Quality

Smith et al. (2008) define meat quality as the characteristics of skeletal muscle that determine the appeal of the product to the consumer. In uncooked products, this refers to the amount of saleable meat, ratio of bone to muscle, meat and fat colour, muscle pH and microbial counts. Eating quality of meat is determined by a complex set of biochemical and biophysical interactions during processing and cooking that combine to create flavour, juiciness and tenderness, which are experienced during consumption (Smith et al., 2008). Although they are complex, an understanding of these interactions is vital, as the perceived eating quality of cooked meats determines repeat purchases (Tornberg, 1996, Smith et al., 2008, Troy & Kerry, 2010).

Despite tenderness being widely recognised as being the most important factor in determining eating quality of red meats (Hood & Tarrant, 1981), changes to the myofibrillar structure will also alter other sensory traits. Thompson et al. (2005b) agree by suggesting that flavour and overall liking are driven to some extent by changes in tenderness and consequently, an untrained consumer panel that represents the everyday consumer, can find it difficult to distinguish between these sensory scores.

2.2.1 Meat Tenderness

Tenderness is recognised as the most important factor in determining consumer perception (Hood & Tarrant, 1981), as a tough steak is unacceptable (Boccard et al., 1981, Wood et al., 2008). Tenderness is defined as the ability of the product to be chewed or cut and is determined by the interaction of connective tissue and myofibrillar structures as well as the links between the two structures, as muscle is converted into meat. Although the types of muscle fibres and connective tissue present in the live animal is determined by genetics, nutrition and environmental factors (Berg & Butterfield, 1974), post mortem processes, such as electrical stimulation, stretching and post mortem storage (‘ageing’) have the potential to change final eating quality (Hedrick et al., 1993), primarily through their effects on the myofibrillar proteins (Lepetit et al., 1986).
2.2.2 Flavour

Flavour which has an impact on the organoleptic properties of meat, is developed through a series of complex interactions that are thermally induced during cooking, as raw meat has almost no aroma and only a blood like taste (Mottram, 1998). Although it is generated by cooking, the characteristics which govern flavour are determined by the raw product. These include type of proteins present in the muscle, dry matter content, fatty acid profile, total amount of lipids, haem iron levels, collagen content and pH (Priolo et al., 2001, Renand et al., 2001, Smith et al., 2008). Smith et al. (2008) also suggest that the types and amounts of proteins which are degraded during ageing also contribute to flavour.

2.2.3 Juiciness

Juiciness has been identified as determining the palatability of meat however it has a lesser importance in determining final eating quality in comparison to tenderness and flavour (Watson et al., 2008). A review by Smith et al. (2008) identified juiciness as being influenced by the amount of intramuscular fat and moisture which remain in the muscle after cooking. Tornberg (1996) proposes myofibrillar contractions during rigor mortis contributes to juiciness, as both lateral and longitudinal contractions alter water holding capacity by forcing water out of the myofibrils and myofibres into extra cellular space. This creates water compartments, which are then expelled as cooking loss when the muscle contracts during cooking.

2.3 Biochemical and Biophysical Structure of Meat

It is well established in literature that the eating quality of meat is a factor of the biophysical and biochemical attributes of the muscle and fat (Hedrick et al., 1993, Tornberg, 1996, Thompson et al., 2008a, Thompson et al., 2008b, Warriss, 2010), therefore a review of meat quality must also explore the literature on the structure and anatomy of muscle.
2.3.1 Topographical Muscle Anatomy

Topographical anatomy of the muscle is important to meat quality as the texture and background toughness is determined by the muscle fibre size and connective tissue type and percentage (Lawrie, 1966). Several authors (Tornberg, 1996, Lawrie, 2006, Warriss, 2010) describe the basic structure of muscles as myofibres (bundles of muscle fibres), bound together by the endomysium, a connective tissue layer, which are arranged into larger bundles by the perimysium that are held intact by the epimysium, as illustrated in Figure. 2.1.

![Illustration of the topographical anatomy of a skeletal muscle, showing the arrangement of the connective tissue layers and bundles of myofibres (Scanes, 2003).](image)

Each myofibre is functionally equivalent to one cell and contains the same organelles as any normal cell; the nuclei (more than one because they form from several myoblasts), mitochondria and an extensive sarcoplasmic reticulum (Warriss, 2010). Embedded within the sarcoplasm there are myofibrils, which are cross-striated with the A-band within the H- Zone and the Z-line in the I-Band (Fig. 2.2.). A sarcomere is the structure between Z-lines (Lawrie, 1966).
The myofibrils consist of smaller units, or filaments, which are classified as either thick or thin. Thick filaments transverse the A-Band (Lawrie, 1966) and are formed mainly by aggregations of the tails of myosin molecules. However, thin filaments are formed by the proteins actin, troponin and tropomyosin (Warriss, 2010) and are a major component of the I-band, although they extend into the A-band (Lawrie, 1966). As illustrated in Figure 2.3, both of these filaments are arranged in-situ so that each thick filament is surrounded by 6 thin filaments and each thin filament is surrounded by 3 thick filaments.
2.3.2 Muscle Proteins

The main functional proteins are involved in metabolic processes (25.5%), cell structure (17%), cell defence (16%) and contractile apparatus (14.5%) (Bouley et al., 2004). However, a large percentage cannot be grouped into one of these categories (25.5%) and a further 1.5% of proteins still have unknown functions. In terms of determining meat quality Hedrick et al. (1993) suggest that of these, contractile, cytoskeletal and regulatory proteins are vital. Contractile proteins are those that are associated with the locomotive function of muscles and include myofibrillar proteins actin, myosin, troponin and tropomyosin (Tornberg, 1996, Hooper & Thuma, 2005), while cytoskeletal proteins, such as desmin, hold together the architecture of the myofibrillar system (Hooper & Thuma, 2005). Regulatory elements, as the name suggests, are components that control processes within the muscle to maintain homogeneity (Hedrick et al., 1993).

2.3.2.1. Myofibrillar Proteins

A majority of the myofibrillar proteins are contractile elements, as 80% of the myofibrillar volume is comprised of thin and thick filaments (Warriss, 2010). Thin filaments are a double helix of the contractile monomer, polymerised globular actin and the regulatory proteins, troponin and tropomyosin (Hooper & Thuma, 2005). Tropomyosin is a rod-like shaped protein that lies between the actin chains, with troponin at the cross over points (Warriss, 2010). During contraction of the muscle, the release of Ca\(^+\) ions into the myofibrillar space is controlled by troponin and tropomyosin (Tornberg, 1996). This release of Ca\(^+\) causes cross linked bridges to be created between actin and the myosin heads where ATPase activity is located (Reed, 2009).

Actin and myosin are a large component of myofibrillar proteins, equalling 55-60% (Tornberg, 1996) which is important for tenderness as formation of cross-linked bridges between myosin and actin is responsible for the toughening of meat that occurs at rigor mortis (Marsh, 1974, Koohmaraie & Geesink, 2006, Hopkins & Geesink, 2009). Furthermore, it is these myofibrillar proteins that play an important role in the determination of texture and water holding capacity during processing and thermal treatment (Chen & Han, 2011).
2.3.2.2 Cytoskeletal Proteins

Cytoskeletal proteins are important components of muscle in terms of meat quality as they provide the scaffold for myofibrillar alignment and sarcomere formation (Laville et al., 2009). Hedrick et al. (1993) highlight that of these cytoskeletal proteins, titin is the most abundant, extending along half the sarcomere from the M Line to the Z-disc. Titin is responsible for the alignment of filaments during myofibril formation and is responsible for maintaining the ordered structure and integrity of the filaments in mature muscle and is anchored in the Z-disks. However there are other cytoskeletal proteins, including nebulin, desmin, synemin, and vinculin, which also play a role in organising filaments during myofibril formation, stabilising filaments and anchoring them to the sarcomere (Hedrick et al., 1993, Ilian et al., 2004, Kemp et al., 2010).

2.3.3 Connective Tissue

The connective tissue network is continuous throughout the entire carcass, surrounding the whole muscle as the epimysium, the sarcolemma as the endomysium and enveloping the muscle fibre bundles as the perimysium (Warriss, 2010). This network also joins the muscle to the skeleton via tendons and joins bones across joints via ligaments (Scanes, 2003). The two main fibrous proteins associated with connective tissue are collagen and elastin (Tornberg, 1996) embedded in an amorphous matrix (Hedrick et al., 1993). However, connective tissue does not contain consistent concentrations of collagen and elastin, rather the contributions of collagen and elastin change based on the connective tissue type (Lawrie, 1974).

Collagen is described by Lawrie (1974) as a large extra-cellular protein comprised of 3 left handed α-helical polypeptide chains. On their own each of these chains is unstable, however when they are wrapped around each other, they create a very stable right handed α-helix, tropocollagen, which is stable. Glycine is a main amino acid of collagen while proline and hydroxyproline are also common (Warriss, 2010).

All collagens also contain varying amounts of the amino acids, lysine and hydroxylysine which are important to meat quality as lysine residues on adjacent but offset molecules form aldehyde cross-links by enzymatic oxidation. These cross-links stabilise the non-covalent bonds into covalent bonds that are further stabilised as animals age and become more
resistant to thermal rupture (Marsh, 1974) and gelatinisation during cooking (Bouton & Harris, 1972).

Elastin is a highly branched thread-like protein that is thinner than collagen fibres. Although it is less prevalent than collagen in most skeletal muscles, as it form sheets in ligaments muscles that have a high ligament content such as m. semitendinosus and m. latissimus dorsi contain up to 30-40% of the connective tissue as elastin (Pearson & Young, 1989).

The connective tissue content and type has been linked to meat quality not only by its effect on tenderness after cooking (Bouton & Harris, 1972) through heat stability and cross-linking of collagen (Marsh, 1974), but also its effect on shear force values of raw meat and water holding capacity (Bouton et al., 1975). Furthermore, connective tissue has a big impact on final eating quality as background toughness due to its long half-life (Pearson & Young, 1989) and relative stability during post-mortem ageing (Hopkins & Geesink, 2009).

### 2.3.4 Fatty Acids

Fatty acid composition and the deposition of fatty acids in either subcutaneous or intramuscular adipose tissue contribute to the nutritive value and meat quality of the carcase. Health concerns during the 1970’s and 1980’s led to consumers seeking leaner meat products (Lang & Heasman, 2004) while more recent studies have provided evidence which suggests that omega-3 fatty acids may reduce cardiovascular disease, inflammatory diseases and mental health disorders (Clayton, 2008, Bocking et al., 2010). Therefore, as described by Bocking et al. (2010), there is distinct benefit in identifying dietary sources that have a major impact on the composition of these fatty acids in the meat that humans eat (Ponnampalam et al., 2010, Najafi et al., 2012, Ponnampalam et al., 2012b). In addition to human health benefits, fatty acid deposition and composition also determines the flavour and juiciness that are important components in the final eating quality of meats (Wood et al., 1998, Smith et al., 2008).

Fatty acids are carboxylic acids with hydrocarbon chains, ranging from 4 to 36 carbons long (Chesworth et al., 1998). Fatty acids of nutritional importance are classified into three classes: saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA), depending on the number of double bonds that are
present. SFAs have no double bonds, while MUFAs have one double bond and PUFAs have two or more double bonds (Clayton, 2008). Further discrimination of PUFAs is based on the number of carbon atoms present and the position of the first of these double bonds as Omega-3 FAs have more than one double bond with the first occurring three bonds from the methyl end of the chain (Nelson & Cox, 2008) whilst Omega-6 PUFAs have two or more double bonds in their structure, with the first occurring six bonds from the methyl end of the chain (Chesworth et al., 1998).

PUFAs are of particular importance as they are considered healthier fats in comparison to SFAs and are essential components of cell membranes and cell function (Nelson & Cox, 2008), while excessive intake of saturated fats has been linked to numerous chronic diseases (World Health Organisation, 2003). Consequently, determining SFA concentrations is important to ensure that animal and meat products meet with the human nutrition and dietary guidelines and do not exceed 8 – 10% of the total dietary energy (Food Standards Australia New Zealand., 2009).

Fatty acid composition also affects the oxidative stability of the meat and consequently the processing and retail characteristics of the product. Meat quality problems, including warmed over flavour that is defined as an adverse flavour found in cooked pork and beef, have been linked to rapid oxidation of fatty acids (Brøndum et al., 2000). This rapid oxidation is a combination of product handling, for example rapid thawing after freezing cooked product, and fatty acid composition, as PUFAs in phospholipids are known to be liable to rapid oxidative breakdown (Wood et al., 2008). Further studies have also connected fatty acid composition, mainly PUFA concentrations, with consequent oxidative stability during shelf life (Insausti et al., 2008) and manufacturing (Fernández-Cabanás et al., 2011). Although, these reactions may be altered by other factors including antioxidant content and forms of haem (Ponnampalam et al., 2012b).
The composition of FAs in meats is highly variable depending on diet (Ponnampalam et al., 2010, Najafi et al., 2012), age, physiological state of the animal (Borys et al., 2012) and breed (Demirel et al., 2004, Demirel et al., 2006), however there are common fatty acids present in muscle foods, as summarised in Table 2.1.

<table>
<thead>
<tr>
<th>Trivial Name</th>
<th>Shorthand Nomenclature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myristic Acid</td>
<td>C14:0</td>
</tr>
<tr>
<td>Palmitic Acid</td>
<td>C16:0</td>
</tr>
<tr>
<td>Palmitoleic Acid</td>
<td>C16:1</td>
</tr>
<tr>
<td>Stearic Acid</td>
<td>C18:0</td>
</tr>
<tr>
<td>Oleic Acid</td>
<td>C18:1</td>
</tr>
<tr>
<td>Linoleic Acid</td>
<td>C18:2</td>
</tr>
<tr>
<td>α-Linoleic Acid</td>
<td>C18:3</td>
</tr>
<tr>
<td>α-Linolenic Acid</td>
<td>C18:3n-3</td>
</tr>
<tr>
<td>Arachidonic Acid</td>
<td>C20:4n-6</td>
</tr>
<tr>
<td>Eicosapentaenoic Acid (EPA)</td>
<td>C20:5n-3</td>
</tr>
<tr>
<td>Docosahexaenoic Acid (DHA)</td>
<td>C22:6n-3</td>
</tr>
<tr>
<td>Docosapentaenoic Acid (DPA)</td>
<td>C22:5n-3</td>
</tr>
</tbody>
</table>

Although all these FAs are contained in the lipid fraction of meats, lipid is not solely comprised of FAs (Clayton, 2014). Lipids fraction themselves are classified into two groups; neutral lipids which includes triacylglycerols (triglyceride), cholesterol esters and free fatty acids or polar lipids which include phospholipids (Christie, 2003). Although all lipids contain FA groups, the number of fatty acid groups per lipid molecule varies depending on the lipid fraction (Borys et al., 2012). Whilst phospholipids contain two fatty acid groups with a glycerol and phosphoric and choline groups (Fig 2.4), triglycerides contain three fatty acid groups with a glycerol group (Fig. 2.5).

![Figure 2.4 Illustration of the chemical conformation of a phospholipid showing the hydrophobic tail consisting of two fatty acid groups and the polar head group consisting of the glycerol, phosphate and choline groups (Clayton, 2014).](image-url)
Due to differences in fat digestion and lipid deposition, subcutaneous and intramuscular fat can vary significantly in their FA composition, between species and between individuals of the same species (Wood et al., 2008, Borys et al., 2012). The review of fat deposition and FA composition by Wood et al. (2008) highlights these differences indicating that the major lipid class of subcutaneous fat is triglycerol or neutral lipid while in muscle a significant proportion is phospholipid, which contains higher concentrations of PUFAs in order to perform the functions required of cellular membranes. Therefore, in animal tissues of the same species phospholipids tend to have a constant composition under normal physiological conditions yet the proportions of the simple lipids, especially triglycerols can vary greatly with diet and physiological state (Christie, 2012).

### 2.4 Changes to the Biochemical and Biophysical Properties of Meat

Meat undergoes several changes in structure during processing, which are important to the final meat quality. The first of these changes occurs at slaughter when the oxygen source of the muscle is terminated and the muscles undergo the biochemical reactions that result in *rigor mortis*. The second phase of changes occurs after rigor mortis if the muscle is stored and degradation is allowed to occur.
2.4.1 Immediate Post Mortem Changes

Under normal conditions skeletal muscle contractions occur as the energy potential reaching the neuromuscular junction causes Ca$^{2+}$ channels to open, facilitating the diffusion of Ca$^{2+}$ ions into the terminal before acetyl-choline is released, diffusing into the synaptic cleft before being bound to receptors which causes ligand gated cation channels to open. This process allows sodium to enter and potassium to exit the muscle fibres. A voltage sensitive protein between the sarcoplasmic reticulum and T-Tubules changes shape allowing the Ca$^{2+}$ ions to flood the sarcoplasm, and Ca$^{2+}$ then binds to troponin which causes troponin to change shape and tropomyosin moves away opening up the actin binding sites (te Pas et al., 2004, Nelson & Cox, 2008). The myosin head is then activated as adenotriphosphate (ATP) binds to it and is hydrolysed to adenosiphosphate (ADP). Consequently, the cross bridge cycle is able to commence and actin and myosin bind, releasing inorganic phosphate before ADP is released and the myosin head pivots providing the power stroke for the contraction. As another ATP binds to the myosin head, the link between actin and the myosin head is weakened and myosin detaches. Further ATP is then needed to reactivate the myosin head before contraction can continue (Pearson & Young, 1989).

Early post mortem events that are related to the formation of rigor mortis occur as a response of circulatory failure, the inability of the cardiovascular system to maintain oxygen delivery to the body and consequent anoxia. Therefore, there is a change in muscle metabolism from aerobic to anaerobic (Bjarnadóttir et al., 2011), however the change is not immediate. During the delay period before the onset of rigor, muscle cells continue normal metabolic processes and ATP levels remain constant. This is achieved in the short term as the body breaks down creatine phosphate (PCr) in order to provide the inorganic phosphate required to continue creating ATP from ADP (Savell et al., 2005). However this is not sustained and once approximately 70% of the PCr pool in the muscle has been depleted ATP levels decrease and muscle glycogen begins to be degraded (Scheffler & Gerrard, 2007).
Without oxidative phosphorylation and the Kreb’s cycle providing the phosphate for the conversion of ADP to ATP, anaerobic glycolysis commences. Anaerobic glycolysis, sees glycogen consumed to phosphorylate ADP, a chemical reaction which creates 2 ATP molecules, 2 pyruvate molecules, 2 NADH and one hydrogen atom. A further chemical reaction then converts the pyruvate molecule and 2 hydrogen atoms into lactate (Lawrie, 1966). As the circulatory system is no longer functioning, these waste products of ATP synthesis are not able to be removed and due to the build-up of H+ ions, pH of the muscle drops (Scheffler & Gerrard, 2007). These processes of anaerobic glycolysis continue until such time as the enzymes which are required can no longer function due to depletion of glycogen, decreases in carcase temperature or the increase in acidity as H+ ions accumulate (Warriss, 2010). However, anaerobic glycolysis produces relatively little ATP and therefore ATP production slows dramatically preventing the actin myosin cross bridges from being broken and consequently the muscle is unable to relax (Blanshard, 1974). These biochemical changes within the muscle are described as the fast onset phase of rigor (Savell et al., 2005), as the muscles quickly become inextensible under isometric conditions (Tornberg, 1996).

2.4.2 Post Mortem Ageing Period

Post mortem storage or ‘ageing’ is a widely used process to improve meat quality as it is well established that proteolysis occurs post mortem (Hedrick et al., 1993, Thompson et al., 2005b, Bekhit et al., 2007) and the strength of the muscle fibres decreases (Clerjon et al., 2011), which is reflected by decreased shear force and degradation of the myofibril measured as decreases in particle size (Toohey et al., 2012b). Histological studies conducted on the structural changes which occur with proteolysis have identified increases in the percentage of fibre breaks (Taylor & Frylinck, 2003, Toohey et al., 2012b) at the junction of the Z-band and I-band which is most likely due to the degradation of titin and nebulin (Taylor & Frylinck, 2003). Whilst the improvements to tenderness with proteolysis are widely accepted, debate still exists as to which proteins degrade and what mechanisms are responsible for the increases in tenderness (Takahashi, 1996, Hopkins & Thompson, 2002a, Koohmaraie & Geesink, 2006, Ouali et al., 2006).
Ouali et al. (2006) proposed that proteolysis is a multi-enzymatic process including cathepsins, calpains and other enzyme systems, whose functions are not well defined. Hopkins & Thompson (2002a) agree that ageing and consequent changes to meat tenderness is the result of a complex system of interactions within the muscle post-mortem, however they disagree with the proposal that cathepsins are one such system. This is supported by an array of studies cited, all that suggest variation in tenderness cannot be explained accurately by differences in cathepsin activity, either due to the experimental treatment or inconsistent results across treatment groups. Koohmaraie & Geesink (2006) disagree with the opinion that aging is a multi-enzymatic process, suggesting instead that calpains, calcium activated proteases are solely responsible.

Jordan et al. (2009) suggest that degradation of myofibrillar proteins is not the only important factor in the post-mortem changes that occur with ageing, as a decrease in soluble proteins was also observed. Although, it would be expected that during ageing, denaturation of the sarcoplasmic reticulum would result in an increase in soluble proteins present, drip losses and existing microfauna diminish soluble proteins and therefore, substantial losses can occur.

While Beattie et al. (2008) agree that the amount of soluble proteins increase with ageing, they suggest that meat is a closed system and the amount of any amino acid does not change with ageing instead it is the location of the amino acid that changes.

Further studies have proposed otherwise, proposing that the small heat shock protein family (sHSP) may play a key role in increasing in tenderness as a result of post-mortem ageing (Bjarnadóttir et al., 2010, Bjarnadóttir et al., 2011). Di Luca et al. (2011) have highlighted the role of sHSPs in stabilising the myofibrillar structure and preventing protein denaturation. sHSPs are the most highly induced proteins of the cellular stress response in mammalian cells, protecting the cells from stress and restoring the function of damaged proteins. A study on the impact of electrical stimulation (ES) on tenderness and proteome changes has hypothesised that ES reduces the abundance of sHSPs. Consequently, in ES treated carcasses the degradation of other myofibrillar proteins occurs earlier and proteolytic enzyme activity PM was greater, correlating low levels of sHSPs (Bjarnadóttir et al., 2011) and earlier activation of μ- calpains (Hwang & Thompson, 2001) to improved tenderness in beef.
Unlike the other theories previously discussed which rely on the action of catabolic enzymes, Takahashi (1996) propose an alternate hypothesis, the calcium theory of meat tenderisation. The calcium theory suggests that the presence of calcium ions causes tenderisation as Z-discs and actin and myosin cross bridges were found to weaken, titin filaments split and nebulin and desmin filaments were found to fragment when 0.1 mM of calcium ions were present in the myofibril regardless of the action of enzymes.

Although none of these mechanisms is fully understood, the impact of proteolysis on increasing tenderness is widely accepted. Hence, ageing is an extensively used tool to improve meat tenderness.

2.5 Traditional Indicators of Meat Quality

As eating quality is a reflection of the biochemical and biophysical characteristics of the muscle, many methods have been established which measure the muscle characteristics that relate to eating quality. However, due to labour, costs and time associated with some of the direct measurements, indirect measurements or indicators are often used to infer these characteristics.

2.5.1 Shear Force Values

Shear force values are a mechanical measure of the breaking strength of meat tissues (Bouton et al., 1975) measured in either kilograms or Newtons per unit area. Therefore, shear force can be referred to as either a measure of meat quality or an indicator of tenderness measured by sensory panels. Although shear force decreases with increasing tenderness, the relationship between shear force values and sensory tenderness can vary as shear force values may be viewed as a simplistic one dimensional measurement in comparison to the determination of tenderness (Perry et al., 2001). This is a result of the ‘halo affect’ where untrained consumers confound sensory traits (Hopkins et al., 2006) and thus sensory scores made by untrained consumers consider the contributions of water and fat content to the perception of tenderness (Perry et al., 2001). Furthermore, untrained consumers are less likely to record extreme values (Tornberg, 1996). Consequently, a few studies have found low correlations between shear force values and sensory scores of some muscles (Shackelford et al., 1997, Silva et al., 1999, Hopkins et al., 2006).
Yet, shear force values are still widely used to determine toughness as they are objective and are a useful predictor of tenderness, enabling meat to be ranked on toughness (Bouton et al., 1975, Tornberg, 1996, Okeudo & Moss, 2005, Thompson et al., 2008a, Oury et al., 2009, Radunz et al., 2009).

Although a discrete value, the interpretation and measurement of shear force values can differ depending on the equipment used (Derington et al., 2011, Hopkins et al., 2011a), laboratory variation (Hopkins et al., 2010), statistical handling of data recorded (Hopkins et al., 2012), muscle and location measured (Shackelford et al., 1997, Tschirhart-Hoelscher et al., 2006), the number of repetitions measured per sample (Holman et al., 2015a), freezing (Duckett et al., 1998) and whether the initial or peak force is recorded (Bouton et al., 1978). Therefore, the measurements reported in literature may vary greatly even for the same species or the same muscle (Tschirhart-Hoelscher et al., 2006, Hopkins et al., 2012) Consequently, Hopkins et al. (2006) have developed a threshold shear force value of 27 N as the target shear force value for meat given that sheep meat classified as good everyday (3 star) within the sheep meat eating quality program required an overall liking score of 63 (Thompson et al., 2005a). Yet, lamb with shear force values over 49 N was unacceptable to consumers (Hopkins et al., 2006).

2.5.2 Sarcomere Length

Due to the biochemical changes associated with the conversion from muscle to meat, measurement of the length of the sarcomere after the carcase has entered rigor mortis is measured as an indicator of shear force values as shorter sarcomeres at rigor may produce tougher meat (Hopkins et al., 2011b). This is largely a consequence of the structural changes associated with rigor and the longitudinal and lateral contractions of the myofibril that result in a denser myofibrillar mass with more fibres per unit cross sectional area (Tornberg, 1996). Yet sarcomere length (SL) alone does not account for toughness (Hwang et al., 2004) as the connective tissue matrix, particularly the perimysium, can prevent the myofibrillar mass contracting (Rowe, 1981, Tornberg, 1996). Thus, shortened muscles free of connective tissue are tougher than those with connective tissue (Lepetit, 2000). It has been found that for pork, at 24 h PM, up to 75% of the variation in tenderness could be accounted for by sarcomere length, proteolysis and collagen (Wheeler et al., 2000). However, after 24 h PM this varies depending on the extent of proteolysis.
Post slaughter processes can also impact on sarcomere length and related tenderness as there is an interaction between sarcomere length and the pH temperature decline during the formation of rigor. The affect is described by Hwang et al. (2004) who found that minimum sarcomere length was achieved when carcases enter rigor at temperatures between 10 – 18°C. Consequently, the position of the muscle of measurement within the carcase also affects sarcomere length as deeper muscles cool at a slower rate in comparison to externally located muscles (Howard & Judge, 1968). However, hanging methods can also reduce the impacts of shortened sarcomeres on tenderness as tenderstretch hung carcases physically constrain the *longissimus thoracis et lumborum* and other hind leg muscles, such as topside, from shortening by placing it under the tension of the leg (Bouton et al., 1973c, Hopkins et al., 2000). Given these relationships between sarcomere length and other factors, it is unsurprising that some authors have suggested sarcomere length explains variation in shear force (Feldhusen & Kühne, 1992, Lepetit, 2000, Wheeler et al., 2000, Hopkins et al., 2006) while others haven’t (Shackelford et al., 1997, Hwang et al., 2004).

Sarcomere lengths can be measured using a laser diffraction method whereby a helium-neon gas laser (wavelength 632.8nm) is used as a light source to obtain the diffraction patterns from muscle fibre samples compressed between glass microscope slides. Using the diffraction pattern displayed on the screen, measurement of the angle 2θ subtended by the diffraction bands equivalent to the 1st, 2nd or higher orders (n = 1, 2, 3…) facilitates the determination of sarcomere lengths from the equation \( n\lambda = s \sin \theta \), where \( \lambda = 632.8 \) (Bouton et al., 1973b). However, sarcomere length can also be measured using the filar micrometre method using phase contrast microscopy with an Olympus BX 40 microscope at 100 x magnification as previously described by Toohey et al. (2012a).
2.5.3 pH Values

As anaerobic glycolysis leads to an accumulation of free H+ ions (Scheffler & Gerrard, 2007), measurement of pH has been widely used in meat science as an indicator of the progression of the state of post mortem metabolism (Bouton et al., 1973a, Thompson et al., 2005a, Choe et al., 2008). Although some authors have found a relationship between ultimate pH (pHu) and pH decline (Warriss, 1990, Okeudo & Moss, 2005) a distinction needs to be made between ultimate pH values (pHu) and pH decline. Choe et al. (2008) outlines this difference stating that the rate of pH decline depends on the metabolic rate and glycolytic potential of the muscle, whilst pHu is a measure of the total extent of decline that is dependent on the concentrations of glycogen present in the muscle at slaughter.

2.5.3.1 pH decline

After slaughter, a muscle with a normal rate of pH decline decreases from a pH value of approximately 7.0 to a pHu of approximately 5.3 – 5.8 (Savell et al., 2005). Beef carcases have the slowest rate of decline typically taking 18 – 40 h to reach pHu and pork has the fastest rates reaching pHu between 6 – 12 h PM whilst lamb carcases are intermediate, reaching pHu by 24 h PM (Savell et al., 2005, Lawrie, 2006). However, as pH decline depends on the metabolic rate and glycolytic potential of the muscles (Choe et al., 2008) these rates of decline vary greatly between species and between animals due to stress prior to slaughter (Tornberg, 1996) muscle fibre type (Kerth, 2013) fat depths and carcase weights (Wheeler et al., 2000).

Since pork carcases contain a majority of type II glycolytic muscle fibres, which have a high glycolytic potential, in extreme cases they may reach pHu within 30 min post slaughter (Savell et al., 2005) and therefore may be affected by the conditions associated with pale soft and exudative (PSE) meat. This occurs as ATP declines rapidly during post mortem metabolism so carcases enter rigor at higher temperatures due to the generation of heat during rapid cellular metabolism and shorter chilling times before rigor. Overall this causes lower water holding capacity, lighter colour and a looser texture as the net charges of the myofibrils change, sarcoplasmic proteins denature due to heat and acidity and the filaments move (Scheffler & Gerrard, 2007, Laville et al., 2009) expulsing fluids in the extra cellular spaces (Tornberg, 1996) and altering the pathway of light from the surface (Hughes et al., 2014).
While a rapid pH decline of pork is more likely to cause PSE affected carcases this is not always the case as normal glycogen concentrations within the muscle will allow the pH to decline to a pHu within normal ranges (Scheffler & Gerrard, 2007). However, due to the rapid pH decline and high temperatures at rigor pork carcases are also highly susceptible to heat toughening, where the combination of high temperatures at low pH causes early exhaustion of the proteolytic activity, higher drip losses and increased toughness (Dransfield, 1993).

Although beef and lamb carcasses, have a slower rate of pH decline they too can be affected by higher temperatures at the onset of rigor and heat toughening, particularly for longer term grain fed animals where carcase size and fat depth are likely to inhibit the carcase from cooling. Furthermore, excessive use of ES can cause increased toughening and reduced tenderisation and increased purge with ageing as a result of elevated temperatures at rigor (Devine et al., 1999, Hopkins et al., 2014b). Consequently, the pH/temperature decline has been included in tenderness quality assurance programs, such as the Meat Standards Australia beef carcase grading system (Thompson, 2002).

Alternatively as lamb and beef typically have a slower rate of decline, they may also be affected by cold shortening as carcasses may cool before the pH decline is complete (Savell et al., 2005). Consequently, ATP may still be available for contraction when the temperatures drop below 10°C resulting in extensive shortening and reduced tenderness (Thompson, 2002). Thus, the relationship between sarcomere length and tenderness may be pH decline related (Feldhusen & Kühne, 1992), for example Hopkins et al. (2011b) found the best models to explain variation in shear force values of lamb at 5 days PM included pH@18 °C, temp at pH 6 and pH of the m. longissimus lumborum at 24 h PM (LL₂₄pH). However, despite different rates of decline, glycolysis may continue until pHu values are similar (Hwang et al., 2004).

### 2.5.3.2 Ultimate pH value

As pHu is an indicator of the glycogen concentration of the muscle at slaughter (Ferguson et al., 2008) it is often used as an indicator of tenderness and meat quality (Thompson, 2002, Thompson et al., 2005a) as it is a measure of the extent to which pH can decline as a result of post mortem metabolic processes (Lawrie, 1966). As a consequence of the extent of pH decline, pork and beef carcases are susceptible to quality issues including pale soft and exudative (PSE) meat, ‘acid meat’ and dry firm and dark (DFD) meat.
Despite several authors finding that relationships exist between pHu and meat quality traits including proteolytic activity and tenderness (Hopkins & Geesink, 2009), colour (Purslow et al., 2008) and water holding capacity (Huff Lonergan & Lonergan, 2005). These relationships are not linear (Devine et al., 1993) and are often reliant on a large range of pHu values including extreme values. Indeed Bouton et al. (1973a) suggest that pHu values need to be above 6 before the improvements in tenderness associated with high pHu values are noted. Thus, intermediate pHu values can affect shear force values with pHu values between pH 5.8 – 6.1 having the largest impacts on tenderness. Yet this relationship changes with ageing and different sarcomere lengths (Purchas, 2004). In contrast, meat with extremely low pHu values is likely to be tougher as the enzymes associated with proteolysis are inhibited by the increased acidification of the myofibril (Maltin et al., 2003). Given the convoluted nature of the relationships between pHu values and tenderness, it is not surprising that some authors have found a relationship between pHu and meat quality (Bouton et al., 1971, Bouton et al., 1973a, Smulders et al., 1990, Devine et al., 1993) while others have not (Hopkins et al., 2011b).

Due to the inexpensive and rapid measurement of pH using probes pH measurements are routinely conducted throughout pH decline periods (Bouton et al., 1973a, O'Neill et al., 2004, Hopkins et al., 2014b). However pHu values are also determined by using a homogenised sample of meat such as the method for pre-rigor determination of pH described by Dransfield et al. (1992) where 2.5 g of meat is homogenised at 19 000 rpm for two bursts of 15 s, in 10 mL of 5 mM iodoacetate/150 mM KCl that has been adjusted to pH 7.0. Between bursts of homogenisation, samples are held on ice for 30s after which they are incubated in a water bath at 20°C prior to being measured for pH (Toohey et al., 2013). Although this method is designed for pre-rigor meat, as the iodoacetate prevents further glycolysis, it also is suitable for pHu determination as the iodoacetate provides a buffer which enables the meat to be homogenised for an accurate measurement.
2.5.4 Particle Size

Particle size (PS) analysis has been developed as an alternative to myofibrillar fragmentation index (MFI) as a measure of post mortem proteolysis (Karumendu et al., 2009) as degradation of the myofibril during ageing causes cleavage of structural proteins (Hopkins & Thompson, 2002b). This was a result of the lack of specifics reported on the homogenisers and times used in studies using MFI (Karumendu et al., 2009) which limits the comparison of results between studies that have used MFI. Furthermore, a new method to determine particle size was reported as being more rapid and simpler than the method to determine MFI (Lametsch et al., 2007). Consequently, Karumendu et al. (2009) developed a method of measuring particle size of myofibres using lamb homogenised at 16 000 rpm using a laser diffraction method based on the principles of the Mie theory of light scattering first described by Lametsch et al. (2007) for the determination of PS in pork loin. However, results of particle size analysis are variable, hence a quality assurance method is required to ensure that the variation does not affect the statistical analysis (Hopkins et al., 2014a).

2.5.5 Histology

Given that the degradation of the myofibril with ageing causes cleavage of the structural proteins of the sarcomere (Hopkins & Thompson, 2002b), it is unsurprising that some authors (Taylor & Frylinck, 2003, Toohey et al., 2012b) have used histological and electron microscopy to detect the ultra-structural changes associated with proteolysis and increased tenderness. Indeed, all of the studies cited found that the number of breaks across the fibres reflected an increase in tenderness. However others have applied the use of electron microscopy to detect the structural changes associated with repeated freezing and thawing (Qi et al., 2012) as well as changes to the architecture of collagen as muscles enter into rigor (Nakamura et al., 2010).
2.5.6 Colour

As highlighted by a review on the research into meat colour by Mancini & Hunt (2005), the appearance of meat is a complex topic which involves genetics, ante- and post mortem conditions, the biochemical processes of cellular metabolism post mortem and muscle oxidation as well as many factors relating to the processing, packaging, distribution, storage and display of meats. However, as colour is vital to consumer acceptance of meat products (Hopkins et al., 2013b) and therefore included in carcase grading systems (Polkinghorne & Thompson, 2010) several instrumental, computer, visual and optic techniques are currently used to measure colour (Geesink et al., 2003, Mancini & Hunt, 2005, Holman et al., 2015b).

The main biochemical reactions responsible for meat colour are due to the reactions of myoglobin, a water soluble protein that contains 8 α-helices linked by short non-helical sections (Mancini & Hunt, 2005). Myoglobin may take on the forms of de-oxymyoglobin (DMb) when no ligand is present on the 6th coordination site, oxymyoglobin (oxyMb) when bound to a dioxygen molecule and metmyoglobin when bound to a water molecule, which gives meat the purple (DMb), red (oxyMb) and brown (MMb) colours (Kerth, 2013). These reactions of myoglobin are affected by other factors as temperature at rigor affects the formation of MMb and oxidative capacity affects the formation of oxyMb (te Pas et al., 2004). Hence, meat discolouration occurs when both the ferrous myoglobin derivatives oxidate to ferric iron (Mancini & Hunt, 2005).

However, myoglobin is not the only compound responsible for meat colour as dietary compounds including carotene and glycogen can affect the yellowness (b*) and lightness (L*) of meat products (Priolo et al., 2001). Since the glycogen concentration is partly responsible for L* values it is unsurprising that some authors have found a relationship between colour and pH (Bekhit et al., 2001, Priolo et al., 2001, Mancini & Hunt, 2005). Indeed, Brewer et al. (2001) suggest that L* is the best indicator of PSE or DFD pork, however this may be more a result of the impacts of these quality defects on the ultra-structure of the myofibril and consequently, the light reflected from the meat’s surface (Hughes et al., 2014).
2.5.7 Water Holding Capacity

Reduced water holding capacity is a problem for industry as it causes losses of weight through increased drip, purge and thaw losses (Huff Lonergan & Lonergan, 2005) and reduces the organoleptic sensations of juiciness and tenderness during consumption (Smith et al., 2008).

Fluids within the myofibril are held in several different fractions; as protein associated water that is bound tightly to the charged hydrophilic groups on the muscle proteins, immobilised water which has a less orderly molecular orientation toward the charged group or as free water which is held only by capillary forces (Pearce et al., 2011). Consequently, the ability of meat to hold fluids within the myofibril is a result of the structural integrity of the intra- and extra-myofibrillar spaces (which hold 85% and 15% of the water respectively (Pearce et al., 2011)), as well as the ability of the proteins to maintain the chemical charges holding the water during processes such as chilling, ageing, packaging, freezing, thawing and cooking.

During the conversion of muscle to meat immobilised water is the most affected. Changes in the net charges as the myofibril gets closer to the isoelectric point and the lateral and longitudinal contractions which characterise the onset of rigor can move immobilised water into extra-cellular spaces within the myofibril (Tornberg, 1996, Huff Lonergan & Lonergan, 2005). Subsequent processes such as ageing, freezing, thawing and cooking can then cause this water to be lost as purge, thaw or cooking losses as the structure of the myofibril changes reducing the amount of extracellular space within the muscle (Tornberg, 1996).

Due to these effects of the protein and myofibrillar denaturation, net charges and myofibrillar structure on the ability of the muscle to retain water, meat science research has studied the relationships between water losses and meat quality indicators. These include the relationships between cooking loss and shear force (Okeudo & Moss, 2005), purge and pH (Boler et al., 2010) as well as thaw losses and shear force values (Leygonie et al., 2012).
2.6 Technologies to Measure Meat Quality

There are many biophysical technologies that have been studied for application in the processing sector to assess meat quality. These technologies can be loosely categorised into mechanical, optic, dielectric, x-ray and nuclear magnetic resonance (NMR) methods.

Mechanical methods, such as Warner-Bratzler shear force, are most widely used to measure meat toughness (Bouton et al., 1975, Tornberg, 1996, Okeudo & Moss, 2005, Thompson et al., 2008a, Oury et al., 2009, Radunz et al., 2009) yet these techniques to measure mechanical force use bulky machines. Whilst development of smaller machines, such as the G2 Tenderometer, have been studied as a potential instrument for commercial use (Graafhuis et al., 1991, Cummings et al., 2008, Hopkins et al., 2011a), measurements of shear force are destructive, time intensive and comparable results are dependent on the sample orientation and blades used (Damez & Clerjon, 2008, Hopkins et al., 2011a). Specific requirements for technologies that overcome these limitations of mechanical measurements has directed research towards the development of potential spectroscopic techniques for measuring meat quality.

Advances in digital camera and laser technologies have facilitated greater research into optic methods as it has become feasible to develop fibre optic components and energy efficient lasers, suitable for portable devices (Damez & Clerjon, 2008). Technological advances in high-speed computers with high-capacity memory and high-speed data transfer, now facilitates real time evaluation and control during online application of optic technologies (Valous et al., 2010). As a result, there are many advantages in applying optical spectroscopic techniques within the meat industry, as outlined in Table 2.

Exciting an organic molecule with scattered light may cause a change in the vibrational state of the chemical bonds or the molecule may remain in a constant energy state (Das & Agrawal, 2011) depending on the type of light and the specific chemical structure of the material. Optical spectroscopic methods utilise these principles of propagation, radiation and absorption as well as the interactions between electromagnetic radiation and organic matter (Damez & Clerjon, 2008). Spectroscopic technologies such as near-infrared (NIR), infrared (IR), visible, ultraviolet (UV) and fluorescence spectroscopy are differentiated on the wavelength of scattered light used and detected (Table 2).
Table 2. Summary of spectroscopic technologies and their advantages and disadvantages.

<table>
<thead>
<tr>
<th>Method</th>
<th>Electromagnetic Wavelength Range</th>
<th>Advantages</th>
<th>Disadvantages</th>
<th>Current Uses</th>
<th>Sources</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infrared Spectroscopy</td>
<td>750nm-1000nm</td>
<td>Rapid, ease of use and versatile, has been developed into a hand-held portable device.</td>
<td>Transmission through liquids is most powerful in this range and gases but is inappropriate for undiluted solids- sample presentation is required for muscle samples.</td>
<td>Forensic and biomedical sciences, quality control, industrial monitoring and chemical measurement applications.</td>
<td>(Damez &amp; Clerjon, 2008)</td>
</tr>
<tr>
<td>Near-Infrared Spectroscopy</td>
<td>800-2500nm</td>
<td>Segregates post rigor ages with good accuracy. Modern instruments are more portable.</td>
<td>Sensitive to accumulation of free water. Samples require preparation such as mincing or biopsy.</td>
<td>Pharmaceuticals, medical diagnosis, food and agrochemical quality control, combustion research.</td>
<td>(Damez &amp; Clerjon, 2008, Fernández-Cabanás et al., 2011, Guy et al., 2011, Elmasry et al., 2012)</td>
</tr>
<tr>
<td>Visible Light Spectroscopy</td>
<td>380-740nm (often extended to the near UV and near IR regions)</td>
<td>Characterises tissues objectively and non-destructively.</td>
<td>Limited to detecting only structural and colourimetric information.</td>
<td>Biomedical sciences, and medical diagnostics.</td>
<td>(Swatland et al., 1995, Damez &amp; Clerjon, 2008)</td>
</tr>
<tr>
<td>Ultra Violet Spectroscopy</td>
<td>10-400nm</td>
<td>Sensitive to conjugated organic compounds and biological molecules such as tryptophan which are common in meat proteins.</td>
<td>Need to be combined with other methods such as fluorescence or Raman as limited transitions are recorded.</td>
<td>Analytical chemistry, some biomedical and meat science research applications.</td>
<td>(Hudson &amp; Mayne, 1986, Austin et al., 1993, Clerjon et al., 2011)</td>
</tr>
<tr>
<td>Fluorescence Spectroscopy</td>
<td>Usually uses polarised light in the UV spectrum (10-400nm)</td>
<td>Connective tissues have high fluorescence and are easily distinguished. Structure can also be determined as polarised light allows preferential excitement.</td>
<td>Works best at a right angle to the incident light which cannot be achieved in foods.</td>
<td>Biomedical, medical, and chemical research, and astronomy.</td>
<td>(Damez &amp; Clerjon, 2008, Clerjon et al., 2011)</td>
</tr>
</tbody>
</table>
Many studies have been conducted to assess the potential use of optic spectroscopic methods in assessing meat quality (Strasburg & Ludescher, 1995, Swatland, 1997a, Swatland, 1997b, Al-Jowder et al., 1999, Swatland, 2001, Alomar et al., 2003, Skjervold et al., 2003, Swatland, 2005, Sahar et al., 2009, Clerjon et al., 2011). Although fluorescent, reflective and UV spectroscopy have been investigated, issues with fluorescence, saturation, reliability when compared to reference analyses and complex processes to identify and quantify components has limited the application of these technologies (Austin et al., 1993, Grau et al., 2000, Graham et al., 2010). There has been much interest in the application of NIR as chemometric analysis has largely been able to overcome issues with fluorescence and saturation (Al-Jowder et al., 1999, Zamora-Rojas et al., 2012).

IR and NIR technologies commonly rely on diffuse transmittance and diffuse reflection of light between 800-1100nm, although transmittance, partial transmission, impedance, and absorption are also frequently used (Huang et al., 2008, Zamora-Rojas et al., 2012). NIR analysis has been used to assess fat, water and protein in meat samples, as NIR spectra arise from the absorption of energy and change of electromagnetic radiation wavelengths during the vibration of C-H, O-H and N-H bonds which are present in organic biomolecules (Huang et al., 2008).

Early NIR studies in meat focused on predicting the proximal analysis, in terms of fat, protein and moisture, of homogenised beef mince (Isaksson et al., 1996). This study concluded that a root mean square error of cross validation (RMSECV) of 0.73% could be achieved for fat, 0.75% for moisture and 0.32% for proteins. However, results were inconsistent and depended on the particle size of the minced meat, as a consequence of the size of the grinding plate with smaller particle size reducing variation of the sample. Correlations comparing the NIR spectra to total fat weight as determined by reference analysis suggested that fat can be accurately determined ($r = 0.97$). Despite low precision (RMSECV = 1.50%), this high accuracy prompted the investigation of NIR as a potential tool to determine the concentrations of fat, water and protein in industrial scale batches (Tøgersen et al., 1999) and in semi-frozen ground beef (Tøgersen et al., 2003). NIR has also been investigated as a potential tool to assess the adulteration of ground beef with offal and to determine whether different beef cuts could be distinguished within mince (Al-Jowder et al., 1999). This research reported it was possible to detect adulteration of beef mince with offal levels at less than 10% w/w addition. The addition of liver to beef mince, was easily identifiable as a distinct spectra which reflected the carbohydrates present in the liver.
The results of these early studies generated a large interest in applying NIR to meat assessment and a large amount of literature now exists on this technology (Campo et al., 2000, Rødbotten et al., 2000, Van Kempen, 2001, Ellis et al., 2002, Alomar et al., 2003, Cozzolino et al., 2003, Geesink et al., 2003, Tøgersen et al., 2003, Cozzolino & Murray, 2004, Barth, 2007, Prieto et al., 2008, Pérez-Marín et al., 2009, Prieto et al., 2009b, Fernández-Cabanás et al., 2011, Guy et al., 2011, Collell et al., 2012, De Marchi et al., 2012). A review of this literature reveals that while informative, a majority of these studies are not relevant to measuring meat quality of intact muscle. Comparison between these studies is difficult due to different NIR equipment, experimental designs and sample treatment. However issues relating to the application of NIR to meat assessment can be categorised into equipment, reference analysis, sample preparation, calibration models, sensitivity, optical and cost issues.

Equipment is limiting for online application given that most NIR research has focused on the use of bench top devices (Alomar et al., 2003, Cozzolino et al., 2003, Geesink et al., 2003, Cozzolino & Murray, 2004, Barlocco et al., 2006, Cozzolino et al., 2006, Savenije et al., 2006). While these studies demonstrate some possible applications of NIR in the meat industry, particularly in manufacturing, the use of bench top devices is not suitable for assessing intact muscle, as a muscle biopsy is required.

The inhomogeneous and complex structure of meat creates a challenge to determine a method of sampling required to generate representative subsamples. Some studies have overcome this by completing NIR scans after the samples have been homogenised (Al-Jowder et al., 1999, Cozzolino et al., 2003, Cozzolino & Murray, 2004, Prieto et al., 2009b). Despite this, it is well recognised that homogenisation of samples prior to scanning severely destroys and randomises the fibre arrangement, which negates the ability to accurately determine traits, like tenderness.

Other studies such as Alomar et al. (2003) have used frozen samples. A review of NIR research by Prieto et al. (2009a) concluded that freezing or freeze drying meats for NIR assessment increases the ability of NIR to predict meat quality. The intensity of NIR bands depends on the change of the dipole moment and light atoms such as hydrogen have the largest molecular vibrations (Blanco & Villarroya, 2002). When measuring meat, a main NIR band is measured that corresponds to the O-H bond of water, as a result of this molecular behaviour of the hydrogen atoms.
The size of this band obscures the intensities of other weaker NIR bands for bonds such as C=O and C-C (Blanco & Villarroya, 2002). Consequently, the spectra need to be mathematically corrected to subtract the contribution of water to the NIR spectra and highlight weaker bands. In non-freeze dried meat samples, there is a high water content (Lawrie, 1974), although it is variable (Hamm, 1974) and mathematical correction for its contribution could reduce the accuracy of the subsequent spectral analysis. The removal of variable water content by freezing drying would therefore improve the ability of NIR to pick up the weaker C=O and C-C bands that are common in proteins of meat (Lanza, 1983, Mitsumoto et al., 1991, Blanco & Villarroya, 2002). Furthermore, it is plausible that freeze drying can make the appearance of the meat more homogenous and reduce the affect of temperature variability on the NIR spectroscopy equipment. While NIR studies have been able to overcome these limitations in laboratory situations (Cozzolino et al., 2003, Prieto et al., 2009a, Fernández-Cabanás et al., 2011, Guy et al., 2011, De Marchi et al., 2012), freezing or freeze drying is destructive. Therefore, NIR techniques that rely on homogenisation or freezing to accurately and precisely predict meat quality are not suitable for online application.

Further developments in NIR equipment has seen development of hand held probes (Shackelford et al., 2005, Rosenvold et al., 2009, Liao et al., 2010, Prieto et al., 2011, Collell et al., 2012, De Marchi et al., 2012). However none of these are yet capable of commercial application as other issues with calibration models, chemometric analysis and time required to scan meat samples are yet to be overcome (Prieto et al., 2009a, Zamora-Rojas et al., 2012).

Disparity between reference analysis and chemometric modelling is also identified as a limitation of current on-line NIR studies. Reviews of current published results conducted by Prieto et al. (2009a) and Huang et al. (2008) have indicated that low predictability of meat quality traits, mainly tenderness, can in part be explained by variability in the trait of interest. Prieto et al. (2009a) propose that the low prediction of shear force values ($R^2 = 0.28$ in some studies) is most likely to have been caused by little variability of shear force measurements in the sample population. Suggestions of increasing the number of scans taken, more reference samples (Prieto et al., 2009a) or taking better control of the factors influencing spectral data and precision of reference methods (Huang et al., 2008) are costly, complex and labour intensive measures.
While these reviews also highlight other issues, such as cost of implementation and lack of sensitivity to the mineral content (Huang et al., 2008), adoption of NIR equipment for online assessment of intact muscle is mainly inhibited by the reaction of intact myofibrils to light energy when NIR absorption is measured. Intact muscle fibres themselves may act like fibre optic conduit during NIR measurement, tending to conduct light along their length by a series of internal reflections. Further exacerbating this issue, intact muscle also absorbs more energy giving less reflectance when compared to homogenised muscle (Prieto et al., 2009a). Therefore, the success of NIR depends on the ability of current technologies to overcome these issues.

2.7 Raman Spectroscopy

Raman spectroscopy is an optic method that has recently been highlighted as having potential to assess meat quality online in commercial applications (Damez & Clerjon, 2008). Raman is based on inelastic scattering of light, which leads to the vibrations of molecular bonds (Li-Chan et al., 1994), providing data on the structure, symmetry, electronic environment and bonding of the molecules (Das & Agrawal, 2011). As it is based on this scattering of light, Raman spectroscopy overcomes the issues associated with the internal reflection of light within intact muscle samples during measurement which can occur with NIR.

As explained by Das & Agrawal (2011) Raman spectroscopy is based on the principle that light, when directed at a molecule, interacts with matter and an exchange of energy occurs (Raman scattering). If the light interacts with the molecule and the net exchange of energy causes the light photon to gain vibrational energy from the molecule and the frequency is greater than the incident beam this is called Anti-Stokes Raman Scattering. However, the reverse may also happen, where matter gains energy from the incident beam resulting in a frequency which is lower than the incident beam and this is called Stokes Raman Scattering (Ozaki, 1999). These interactions with the excitation photons are graphed for wavenumber and intensity of the frequencies measured as a spectrum where the peaks indicate Anti-Stokes Raman Scattering and the troughs indicate Stokes Raman Scattering (Fig. 2.6). Furthermore, vibrations for different chemical bonds are distinct due to the wavenumber at which energy excites them and the movement of atoms which is generated by the exchange. Consequently, vibrations are characterised by their action including stretch (symmetric and asymmetric),
scissor, rock, deformation and the out of phase bends twist and wagging as illustrated in Figure. 2.7.

![Diagram of chemical bond vibrations](image)

**Figure. 2.6** An example Raman spectra showing the peaks and troughs associated with Anti-Stokes and Stokes Raman Scattering.

**Figure. 2.7** Examples of the chemical bond vibrations which are characterised by Raman spectroscopy.
Although the structure and force field of molecules cannot be obtained directly from the spectrum, approximations have been determined using the atomic displacements of vibrational modes of large molecules (Krimm & Bandekar, 1986a). Early Raman studies ascertained the character of the frequencies (intensity, height and symmetry) and the determination of their relation to the structure of the molecule using various pure and denatured samples, isotopic substitution and excitation at various wavelengths (Carey, 1982, Hudson & Mayne, 1986, Krimm & Bandekar, 1986a, Krimm, 1987).

Historically, Raman spectroscopy has many applications including chemical identification of materials in artworks (Breitman et al., 2007), quantitative analysis of fungicides, three dimensional imaging of chemical composition, rapid identification of biological samples, pharmacology, dermatology, cosmetic development, characterising pigments as well as atomic and element composition (Hudson & Mayne, 1986, Das & Agrawal, 2011). With recent advances in charge coupled devices and laser technologies, the use of Raman Spectroscopy is expanding and its uses within commercial food systems are being explored.

Raman spectroscopy is suitable for use in food systems as it can provide information about the structure, function and kinetics of biomolecules including proteins and lipids by identification of the vibrational bands (Das & Agrawal, 2011). Advantages for using Raman Spectroscopy, in lieu of other technologies, include no sample preparation so measurements can be taken in any physical state including gases, liquids, gels, amorphous solids and crystals. Raman is not affected by varying water content, it is non-destructive, non-invasive and can detect substances even if only small amounts are present. Furthermore, it is sensitive to structural changes that are induced by freezing, heating, mixing, aeration, fibre formation, denaturation and gelatination (Li-Chan et al., 1994, Li-Chan, 1996, Ozaki, 1999, Beattie et al., 2004b, Afseth et al., 2006, Chen & Han, 2011).

Although there are some strong advantages in using Raman within food systems, the presence of fluorescence and background noise are current challenges when measuring biological samples. As the level of light scattered by the Raman process is generally weak in comparison to other spectroscopic methods the vibrational spectrum can be completely obscured by other energy contributions including broad featureless fluorescence emission (Hudson & Mayne, 1986). Fluorescence occurs when a majority of light is fully absorbed and emitted at a longer wavenumber and therefore lower energy (Austin et al., 1993).
However, as described by Hudson and Mayne (1986) the use of highly effective filters and measuring at a wavelength greater than 250nm can, to some extent, overcome these challenges.

Like fluorescence, the presence of non-Raman background signals can completely obscure the Raman vibration modes (Beattie, 2011). Any photons that aren’t generated by the sample at the frequency of interest are considered background noise (McCreery, 2005). Background noise may arise from a range of optical phenomena (Beattie, 2011) including luminescence of cells, samples or optic equipment; stray light from Rayleigh scattering; and reflections from optics or dust (McCreery, 2005).

2.7.1 Use of Raman Spectroscopy in Previous Meat Studies

The potential of Raman Spectroscopy in food systems previously outlined has not been overlooked in recent meat science research. Preliminary investigations have been conducted on pork focussing on estimation of composition, tenderness, flavour and processing techniques, such as the addition of salt or heating, on the structure of manufactured and minced products (Olsen et al., 2007, Beattie et al., 2008, Olsen et al., 2010, Chen & Han, 2011, Lyndgaard et al., 2011, Wang et al., 2012). Furthermore, the prediction of the sensory quality of beef silverside has also been investigated using Raman (Beattie et al., 2004a) as well as the relationship between shear force, cooking loss and spectra in sheep meat (Schmidt et al., 2013).

Beattie et al. (2004a) suggested that Raman spectroscopy is a useful tool for predicting the sensory traits in beef silverside. This study reported a good coefficient of determination between the Raman spectra and texture ($R^2 = 0.71$), tenderness ($R^2 = 0.65$), juiciness ($R^2 = 0.62$) and overall liking ($R^2 = 0.67$). However, the commercial application of this finding is limited as the study was conducted using a bench top Raman device.

Further studies have been conducted to ascertain the potential of Raman spectroscopy as a tool to measure and predict the fatty acid content and iodine value of pork adipose tissue (Olsen et al., 2007, Olsen et al., 2010, Lyndgaard et al., 2011), determine whether porcine meat has been affected by warmed over flavour (Brøndum et al., 2000) and analyse fatty acid methyl esters (Beattie et al., 2004b).
Lynggaard et al. (2011) demonstrated Raman spectroscopy is capable of predicting the percentage of PUFA and Iodine Value (IV) with good accuracy ($R^2 = 0.72$ and $R^2 = 0.69$, respectively) and good precision (RMSECV = 1.17 and RMSECV = 2.00, respectively). These results agree with Olsen et al. (2010) who obtained an $R^2 = 0.89$ and an RMSECV = 1.97. This research aimed to validate the statistical model used by Olsen et al. (2007) and demonstrated the influence of pre-processing and statistical modelling on the precision and accuracy of the prediction. Limited pre-processing using only the first derivative Savitzky-Golay filter and a 2nd order polynomial resulted in a $R^2 = 0.89$ and a RMSEP = 3.70, while pre-processing with multiplicative scatter correction (MSC) increased the accuracy ($R^2 = 0.95$) and decreased the error prediction (RMSEP = 2.04) (Olsen et al., 2010). This indicates that the routine use of pre-processing tools, such as MSC, have an impact on the predictions reported. Regardless of data analysis, these studies provide good insight to measurement of fatty acids with Raman spectroscopy and bands indicative of the fatty acid profile.

The most common Raman equipment currently in use are the bench top Raman devices. A schematic diagram (Fig. 2.8) illustrates the arrangement, where a laser passes through the sample, and the scattered light is collected through a Charged Coupled Device (CCD) detector, after it has passed through a notch filter, and focused onto the CCD detector (Yang & Ying, 2011). While the bench top devices can be useful in suppressing fluorescence, which can be limiting when measuring biological samples (Hudson & Mayne, 1986), they are limited for in-situ measurement by their size, weight and the power consumption of the lasers (Schmidt et al., 2009).
Miniaturisation of the laser and implementation of fibre optics into Raman devices has enabled the development of ball probes which can be connected to Raman Spectroscopy instruments via a fibre optic cable (Olsen et al., 2007). However, the CCD and spectrograph technologies used with this probe are bulky and not suitable for in-situ use as collected light needs to be filtered before it can be focused and collected into the CCD.

Schmidt et al. (2009) have overcome the limitation of Raman bench top devices by developing a hand held Raman sensor head for in-situ characterisation of meat quality (Fig. 2.9). This technology uses the principles of back-scattering geometry, lens optics, integration of laser and Raman filter stage in one optical bench device. Miniaturisation of the bench top devices required the laser and band filters to be combined into a single module, along with two mirrors and a Raman edge filter as illustrated in Figure 2.10 (Schmidt et al., 2009). The back scattered light can also be detected, the Rayleigh scattered radiation can be removed and the Raman signal can be launched into an optical fibre within the same device (Schmidt et al., 2009).
Laboratory assessment of this technology showed that a wavelength of 671nm is optimal for measuring meat, as it is a trade-off between the short wavelengths required for high scattering intensities in meat and the long wavelengths required to reduce fluorescence of the biological molecules (Schmidt et al., 2010). However, other Raman spectroscopy measurement parameters including integration time which is the length of time used to collect photons on the detector and the number of integrations acquired (accumulations) can also be altered between samples to improve spectra, dependant on factors such as the rigor status of the meat.
A preliminary investigation of the hand held Raman sensor head illustrated that prediction for low and medium shear forces was high ($R^2 = 0.72$) but reduced for samples with high shear force, due to the low numbers of samples that had high shear force in the study. Although, the findings were similar to Beattie et al. (2004a) and it overcame the use of bench top devices, the application of this research to industry is limited. This is due to the freezing of samples prior to measurement, as Raman is sensitive to the structural changes that are induced by freezing (Li-Chan et al., 1994, Herrero, 2008b, Chan et al., 2011a).

2.7.2 Assignment of Bands

Early Raman spectroscopic studies by Krimm & Bandekar (1986a) and Carey (1982) determined the character of frequencies from Raman scattering. They also ascertained the empirical relationships between the structure of the molecule and the wavenumber and intensity of the spectrum to assign bands to specific vibrational states of bonds (Krimm & Bandekar, 1986a). Traditionally, these bands relied on group frequencies, symmetry of the peaks and isotopic substitution (Krimm, 1987).

2.7.2.1 Protein

Many studies have been completed to correlate the structure of proteins to Raman spectra (Carey, 1982, Harada et al., 1982, Hudson & Mayne, 1986, Krimm & Bandekar, 1986a, Tu, 1986, Austin et al., 1993, Ozaki, 1999). Some studies, such as that by Hudson & Mayne (1986), measured ribonucleotides and simple peptides with varying wavenumbers to create a Raman excitation profile for the proteins, while others including that by Krimm & Bandekar (1986a) compared the Raman spectra of chemically similar molecules. Further studies have correlated the Raman spectra of proteins engineered to have specific conformational traits denatured or substituted, to the spectra of the native protein (Tu, 1986, Krimm, 1987, Bandekar & Krimm, 1988). Despite the different approaches the results of these studies show clear peaks in the Raman spectra, where the electromagnetic energy is changed compared to the excitation wavenumber. For chemical bonds including C-H, N-H and C=O, the molecular vibration of these atoms is specific and does not change from one molecule to another (Krimm & Bandekar, 1986b, Krimm, 1987).
Although all chemical bonds vibrate when excited (Krimm, 1987), those that have a stretching mode vibrate with a more intense Raman signal than others (Ozaki, 1999). The stretching mode gives a more intense Raman signal when chemical bonds that include a heavy atom, such as S-S, or a double or triple chemical bond is present, such as C=S and C=N. More intense Raman signals are also given by symmetric vibrations, breathing modes of ring compounds such as phenylalanine and tryptophan side chains, as well as from the vibration of larger molecules or larger parts of molecules such as the stretching mode of a whole alkyl chain (Ozaki, 1999).

Based on extrapolation of the Raman spectra of peptides it was concluded that several distinct frequency peaks or ‘amide bands’ can be used to characterise the vibrational modes of the polypeptide backbone and the secondary structure of side chains (Carey, 1982, Harada et al., 1982, Tu, 1986). Of the possible amide bands, amide bands I and III are recognised as being the most important when examining proteins, as they reflect the structure of the –CO-NH-peptide bonds and have high Raman intensity (Carey, 1982).

Tu (1986) describes the amide I band as a reflection of the in plane carbonyl stretching vibration of C=O, however Austin et al. (1993) suggest that there is also small contribution from the N-H in plane bending. The location of this band isn’t set as it is affected by the secondary structures, as a consequence of the strength of the hydrogen bonds involved in maintaining the protein’s structure (Tu, 1986). A protein that is high in α-helices will cause this band to be centred in smaller wavenumbers (near the 1645- 1658cm\(^{-1}\)), although it will shift to be centred at larger wavenumbers if the protein has a high β- sheet content (1665-1680cm\(^{-1}\)) and random coils or the structure is unordered (1660-1665cm\(^{-1}\)) (Chen & Han, 2011).

The amide III band signifies the C-N stretch and N-H plane bend as well as the CH\(_3\)-C stretch and a small proportion of the C=O stretch (Tu, 1986). Unlike the amide I band, correlations between the amide III band and the secondary structure of proteins are not easily determined because amide III band has a maximum intensity between 1200-1300cm\(^{-1}\), which is an area of the spectrum which also includes the CH bending and the ring vibrations of tyrosine and phenylalanine side chains (Austin et al., 1993).
As with the location of the bands, the relationships between amide bands are also heavily reliant on the hydrogen bonds between C=O. When the C=O bond in a peptide is involved with a hydrogen bond to maintain stability of the polypeptide backbone, the frequency intensity of the amide I band is decreased, but the amide III band is increased (Hudson & Mayne, 1986). Furthermore, increasing the donation of a hydrogen bond to the carbonyl group also causes a decrease in the amide band I, but an increase the amide III band (Austin et al., 1993).

Moreover, Raman spectra can also provide greater information on proteins such as the presence of di-sulphide bonds, through the S-S stretching vibration at 500-550cm\(^{-1}\) and the presence of tyrosine, phenylalanine and tryptophan via the vibrations of the indole and aromatic side chains (Krimm & Bandekar, 1986b). The contribution of tyrosine and phenylalanine to the spectra is not well defined and they have many bands assigned to them. Tyrosine may have maximum intensity at 646, 830, 856 or 1208cm\(^{-1}\). While other bands such as 1605-1613cm\(^{-1}\), 1500-1510cm\(^{-1}\), 1265-1290cm\(^{-1}\) and 1170-1180cm\(^{-1}\) are also attributed to the phenolate ring vibrations of tyrosine residues (Tu, 1986). Tu (1986) suggests that some of this variation is caused by the arrangement of the tyrosine in relation to the hydrogen bonds and the strength of the bonds. If the tyrosine is both a hydrogen bond acceptor and donor and therefore classed as ‘exposed’, the vibration has a shorter wavenumber at maximum intensity in comparison to a tyrosine that is only a bond donor, a ‘buried’ tyrosine. Similarly phenylalanine may have peaks at 622cm\(^{-1}\), 1006cm\(^{-1}\), 1033cm\(^{-1}\), 1183cm\(^{-1}\), 1207cm\(^{-1}\), 1585cm\(^{-1}\) and 1605cm\(^{-1}\), depending on the strength of the whole molecules ‘breathing’ vibration.

A review by Tu (1986) proposes that despite having narrow frequencies, the location of these characteristic bands may vary based on temperature, pH, solvents used and the wavelength of scattered light used to excite the molecule. While some of these relationships are established in literature, such as the enhanced intensity of tryptophan at 1320cm\(^{-1}\) when measuring cryoimmunoglobins at -8°C compared to measurements at 35°C or at several bands between 757-1582cm\(^{-1}\) when exciting it at 363.8nm (Tu, 1986), it is unclear as to how improvements in laser and CCD technologies have affected band assignment. Likewise, no relationship has been established in the literature between these early studies at lower wavelengths and the current Raman spectroscopy studies of meat, which have been conducted at 671nm (Schmidt et al., 2013) and 785nm (Beattie et al., 2008).
2.7.2.2. Fatty Acids

Assignment of wavenumber bands to identify conformational traits of fatty acids has traditionally been done in a similar way to proteins, where assignments rely on the group frequencies and symmetry of the peaks (Krimm, 1987). However, unlike assignment of protein bands that have relied on isotopic substitution, assignment of fatty acid bands has relied on the effects of temperature on the conformation and fluidity of fatty acids (Susi et al., 1979, Zerbi et al., 1987, Bresson et al., 2005, Bresson et al., 2006). This is possible because the length of the fatty acid chain, position of the double bond and degree of unsaturation of the hydrocarbon chain affects the melting, hardening and transitional phases (Susi et al., 1979). Consequently, the vibrational behaviour of the carbonyl and methylene groups depends on the temperature (Bresson et al., 2006).

Comparisons between the Raman spectra and conformation of fatty acids have identified several important peaks. These occur because the stretching modes of the C-C, C=C, C=O and =CH double bonds as well as the scissor and twist deformations of the methylene group, which give a more intense Raman signal (Ozaki, 1999). While these chemical bonds give specific Raman peaks, such as the ester or olefinic C=C bonds at 1640-1660cm\(^{-1}\), they are subject to a range of external and internal conditions, which can change the location and intensity (Beattie et al., 2004b). Zerbi et al. (1987) suggest that this effect is greatest in the wavenumbers between 1000cm\(^{-1}\) and 1500 cm\(^{-1}\) which is dominated by the C-C stretching and CH\(_2\) scissor vibrations, respectively. It is proposed that this is a consequence of the polarity of the carbon bonds, where the carboxyl group induces a strong polarity of the C-C bonds close to it on the hydrocarbon chain. A more intense Raman signal is measured for the CH\(_2\) scissor vibration in comparison to a C-C bond that is further from the carboxyl group (Zerbi et al., 1987). When the sample is heated, the intensity of the bands starts to decrease and collapses as the fatty acid approaches its melting point.

If the effect of temperature on the conformation and fluidity of the fatty acid is known, the intensity of the CH\(_2\) and C=O stretching modes at 2845cm\(^{-1}\) can be extrapolated to provide information on the polymorphic form (Bresson et al., 2006). However, there is a limited ability to do so when mixed fats are present as there will always be some components closer to their normal melting temperatures in comparison to others (Beattie et al., 2004a). Therefore, the effect of the transitional phases needs to be considered when comparing between studies to determine group frequencies.
Socrates (2001) has overcome this by establishing the relationship between Raman spectra of melted fat with the spectra of adipose tissue. This highlighted group frequencies at 1263 cm\(^{-1}\) (=C-H unconjugated cis- bonds), 1306 cm\(^{-1}\) (in-phase methylene twist), 1443 cm\(^{-1}\) (methylene scissoring deformation), 1655 cm\(^{-1}\) (HC=CH stretch), 1743 cm\(^{-1}\) (C=O stretch) and 2853-2898 cm\(^{-1}\) (methylene and methyl symmetric stretch) that have good correlations with prediction of PUFA, MUFA and SFA \((R^2 = 0.98 – 0.73)\) when compared to gas chromatography (GC), Fourier Transform Infra-red (FTIR) and NIR fatty acid analysis (Olsen \textit{et al.}, 2007). However, interpretation of these frequencies and what they mean for the composition of lipids has not been well defined.

2.7 Limitations of Current Literature

From the literature, it is clear that Raman Spectroscopy has the potential to be used for online measurement of eating quality traits in intact muscle in a rapid, non-destructive way. However, the relationship between Raman spectra and the biophysical/biochemical structure of the muscle has not yet been determined. Research questions that have emerged from the literature with respect to eating quality traits and their measurement include;

- What is the potential of the Raman hand held probe as an online tool to measure and predict meat quality traits?

- What are the relationships between the Raman spectra and the biophysical and biochemical structures of the muscle?

- What are the relationships between the Raman spectra and biochemical processes of the muscle during early post mortem events, ageing, freezing and thawing?

- What is the impact of improvements in spectroscopic technologies and different parameters on the Raman band assignments?
2.8 Aims and Objectives

Research to begin to address the limitations identified in literature should establish whether Raman Spectroscopy measurement of meat can be useful to predict eating quality traits before and after aging periods. As the proposed research aims to develop and validate a probe for assessing meat quality for online application, it is first essential to define meat quality and describe the factors which contribute to variation of meat quality between animals and between muscles using traditional indicators of meat quality including shear force, intramuscular fat and fatty acid composition. Comparisons of Raman spectra and reference analyses will then be undertaken to determine the potential use of Raman spectroscopy in predicting these. To further understand any derived relationships the biophysical and biochemical changes that occur during the ageing period will be investigated in reference to Raman spectra. Sheep meat was selected for the study, although the technology is relevant to beef meat.
3. Raman spectroscopy compared against traditional predictors of shear force in *m. longissimus lumborum*

3.1 Introduction

Although tenderness may not be as significant in determining the eating quality of lamb in comparison to other sensory qualities, tough lamb is still associated with poor eating performance for the consumer (Thompson et al., 2005b). Due to the number of factors, from production, processing, value-adding and cooking, which impact on tenderness and eating quality of red meat products, it is unsurprising that a significant amount of research has concentrated on technologies which may have the potential to objectively measure tenderness. A review of these technologies demonstrated the potential of Raman Spectroscopy (RS) for online measure of meat quality (Damez & Clerjon, 2008).

Raman spectroscopy has several distinct advantages for online measurement of meat products as it is rapid, non-destructive, not affected by varying water content and is not based on the absorption of light (Yang & Ying, 2011). Meat science research has not overlooked these advantages and studies have revealed Raman spectroscopy is able to explain variation in shear force values ($R^2 = 0.77$) of pork (Beattie et al., 2008), predict sensory traits of cooked beef silverside (Beattie et al., 2004a). However, the Raman bench top device that was used for these studies is not suitable for industrial application and the integration times used are too long for online scenarios. Alternatively, Schmidt et al. (2013) reported that Raman spectra taken with a hand held device enabled the prediction of shear force values of lamb *m. longissimus thoracis et lumborum* ($R^2 = 0.79$ and 0.86 for two sample groups), although these samples were frozen and thawed. A further study by Bauer et al. (2013) demonstrated the potential of the same hand held Raman device to predict shear force values of aged bovine *m. gluteus medius muscles* ($R^2 = 0.87$).

Given the economic value of lamb *m. longissimus lumborum* (LL) to a carcase and its common measurement for a range of traits, the aim of this research was to assess the potential of the hand held Raman device to predict the tenderness of fresh intact *m. longissimus lumborum* (LL).
3.2 Materials and Methods

3.2.1 Samples

Samples of LL (Product identification number HAM 4866; Anonymous, 2005) were collected from 80 sheep, over 4 consecutive days (20 samples per muscle per day) from the same abattoir. All lambs were slaughtered in the same commercial abattoir without electrical stimulation, and the loins were removed at 24 h post mortem. Samples were randomly selected from different consignments and thus, were of varying backgrounds, sex and age, representing animals typically processed by the abattoir to achieve a spread in shear force values. The *m. semimembranosus* (SM) was also removed from the same carcases for a similar study to assess the ability of Raman spectroscopy to predict shear force on a different muscle (data reported in Chapter 4).

3.2.2 Raman Spectroscopy

Prior to measurement, the Raman spectroscopic device was calibrated on polystyrene. Raman spectra were then collected on chilled intact muscle with the silver skin removed (Fig. 3.1) at a room temperature of 16 °C. A hand held Raman spectroscopic device with a 671nm laser using 70mW of power and an integration time of 3 s was used for measurement with 1 accumulation.

![Hand held Raman spectroscopic device showing the measurement of fresh intact LL with the epimysium removed.](image)

Figure 3.1 Hand held Raman spectroscopic device showing the measurement of fresh intact LL with the epimysium removed.
As illustrated in Fig 3.1, 12 Raman measurements of the muscle were taken along each of the caudal and medial portions of the LL, perpendicular to the muscle fibre direction 24 h PM. Ageing treatments (24 h and 5 days PM) were allocated across the caudal and medial portions of LL in a balanced manner across carcases and the remaining cranial portion was removed and frozen at -20 °C for IMF analysis (data reported in Chapter 8).

After Raman spectra were collected 24 h PM, LL portions allocated to 5 days post slaughter ageing were vacuum packed and held at -1°C for 4 days. At 5 days PM, the vacuum packs were opened for 2 hours, allowing samples to ‘bloom’ so the metmyoglobin could re-bind to oxygen (Lee et al., 2008) and a freshly cut (very fine surface removed) area was scanned to avoid losing the weak Raman signal due to fluorescence and saturation (McCreery, 2000). As with previous Raman spectroscopic measurements, scans at day 5 post mortem were also conducted perpendicular to the muscle fibre direction, with the same parameters used 24 h PM.

3.2.3 Traditional Indicators of Tenderness

3.2.3.1 24 h Aged LL

LL portions allocated to an ageing period of 24 h PM had sections excised after initial Raman measurement to test for the traditional indicators of tenderness; shear force (SF), cooking loss (CL), ultimate pH (pHu), sarcomere length (SL) and particle size analysis (PS).

SF tests were conducted on blocks (mean weight 64g), cut from the middle of the muscle and cooked in a waterbath at 71°C for 35 mins. Measurement was completed using a Lloyd texture analyser with a vee-blade, as described by Hopkins et al. (2011a). SF measurements used were the mean of 6 replicate values, except where the co-efficient of variation exceeded 24% then the median value of the 6 replicates was used (Hopkins et al., 2012). Blocks were weighed before and after cooking to determine CL expressed as a percentage of raw muscle weight.

pHu was determined using 2.5g of muscle homogenate in 10 ml of 5 mM iodoacetate/150 mM KCl (pH adjusted to 7.0), as explained by Dransfield et al. (1992) and the laser diffraction method was used to measure SL, as previously described by Bouton et al. (1973c). PS was measured as previously described by Karumendu et al. (2009).
3.2.3.2 Day 5 Post Mortem Aged LL

LL portions allocated to 5 day post slaughter ageing had 1-2 g sections removed at 24 h PM to measure PS (Karumendu et al., 2009) and SL (Bouton et al., 1973c), as well as a 1 cm³ section for histological imaging to quantify myofibrillar breaks.

Sections excised for histological imaging were fixed in a solution of 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1M phosphate buffer (adapted from Taylor & Frylinck (2003)). Embedding and staining was completed before images were taken on a Leica DMR microscope and Nikon DXM 1200F digital camera, and total and partial breaks across the myofibril were quantified over 45 fibres per sample (Hopkins et al., 2007).

At 5 days post mortem, after Raman spectroscopy measurements, further sections were removed to determine PS, histological imaging, SF and CL, as previously described for 24 h PM aged LL portions.

3.2.4 Data Analysis

Before scanning each sample, a measurement without using the laser or ‘dark scan’ was completed to determine the background interference. This background was removed from the spectra before they were saved as raw data. Principle Component Analysis (PCA) was performed using MATLAB 7.9.0 (R2009b) software (TheMathWorks Inc., Natick, MA, USA) on muscle spectral data to identify spectra that did not relate to meat and ascertain ‘clean’ meat spectral data without interference that were not detected and removed at measurement. Spectra which had high PCA values were checked and removed if intensities correlating to lamb were not present in the spectra. Sub-sets of spectra for each sample were then averaged and the wavenumber frequencies were reduced to a range of 500-1800cm⁻¹.

Alternative pre-processing methods were compared including no pre-processing, standardisation using the l²-norm (square root of sum of squared intensities), two versions of the Savitzky-Golay filter and Multiplicative normalisation at 1517cm⁻¹.

Standardisation using the l²-norm or the square root of sum of squared intensities is described by the equation:

\[
\text{Standardisation l²-norm}(Y_{i,j}) = Y_{i,j} / \sqrt{\sum Y_{i,j}^2}
\]

Where \( Y_{i,j} \) denotes the average Raman intensity for the \( i^{th} \) sample at the \( j^{th} \) frequency.
The two versions of the Savitzky-Golay filter are described by the equations:

a. Savitzky-Golay filter with \( p = 2, m = 0, w = 15 \) (SGF.2.0.15)

b. Savitzky-Golay filter with \( p = 2, m = 2, w = 15 \) (SGF.2.2.15)

Including the parameters for polynomial degree (\( p \)), the order of differentiation (\( m \)), and the window width (\( w \)).

Multiplicative normalisation at 1517cm\(^{-1}\) is defined by the equation:

\[
\text{Norm1517}(Y_{i,j}) = \frac{Y_{i,j}}{Y_{ic}}
\]

Where \( Y_{i,j} \) denotes the average Raman intensity for the \( i^{th} \) sample at the \( j^{th} \) frequency and \( Y_{ic} \) the average Raman intensity for the \( i^{th} \) sample at frequency 1517 cm\(^{-1}\).

The k-fold (\( k=8 \)) cross validation method was used to determine the number of latent vectors to include in the model. The number of latent vector was chosen based on the number of components with the minimum average cross validated root mean square error of prediction (optimal RMSEP) across 20 replicates of the k-8 cross validation. Once the number of latent vectors had been established, partial least squares regression analysis (PLS) was fitted using leave one out (LOO) cross validation to determine the coefficient of determination between the predicted shear force values based on the spectra and observed shear force values. A Null model of the observed shear force values was obtained by predicting the average shear force value leaving one observation out at a time. Spectra for the assignment of vibrational bands were background corrected at 510 cm\(^{-1}\), 766 cm\(^{-1}\), 968 cm\(^{-1}\), 1138 cm\(^{-1}\), 1528 cm\(^{-1}\), 1719 cm\(^{-1}\) and 1999 cm\(^{-1}\). Further models combining Raman spectra and the traditional indicators of shear force was also fitted using PLS regression.

Models for the prediction of shear force based on traditional indicators of SF, SL, pHu, CL, breaks across the myofibril and/or PS were fitted using simple linear regression.

### 3.3 Results

#### 3.3.1 Raman Spectroscopy

Spectra collected in this data set exhibit the typical Raman signal for muscle tissue with peaks in intensity representing key chemical bond vibrations of amino acids including tryptophan, tyrosine and phenylalanine, as well as those of protein backbone conformations
and secondary protein structures (Pézolet et al., 1988). The main intensity peaks for lamb are illustrated in Figure 3.2 and outlined in Table 1.

Figure 3.2. An example of the unprocessed Raman spectra of lamb muscle, illustrating the main intensity peaks of the aromatic amino acid (tryptophan, tyrosine and phenylalanine) side chains and the peptide backbone signals (α-helix, amide I and III as well as C-H deformations) (Pézolet et al., 1988).
Table 3.1. The position and band assignments of the main peaks in intensity in the Raman spectra of muscle tissue (adapted from Beattie et al, 2004; Beattie et al, 2008; Schmidt et al, 2013).

<table>
<thead>
<tr>
<th>Approx. Wavenumber (cm(^{-1}))</th>
<th>Assignment and Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>410-480</td>
<td>Pro and Hyp</td>
</tr>
<tr>
<td>500-507</td>
<td>Phe and Tyr</td>
</tr>
<tr>
<td>520</td>
<td>S-S bond</td>
</tr>
<tr>
<td>600-800</td>
<td>Cys and Met modes with Phe, Val Ile and Tyr</td>
</tr>
<tr>
<td>650-700</td>
<td>Maximum fluorescence</td>
</tr>
<tr>
<td>660-720</td>
<td>C-S bond</td>
</tr>
<tr>
<td>757</td>
<td>Trp</td>
</tr>
<tr>
<td>826 &amp; 853</td>
<td>Tyr doublet</td>
</tr>
<tr>
<td>877</td>
<td>Trp</td>
</tr>
<tr>
<td>820-860</td>
<td>Tyr – Fermi resonance</td>
</tr>
<tr>
<td>880-965</td>
<td>C-C stretching modes</td>
</tr>
<tr>
<td>900</td>
<td>C-C stretch ((\alpha)-helix)</td>
</tr>
<tr>
<td>1000 (1003)</td>
<td>Phe (insensitive to environment*)</td>
</tr>
<tr>
<td>1020-1130</td>
<td>C-C, N-stretch (lipids and proteins)</td>
</tr>
<tr>
<td>1214</td>
<td>Tyr + Phe</td>
</tr>
<tr>
<td>1225-1305</td>
<td>Amide III (CH(_2) residues also and sensitive to secondary and tertiary structures)</td>
</tr>
<tr>
<td>1250-1300</td>
<td>Amide III</td>
</tr>
<tr>
<td>1260</td>
<td>His, tautomers II</td>
</tr>
<tr>
<td>1270</td>
<td>(=) C – H deformations (lipids)</td>
</tr>
<tr>
<td>1300</td>
<td>CH(_2) twist (lipids)</td>
</tr>
<tr>
<td>1320</td>
<td>C-H deformations</td>
</tr>
<tr>
<td>1338</td>
<td>Trp</td>
</tr>
<tr>
<td>1400</td>
<td>COO(^{-}) stretch for Asp + Glu</td>
</tr>
<tr>
<td>1450</td>
<td>C-H deformations (decreases with increasing hydrophobicity)</td>
</tr>
<tr>
<td>1548</td>
<td>Trp</td>
</tr>
<tr>
<td>1587</td>
<td>Phe and Arg</td>
</tr>
<tr>
<td>1607-1615</td>
<td>Phe and Tyr</td>
</tr>
<tr>
<td>1640-1685</td>
<td>Amide I- COONH (sensitive to secondary structure)</td>
</tr>
<tr>
<td>1647</td>
<td>(\alpha)- helix</td>
</tr>
<tr>
<td>1650</td>
<td>Amide I</td>
</tr>
</tbody>
</table>

Pre-processing LL spectra at 24 h and 5 days PM by subtracting a 5\(^{th}\) order polynomial, shows that spectral changes associated with ageing occur between 500 – 800cm\(^{-1}\) and 1150 – 1700cm\(^{-1}\). However, smaller changes in intensity also occur between 790- 1125cm\(^{-1}\) (Fig. 3.3).
Chemometric analysis using the Raman spectral data to predict shear force values of the LL at 24 h and 5 days PM are summarised in Table 3.2. The predictability of these models varied, producing $R^2_{cv}$ values of 0.00 – 0.09 (PLS). Although there was no significant reduction in the optimal RMSEP or cross validated coefficients of determination for models using any alternative pre-processing approach.
Table 3.2 Summary of the PLS regression analysis results using Raman Spectroscopy measured at 24 h and 5 days PM to predict shear force values of fresh intact LL measured at 5 days PM.

<table>
<thead>
<tr>
<th>Pre-processing Method</th>
<th>Spectra Measurement Time</th>
<th>No. Latent Vectors</th>
<th>Optimal RMSEP (Latent Variables)</th>
<th>$R^2_{cv}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>24 h</td>
<td>3</td>
<td>13.3</td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td>5 Day</td>
<td>0</td>
<td>10.0</td>
<td>0.00</td>
</tr>
<tr>
<td>Standardisation using l2-norm</td>
<td>24 h</td>
<td>2</td>
<td>13.2</td>
<td>0.09</td>
</tr>
<tr>
<td></td>
<td>5 Day</td>
<td>1</td>
<td>9.9</td>
<td>0.02</td>
</tr>
<tr>
<td>Savitzky-Golay filter a.</td>
<td>24 h</td>
<td>3</td>
<td>13.3</td>
<td>0.07</td>
</tr>
<tr>
<td>(SGF.2.0.15)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Savitzky-Golay filter b.</td>
<td>24 h</td>
<td>0</td>
<td>13.8</td>
<td>0.00</td>
</tr>
<tr>
<td>(SGF.2.2.15)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Multiplicative Normalisation</td>
<td>24 h</td>
<td>2</td>
<td>13.3</td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td>5 Day</td>
<td>0</td>
<td>10.0</td>
<td>0.00</td>
</tr>
</tbody>
</table>

Based on the $R^2_{cv}$ criterion, the best model was the prediction of shear force values 24 h PM using Raman spectra collected 24 h PM that have been standardised using the l2-norm ($R^2_{cv} = 0.09$; Fig 3.4). This model gave a RMSEP of 13.2, using 2 latent vectors.

![Graph](attachment:image.png)

Figure 3.4. Cross validated prediction of shear force values (N) at 24 h post mortem based on Raman spectra collected 1d post mortem and analysed with 2 latent vectors.
As illustrated in Figure 3.5, prediction of the difference in LL shear force values between 1 and 5 days post mortem based on the difference between Raman spectra collected on day 1 and day 5, yielded an $R^2_{cv} = 0.17$. This model gave a RMSEP of 11.7, based on 6 latent variables.

![Figure 3.5](image.png)

**Figure 3.5.** Cross validated prediction of the difference between 1 and 5 day post mortem LL shear force values (N) based on the difference between Raman spectra taken 1 and 5 days post mortem and analysed with 6 latent vectors.

### 3.3.2 Traditional Indicators of Shear force

Summary results for the traditional indicators of tenderness (shear force, cooking loss, sarcomere length, pHu, particle size analysis and myofibrillar breaks) are given in Table 3.3.
Table 3.3. Mean, standard deviation (SD) and range of shear force (N), cooking loss (%), sarcomere length (µm), pH24, Particle Size Analysis (µm) and myofibrillar breaks (%) number of full and partial breaks) of lamb m. longissimus lumborum (n = 80).

<table>
<thead>
<tr>
<th>Trait</th>
<th>Ageing (days)</th>
<th>Mean</th>
<th>SD</th>
<th>Range (min, max)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shear Force (N)</td>
<td>1</td>
<td>60</td>
<td>13.7</td>
<td>34.8 - 87.1</td>
</tr>
<tr>
<td>Shear Force (N)</td>
<td>5</td>
<td>38.8</td>
<td>9.9</td>
<td>20.9 - 64.6</td>
</tr>
<tr>
<td>Cooking Loss (%)</td>
<td>1</td>
<td>17.3</td>
<td>2.7</td>
<td>11.3 - 25.9</td>
</tr>
<tr>
<td>Cooking Loss (%)</td>
<td>5</td>
<td>19.1</td>
<td>4.3</td>
<td>9.8 - 28.8</td>
</tr>
<tr>
<td>Sarcomere Length (µm)</td>
<td>1</td>
<td>1.65</td>
<td>0.09</td>
<td>1.36 - 1.86</td>
</tr>
<tr>
<td>pH24</td>
<td>1</td>
<td>5.69</td>
<td>0.16</td>
<td>5.54 - 6.29</td>
</tr>
<tr>
<td>PS (µm)*</td>
<td>1</td>
<td>176</td>
<td>38.2</td>
<td>94 - 285</td>
</tr>
<tr>
<td>PS (µm)*</td>
<td>1</td>
<td>196</td>
<td>66.6</td>
<td>83 - 433</td>
</tr>
<tr>
<td>PS (µm)</td>
<td>5</td>
<td>127</td>
<td>24.2</td>
<td>84 - 236</td>
</tr>
<tr>
<td>Myofibrillar breaks (%)</td>
<td>1</td>
<td>0.13</td>
<td>0.22</td>
<td>0 - 1.4</td>
</tr>
<tr>
<td>Myofibrillar breaks (%)</td>
<td>5</td>
<td>0.67</td>
<td>0.57</td>
<td>0 - 2.2</td>
</tr>
</tbody>
</table>

*PS measurement at 24 h for loin portion allocated to 24 h PM ageing period.
*PS measurement at 24 h for loin portion allocated to 5 day PM ageing period.

In Table 3.4, the RMSEP values for the cross validated prediction models using the traditional indicators (sarcomere length, cooking loss and/or pHu) and Raman spectra to predict shear force values of the LL at 24 h and 5 days PM are summarised.

Table 3.4. The RMSEP for models using traditional indicators and Raman spectra to predict shear force values (N) of lamb LL 24 h and 5 days post mortem.

<table>
<thead>
<tr>
<th>Model Covariates</th>
<th>Shear Force (N)</th>
<th>Relative Reduction in RMSEP (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(24 h)</td>
<td>(5 Day)</td>
</tr>
<tr>
<td></td>
<td>(24 h)</td>
<td>(Day 5)</td>
</tr>
<tr>
<td>Null</td>
<td>14.0</td>
<td>10.0</td>
</tr>
<tr>
<td></td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Cooking loss (CL)</td>
<td>13.5</td>
<td>9.7</td>
</tr>
<tr>
<td></td>
<td>3.6</td>
<td>3</td>
</tr>
<tr>
<td>Sarcomere length (SL)</td>
<td>12.9</td>
<td>10.1</td>
</tr>
<tr>
<td></td>
<td>7.9</td>
<td>- 1.0</td>
</tr>
<tr>
<td>pH24</td>
<td>14.0</td>
<td>10.1</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>- 1.0</td>
</tr>
<tr>
<td>PS</td>
<td>14.0</td>
<td>10.2</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>- 2.0</td>
</tr>
<tr>
<td>Myofibrillar breaks (MB)</td>
<td>13.8</td>
<td>9.8</td>
</tr>
<tr>
<td></td>
<td>1.4</td>
<td>2.0</td>
</tr>
<tr>
<td>CL, SL, PS, pH24 and MB (all available)</td>
<td>13.4</td>
<td>9.9</td>
</tr>
<tr>
<td></td>
<td>4.3</td>
<td>1.0</td>
</tr>
<tr>
<td>Raman Spectra (24 h)</td>
<td>13.6</td>
<td>10.0</td>
</tr>
<tr>
<td></td>
<td>2.9</td>
<td>0</td>
</tr>
<tr>
<td>Raman Spectra (5 day)</td>
<td>—</td>
<td>10.0</td>
</tr>
<tr>
<td></td>
<td>—</td>
<td>0</td>
</tr>
<tr>
<td>Raman Spectra (24 h) + CL, SL, PS, MB and pH24*</td>
<td>13.6</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>2.9</td>
<td>—</td>
</tr>
<tr>
<td>Raman Spectra (24 h) + CL, SL, PS, MB and pH24*</td>
<td>—</td>
<td>10.0</td>
</tr>
<tr>
<td></td>
<td>—</td>
<td>0</td>
</tr>
<tr>
<td>Raman Spectra (5 day) + CL, SL, PS, MB and pH24</td>
<td>—</td>
<td>10.0</td>
</tr>
<tr>
<td></td>
<td>—</td>
<td>0</td>
</tr>
</tbody>
</table>

* Model 24 h data for LL portion allocated to 24 h PM ageing period.
* Model 24 h data for LL portion allocated to 5 day PM ageing period.
Of the indicators reported (Table 3.4) the most precise model for predicting shear force at 24 h used sarcomere length (RMSEP = 12.9), however although overall the relationship between shear force values and sarcomere length is weak ($R^2 = 0.14$; Fig. 3.6). There was small improvements in the precision of models that included the traditional indicators and Raman Spectra as covariates, although these improvements (RMSEP = 13.2 – 13.4) were marginal in comparison to the null model (RMSEP = 13.8).

![Figure 3.6. The relationship between LL shear force values (N) and sarcomere length (µm) 24 h PM.](image)

The prediction error of the models to predict LL shear force at 5 days post mortem (Table 3.4) show that the most precise model is gained by using cooking loss (RMSEP = 9.7; Null RMSEP = 10.0), although this is only marginally more precise than the combination of all three traditional indicators and Raman spectra collected 5 days post mortem (RMSEP = 9.8). The relationship between shear force and cooking loss is weak at both 1 and 5 days ($R^2= 0.07$ and $R^2= 0.06$, respectively).

There was no significant linear trend between shear force values and full breaks across the myofibril, with or without adjustment for partial breaks. However, after adjusting for ageing period, shear force significantly decreased ($P < 0.05$) with increasing partial breaks across the myofibril (Fig. 3.7). Shear force values were predicted to decline by 5.9 N (s.e. = 2.8 N) with each percentage increase in partial breaks.
Figure 3.7 Electron microscopy 50 x image of ovine *m. longissimus lumborum* showing partial breaks across the myofibril (indicated by arrows).

### 3.4 Discussion

#### 3.4.1 Prediction of Shear force with Raman Spectroscopy

There is a poor ability to use Raman spectra to predict the shear force values of LL, despite prediction models varying slightly with different pre-processing approaches. Despite Schmidt *et al.* (2013) using the same hand held Raman spectroscopic device to predict shear force values of raw lamb LL with good accuracy ($R^2 = 0.79$ and $R^2 = 0.83$, from samples obtained from two sites), there is little accordance between studies. While both studies were conducted on intact and raw lamb LL, Schmidt *et al.* (2013) conducted Raman Spectroscopic measurement on 3 cm thick sections of LL that were aged, before frozen at $-20^\circ$C and thawed prior to measurement. This is problematic as Raman Spectroscopy determines the “chemical fingerprint” of substances by measuring the exchange in energy of the chemical bonds from the amino acid side chains and peptide backbone when excited and is therefore sensitive to the changes which occur to the myofibril when meat is frozen and thawed (Li-Chan *et al.*, 1994, Herrero, 2008b). Furthermore, this study is the first to conduct both Raman Spectroscopy, shear force and traditional indicator measurements on the same part of the muscle as Schmidt *et al.* (2013) conducted Raman Spectroscopy measurements and shear force tests on different portions of the muscle for logistical reasons.
Previous studies using Raman spectroscopy to predict shear force values of porcine \textit{m. longissimus} and beef silverside also produced relatively high $R^2_{cv}$ values of 0.77 and 0.75 (Beattie \textit{et al.}, 2004a, Beattie \textit{et al.}, 2008). However, similar to the studies conducted on lamb, a direct comparison between results reported in literature and models created in this research cannot be made as there is little accordance between experimental design and parameters for Raman spectroscopic measurement. Although all studies aim to predict shear force values measured using a Warner-Bratzler texture analyser with Raman spectra, like Schmidt \textit{et al.} (2013) both Beattie \textit{et al.} studies (Beattie \textit{et al.}, 2004a, Beattie \textit{et al.}, 2008) conducted Raman measurements on meat samples that had been subjected to freezing and thawing prior to measurement. Further complicating any direct comparison between prediction models, the number of independent samples reported in these studies was small, chemometric analysis of spectral data varies, spectra have been collected using a bench top Raman device with different wavelengths and the samples have been measured on a rotating stage using various integration times and accumulations.

Of these factors, sampling is critical as it determines the ways in which the scattered light can be detected and the chemometric data can be analysed. Bonnier & Byrne (2012) suggest that despite the widespread use of PCA, the clustering of data based on spectral similarity and spatial distributions, results in the mean spectra representing each cluster and under representing subtle differences between spectral regions of the samples. It is expected that Raman spectra obtained from numerous sub-sections removed from a small number of total samples would substantially impact the variance and loadings which differentiate groups in further data analysis. For example in Beattie \textit{et al.} (2004a) all 168 pork LL sections measured with Raman spectroscopy were sub-samples from only 18 pigs. As the strength of this analysis technique is in the loadings given that they represent the spectral origin of the variations (Bonnier & Byrne, 2012), it is hypothesised that such data sets with few truly independent samples are at risk of artificially biasing the results from chemometric analysis and consequently under representing subtle differences within more complex data sets. The models generated may then have a better accuracy of prediction that if the true spectral differences were represented. However, as the models for prediction of shear force previously reported by Beattie \textit{et al.} (2008), Beattie \textit{et al.} (2008) and Schmidt \textit{et al.} (2013) have not been validated on independent data sets, the effect of sub-sampling and sample size on the accuracy of prediction is unknown.
It is interesting to note that while shear force prediction models on fresh intact lamb are poor in comparison to previous Raman studies, a better prediction was found for predicting shear force values of \( R^2_{cv} = 0.27 \) intact fresh lamb SM (Table 4.1) in comparison to LL samples found in this study \( R^2_{cv} = 0.09 \) at best. In a review of the application of Raman spectroscopy to analysing biological tissues, Matousek & Stone (2009) suggested that conducting Raman spectral measurements on the surface or close to the surface of tissues limits Raman scattering as tissue has a diffuse scattering nature which leads to random propagation of the photons within the matrix of the tissue, preventing the formation of sharp signals and the discrimination of signals emerging from deeper areas. Furthermore, this is compounded by additional background noise and fluorescence, which further swamp the weak Raman signal. Due to the narrower shape and smaller size of the LLs in comparison to the SMs, when removed from the carcase, the prediction models from the LLs are poorer potentially as a result of random propagation and diffuse scattering. To avoid the myofibrils conducting the scattered light away from the Raman head like fibre optics cables, all Raman measurements were conducted perpendicular to the direction of the muscle fibres. As a consequence Raman measurements on the LL were conducted on the thinnest side (Fig. 3.1), which may have emphasised the diffuse nature of the tissue in comparison with the SM due to the proximity of the measurements to the surface of the tissue. It is also possible that measurement on the thinnest side also resulted in greater background noise being recorded, further reducing the Raman signal.

Although Figure 3.3 suggests that Raman Spectroscopy is able to determine the changes associated with ageing and improvements in shear force values as a result of protein degradation, the poor prediction models found by this study suggest that there is little significant difference between the spectra of tough and tender LL samples. Therefore, completing tentative interpretation of Raman signal based on spectra collected in this study would not be informative in terms of determining what protein composition and structure characteristics of the muscle determine shear force values.
3.4.2 Prediction of Shear force with Traditional Indicators

Traditionally, objective measurements of SL, pHu and CL have often been used as indicators of shear force values (Bouton et al., 1971, Bouton et al., 1975, Thompson et al., 2005b, Hopkins et al., 2006, Hopkins et al., 2011b). Despite some studies demonstrating links between these indicators and shear force (Bouton et al., 1973a, Hopkins et al., 2006), results presented in this research agree with others that suggest these factors do not explain large amounts of variation in shear force (Geesink et al., 2001, Hopkins et al., 2011b).

Using these traditional predictors of shear force suggested that sarcomere length at 24 h PM explained the most variation in shear force values ($R^2 = 0.14$; Fig 3.12), which highlights the significance of early post mortem events in determining tenderness. During the early post mortem period, actomyosin crosslink bridges are formed and broken until glucose is no longer available to fuel muscle cell metabolism or ATP is no longer available to break them (Blanshard, 1974, Huff Lonergan et al., 2010). When this occurs and the muscle becomes 'set' in rigor mortis (Tornberg, 1996) and the negative relationship between sarcomere length and shear force is established (Howard & Judge, 1968). Although this relationship has been widely used as an indicator of shear force (Howard & Judge, 1968, Bouton et al., 1973c, Cross et al., 1981, Smulders et al., 1990, Lepetit, 2000, Wheeler et al., 2000, Hopkins et al., 2011b), there is no agreement on the strength of the relationship and consequently it is difficult to determine the extent to which sarcomere length impacts the variability of shear force.

Previously reported figures using various statistical models for determining the relationship between shear force and sarcomere length range from a correlation of $r = 0$ (Geesink et al., 2001) to a co-efficient of 0.11 µm decrease in sarcomere length per Newton (N) increase in shear force (Hopkins et al., 2007). Based on these reported results, the variation explained in this study is low by comparison. Despite different studies reporting different statistics collected from samples that have undergone a variety of treatments, previous studies have suggested correlations range between decreases in sarcomere length of 0.11 µm to 0.90 µm per Newton (N) increase in shear force, (Smulders et al., 1990, Wheeler et al., 2000, Rhee et al., 2004, Maher et al., 2005, Hopkins et al., 2007, Christensen et al., 2011, Pen et al., 2012). However, other studies have reported poorer correlations of between 0 to 0.04 µm decreases in sarcomere length per N increase in shear force (Tschirhart-Hoelscher et al., 2006, Hopkins et al., 2011b).
Differences between the relationship of sarcomere length in studies can be attributed to a variety of factors, including species (Wheeler et al., 2000), breed (Christensen et al., 2011), gender (Maher et al., 2004), individual muscle (Tschirhart-Hoelscher et al., 2006), muscle location (Rhee et al., 2004), pHu (Purchas, 2004), rate of glycolysis (Smulders et al., 1990), temperature decline and experimental treatments including hanging method (Hostetler et al., 1972), stretching (Pen et al., 2012), protease inhibition injections (Hopkins & Thompson, 2001) and ageing period (England et al., 2012); all of which can impact on the relationship between shear force and sarcomere length. Furthermore, methods used to determine shear force can alter correlations found as Silva et al. (1999) demonstrated that evaluation of shear force using sensory panel scores or Warner Bratzler shear force can change prediction relationship. The 0.14 coefficient correlation found in this study does not reflect the accuracy of other studies that have determined the relationship between sarcomere length and shear force measured using the Warner-Bratzler texture in lamb. For similar ageing periods, Hopkins et al. (2007) have reported mixed model coefficients of -0.96 and -0.58 at 1 and 5 days post mortem respectively, while Tschirhart-Hoelscher et al. (2006) reported a correlation coefficient of 0.28 after 7 days ageing. Although the species, muscle, pHu, hanging and ageing are known or controlled in the experimental design of this research, due to the measurement of LLs removed from the carcase at 24 hours post slaughter from mixed consignments representing different ages, gender and breeds with varying fat thickness and carcase weights, the impact of all of these factors are unable to be determined for this data set. Consequently, prediction models for shear force using sarcomere length as an indicator may be improved if interrelationships with factors such as temperature and pH decline, muscle location, GR fat depth and carcase weight were to be included (Wheeler et al., 2000, Hopkins et al., 2006, Hopkins et al., 2011b) or by controlling the genotype and phenotype of carcases included in the study (Hopkins et al., 2007).

While sarcomere length was significant in determining shear force of the LL at 24 h PM, by 5 days post mortem, it was not significant (P > 0.05). Given that the process of proteolysis results in ultra-structural changes through myofibrillar degradation which causes breaks in the sarcomere at the junction between the Z-disc and I-band (Hopkins et al., 2000), it is unsurprising that the variation in sarcomere length is increased with ageing and as a result the relationship between sarcomere length and shear force is weakened (Hopkins et al., 2011b).
Despite cooking loss being significant in determining shear force and reducing inter-laboratory differences in Warner-Bratzler measurements (Hopkins et al., 2010), few studies have described the relationship. Data found in this study indicates that cooking loss explains less variation ($R^2 = 0.07$ and $R^2 = 0.06$ at 1 and 5 days post mortem respectively) compared to previous studies that have reported correlations of between 0.35 and 0.81 (Shackelford et al., 1997, Silva et al., 1999, Rhee et al., 2004). Furthermore Purchas (2004) suggest that the variation in cooking loss can be partially explained by an inter-relationship between pHu, ageing, cooking loss and shear force where the impact of cooking loss on shear force may vary based on the combination of ageing and pHu. Although these studies have been conducted on beef, the location and muscle effects reported suggest that an inter-relationship between collagen, cooking loss and shear force values may explain more variation because the total amount of collagen reflects the ability of the contract, both laterally and longitudinally, during cooking which results in the expulsion of fluid (Tornberg, 1996).

The significance of cooking loss contributing to the variation of shear force within this study may be a reflection of the amount and stability of the connective tissue matrix. Because cooking loss is an indication of the expulsion of fluids and the loss of water binding capacity through protein denaturation and shrinkage of connective tissue during cooking (Geesink et al., 2011), the reaction of the myofibril to cooking varies between muscles and animals. When meat is cooked the contraction of the myofibrillar mass is most limited by the perimysium, due to its mechanical integrity which is maintained by heat stable collagen (Purslow, 2005). Therefore, cooking loss is likely to be a better indicator of shear force in LLs, which are have relatively low amounts of perimysium compared to other muscles. Therefore, the relationship between cooking loss and shear force through shrinkage of the myofibrillar mass is not affected by an inter-relationship with the amount or stability of the connective tissue matrix, namely the perimysium (Tschirhart-Hoelscher et al., 2006).

As there are no extreme pH values for this data set, it is unsurprising that pH was not significant in determining shear force. pHu has been widely regarded as an indicator of shear force values (Bouton et al., 1971, Bouton et al., 1973a, Hood & Tarrant, 1981, Devine et al., 1993, Hopkins & Fogarty, 1998, Silva et al., 1999, Young et al., 2004, Hopkins et al., 2006, Young et al., 2006, Addis et al., 2010), as improvements to eating quality and shear force are evident when pHu is below the threshold level of 5.7 (Thompson, 2002). However, the relationship is not linear, as below a pHu value of 6, many factors impact on the relationship between pHu and shear force (Devine et al., 1993).
Previous studies by Taylor & Frylinck (2003) and Toohey et al. (2012a) demonstrated that ageing increased the percentage of myofibrillar breaks as found by this study and the decreasing particle size with ageing in the current study was also consistent with the findings of others (Toohey et al., 2012a). Combining the significant factors cooking loss, sarcomere length, partial myofibrillar breaks and ageing period explained 10% of the variation in shear force values, but did not significantly reduce the RSMEP in comparison to the null model when included as co-variates for predicting shear force. While myofibrillar breaks reflect the impact of enzymes like calpains which lead to proteolysis that breaks the junction of the I-band and Z-line increasing tenderness (Hopkins & Thompson, 2002a), they don’t account for variation in other factors such as fibre to fibre adhesion, fibre diameter, sarcomere attachment or fat and collagen amounts that may affect the ability to reproduce the shear force prediction across the muscle (Taylor & Frylinck, 2003, Tschirhart-Hoelscher et al., 2006). It is also interesting to note that distinction between partial and complete myofibrillar breaks indicated that partial breaks had a significant impact on shear force values but total breaks across the myofibril did not, which has not been reported before.

Despite the wide use of linear correlations to describe the relationship between these traditional indicators and shear force, they are not a good measure of the merit of calibration for spectroscopic data sets because they are dependent on the range of the reference data set (Davies & Fearn, 2006). Therefore, comparing reductions in the error of the prediction by comparing between the root mean square error of prediction (RMSEP) values gives a more robust comparison between indicators. This is because the RMSEP is a measure of the variability of the difference between the predicted and reference values and therefore is an estimate of the typical difference between them (Davies & Fearn, 2006). Based on this RMSEP criterion, cooking loss for LLs at 5 days post mortem was the best model for predicting shear force values (Table 3.3). Although cooking loss was the best model (RMSEP = 9.7 N), it did not provide a significant reduction in the variability of prediction in comparison to models using Raman spectra at day 1 post mortem or a combination of Raman Spectra at either 1 or 5 days, plus the traditional indicators, which all yielded RSMEP values of approximately 9.9 N (Table 3.2). Inclusion of the traditional indicators of shear force into models with Raman spectra at day 1 did not significantly improve this outcome (RMSEP = 13.3 N, Null RMSEP = 13.8 N; Table 3.3). When this is considered with the accuracy of prediction models for Raman spectra, measurement of the LL using these experimental parameters would not give the accuracy and precision required by industry.
3.5 Conclusion

While this data indicates a poor ability to predict shear force based on Raman spectra from 80 loin samples, the Raman spectra at day 1 demonstrated a slightly better accuracy in comparison to predicting shear force using Raman spectra measured at 5 days post mortem. Using the traditional indicators to predict shear force values only marginally reduced the prediction error, despite the percentage myofibrillar breaks, sarcomere length and cooking loss being significant in explaining part of the variation in shear force. Although measurement of LL online in lamb carcases using these experimental parameters would not give the accuracy required by industry, these conclusions are restricted to the LL and excludes other muscles, traits such as intramuscular fat, and the impact of other experimental parameters such as longer accumulation times are yet to be determined. Therefore, future research will need to address these limitations.
4. Prediction of intact ovine *m. semimembranosus* shear force using Raman Spectroscopy

### 4.1 Introduction

Tenderness, juiciness and flavour are all factors which influence the eating quality of meat. Of these, tenderness is critical as in Western cultures a tough steak is unacceptable (Wood *et al.*, 2008), although tenderness of lamb may have a lesser importance than other sensory attributes (Thompson *et al.*, 2005b). It has been established that the tenderness of meat is determined by interaction between the connective tissue that creates ‘background toughness’, the myofibrillar structure (Damez & Clerjon, 2008) and the changes to these structures post mortem (Hopkins & Geesink, 2009). Consequently, considerable research has focused on the ability of technologies to objectively measure tenderness. A review of such technologies has highlighted Raman Spectroscopy as having potential to be used for online measurement of meat quality traits (Damez & Clerjon, 2008).

Based on the inelastic scattering of light which can provide information about molecular composition and structure, Raman spectroscopy has potential for use in muscle food systems as it is rapid, non-destructive, non-invasive, not sensitive to varying water content and is not based on the absorption of light (Li-Chan, 1996). Previous research has not overlooked these advantages and several studies have demonstrated Raman is a useful tool in predicting sensory traits in cooked beef (Beattie *et al.*, 2004a) and explaining a large variation ($R^2 = 0.77$) in shear force of pork (Beattie *et al.*, 2008). However, these studies used a bench top instrument that is not suitable for industrial application. Alternatively, Schmidt *et al.* (2013) used a hand held Raman device suitable for online application to predict shear force of lamb meat with good predictability ($R^2 = 0.79$ and 0.86 for two sample groups). Despite use of the hand held Raman device, the industrial application of this study is limited as samples in the study were frozen and then thawed prior to measurement. Further to this, none of these studies cited have reported on the measurement of Raman spectroscopy, shear force and other predictors of tenderness on the same piece of meat.
Although Schmidt et al. (2013) overcome the limitations of similar studies, such as Beattie et al. (2004a) who used bench top devices, the online application of this research is limited as the samples were frozen prior to scanning and Raman is sensitive to the structural changes that are induced by freezing (Li-Chan et al., 1994, Herrero, 2008b, Chan et al., 2011b). Therefore, the aim of this research was to assess the potential of a hand held Raman probe to predict the tenderness of fresh intact *semimembranosus* (SM).

### 4.2 Materials and Methods

#### 4.2.1 Samples

Topside (Product identification number HAM 5077; Anonymous, 2005) samples were collected from 80 lamb carcases, over 4 consecutive days (20 samples per muscle per day) from the same abattoir. Samples were randomly selected from different consignments and were of unknown backgrounds, sex and age, representing the variety of animals typically processed by the abattoir to achieve a spread in shear force values. The cap muscle (*m.gracilis*) and *m.adductor* were removed to leave the *m. semimembranosus* (SM) which was the muscle of measurement. The carcases also had the loin removed to compare Raman spectroscopy against traditional indicators of shear force in lamb *m. longissimus lumborum* (data reported in Chapter 3) and fatty acid composition (data reported in Chapter 8).

#### 4.2.2 Raman Spectroscopy

Raman spectroscopic measurements were conducted at a room temperature of 16 °C at 24 h PM on a fresh cut surface of the intact SM with the epimysium removed (Fig.4.1). Twelve (12) positions were scanned using a Raman hand held device (Schmidt et al., 2010) perpendicular to the muscle fibre, over the face where the *m. adductor* had been removed. Spectra were recorded using 70mW of laser power and an integration time of 3.75 seconds and 1 accumulation.

After scanning with the Raman probe and removal of sections for measurement of traditional indicators, SM samples were vacuum packed and held at 1°C for 4 days. At 5 days PM, SM samples were removed from the vacuum packs and allowed to ‘bloom’ for 2 hours before a freshly cut surface was rescanned.
4.2.3 Traditional Indicators of Tenderness

At 24 h PM, sections were removed for sarcomere length analysis using the laser diffraction method (Bouton et al., 1973c) and particle size analysis (PS) (Karumendu et al., 2009).

At 5 days PM, shear force tests were conducted on blocks (mean weight 65g) cut from the middle of the muscle after Raman scanning. Blocks were cut and cooked at 71°C for 35mins and analysed using a Lloyd texture analyser with a vee-blade as described by Hopkins et al. (2011a). The average of 6 repetitions was reported except when the coefficient of variation exceeded 24% in which case the median of the 6 repetitions was used (Hopkins et al., 2012). Shear force blocks were weighed before and after cooking to determine cooking loss. The pHu was determined using 2.5g of muscle homogenate in 10ml of 5mM iodoacetate/150mM KCl (pH adjusted to 7.0), as described by Dransfield et al. (1992). At 5 days PM, another section was taken for particle size analysis (Karumendu et al., 2009).
4.2.4 Data Analysis

Data analysis was conducted as previously described for LL samples (Chapter 3.2.4).

4.3 Results

4.3.1 Raman Spectroscopy

As illustrated by Fig 4.2., Raman Spectroscopy can determine the difference between the extremes of tenderness of SM samples. When normalised, spectra collected at 24 h for the 10 SM samples highest above the shear force median (66 – 78N; red) tended to have a lower intensity than spectra for the 10 samples lowest (black) below the shear force median (29 – 36N; black). The more tender samples had clearer more defined and symmetrical peaks, with large peaks at 1001 cm\(^{-1}\) and 1450 cm\(^{-1}\). However, smaller peaks are also evident at 757 cm\(^{-1}\), 826 cm\(^{-1}\), 853 cm\(^{-1}\), 1250-11300 cm\(^{-1}\) and between 1650 cm\(^{-1}\). The tougher samples had largely indistinct spectra, except for the three peaks at 1001 cm\(^{-1}\), 1450 cm\(^{-1}\) and 1650 cm\(^{-1}\).

![Raman Spectra of the toughest SM samples (66-78N) and the tenderest SM samples (29-36N) collected 24 h PM.](image)

Figure 4.2. Raman Spectra of the toughest SM samples (66-78N) and the tenderest SM samples (29-36N) collected 24 h PM.
Furthermore, Raman spectra are able to differentiate between ageing periods. The average SM spectra at 24 h PM had a lower overall intensity than the average spectra at 5 days PM, resulting in a net difference between spectra (Fig. 4.3). Peak intensities occurred at similar wavenumbers despite the difference in overall intensity. The large peaks occurred at 1001 cm\(^{-1}\), 1450 cm\(^{-1}\) and 1650 cm\(^{-1}\), while smaller peaks are also evident at 757 cm\(^{-1}\), 826 cm\(^{-1}\), 853 cm\(^{-1}\) and 900 cm\(^{-1}\).

Further processing of these spectra using a 6\(^{th}\) polynomial baseline correction and normalisation by dividing the intensity at each wavenumber by the excitation power (mW) multiplied by the integration time, showed that between tough and tender SM samples at 24 h PM the differences between peak intensity positions occur mainly between 800-1200 cm\(^{-1}\) (Fig. 4.4).
The chemometric analysis using the Raman spectral data to predict shear force values of the SM samples at 5 days post slaughter are summarised in Table 4.1. The predictability of these models varied, producing $R^2$ values of 0.00 – 0.27 (PLS). However, there was no significant reduction in the optimal RMSEP or coefficients of determination for models using any alternative pre-processing approach.

Table 4.1 Summary of the chemometric analysis using Raman Spectroscopy to predict 5 day shear force in SM samples.

<table>
<thead>
<tr>
<th>Pre-processing Method</th>
<th>Spectra Measured</th>
<th>No. Latent Vectors</th>
<th>Optimal RMSEP</th>
<th>$R^2_{cv}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>24 h</td>
<td>4</td>
<td>11.5</td>
<td>0.26</td>
</tr>
<tr>
<td></td>
<td>5 Day</td>
<td>13</td>
<td>12.3</td>
<td>0.16</td>
</tr>
<tr>
<td>Standardisation using $l_2$-norm</td>
<td>24 h</td>
<td>3</td>
<td>11.5</td>
<td>0.27</td>
</tr>
<tr>
<td></td>
<td>5 Day</td>
<td>9</td>
<td>12.4</td>
<td>0.17</td>
</tr>
<tr>
<td>Savitzky-Golay filter a. (SGF.2.0.15)</td>
<td>24 h</td>
<td>4</td>
<td>11.5</td>
<td>0.26</td>
</tr>
<tr>
<td></td>
<td>5 Day</td>
<td>6</td>
<td>12.9</td>
<td>0.071</td>
</tr>
<tr>
<td>Savitzky-Golayfilter b. (SGF.2.2.15)</td>
<td>24 h</td>
<td>1</td>
<td>12.0</td>
<td>0.17</td>
</tr>
<tr>
<td></td>
<td>5 Day</td>
<td>0</td>
<td>13.2</td>
<td>0.00</td>
</tr>
<tr>
<td>Multiplicative Normalisation</td>
<td>24 h</td>
<td>3</td>
<td>11.7</td>
<td>0.22</td>
</tr>
<tr>
<td></td>
<td>5 Day</td>
<td>10</td>
<td>12.5</td>
<td>0.15</td>
</tr>
</tbody>
</table>
Although there was no significant improvement between alternative pre-processing approaches, a marginal improvement was evident when spectra were standardised using the $l_2$-norm (Table 4.1). Using this pre-processing method to predict shear force values measured 5 days post mortem using Raman spectra taken 24 h PM was the most accurate based on the coefficient of determination between cross validated predicted and observed shear force values ($R^2_{cv} = 0.27$; Fig. 4.5). This gave a RMSEP of 11.5, based on 3 latent vectors.

![Figure 4.5. Cross validated prediction of SM shear force values (N) measured 5 days post mortem using Raman spectra taken 24 h post mortem and analysed with 3 latent vectors.](image)

### 4.3.2 Traditional Indicators of Shear Force

Summary statistics for the traditional indicators of tenderness (shear force, cooking loss, sarcomere length, pHu, particle size analysis and myofibrillar breaks) are given in Table 4.2.

<table>
<thead>
<tr>
<th>Trait</th>
<th>Days Post Mortem</th>
<th>Mean</th>
<th>SD</th>
<th>Range (min, max)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shear Force (N)</td>
<td>5 days</td>
<td>51.4</td>
<td>13.1</td>
<td>29.2 - 78.4</td>
</tr>
<tr>
<td>Cooking Loss (%)</td>
<td>5 days</td>
<td>19.2</td>
<td>3.7</td>
<td>0.2 - 28.8</td>
</tr>
<tr>
<td>pHu</td>
<td>5 days</td>
<td>5.6</td>
<td>0.1</td>
<td>5.5 - 6.2</td>
</tr>
<tr>
<td>Sarcomere Length (µm)</td>
<td>24 h</td>
<td>1.7</td>
<td>0.03</td>
<td>1.46 – 2.0</td>
</tr>
<tr>
<td>Particle Size Analysis (µm)</td>
<td>24 h</td>
<td>229</td>
<td>46.8</td>
<td>159 - 455</td>
</tr>
<tr>
<td></td>
<td>5 days</td>
<td>166</td>
<td>40.8</td>
<td>95 - 322</td>
</tr>
</tbody>
</table>
Prediction errors (RMSEP) for models using the traditional indicators sarcomere length, cooking loss and/or pHu, and Raman spectra to predict shear force values of the SM at 24 h and 5 days PM are summarised in Table 4.3.

<table>
<thead>
<tr>
<th>Model Covariates</th>
<th>RMSEP (N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Null</td>
<td>13.2</td>
</tr>
<tr>
<td>Cooking Loss (CL)</td>
<td>13.3</td>
</tr>
<tr>
<td>Sarcomere Length (SL)</td>
<td>13.2</td>
</tr>
<tr>
<td>pHu</td>
<td>13.8</td>
</tr>
<tr>
<td>CL, SL and pHu</td>
<td>13.9</td>
</tr>
<tr>
<td>Raman Spectra (24 h)</td>
<td>11.5</td>
</tr>
<tr>
<td>Raman Spectra (5 day)</td>
<td>12.2</td>
</tr>
<tr>
<td>Raman Spectra (24 h) + CL, SL and pHu</td>
<td>12.0</td>
</tr>
<tr>
<td>Raman Spectra (5 day) + CL, SL and pHu</td>
<td>13.0</td>
</tr>
</tbody>
</table>

Based on the RMSEP criterion, the most precise model for predicting shear force values of SM at 5 days PM (Table 4.3) is Raman spectra collected 24 h PM (RMSEP = 11.5). Combining the traditional indicators sarcomere length, cooking loss and pHu with Raman spectra taken 24 h PM did not significantly improve the prediction error (RMSEP = 12.0). However, the prediction models which included Raman spectra had marginal improvements in precision in comparison to those without, as RMSEP values were between 11.5 – 13.0 when Raman spectra was included, but were 13.2 – 13.9 when Raman spectra were not included.
4.4 Discussion

4.4.1 Prediction of Shear force with Raman Spectroscopy

The best $R^2$ between cross validated predictions and observed values ($R^2_{cv}$) was the prediction of shear force values for SMs at 5 days PM using the Raman spectra measured 24 h PM (Table 4.1), pre-processed by standardisation using the $l_2$ norm to give a RMSEP of 11.5 using 3 latent variables, with an $R^2_{cv} = 0.27$. Comparison of this model to the Raman study conducted on frozen and thawed lamb loin indicates that the prediction accuracy is lower, as Schmidt et al. (2013) reported $R^2$ coefficients of 0.72 and 0.86 when predicting shear force values on data sets collected from carcases from 2 different sites (RMSEC = 3.2% and RMSECV = 8.7% and RMSEC = 2.6% and RMSECV = 7.6%, respectively). While both studies were conducted on intact lamb samples, it must be stressed that there is a limited ability to compare between studies. Sample handling varies greatly between the current study and that of Schmidt et al. (2013) who conducted Raman measurements on a 3cm thick section of LL that was aged for 5 days PM, frozen at -20˚C and thawed prior to measurement.

Several reviews of Raman spectroscopy applications in food science indicate that Raman is sensitive to the freezing and thawing of proteins (Li-Chan et al., 1994, Herrero, 2008b). During the freezing and thawing of meat, changes to the biochemical characteristics of the muscle occur as water is moves out of cells and cellular membranes during freezing, resulting in an increase in solutes and a loss in extractability of the myofibrillar fraction (Sikorski, 1978). Furthermore, cell structures disintegrate and oxidation of proteins and lipids occurs (Leygonie et al., 2012). This is the result of damage to organised water structures within the myofibril which alters their hydrophobic adherences as water crystals move during freezing. This movement of water crystals also damages surrounding protein side chains by rupturing hydrophilic/hydrophobic bonds, causing further mechanical damage to muscle structures as the ice crystals form and move. Loss of water during freezing also increasing salt levels that causes a reduction in the number of hydrophilic protein groups, further increasing surface tension and promoting oxidation of the protein fraction (Sikorski, 1978). Such damage of the protein structures and myofibrillar environment is problematic when comparing between Raman studies that have used frozen and fresh meat because Raman bands are characterised by the vibrations of chemical bonds of the peptide backbone and side chains of amino acids (McCreery, 2000). Herrero (2008a) and Li-Chan (1996) agree suggesting that changes in the
amide I and III bands, (1650cm$^{-1}$ and 1250-1300cm$^{-1}$ respectively) are evident as a result of freezing when myosin denatures resulting in a loss of α-helical structures. Furthermore, aliphatic residues (2490cm$^{-1}$) have also been associated with changes in hydrophobic interactions after frozen storage (Herrero, 2008a). However, changes to Raman spectra due to increased oxidation, water movement and decreased water holding capacity and colour stability, which occur when lamb is frozen (Muela et al., 2010) have not yet been characterised. It is hypothesised that movement of water within the myofibril during freezing removes some of the overlap effect of solvent water within Raman spectra that reflect the peptide backbone conformation and reveal the secondary structure of proteins in the amide I band as well as some of the miscellaneous side chain vibrations that contribute to the peptide backbone vibrations of the amide III band (Li-Chan, 1996). Consequently, the loss of these overlapping vibrations in the Raman spectra of the secondary protein structures creates more distinct Raman signals within the peptide backbone bands, giving a better prediction for shear force values.

As the spectral data reported in this study was measured with an integration time of 3.75 seconds for SMs and 3 seconds for LLs, it is plausible that the longer integration and longer accumulations used in other studies (Beattie et al., 2004a, Beattie et al., 2008) has contributed to the better models by reducing the signal to noise ratio (SNR). As Raman spectroscopy is based on inelastic scattering of photons, it is highly sensitive to spectral acquisition parameters in comparison to absorption methods, which has led to more dependence on optical and experimental design because often small variations can lead to large effects on the Raman signal and SNR (McCreery, 2000). Subsequently, SNR and therefore the accuracy of the spectra are improved with larger numbers of measurements averaged. Previous Raman studies on pork (Beattie et al., 2008) and beef (Beattie et al., 2004a) have used integration times of 6 minutes and 3 minutes, respectively. While the previous study conducted on lamb (Schmidt et al., 2013) used a similar and shorter integration times of 5 seconds and 4 seconds to the current study, more spectra were taken per sample resulting in more accumulations. While a 3 or 6 minute total measurement time is not suitable for online application, it is hypothesised that increasing the integration time from 3 seconds or increasing the accumulations would improve the prediction coefficients by reducing the SNR.
Using the RMSEP criterion to give a more robust explanation of the variation of predictions using different covariates (Davies & Fearn, 2006) indicates that there is no significant improvement to models gained by including traditional indicators as covariates in the prediction of shear force using Raman spectra (Table 5.3). When this is considered with the accuracy of prediction models, Raman spectra measured 24 h PM is the most accurate and precise predictor of tenderness. As muscle is a closed system, attributes of muscle which determine the tenderness of aged meat through proteolysis and the subsequent amount of myofibrillar degradation are predetermined by the biophysical and biochemical properties of the sample at processing. It is hypothesised that changes to the myofibrillar structure weakens the Raman signal when spectra are measured at 5 days PM. If changes to ionic strength during ageing are affecting the ability of Raman to predict shear force values, it is hypothesised that collecting spectra as the muscle enters rigor would improve the prediction, as ionic strength of sarcoplasmic fluid rapidly rises as the muscle enters rigor (Ouali, 1992).

4.4.2 Traditional Indicators and shear force variation

Lack of significance between shear force and traditional indicators within this research indicates that interrelationships with these other factors may have a greater contribution to shear force values of lamb m. semimembranosus. However, it is interesting to note that prediction coefficients for models using Raman spectra demonstrated a greater accuracy than models using traditional indicators when predicting shear force for this data set (Table 4.3).

Using these traditional predictors of shear force for SMs suggested that none were significant in determining shear force values ($P >0.05$). Many previous studies have reported coefficients of determination ($R^2$) to describe the significance and the relationships between traditional measures, such as pH, sarcomere length or particle size and shear force (Bouton et al., 1973c, Hood & Tarrant, 1981, Smulders et al., 1990, Hopkins et al., 2007). However, it needs to be recognised that as linear relationships these are dependent on the range of the data set. Consequently, they may not be a good measure of the merit of the calibration between data sets that have different ranges (Davies & Fearn, 2006). Therefore it is problematic to compare between studies that have used different experimental treatments and designs to highlight the interrelationships between shear force and the traditional predictors. This extends to influencing pH to gain a wider range of values (5.6 – 7.0), as Bouton et al. (1971) did, using different muscles (Karumendu et al., 2009, Geesink et al., 2011), species (Smulders et al., 1990) and processing practices (Bouton et al., 1973c).
While these studies are informative on the impact on shear force of extreme values of these indicators, these studies may not be reflective of the ranges that would be expected normally and as a result $R^2$ values reported by literature may not be accurate estimates of the differences which would be found in standard carcasses commonly processed (Davies & Fearn, 2006).

Relationships between traditional indicators of tenderness, such as pH or sarcomere length and shear force values are not always linear and may be confounded by other factors. Hopkins et al. (2006) agree, highlighting that different processing conditions, such as electrical stimulation and ageing, can constrain shear force values which then allows the full impact of other factors, such as age, nutrition and breed, on eating quality measurements to be established, therefore the prediction and accuracy of models were improved when factors like age, were included in models. Likewise, Hopkins et al. (2011b) found that the combination of temperature at pH6, the pH at 18 °C and pH at 24 h provided a larger reduction in shear force variation for lamb LL in comparison to sarcomere length alone, at both 24 h and 5 days PM. Given the these relationships are not clear, it is not surprising that while the variation in shear force values explained by traditional indicators in this study agrees with some studies (Hopkins et al., 2011b), it does not agree with others (Bouton et al., 1973c, Hopkins et al., 2006, Karumendu et al., 2009).

4.4.3 Raman Band Assignments

Raman spectroscopy demonstrated a better ability to predict shear force based on the RMSEP values in comparison to the traditional models (Tables 4.1 and 4.3), however as it relies on vibrational spectroscopy, spectral band assignments must be completed to determine what biophysical and biochemical characteristics of the muscle are reflected in the spectral differences between samples. As with previous Raman studies on meat (Beattie et al., 2004a, Beattie et al., 2008, Schmidt et al., 2013) it is not possible to assign changes in the Raman spectra with certainty, although a tentative interpretation of spectra is plausible based the relationship between Raman spectra and the composition and structure of proteins.
Peaks in the intensity count of the Raman spectra found in this research are similar to the previous Raman study conducted on lamb meat by Schmidt et al. (2013). Peaks corresponding to aromatic amino acid side chains are evident (Fig. 4.3), including phenylalanine (Phe) at 1000 cm\(^{-1}\), the tyrosine doublet (Tyr) at 826 cm\(^{-1}\) and 853 cm\(^{-1}\) and tryptophan (Trp) at 758 cm\(^{-1}\) (Pézolet et al., 1988, Herrero, 2008a). The peptide backbone is also a main feature with strong symmetrical peaks at 1650 cm\(^{-1}\) (amide I), 1450 cm\(^{-1}\) (C-H deformations), 1250-1300 cm\(^{-1}\) (amide III) and 930 cm\(^{-1}\) (C-C stretch) (Pézolet et al., 1988).

Although these vibrational bands appear similar in all lamb samples, they are not constant and differences in intensity as well as shifts in wavenumber values are evident. An initial evaluation of spectra indicate that a difference in overall intensity exists between spectra of tough and tender SMs, as tender samples tended to have a greater intensity than tough spectra (Fig. 4.5). This correlates well with the previous Raman study conducted on lamb that reported a difference in background between tough and tender samples due to altered scattering properties (Schmidt et al., 2013). As a muscle involved in locomotion, the biophysical properties and therefore the meat quality, of \textit{m. semimembranosus} is related to the contractile properties of the muscle and the need for the muscle to exert and transfer force during contraction (Tschirhart-Hoelscher et al., 2006). While the epimysium would have no impact on the Raman spectra as it is removed prior to measurement, the connective tissue matrix of the perimysium and endomysium may impact the scattering of photons through the myofibril. The perimysium consists of two sets of wavy collagen fibres in a cross ply arrangement, which surround large (primary perimysium) and small (secondary perimysium) (Rowe, 1981). While these fibres are together, they are at an angle to the muscle fibre axis that changes depending on muscle length as reorientation of the connective tissue is required to allow for the elongation or contraction of the muscle (Purslow, 2005). While the angle is typically ±54° in resting muscle (Purslow, 2005), as the muscle enters \textit{rigor mortis} the length of the muscle is shortened longitudinally and laterally as determined by the cross linking action of actin and myosin that cause the muscle to ‘set’ into the contracted state associated with rigor (Tornberg, 1996). The extent to which the muscles are able to contract during rigor is determined by the availability of glycogen to fuel cellular metabolism, the levels of Ca\(^{2+}\) being released from the sarcoplasm and the amount of ATP available to break actomyosin bridges (Blanshard, 1974).
Therefore, the level of contraction which the muscle experiences at rigor will vary between animals and muscles, as will the angle at which the connective tissue is set. Nakamura et al. (2010) suggest that this architecture of the connective tissue particularly the perimysium is an important contribution to high shear force values of beef. High magnification scanning electron microscope images illustrated increasing density of the perimysium, with several bands of connective tissue crossing over each other and stronger connection to the endomysium in beef M. longissimus thoracis with high shear force values. It is plausible that a similar effect would be present in lamb. Therefore, the architecture and density of the perimysium in tough SMs could reduce the intensity of the Raman signal by blocking the excitation photons from entering the myofibril and preventing back scattering photons from being detected by the sensor head. It is hypothesised that comparing the Raman spectra with a collagen assay could be useful in comparing the differences in intensity of the Raman signal between samples and the contribution of collagen to the shear force value. However, it is unclear from literature whether the density and architecture of the collagen in the perimysium can be extrapolated from a collagen assay or whether electron microscopy work would need to be undertaken to provide enough information to determine the effects muscle connective tissue elicits on the spectra.

Further processing spectra using a 6th order polynomial baseline correction and normalising by dividing intensity at each wavenumber by the excitation wavelength multiplied by the integration time removes the impact of the laser energy and background noise on the Raman spectra, and leaves only the Raman signal. An average of 5 baseline corrected and normalised spectra from tender SMs plotted against the average of 5 tough SM spectra that have undergone the same processing demonstrated a difference in intensity and some peak positions, despite the difference in background noise and fluorescence (Fig. 4.4).

Spectra representing tough and tender samples are similar at the C-H deformation and Amide I bands (Fig. 4.2, Table 3.1), although there is a large difference between the 778 – 1122cm\(^{-1}\) bands and smaller differences between 550 – 600cm\(^{-1}\), 650 – 750cm\(^{-1}\) and 1150 – 1350cm\(^{-1}\). Due to the complex nature and environment of meat it is difficult to assign Raman spectral bands with certainty as protein Raman studies have been conducted on simple proteins, however from this literature a limited interpretation can be made.
Within the wavenumber range of 778 – 870 cm\(^{-1}\) peaks found correspond to the aromatic amino acid side chains of tryptophan and tyrosine at 757 cm\(^{-1}\), 826 cm\(^{-1}\) and 853 cm\(^{-1}\) (Table 4.1) are lower in intensity in Raman spectra of tough samples (Fig. 4.4). Weakening of the tyrosine doublet signal at 826 cm\(^{-1}\) and 853 cm\(^{-1}\) in tough lamb was also found by Schmidt et al. (2013) who hypothesised that the presence of this doublet signal may be used to classify tender lamb. Beattie et al. (2008) agreed, suggesting that shifts in the tyrosine bands are a direct reflection of proteolysis, as tyrosine being a free amino acid, increases with aging in normal quality pork. However, Figure 4.4 suggests that there is also a change in the intensity ratio between the two peaks that combine to create the tyrosine doublet. In the tender SMs, the tyrosine doublet peak intensities at 826 cm\(^{-1}\) to 853 cm\(^{-1}\) have a ratio of 0.56:0.61 c/s while in tough SMs the intensities have a ratio of 0.49:0.48 c/s. This equates to a 12% difference in the peak height at 853 cm\(^{-1}\) between tough and tender, as there was a 9% increase for tender samples and a 3% decrease for tough SMs. Protein structural studies on L-tyrosine crystals have indicated that this intensity at 826 cm\(^{-1}\) is a the chemical breathing mode of the phenyl group of tyrosine (Tsuboi & Thomas, 1997). While tyrosine is an amino acid and therefore present in many proteins, it is known that the relative loss of intensity of tyrosine at approximately 830 cm\(^{-1}\) is indicative of the environment of hydrogen bonds in the aromatic side chain of tyrosine (Tsuboi et al., 1998). As the phenolic hydroxyl group binds tightly when oxygen is the donor, but relatively loosely when hydrogen is the donor (Nelson & Cox, 2008), a change in what functions as the donor in hydrogen bonds present in the phenyl group of tyrosine may be indicative of the hydrophobicity of the proteins as well as their ability to resist oxidation and reduction (Nelson & Cox, 2008). Herrero (2008a) suggests that such changes in bond donors are reflected in the Raman spectra indicating that where OH functions as a H-bond donor the intensity ratio of I\(_{850}/I_{830}\) (intensity at 850 cm\(^{-1}\) divided by intensity at 830 cm\(^{-1}\)) is less than 0.3 and is largest (may be < 2.5) when tyrosine functions as a receptor in a strong H-bond. Calculating intensity ratio for this data set demonstrates that there is little difference between tough and tender with 1.08 and 0.97, respectively. Therefore, despite the differences in intensity and peak height there is no significant change in phenyl group hydrogen bonds to determine if any correlations exist between the tyrosine breathing modes and shear force values for this data set.
Nevertheless, it is informative as the doublet ratio is also indicative of the structure of tyrosine and whether it is buried or exposed. Herrero (2008a) proposes that if $I_{850} < I_{830}$ than tyrosine residues are exposed and changes to tertiary structure are present, however if $I_{850} > I_{830}$ tyrosine residues are buried, with increases in $I_{850}$ compared to $I_{830}$ being positively related to the how well within the protein network the residues are buried. It is hypothesised that the difference in this region of the wavenumber demonstrates the susceptibility of the hydrogen bonds to breakdown during proteolysis processes through the structure of the residues. Buried tyrosine residues may be less susceptible to breakdown during proteolysis as $I_{830} = I_{850}$ in tough SMs, even though they may be weaker hydrogen bonds.

Despite potential implications of the tyrosine doublet on shear force, larger differences between intensities exist at the wavenumber range 870-1122 cm$^{-1}$, which represents a combination of bands including tryptophan (Trp), the N-stretch and C-C stretch vibrations (Table 4.1). Although interpretation of these bands is complicated by contributions of aliphatic side chains, previous Raman studies have identified the importance of the C-C stretch vibrations at approximately 930 cm$^{-1}$ as they represent the α-helical secondary structures of proteins (Herrero, 2008a). Although it has been shown that loss of these structures leads to broadening and weakening of the intensity of this band (Herrero, 2008a), links between this band and the shear force of fresh meat have not been established (Beattie et al., 2004a, Beattie et al., 2008, Schmidt et al., 2013).

Data presented in Figures 4.3 and 4.4 suggests a decrease in the number of α-helical protein structures of tough lamb as the band disappears. When secondary protein structures convert from α-helices into β-sheets or random coils due to denaturation the intensity of the Raman signal corresponding to α-helices at approximately 930 cm$^{-1}$ becomes broader and weaker (Tu, 1986), which could contribute to the loss of intensity peak and symmetry in spectra of tough SMs. This agrees with Beattie et al. (2004a), who suggested tough meat has a larger number of β-sheets in comparison to tender meat. However, the presence of the α-helical protein backbone structures is dependent on sample orientation as organisation of the proteins that form the cytoplasm of muscle cells, such as myosin and tropomyosin, are orientated such that the axis of approximately 50% of the α-helical segments run parallel to the muscle fibre. Therefore inducing polarized changes parallel to the bonds results in a different excitation pattern in contrast to changes induced perpendicular to the bonds. For example, amide I and III bands are stronger when the incident and scattered light is parallel to the muscle fibre as they are orientated at 39° to the α-helical axis (Pézolet et al., 1988).
Consequently, by placing samples on a rotating stage, Beattie \textit{et al.} (2004a) and Beattie \textit{et al.} (2008) would have artificially reduced the contribution of $\alpha$- helices to the spectra by continually changing the angle at which the laser enters the sample in comparison to the fibre orientation and reducing the time during which $\alpha$-helical segments would be polarised.

Although it is likely that fibre orientation to the laser has affected the contribution of $\alpha$-helical secondary structures to the Raman spectra in previous studies, the influence of $\beta$-sheet structures cannot be dismissed as a contributor to spectral differences of tough meat. Pézolet \textit{et al.} (1988) have suggested that although $\beta$-sheets are relatively low in muscle fibres and they are not orientated, the band at 902cm$^{-1}$ is characteristic of their presence. This band may be important to determining meat shear force, as it has been previously recorded to increase in intensity when Ca$^{2+}$ ions are added to myosin or when myosin is aggregated. It is interesting to note that in the spectra of tough SMs (Fig. 4.4), a peak is evident at 902cm$^{-1}$ while tender SMs have no equivalent peak. It is hypothesised that this peak is representative of a relative increase in Ca$^{2+}$ ions in tough lamb resulting in a greater number of actin-myosin crosslink bridges forming during rigor.

The other main changes in bands between 1120 – 1350cm$^{-1}$ are difficult to assign as these wavenumbers are complex (Krimm & Bandekar, 1986b), representing mixed vibrational bands of C-H bending, tyrosine and phenylalanine ring vibrations as well as the conformational peptide backbone signals associated with the amide III and I vibrational bands (Tu, 1986). Consequentially, the lack of changes to the amide I and III band ratio (Fig 4.4) indicates that the peptide backbone conformation is not a major contributor to the difference between tough and tender lamb spectra in this study.

Raman spectral studies of collagen have shown that the amide I, amide III and skeletal bands are observed in all collagens at approximately 940, 1250 and 1670cm$^{-1}$ (Cardona-Gómez \textit{et al.}, 2002). However, Bonifacio & Sergo (2010) have demonstrated that these wavenumber locations and intensities change with collagen fibre orientation, particularly around 1268cm$^{-1}$. Due to the molecular structure, the C=O group rotates and stretches in different directions depending on whether the bond axis is perpendicular to the laser polarisation. Consequently, when the collagen fibres are parallel to the excitation source, the peak at approximately 1268cm$^{-1}$ becomes weaker, as evident for tender lamb (Fig. 4.4).
This suggests that the impact of the perimysium architecture and density during muscle contraction as *rigor* is established, may extend beyond impacting on overall intensity of the Raman signal, as previously discussed. While the angle of the perimysium is typically ± 54º in resting muscle (Purslow, 2005), longitudinal and lateral contraction during *rigor mortis* shortens the muscles (Tornberg, 1996) and changes the angle of the cross ply connective tissue matrix (Purslow, 2005). With increasing muscle shortening this angle changes becoming more obtuse with a gradient closer to 75º as the sarcomere length decreases (Purslow, 1989). It is possible that as a result of this change in angle less of the perimysium would run parallel to the laser excitation, resulting in the increase in intensity evident for tough meat at 1268cm⁻¹.

### 4.5 Conclusion

Overall, it is difficult to determine the ability of Raman spectroscopy to predict shear force values of intact lamb SM, as there is currently no opportunity to compare these results with other studies using the same experimental design. Although, there is some potential for Raman spectra to predict shear force values of SM at 24 h PM, the accuracy and precision of the predictions found in this study need to be validated.

Direct comparison of the Raman spectra demonstrated that the discrimination between tough and tender fresh intact SM can be made using the intensity of spectral peaks that correspond to key amino acid side chain vibrations. But the impact of early post mortem events and collagen on the prediction of shear force using Raman Spectroscopy are yet to be determined and Raman band assignments for meat need to be clarified. Consequently, the next phase of work will need to address these issues.
5. Can confocal Raman microscopy help us understand variation in shear force?

5.1 Introduction

Studies previously conducted on intact lamb using a hand held Raman spectroscopic device, have determined that Raman spectra correlated well with shear force values of lamb *m. longissimus thoracis et lumborum* \( (R^2 = 0.78; \text{Schmidt et al., 2003}) \) and explained more variation in shear force than traditional indicators of shear force in fresh intact lamb *m. semimembranosus* \( (R^2_{cv} = 0.27; \text{Chapter 4}) \). However despite the positive relationships found, due to the complexity and inhomogeneous nature of intact muscle, changes in the Raman spectra of tough and tender lamb have not been assigned with any certainty \( (\text{Beattie et al., 2008, Schmidt et al., 2013}) \). While Raman spectra in these studies exhibit a signal with peaks which are typical for meat, extrapolation of the key intensity peaks to evaluate an entire muscle is difficult, as these peaks represent chemical bond vibrations of amino acid side chains and the peptide backbone structure of proteins at an atomic level \( (\text{Pézolet et al., 1988}) \). Therefore, while tentative interpretations have been made based on the relationship between Raman spectra and the structure and composition of simple proteins \( (\text{Beattie et al., 2008, Schmidt et al., 2013}) \), the implications of shifts in the Raman spectra have not been well defined for intact muscle in its native state.

Confocal Raman microscopy is an ideal tool for elucidating the chemical composition, morphology and structure of cells in their native state \( (\text{Gierlinger et al., 2012}) \), as it combines the advantages of Raman spectroscopy that determine the ‘chemical finger print’ of the sample \( (\text{Das & Agrawal, 2011}) \) with the high spatial resolution of confocal microscopy to give spatially resolved chemical information \( (\text{Gierlinger et al., 2012}) \).

Therefore, the aim of this research was to use Raman confocal microscopy and chemical imaging to determine the impact of chemical and spatial differences in Raman spectra of ovine *m. semimembranosus* (SM) on indicators of tenderness and the potential to classify SMs based on shear force values using key intensity peaks previously identified in spectroscopic studies on meat.
5.2 Materials and Methods

5.2.1 Samples

Eighty (80) carcases from the same abattoir were randomly selected from different consignments over 4 consecutive days (20 per day) to represent animals typically processed by the abattoir. Therefore, carcases were from different backgrounds, age, and gender, in order to obtain a spread of shear force values.

Lambs were processed following standard commercial slaughter processes and were electrically stimulated pre-dressing with a mid-voltage unit (2000mA with variable voltage to maintain a constant current, for 25 s at 15 pulses/s, 500 microsecond pulse width, unipolar waveform) (Toohey et al., 2008).

Twenty (20) minutes post slaughter body number, lot number and hot carcase weight (HCW) were recorded. At 4 h post slaughter GR fat or the total tissue depth over the 12th rib, 110mm from the midline, was measured using a GR knife.

At 24 h PM, the topsides were removed (‘boned out’) from the carcase and the cap muscle (m. gracilis) and m.adductor were removed to leave the m. semimembranosus (SM) which was the muscle of measurement.

The 80 SMs were classified into tough and tender samples based on shear force values and 14 of the most tender (26 – 35 N) and 14 of the toughest (54 – 74 N) were selected (28 SMs total) for further analysis, although useful Raman microscopic data could not be obtained from two samples due to issues with high fluorescence and these samples were excluded from the study (26 samples were further analysed).
5.2.2 Raman Confocal Microscopy

A 1-2 g section was removed from each SM 24 h PM, fixed in a solution of 2.5% gluteraldehyde and 2% paraformaldehyde in 0.1M phosphate buffer.

Once fixed, these sections were rinsed in normal saline and placed into 70% ethanol and processed using the standard processing schedule for a Leica ASP200 tissue processor (70% ethanol for 2 h, 90% ethanol for 2 h, 100% ethanol for 6 h, toluene for 2 h and paraffin wax for 2.5 h), before being embedded in paraffin wax. Once embedded, sections were cut to a 15 µm thickness using a rotary microtome, dewaxed with toluene and mounted on gold slides.

Raman confocal measurements were conducted using a WiTec Raman microscope with 80 mW of laser power and a 3.45 s integration time. Spectral measurements were mapped over a 40 µm area (20 µm x 20 µm and 20 scans over 20 lines) of the myofibril using WiTec project software (WiTec, 2012) and a 50 x confocal microscopic image of the scan area was taken.

5.2.3 Traditional Indicators of Variation in Tenderness

Prior to the onset of rigor mortis, pH (pHPR) and temperature (temp20) at 20 mins post slaughter was measured on the topside (Product identification number HAM 5077; Anonymous, 2005) in-situ after subcutaneous fat was removed using a TPS® Intermediate Junction pH electrode with a BNC plug calibrated using pH buffers (pH 4.0 and 6.0) held at a room temperature of 16 °C.

At 24 h post mortem, pH (pH24) was re-measured using the pH electrode and sections of approximately 1-2g were excised from the muscle face where the m. adductor was removed for sarcomere length (SL) analysis, particle size analysis (PSA) and Raman microscopy. The sections excised for SL and PSA were frozen at -20°C until being analysed. SL was determined using the laser diffraction method (Bouton et al., 1973c), while PSA was conducted using the method previously described by Karumendu et al. (2009). The remaining SM was then vacuum packed and held at -1°C for 4 days.

At 5 days post mortem, shear force (SF) tests were conducted on blocks (mean weight 65g) that were cut to a length of 60 – 70 mm, a width of 40 – 50 mm and 20 – 25 mm thick. These blocks were cooked and cooking loss (CL) determined as previously described by Hopkins & Thompson (2001). From each block, 6 sub-samples of 1 cm² were cut parallel to the muscle fibres and shear force measured using a Warner- Bratzler texture meter with a vee-blade. The
mean value was recorded unless the co-efficient of variation (CV) was greater than 24%, in which case the median value of the repetitions was used (Hopkins et al., 2012).

Further sections were removed from each SM at 5 days post mortem to determine PSA after ageing as well as total (TC) and soluble collagen (SC). TC was determined using 0.1 g of freeze dried homogenised meat, hydrolysed in 3 ml of 3.5 M H$_2$SO$_4$, diluted with 3.75 ml water and 0.25 ml of 2 M NaOH, oxidised, heated at 60º C in a water bath for 15 min and cooled, before the absorbance of the solution was measured at 558nm, as described in the AOAC standard (AOAC, 2000). SC was measured using 0.5 ml of filtered supernatant made of 1.5 g freeze dried meat in 10 ml of water, heated and mixed, with the insoluble material removed. This supernatant was hydrolysed in 3 ml of 3.5 M H$_2$SO$_4$ and measured as per total collagen (AOAC, 2000).

5.2.4 Statistical Analysis

Chemical images (Figure 5.1) of the Raman spectral maps were generated using WiTec project software (WiTec, 2012), using the total integrated intensity of Raman bands between 500 – 2100cm$^{-1}$. To facilitate a comparison between single chemical images and specific Raman signals for individual wavenumbers, further chemical images were generated of spectral maps for the total integrated intensity of intensity peaks that are characteristic of the tryptophan (750cm$^{-1}$), tyrosine doublet (826cm$^{-1}$ and 853cm$^{-1}$), α- helix (930cm$^{-1}$), amide I (1245cm$^{-1}$, 1268cm$^{-1}$ and 1330cm$^{-1}$), C-H deformation (1450cm$^{-1}$) and amide III (1650cm$^{-1}$) chemical vibrations.

Figure 5.1. An example of a Raman chemical map generated using the total integrated intensity of Raman bands between 500 – 2100 cm$^{-1}$ measured over a 40µm area of the myofibril of ovine m. semimembranosus.
Once generated, analysis was conducted using ImagePro Plus© image analysis software (Media Cybernetics Inc, 2011) to distinguish the proportion of the spectral maps which have high (10 to 20 CCD counts), moderate (-10 to 10 CCD counts) and low (-10 to -20 CCD counts) intensities at each of these wavenumbers, as illustrated in Figure 5.2.

![Figure 5.2](image)

**Figure 5.2.** An example of a Raman chemical image for the integrated intensities for the Amide I peak centred at 1330 cm⁻¹ (A), and the chemical image showing the proportions of high (green), moderate (pink) and low (purple) integrated intensity classifications used for image analysis (B).

A linear discriminate analysis (LDA) was used to determine whether these percentages of high, moderate and low intensities at each of the 8 wavenumbers can be used to classify SMs into tough and tender groups.

LDA uses the equation:

\[ \beta_0 + \beta_1 \text{High} + \beta_2 \text{Mod} + \beta_3 \text{Low} \]

The co-efficients \( \beta_i \) (i = 0, 1, 2, 3) are given in Table 5.1.
Table 5.1 Co-efficients used for linear discriminate analysis (LDA) to determine whether intensities at 8 wavenumbers can be used to classify *m. semimembranosus* into tough or tender shear force groups.

<table>
<thead>
<tr>
<th>Wavenumber</th>
<th>Intercept ($\beta_0$)</th>
<th>High Intensity ($\beta_1$ High %)</th>
<th>Moderate Intensity ($\beta_2$ Mod %)</th>
<th>Low Intensity ($\beta_3$ Low %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha$-Helix (930 cm$^{-1}$)</td>
<td>-2723.506</td>
<td>27.242</td>
<td>27.237</td>
<td>27.203</td>
</tr>
<tr>
<td>Amide I (1245 cm$^{-1}$)</td>
<td>3346.242</td>
<td>-33.859</td>
<td>-33.499</td>
<td>-33.458</td>
</tr>
<tr>
<td>Amide I (1268 cm$^{-1}$)</td>
<td>81.540</td>
<td>-0.857</td>
<td>25.097</td>
<td>-25.079</td>
</tr>
<tr>
<td>Amide I (1330 cm$^{-1}$)</td>
<td>2509.021</td>
<td>25.097</td>
<td>25.079</td>
<td>-25.380</td>
</tr>
<tr>
<td>Amide III (1650 cm$^{-1}$)</td>
<td>3544.872</td>
<td>-35.479</td>
<td>35.439</td>
<td>-35.445</td>
</tr>
<tr>
<td>Tyrosine Doublet (826 cm$^{-1}$)</td>
<td>3174.556</td>
<td>-31.746</td>
<td>-31.773</td>
<td>-31.740</td>
</tr>
<tr>
<td>Tyrosine Doublet (853 cm$^{-1}$)</td>
<td>-1225.278</td>
<td>12.224</td>
<td>12.257</td>
<td>12.275</td>
</tr>
<tr>
<td>Tryptophan (750 cm$^{-1}$)</td>
<td>-2754.808</td>
<td>27.520</td>
<td>27.546</td>
<td>27.553</td>
</tr>
</tbody>
</table>

If the expression was $\leq 0$ the sample was classified as having a tender shear force value and if it was $> 0$ the sample was classified as having a tough shear force value.

Spectra were also extracted using WiTec project software before being individually background corrected using a 6th order polynomial (Table 5.2) and bands assigned using Opus® chemometric software (Bruker, 2013) and Microsoft Excel®.

Table 5.2 Wavenumbers used to fit a 6th order polynomial through unprocessed spectra to remove contributions of background noise and fluorescence.

<table>
<thead>
<tr>
<th>Raman Spectra</th>
<th>Wavenumbers (cm$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tender SM</td>
<td>511, 684, 801, 1147, 1411, 1497, 1731</td>
</tr>
<tr>
<td>Tough SM</td>
<td>503, 596, 782, 1136, 1389, 1499, 1762</td>
</tr>
</tbody>
</table>

An alternative analysis using Support Vector Machines (SVM) methods (Vapnik, 1998) was also trialled. This was applied to the percentages of high, moderate and low intensities at each of the identified wavenumbers (Table 5.1) with a linear kernel function.

Both classification methods were performed using R statistical software (R Core Team, 2013) with MASS package for LDA (Venables & Ripley, 2002) and kernlab package to complete SVM classifications (Karatzoglou *et al.*, 2004).

The relationship between SF and the other traditional indicators of tenderness (CL, SL, PS TC, SC, pH$_{PR}$ and pH$_{24}$) were derived using pair wise plots using R statistical software (R Core Team, 2013).
5.3 Results

5.3.1 Traditional Indicators of Variation in Tenderness

As outlined in Table 5.3., there was only small differences between SMs classified into tough (54.4 – 74.3 N) and tender (26.3 – 35.3 N) groups for some of the traits commonly used as indicators of tenderness. Pre-rigor pH (pHPR) tended to be slightly lower and less variable for tough SMs (6.3 ± 0.17 for tender and 6.2 ± 0.26 for tough), as was pHu (5.57 ± 0.18 and 5.52 ± 0.07, respectively for tender and tough SMs). Cooking loss was greater for SMs classified as tough (23.4% for tough and 21.0% for tender SMs) as was particle size at 1 and 5 days (274 µm tough, 256 µm tender and 166 µm tough and 150 µm, respectively).

There was slightly less total collagen in tender SMs (12.2 mg/ g tender, 13.0 mg/ g tough), however tender SMs had more soluble collagen compared to tough SMs (1.27 mg/g tender, 1.15 mg/ g tough).

<table>
<thead>
<tr>
<th>Trait</th>
<th>Time Measured</th>
<th>Tender</th>
<th>Tough</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shear Force (N)</td>
<td>5 days</td>
<td>32.78 (+2.5)</td>
<td>60.4 (+5.5)</td>
</tr>
<tr>
<td>Carcase Weight (kg)</td>
<td>pre-rigor+</td>
<td>22.7 (+1.79)</td>
<td>22.6 (+1.72)</td>
</tr>
<tr>
<td>GR Fat (mm)</td>
<td>pre-rigor+</td>
<td>11.7 (±3.6)</td>
<td>11.6 (±3.09)</td>
</tr>
<tr>
<td>Cooking Loss (%)</td>
<td>5 days</td>
<td>21.0 (±2.4)</td>
<td>23.4 (±2.2)</td>
</tr>
<tr>
<td>pH</td>
<td>pHPR</td>
<td>6.26 (±0.17)</td>
<td>6.17 (±0.26)</td>
</tr>
<tr>
<td>pHu</td>
<td>5.62 (±0.16)</td>
<td>5.61 (±0.07)</td>
<td></td>
</tr>
<tr>
<td>pH24</td>
<td>5.57 (±0.18)</td>
<td>5.52 (±0.07)</td>
<td></td>
</tr>
<tr>
<td>Sarcomere Length (µm)</td>
<td>24 h</td>
<td>1.79 (±0.07)</td>
<td>1.61 (±0.1)</td>
</tr>
<tr>
<td>Particle Size (µm)</td>
<td>24 h</td>
<td>256.7 (±65.7)</td>
<td>273.5 (±56.0)</td>
</tr>
<tr>
<td>Total Collagen (mg/ g)</td>
<td>5 days</td>
<td>150.3 (±38.5)</td>
<td>166.1 (±30.8)</td>
</tr>
<tr>
<td>Soluble Collagen (mg/ g)</td>
<td>5 days</td>
<td>1.27 (±0.3)</td>
<td>1.15 (±0.42)</td>
</tr>
</tbody>
</table>

* 20 minutes post slaughter
* 4 hours post slaughter

Further analysis indicated that none of these factors were significant in explaining variation in the shear force values (P > 0.05).
5.3.2 Chemical Imaging

As illustrated by Figures 5.3 and 5.4, chemical imaging through Raman mapping was able to pick up differences in the ultra-structure and integrated intensities across the myofibril of tender (Fig. 5.3.) and tough (Fig. 5.4.) SMs.

![Figure 5.3. Chemical image of the most tender ovine m. semimembranosus (26 N) showing the integrated intensities of wavenumbers between 500 – 1900 cm⁻¹.](image)

Further analysis of the chemical images for the 8 key Raman spectral bands (Table 5.4) suggests that the integrated intensities are greater in the amide I band at 1330 cm⁻¹, tyrosine doublet band at 853 cm⁻¹ and the tryptophan band at 750 cm⁻¹ for tender SMs. However, tender SMs also had larger proportions of areas across the myofibril with low integrated intensities at the α-helix band characterised by the vibration at 930 cm⁻¹ and moderate integrated intensities for the tyrosine doublet band at 826 cm⁻¹.

![Figure 5.4. Chemical image of the toughest ovine m. semimembranosus (74 N) showing the integrated intensities of wavenumbers between 500 – 1900 cm⁻¹.](image)
SMs classified as tough had greater proportions of moderate integrated intensities for the amide I band at 1330 cm$^{-1}$ as well as the tyrosine doublet band at 853 cm$^{-1}$ in comparison to tender SMs.

### Table 5.4. Summary statistics for the proportion of the Raman chemical images from tender (26 – 35N) and tough (54 – 74 N) ovine m. semimembranosus with high, moderate and low integrated intensities across the 8 wavenumbers important to meat quality assessment.

<table>
<thead>
<tr>
<th>Raman Band (Wavenumber cm$^{-1}$)</th>
<th>Intensity Classification</th>
<th>Tender Mean (SD)</th>
<th>Range (min, max)</th>
<th>Tough Mean (SD)</th>
<th>Range (min, max)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\alpha$-helix (930 cm$^{-1}$)</td>
<td>High</td>
<td>6.9 ± 23.4</td>
<td>0 – 81.2</td>
<td>10.1 ± 23.6</td>
<td>0 – 73.4</td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
<td>76.36 ± 38.4</td>
<td>1.1 – 100</td>
<td>88.3 ± 23.0</td>
<td>26 – 100</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>16.75 ± 34.3</td>
<td>0 – 98.9</td>
<td>1.6 ± 2.6</td>
<td>0 – 7.2</td>
</tr>
<tr>
<td>Amide I (1245 cm$^{-1}$)</td>
<td>High</td>
<td>0.6 ± 2.1</td>
<td>0 – 7.4</td>
<td>0</td>
<td>0 – 0</td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
<td>5.11 ± 7.7</td>
<td>0 – 22.3</td>
<td>2.6 ± 6.9</td>
<td>0 – 24.4</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>94.3 ± 8.9</td>
<td>75.9 – 100</td>
<td>97.4 ± 6.9</td>
<td>75.6 – 100</td>
</tr>
<tr>
<td>Amide I (1268 cm$^{-1}$)</td>
<td>High</td>
<td>6.9 ± 22.9</td>
<td>0 – 79.5</td>
<td>0.7 ± 1.3</td>
<td>0 – 3.8</td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
<td>84.1 ± 34.8</td>
<td>0 – 100</td>
<td>98.7 ± 1.9</td>
<td>94.5 – 100</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>9.0 ± 28.7</td>
<td>0 – 100</td>
<td>0.6 ± 1.6</td>
<td>0 – 5.5</td>
</tr>
<tr>
<td>Amide III (1650 cm$^{-1}$)</td>
<td>High</td>
<td>75.2 ± 30.4</td>
<td>0 – 100</td>
<td>59.0 ± 38.5</td>
<td>0 – 99.1</td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
<td>24.0 ± 29.7</td>
<td>0 – 100</td>
<td>41.0 ± 38.5</td>
<td>0.9 – 100</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>0.9 ± 2.7</td>
<td>0 – 9.4</td>
<td>0.1 ± 0.1</td>
<td>0 – 0.4</td>
</tr>
<tr>
<td>Tyrosine Doublet (826 cm$^{-1}$)</td>
<td>High</td>
<td>13.7 ± 28.2</td>
<td>0 – 78.1</td>
<td>4.5 ± 15.3</td>
<td>0 – 53.2</td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
<td>16.1 ± 29.0</td>
<td>0 – 98.6</td>
<td>21.3 ± 38.7</td>
<td>0 – 99.4</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>72.7 ± 40.1</td>
<td>0 – 100</td>
<td>74.3 ± 44.5</td>
<td>0.2 – 100</td>
</tr>
<tr>
<td>Tyrosine Doublet (853 cm$^{-1}$)</td>
<td>High</td>
<td>8.4 ± 28.9</td>
<td>0 – 100</td>
<td>8.31 ± 28.8</td>
<td>0 – 99.8</td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
<td>15.3 ± 28.9</td>
<td>0 – 100</td>
<td>6.1 ± 9.9</td>
<td>0 – 28.6</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>76.4 ± 37.3</td>
<td>0 – 100</td>
<td>85.6 ± 28.7</td>
<td>0 – 100</td>
</tr>
<tr>
<td>Tryptophan (750 cm$^{-1}$)</td>
<td>High</td>
<td>29.4 ± 31.6</td>
<td>0.1 – 99.5</td>
<td>6.6 ± 7.4</td>
<td>0 – 22.1</td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
<td>69.3 ± 32.4</td>
<td>0.5 – 99.9</td>
<td>79.2 ± 31.3</td>
<td>0.6 – 99.8</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>1.3 ± 3.9</td>
<td>0 – 13.0</td>
<td>14.2 ± 33.6</td>
<td>0 – 99.4</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>22.1 ± 34.2</td>
<td>0 – 95.3</td>
<td>3.2 ± 10.3</td>
<td>0 – 35.9</td>
</tr>
<tr>
<td></td>
<td>Moderate</td>
<td>24.7 ± 33.4</td>
<td>0 – 91.3</td>
<td>22.5 ± 40.1</td>
<td>0 – 99.8</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>53.2 ± 49.5</td>
<td>0 – 100</td>
<td>74.4 ± 44.9</td>
<td>0 – 100</td>
</tr>
</tbody>
</table>

The results of LDA using the proportions of the myofibril with high, moderate and low integrated intensities as determined by the Raman chemical images to classify SMs into tough and tender groups is outlined in Table 5.5. This demonstrates that despite the high percentages of samples correctly classified using some chemical vibrations, use of the spectral locations alone cannot significantly improve the categorisation of carcases into high and low shear force groups ($P > 0.05$).
Table 5.5. The percentages and probabilities of correctly classifying ovine m. semimembranosus into tough and tender categories using key Raman spectroscopy vibrations.

<table>
<thead>
<tr>
<th>Key Raman Peak (Wavenumber)</th>
<th>Percentage Classified using Intensity at that Location</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Tender</td>
<td>Tough</td>
</tr>
<tr>
<td>α-helix (930 cm(^{-1}))</td>
<td>33%</td>
<td>83%</td>
</tr>
<tr>
<td>Amide I (1245 cm(^{-1}))</td>
<td>33%</td>
<td>92%</td>
</tr>
<tr>
<td>Amide I (1268 cm(^{-1}))</td>
<td>17%</td>
<td>100%</td>
</tr>
<tr>
<td>Amide I (1330 cm(^{-1}))</td>
<td>83%</td>
<td>50%</td>
</tr>
<tr>
<td>Amide III (1650 cm(^{-1}))</td>
<td>27%</td>
<td>92%</td>
</tr>
<tr>
<td>Tyrosine Doublet (826 cm(^{-1}))</td>
<td>42%</td>
<td>67%</td>
</tr>
<tr>
<td>Tyrosine Doublet (853 cm(^{-1}))</td>
<td>55%</td>
<td>83%</td>
</tr>
<tr>
<td>Tryptophan (750 cm(^{-1}))</td>
<td>27%</td>
<td>83%</td>
</tr>
</tbody>
</table>

Applying SVM methods with linear kernels indicated that the tyrosine doublet vibration at 853 cm\(^{-1}\) gave a significantly better chance of classifying SMs into tender and tough categories (P < 0.05). However, using the tyrosine doublet band at 853 cm\(^{-1}\) (Fig 5.5) it was also possible to group the SMs based on the content of soluble collagen, as 18 SMs were correctly classified (P < 0.05).

A promising classification was also achieved using SVM methods with linear kernels for the classification of SMs based on high and low sarcomere lengths, as 16 SMs were correctly classified (P < 0.05) using the α-helix (930 cm\(^{-1}\)) signal as highlighted in Figure 5.5.

Figure 5.5. An example of a background corrected spectra extracted from the Raman map of the most tender (26 N; black) and the toughest (74 N; grey) ovine m. semimembranosus highlighting the bands centred at 853 cm\(^{-1}\) and 930 cm\(^{-1}\).
5.4 Discussion

Overall the image analysis of the chemical images created using Raman microscopy demonstrated that integrated intensities at key Raman spectral peaks for meat cannot explain the variation in shear force \( (P > 0.05) \). However, integrating the intensity using the signal centred at 853 cm\(^{-1}\) it was possible to categorise the SMs into categories based on their shear force values and soluble collagen content. If predicting the traits of interest relies on the integration of the intensity at only one wavenumber in the 1300 cm\(^{-1}\) frequency measured, this finding may suggest that the prediction of meat quality traits using Raman spectroscopy is being convoluted by the large data set which is collected, little of which has relevance to the traits of interest. Indeed, a previous study conducted by Scheier et al. (2014) demonstrated that the early PM metabolic changes of pork associated with the development of pH\(_{24}\) could be explained by the summation of five Raman spectral differences that were measured in pure compounds in solution. Thus, the understanding of tenderness using Raman microscopy may be improved by collecting more detailed combinations of reference spectra for components of meat including mixtures of collagen, myofibrillar proteins, pyruvate, lactate and glycogen.

This study demonstrated that using the integrated intensity of the tyrosine peak centred around 853 cm\(^{-1}\), which had a reduced intensity in tough SMs (Fig. 5.5) it was possible to correctly classify 83\% of the tough samples (Table 5.5). Previous Raman spectroscopic studies on the tenderness meat have suggested that this band centred at 853 cm\(^{-1}\) forms part of the tyrosine doublet (Beattie et al., 2004a, Beattie et al., 2008, Schmidt et al., 2013) based on the findings of Raman spectroscopic studies of pure proteins, such as that by Tsuboi et al. (1998). It has been proposed that the presence of this tyrosine band is indicative of proteolysis as the presence of free tyrosine as an amino acid increases with ageing in normal quality pork (Beattie et al., 2008). Thus, weakening of the tyrosine band has been linked with tougher meat samples which have not undergone proteolysis to the same extent as tender samples (Schmidt et al., 2013). Although Figure 5.5 suggests that the band did weaken in tougher samples as with previous studies, the significant correlation between this band and soluble collagen content \( (P < 0.05) \) does not correlate with the previous findings for tenderness. Furthermore, these two studies cited are Raman spectroscopic studies which have been conducted on aged meat, while the present study has been conducted on meat which is
set in gluteraldehyde at 24 h PM thereby stoping any further glycolysis and fixed preventing proteolysis from occurring.

Alternative band assignments for the peak at 853 cm\(^{-1}\) suggest that glycogen (Binoy et al., 2004), hydroxyproline (Pedersen et al., 2003, Cheng et al., 2005) and polysaccharides (Movasaghi et al., 2007) also can contribute to this Raman signal. These molecules have similar chemical conformation which includes a phenol group which would contribute to the vibration at 853 cm\(^{-1}\) as a result of the C-C ring vibration (Merlino et al., 2008) as it does in tyrosine (Tsuboi et al., 1998). Therefore, it is plausible that hydroxyproline and proline are contributing to the signal as they are main components of collagen within the connective tissue network, as well as glycine, proline and varying amounts of lysine and hydroxylysine (Warriss, 2010). Therefore, the reduction in intensity peak at 853 cm\(^{-1}\) may indicate a reduced proportion of proline and hydroxyproline in collagen compared to an increased proportion of other residues such as lysine, which occur in the connective tissue matrix of tough SMs.

This proportion of proline and hydroxyproline to other connective tissue components, such as lysine, may be important to tenderness as it is the lysine residues on adjacent, but offset molecules that form aldehyde crosslinks by enzymatic oxidation which stabilises the non-covalent bonds into covalent bonds joined by hydrogen. These crosslinks make collagen resistant to thermal rupture and gelatinisation during cooking, creating insoluble collagen (Bouton & Harris, 1972, Marsh, 1974). Since gelatinisation of soluble collagen at cooking temperatures above 50 °C contributes to shrinkage of the myofibrillar mass and increases in toughness (Purslow, 2005), it is plausible that the relationship between the intensity at 853 cm\(^{-1}\) and shear force values may also reflect the impacts of soluble collagen on tenderness.

However, the impact of spectral overlap with other phenol groups is unknown, as any other phenol groups present will also contribute to this peak intensity at 853 cm\(^{-1}\) (Binoy et al., 2004). Consequently this peak may also include contributions from glycogen because the tissue was set in gluteraldehyde at 24 h. Consequently, anaerobic glycolysis may not have completely exhausted glycogen, despite no variation in final pHu values (Hwang et al., 2004).

Despite further analysis indicating that it was possible to classify SMs based on sarcomere length using the α- helical signal centred at 930 cm\(^{-1}\) as 16 SMs were correctly categorised, it is difficult to determine the biological processes which contribute to this classification since many proteins present in the myofibril have an α- helical structure. A previous spectroscopic
study conducted by Pezolet *et al.* (1978) on muscle fibres collected from giant barnacles suggests that \( \alpha \)-helical proteins generated chemical vibrations in the skeletal C-C region with a strong band at 939 cm\(^{-1}\) were characteristic of the contractile protein tropomyosin, which has an \( \alpha \)-helical content of 90%. Therefore it is plausible that differences to this intensity reflect changes to the orientation of tropomyosin and exposure of the actin binding sites due to the presence of calcium ions, facilitating the contraction of muscles post mortem which determine sarcomere length post mortem (Warriss, 2010). However, as tropomyosin is not the only \( \alpha \)-helical protein present in muscle which contributes to this band this may also reflect the orientation and state of myosin (Pézolet *et al.*, 1980) and connective tissue components elastin (Debelle *et al.*, 1995) and tropocollagen (Frushour & Koenig, 1975), which can prevent the longitudinal and lateral contraction during rigor (Tornberg, 1996).

The little coherence between the spectra and meat tenderness after 5 days ageing may also be the result of setting the blocks in gluteraldehyde to facilitate embedding of the histology blocks due to the delay between collection of samples and measurement with the Raman microscope. Consequently, it is unclear from this study whether Raman spectroscopy is able to predict shear force values via the ability of proteolysis to cleave the chemical bonds such as the hydroxyl group bond of tryosine or through differences in the amide bands due to changes in the architecture and therefore orientation of the connective tissue matrix around the myofibril as discussed in Chapter 4. Therefore, a Raman microscopic study conducted using this protocol with fresh intact samples may be more informative on the links between Raman spectra and meat quality traits.
Overall this study does suggest that despite the integration of key signals the Raman band assignments are still complex. Whilst the Raman microscopy data is informative on the Raman spectral signals which may help to further explain the variation in shear force values, it does highlight the difficulties of assigning band interpretations to Raman spectra from complex samples such as meat. Since functional groups, such as the phenol group and α-helical proteins, give rise to a common group frequency (Carey, 1982), the intensity of the peak will be a representation of total concentration of the functional group regardless of the compound of origin. Therefore, it is problematic to draw conclusions on the biochemical and biophysical properties of the meat when several vibrations, some which may be unrelated to the trait of interest, also may be eliciting an effect. Furthermore, due to the complex nature of the data set and the volume of data, numerous models need to be considered. In doing so by chance alone some models will be identified as having significant results yet overall they may not be informative. Consequently, better data handling and data analysis techniques need to be developed to fully utilise the large data set which is generated.

5.5 Conclusion

Overall Raman microscopy was able to provide some information on the wavenumbers which may explain variation in meat quality traits. Using the intensity peak centred at 853 cm$^{-1}$ it was possible to classify the *m. semimembranosus* into tender and tough samples based on shear force values as well as samples containing high and low concentrations of soluble collagen. Using the α-helical band centred at 930 cm$^{-1}$ it was possible to classify *m. semimembranosus* into groups based on sarcomere lengths. However, despite integrating intensities, the Raman spectra were still complex and the impact of spectral overlap from other compounds with similar chemical composition is still unknown. Furthermore, the transference of these findings to other data sets is unknown, consequently future research will need to address this.
6. Predicting meat quality traits of fresh intact ovine *m. semimembranosus* using a Raman spectroscopic handheld device

6.1 Introduction

In recent years there has been an increased focus on the management of meat quality of prime lambs in Australia, due to advances in meat grading systems for sheep meat products (Thompson *et al.*, 2005a). However, as lamb carcases are assessed for market suitability using only weight, age, sex and fat scores, better carcase assessment tools are required by industry to determine whether lamb carcases meet the quantitative and qualitative attributes vital to meeting the needs of domestic and export markets. Although there are numerous technologies which have been developed to assess carcase quality (Damez & Clerjon, 2008) few of these methods are suitable for measurement in commercial situations as they are destructive, time and labour intensive, require extensive sample preparation or bulky equipment. Recent advances in digital camera and laser technologies has facilitated greater research into optic methods as it has become possible to develop fibre optic components and energy efficient lasers suited for portable devices (Damez & Clerjon, 2008).

Raman spectroscopy (RS) is one such optic technology as it based on the inelastic scattering of light which provides information on the chemical composition of matter (Das & Agrawal, 2011). Therefore RS is a non-destructive tool which is potentially suitable for online measurement (Damez & Clerjon, 2008), consequently recent meat science studies have focused on its potential to measure meat quality traits (Beattie *et al.*, 2008, Bauer *et al.*, 2013, Schmidt *et al.*, 2013).

Such studies have demonstrated high coefficients of determination ($R^2$) of between 0.71 to 0.98 when using Raman spectra to predict drip loss (Pedersen *et al.*, 2003), early PM pH values (Scheier & Schmidt, 2013) and shear force in pork (Beattie *et al.*, 2008) and beef (Bauer *et al.*, 2013). However, findings for the prediction of shear force values of lamb have been inconsistent. While Schmidt *et al.* (2013) suggested there was good potential to predict shear force values of lamb loins from two different sample groups ($R^2 = 0.79$ and 0.86), previous research presented in this thesis (data reported Chapter 4) demonstrated that cross validated correlations of determination ($R^2_{cv}$) values for prediction of shear force in lamb may be lower ($R^2_{cv} = 0.27$). However, it was acknowledged that due to differences in sample
handling compared with previous studies using the same device, these findings needed to be
validated. Furthermore, it was hypothesised that longer integration times and measurement
pre-rigor may improve the predictions found.

Despite previous studies indicating Raman spectra is capable of measuring other meat quality
traits including pHu ($R^2 = 0.94$) (Scheier & Schmidt, 2013) as well as water holding capacity
($r = 0.79$) (Pedersen et al., 2003) of fresh intact pork, previous RS studies on lamb have
focused on the prediction of shear force values (Schmidt et al., 2013, Fowler et al., 2014b,
Fowler et al., 2014a). Consequently the potential to use RS to predict other indicators of meat
quality of lamb has not been determined.

Therefore, this study aims to validate the findings of previous research (data reported in
Chapter 4) and determine the potential for RS to predict meat quality indicators other than
tenderness in fresh intact lamb. Furthermore, this study also aims to determine whether
improvements to the prediction of shear force values of fresh intact m. semimembranosus can
be made by increasing accumulations and collecting Raman spectra of SM pre-rigor.

6.2 Materials and Methodology

6.2.1 Samples

Eighty lamb carcases were measured over 4 consecutive days (20 per day) from the same
abattoir. Carcases were randomly selected from different consignments and were of unknown
backgrounds, sex and age, representing animals that were typically processed at the abattoir
in order to obtain a spread in shear force values. Lambs were processed following standard
commercial slaughter processes and were electrically stimulated pre-dressing with a mid-
voltage unit (2000mA with variable voltage to maintain a constant current, for 25 s at 15
pulses/s, 500 microsecond pulse width, unipolar waveform) (Toohey et al., 2008).

At 25 min post slaughter hot carcase weight (HCW) was recorded. Approximately 4 hours
post slaughter GR tissue depth (depth of the tissue over the 12th rib, 110 mm from the
midline) using a GR knife.
At 24 h post slaughter, topsides (Product identification HAM 5077; Anonymous, 2005) were removed (boned out) from the carcases. The cap muscle (m. gracilis) and m. adductor were removed to leave the m. semimembranosus (SM) which was the muscle of measurement.

### 6.2.2 Raman Spectroscopy

Raman spectroscopic measurements were conducted prior to the onset of rigor mortis (pre-rigor), at 24 h and 5 days post mortem.

Pre-rigor measurements were conducted 25 min PM on the SM in-situ with the subcutaneous fat and silverskin (epimysium) removed (Fig 6.1). In processing plant conditions at a room temperature of 2º C, 10 positions were measured perpendicular to the muscle fibre. All measurements were taken with a 671 nm hand held Raman spectroscopic device (Schmidt et al., 2013) and spectra were recorded using 70 mW of laser power and an integration time of 2 s. This shorter integration time was chosen to avoid fluorescence and saturation issues arising from the high carcase temperatures pre-rigor and ongoing metabolic processes. To facilitate the comparison between different total measurement times, the first integration (total measurement 2 s) from each Raman spectroscopic measurement was saved separately to a scan which included 10 accumulations (total measurement 20 s) at the same position on the muscle. After measurement, the carcases were chilled at a mean temperature of 3º C for 24 h.

![Figure 6.1 Pre-rigor measurement of the m. semimembranosus in-situ in processing plant conditions.](image)
At 24 h post slaughter, Raman spectroscopic measurements were conducted at a room temperature of 16 °C temperature on the SM once it had been removed from the carcase. A freshly cut surface of the intact SM was made where the m. adductor was removed, perpendicular to the muscle fibres. Ten positions were measured across this face of the muscle and spectra were recorded using 70 mW of laser power and an integration time of 3 s. The first integration (total measurement 3 s) from each Raman spectroscopic measurement was saved separately to a scan which averaged 5 accumulations (total accumulation 15 s) at the same position on the muscle. After scanning with the Raman spectroscopic hand held device and removal of sections for the measurement of traditional indicators, the remaining SM samples were vacuum packed and held at a mean temperature of 1.5º C for 4 days.

At 5 days post mortem, SM samples were removed from the vacuum packs and oxygenate (‘bloom’) for 2 hours prior to rescanning a freshly cut surface at a room temperature of 16°C using the same parameters described for 24 h PM Raman spectroscopic measurements.

6.2.3 Traditional Indicators

Immediately prior to pre-rigor Raman spectroscopic measurements at 25 min PM, pH (pHPR) and temperature (temp20) of the SM was measured in-situ using a TPS intermediate junction pH electrode with a BNC plug calibrated using buffers (pH 4.01 and pH 6.86) held at a room temperature of 16 °C.

At 24 h post mortem prior to measurement for Raman spectra, a fresh cut was made on the muscle face where the m. adductor had been removed, pH remeasured with the pH electrode and a colour reading taken. Colour readings were completed using a Minolta® CR- 400 Colourmeter (Milota Camera Co., Japan) under a D65 illuminant with an 8 mm aperture size, 10 degree observation angle and a closed cone that was calibrated using a white tile (Y = 92.8, X = 0.3160, Y = 0.3323).

Once Raman Spectroscopy measurements were taken, 2 sections (1 – 2 g) were excised for sarcomere length (SL) and particle size (PS) testing. SL tests were conducted using the laser diffraction method described by Bouton et al. (1973c), while PS measurements were conducted by homogenising samples at 16 000 rpm as described by Karumendu et al. (2009). Remaining samples were weighed into vacuum packaging prior to ageing.
At 5 days post mortem SMs were removed from vacuum packaging, patted dry with paper towel and weighed to determine purge. SMs were then allowed to ‘bloom’ for two hours before a finely cut surface was cut off the same face previously measured and another colour reading was taken on the freshly cut surface immediately prior to the Raman Spectroscopic measurement.

Once Raman spectra had been collected at 5 days PM, further 1 – 2 g sections were excised for PS (Karumendu et al., 2009) and pHu determination. pHu was determined using 2.5g of muscle homogenate in 10 ml of 5 mM iodoacetate/ 150 mM KCl (pH adjusted to 7.0), as previously described by Dransfield et al. (1992). Total collagen was determined using 0.1g of freeze dried and homogenised meat which was hydrolysed in 3 ml of 3.5 M H$_2$SO$_4$ that had been diluted in 3.75 ml water and 0.25 ml of 2 M NaOH, as described in the AOAC standard (AOAC, 2000). Soluble collagen was measured using 0.5 ml of supernatant made of 1.5 g freeze dried meat in 10 ml of water, heated and mixed, with the insoluble materials removed, which was then hydrolysed and measured the same as total collagen (AOAC, 2000).

Furthermore, a shear force block was also excised at 5 days post mortem. Shear force was measured as previously described by Hopkins et al. (2011a). The average of 8 shear force repetitions was used except where the co-efficient of variation exceeded 24%. In this instance the median was reported instead of the average (Hopkins et al., 2012). Shear force blocks were weighed before and after cooking to determine cooking loss.

6.2.4 Data Analysis

Raw spectra were prepared for chemometric analysis as previously described in Chapter 3 (3.2.4). Sub-sets of spectra for each sample were averaged and wavenumbers reduced to a range of 500 – 1900cm$^{-1}$. Once the wavenumber was reduced, spectra were not subjected to alternative pre-processing techniques as previous studies have demonstrated that there was no significant improvement to the prediction outcomes when pre-processing was applied (Sections 3.3.1 and 4.3.1).

Models to predict traditional indicators of meat quality were fitted using partial least squares (PLS) regression analysis. The Monte-Carlo K fold cross validation method (Arlot & Celisse, 2010) with K = 8 and 50 random repeats, was used to select the most appropriate model to report. The optimal PLS model was selected based on selecting the simplest model having the minimum cross validated average mean squared prediction error (MSPE$_{cv}$) subject to the one
standard error rule (Hastie et al., 2008). Using this rule, the “optimal” model selected is the most parsimonious model having average mean MSPE$_{cv}$ less than the average MSPE$_{cv}$ plus associated error for the model with minimum average mean MSPE$_{cv}$. Summaries for PLS models, where given, include the Square-root MSPE$_{cv}$ (RMSPE$_{cv}$) for the null and optimal models, relative reduction in the RMSPE$_{cv}$ (null and optimal model), and the squared correlation between the observed trait and the Leave-One-Out (LOO) cross validated predictions ($R^2_{cv}$). Least Angle Regression (LAR) as a model selection method was also trialled to determine which variables to include without overfitting (Efron et al., 2004). All calculations were conducted using R statistical software (R Core Team, 2013) using the pls package (Mevik et al., 2011) and lars package (Hastie & Efron, 2013).

Models to determine relationships between shear force and traditional indicators (likewise pHu and traditional indicators) were fitted using simple linear regression and simplified using stepwise regression.

Background was removed from the spectra for band assignment by fitting the spectra to a 7th order polynomial at wavenumbers outlined in the Table 6.1.

Table 6.1. Wavenumbers used to baseline correct raw Raman spectra measured pre-rigor, 24 h and 5 days post mortem.

<table>
<thead>
<tr>
<th>Raman Spectra</th>
<th>Wavenumbers used for Baseline Correction (cm$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-rigor</td>
<td>533, 626, 764, 1138, 1370, 1523, 1743 and 1846</td>
</tr>
<tr>
<td>24 h</td>
<td>538, 628, 764, 1142, 1370, 1525, 1743 and 1846</td>
</tr>
<tr>
<td>5 Day</td>
<td>538, 630, 764, 1138, 1370, 1523, 1743 and 1846</td>
</tr>
</tbody>
</table>

6.3 Results

6.3.1 Traditional Indicators of Meat Quality

Summary statistics for HCW, GR Fat, shear force, cooking loss, purge, pH, particle size analysis and colour are given in Table 6.2.
Table 6.2 Mean, standard deviation (SD) and range for carcase traits and meat quality indicators of the m. semimembranosus.

<table>
<thead>
<tr>
<th>Trait</th>
<th>Mean</th>
<th>SD</th>
<th>Range (min, max)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCW (kg)</td>
<td>22.7</td>
<td>2.0</td>
<td>16.9 – 28.9</td>
</tr>
<tr>
<td>GR Fat (mm)</td>
<td>15</td>
<td>3.9</td>
<td>4 – 26</td>
</tr>
<tr>
<td>Shear Force (N)</td>
<td>40.2</td>
<td>9.4</td>
<td>24.7 – 76.8</td>
</tr>
<tr>
<td>Cooking Loss (%)</td>
<td>23.9</td>
<td>2.6</td>
<td>14.8 – 34.9</td>
</tr>
<tr>
<td>Purge (%)</td>
<td>2.7</td>
<td>1.2</td>
<td>1.1 – 6.4</td>
</tr>
<tr>
<td>pH PR</td>
<td>6.02</td>
<td>0.20</td>
<td>5.47 – 6.80</td>
</tr>
<tr>
<td>pH24</td>
<td>5.68</td>
<td>0.15</td>
<td>5.44 – 6.12</td>
</tr>
<tr>
<td>pH 5u</td>
<td>5.71</td>
<td>0.10</td>
<td>5.59 – 6.16</td>
</tr>
<tr>
<td>temp @ 25 min PM (°C)</td>
<td>36.3</td>
<td>1.1</td>
<td>33.5 – 38.7</td>
</tr>
<tr>
<td>Sarcomere Length (µm)</td>
<td>1.59</td>
<td>0.16</td>
<td>1.26 – 1.89</td>
</tr>
<tr>
<td>Particle Size (µm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24 h</td>
<td>254</td>
<td>52.4</td>
<td>128 – 376</td>
</tr>
<tr>
<td>5 day</td>
<td>182</td>
<td>46.3</td>
<td>83 – 299</td>
</tr>
<tr>
<td>L*</td>
<td>39.9</td>
<td>2.23</td>
<td>36.2 – 47.7</td>
</tr>
<tr>
<td>a*</td>
<td>16.0</td>
<td>1.39</td>
<td>12.6 – 19.3</td>
</tr>
<tr>
<td>b*</td>
<td>-1.2</td>
<td>1.31</td>
<td>-3.3 – 3.3</td>
</tr>
<tr>
<td>L*</td>
<td>38.3</td>
<td>2.12</td>
<td>33.8 – 44.2</td>
</tr>
<tr>
<td>Colour 24 h PM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a*</td>
<td>16.9</td>
<td>1.26</td>
<td>14.1 – 19.5</td>
</tr>
<tr>
<td>b*</td>
<td>-0.3</td>
<td>1.12</td>
<td>-2.8 – 3.6</td>
</tr>
<tr>
<td>Colour 5 day PM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a*</td>
<td>13.1</td>
<td>2.2</td>
<td>9.1 – 22.3</td>
</tr>
<tr>
<td>b*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Collagen (mg/g)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>soluble</td>
<td>0.49</td>
<td>0.08</td>
<td>0.3 – 0.8</td>
</tr>
<tr>
<td>total</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

A summary of the RMSEP<sub>CV</sub> for models to predict shear force (Table 6.3) indicates that there is no ability to predict shear force values using Raman spectra measured pre-rigor, 24 h or 5 days PM. Furthermore, the potential to predict shear force values was not improved by increasing the total measurement time from 3 s to 15 s.

Table 6.3 Summary chemometrics for the prediction of shear force values using Raman spectra measured 25 mins (pre-rigor), 1 and 5 days post mortem using the first integration (3 s) and the full accumulation (15 s).

<table>
<thead>
<tr>
<th>Age</th>
<th>First Integration</th>
<th>Full Accumulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>PR</td>
<td>9.42 (0 LV)</td>
<td>9.42 (0 LV)</td>
</tr>
<tr>
<td>24 h</td>
<td>9.41 (0 LV)</td>
<td>9.43 (0 LV)</td>
</tr>
<tr>
<td>5 Day</td>
<td>9.43 (0 LV)</td>
<td>9.42 (0 LV)</td>
</tr>
</tbody>
</table>

Including any other indicator or combination of indicators as regressors into the prediction model for shear force using spectra measured did not improve the prediction model.

Univariate regression analysis (Table 6.4) indicates that particle size measured at 5 days was highly significant in explaining the variation in shear force values at 5 days post mortem ($P < 0.01$; Fig 6.2). No other trait was significant ($P > 0.05$).
Table 6.4 Summary of univariate regression parameters and P values of the variation in shear force values explained by the traditional indicators including pH, sarcomere length, particle size analysis, collagen content, purge and cooking losses.

<table>
<thead>
<tr>
<th>Indicator</th>
<th>$a , (\pm \text{s.e.})$</th>
<th>$b , (\pm \text{s.e.})$</th>
<th>Variance</th>
<th>$P$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sarcomere Length</td>
<td>6.25 (± 5.15)</td>
<td>25.97 (± 10.77)</td>
<td>86.61</td>
<td>0.187</td>
</tr>
<tr>
<td>Cooking Loss</td>
<td>6.81 (± 6.89)</td>
<td>27.04 (± 9.59)</td>
<td>86.45</td>
<td>0.171</td>
</tr>
<tr>
<td>pH</td>
<td>8.97 (± 6.74)</td>
<td>2.59 (± 31.00)</td>
<td>86.93</td>
<td>0.228</td>
</tr>
<tr>
<td>pH$_{24}$</td>
<td>0.55 (±0.40)</td>
<td>1.54 (± 39.11)</td>
<td>87.48</td>
<td>0.325</td>
</tr>
<tr>
<td>pH$_{Hu}$</td>
<td>13.03 (± 12.68)</td>
<td>-42.02 (± 57.76)</td>
<td>86.33</td>
<td>0.158</td>
</tr>
<tr>
<td>Purge</td>
<td>0.09 (± 0.02)</td>
<td>37.77 (± 2.68)</td>
<td>87.45</td>
<td>0.320</td>
</tr>
<tr>
<td>PS 24 h</td>
<td>0.33 (± 0.47)</td>
<td>29.98 (± 5.38)</td>
<td>85.23</td>
<td>0.053</td>
</tr>
<tr>
<td>PS 5 Day</td>
<td>0.91 (± 0.92)</td>
<td>22.87 (± 4.09)</td>
<td>74.29</td>
<td>0.000***</td>
</tr>
<tr>
<td>Collagen</td>
<td>0.04 (± 0.02)</td>
<td>35.93 (± 6.26)</td>
<td>88.03</td>
<td>0.488</td>
</tr>
<tr>
<td>Collagen Soluble</td>
<td>14.40 (± 10.11)</td>
<td>33.90 (± 6.25)</td>
<td>87.39</td>
<td>0.307</td>
</tr>
</tbody>
</table>

*where the model equation is indicator = $a + bx + \text{error}$

Figure 6.2. The correlation between particle size ($\mu$m) and shear force values (N) of ovine m. semimembranosus measured at 5 days post mortem determined by univariate regression analysis ($R^2 = 0.21$) with a 95% confidence interval (dotted line).

Results of the chemometric analysis to predict the traditional indicators of shear force using Raman spectra measured 25 min PM (Table 6.5) demonstrates that there is potential to use Raman spectra measured pre-rigor to predict pH$_{24}$ and purge.
Table 6.5. Chemometric analysis using Raman spectra measured 25 min post mortem to predict of meat quality indicators of ovine *m. semimembranosus*.

<table>
<thead>
<tr>
<th>Indicator</th>
<th>Null Model RMSEP&lt;sub&gt;cv&lt;/sub&gt;</th>
<th>Optimal RMSEP&lt;sub&gt;cv&lt;/sub&gt; (Latent Variables)</th>
<th>Relative Reduction RMSEP&lt;sub&gt;cv&lt;/sub&gt; (%)</th>
<th>( R^2_{cv} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH&lt;sub&gt;pH&lt;/sub&gt;</td>
<td>0.205</td>
<td>0.205 (0 LV)</td>
<td>0</td>
<td>0.00</td>
</tr>
<tr>
<td>pH&lt;sub&gt;24&lt;/sub&gt;</td>
<td>0.15</td>
<td>0.14 (6 LV)</td>
<td>6.6</td>
<td>0.26</td>
</tr>
<tr>
<td>pHu</td>
<td>0.10</td>
<td>0.10 (0 LV)</td>
<td>0</td>
<td>0.00</td>
</tr>
<tr>
<td>Sarcomere Length</td>
<td>0.16</td>
<td>0.16 (0 LV)</td>
<td>0</td>
<td>0.00</td>
</tr>
<tr>
<td>24 h PS</td>
<td>52.69</td>
<td>52.69 (0 LV)</td>
<td>0</td>
<td>0.00</td>
</tr>
<tr>
<td>5 Day PS</td>
<td>46.60</td>
<td>46.60 (0 LV)</td>
<td>0</td>
<td>0.00</td>
</tr>
<tr>
<td>Purge</td>
<td>1.15</td>
<td>0.96 (6 LV)</td>
<td>16.5</td>
<td>0.32</td>
</tr>
<tr>
<td>Total Collagen</td>
<td>2.25</td>
<td>2.25 (0 LV)</td>
<td>0</td>
<td>0.00</td>
</tr>
<tr>
<td>Soluble Collagen</td>
<td>0.08</td>
<td>0.08 (0 LV)</td>
<td>0</td>
<td>0.00</td>
</tr>
<tr>
<td>Cooking Loss</td>
<td>2.64</td>
<td>2.64 (0 LV)</td>
<td>0</td>
<td>0.00</td>
</tr>
<tr>
<td>24 h L*</td>
<td>2.25</td>
<td>1.98 (7 LV)</td>
<td>12.0</td>
<td>0.27</td>
</tr>
<tr>
<td>24 h a*</td>
<td>1.40</td>
<td>1.40 (0 LV)</td>
<td>0</td>
<td>0.00</td>
</tr>
<tr>
<td>24 h b*</td>
<td>1.32</td>
<td>1.26 (2 LV)</td>
<td>4.5</td>
<td>0.08</td>
</tr>
<tr>
<td>5 day L*</td>
<td>2.13</td>
<td>2.13 (0 LV)</td>
<td>0</td>
<td>0.00</td>
</tr>
<tr>
<td>5 day a*</td>
<td>1.27</td>
<td>1.27 (0 LV)</td>
<td>0</td>
<td>0.00</td>
</tr>
<tr>
<td>5 day b*</td>
<td>1.13</td>
<td>1.13 (0 LV)</td>
<td>0</td>
<td>0.00</td>
</tr>
</tbody>
</table>

The prediction of purge yielded a 16.5% reduction in the error of the prediction (RMSPE<sub>cv</sub>) and a squared correlation between cross validated predicted values using Raman spectra measured 25 min post slaughter and observed values (\( R^2_{cv} \)) of 0.32 (Fig 6.3) while prediction of pH<sub>24</sub> gave a 6.6% reduction in RMSPE<sub>cv</sub> and an \( R^2_{cv} \) value of 0.26.

![Figure 6.3. The cross validated correlation between purge (%) values predicted using Raman spectra collected 25 min post slaughter and observed values measured from ovine *m. semimembranosus* after 4 days ageing.](image-url)
As evident in Table 6.6, the predictions of pH$_{24}$ and pHu using Raman spectra measured 24 h PM were improved in comparison to Raman spectra measured pre-rigor. The prediction of pH$_{24}$ using Raman spectra measured 24 h PM gave a 20% reduction in RMSPE$_{cv}$ while the prediction for pHu gave a 30% reduction. The squared correlations between cross validated predicted values using Raman spectra and observed values were 0.48 and 0.59 (Fig. 6.4), respectively. There was also a greater ability to predict purge using Raman spectra measured 24 h PM (21.7% reduction in RMSPE$_{cv}$, $R^2_{cv} = 0.42$).

<table>
<thead>
<tr>
<th>Trait</th>
<th>Null Model RMSEP$_{cv}$</th>
<th>Optimal RMSEP$_{cv}$ (Latent Variables)</th>
<th>Relative Reduction RMSEP$_{cv}$ (%)</th>
<th>$R^2_{cv}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH$_{24}$</td>
<td>0.15</td>
<td>0.12 (8 LV)</td>
<td>20.0</td>
<td>0.48</td>
</tr>
<tr>
<td>pHu</td>
<td>0.10</td>
<td>0.07 (7 LV)</td>
<td>30.0</td>
<td>0.59</td>
</tr>
<tr>
<td>Sarcomere Length</td>
<td>0.16</td>
<td>0.16 (0 LV)</td>
<td>0</td>
<td>0.00</td>
</tr>
<tr>
<td>24 h PS</td>
<td>52.77</td>
<td>52.77 (0 LV)</td>
<td>0</td>
<td>0.00</td>
</tr>
<tr>
<td>5 Day PS</td>
<td>46.58</td>
<td>46.58 (0 LV)</td>
<td>0</td>
<td>0.00</td>
</tr>
<tr>
<td>Purge</td>
<td>1.15</td>
<td>0.90 (7 LV)</td>
<td>21.7</td>
<td>0.42</td>
</tr>
<tr>
<td>Cooking Loss</td>
<td>2.64</td>
<td>2.64 (0 LV)</td>
<td>0</td>
<td>0.00</td>
</tr>
<tr>
<td>Total Collagen</td>
<td>2.25</td>
<td>2.25 (0 LV)</td>
<td>0</td>
<td>0.00</td>
</tr>
<tr>
<td>Soluble Collagen</td>
<td>0.08</td>
<td>0.08 (0 LV)</td>
<td>0</td>
<td>0.00</td>
</tr>
<tr>
<td>24 h $L^*$</td>
<td>2.45</td>
<td>1.96 (8 LV)</td>
<td>20.0</td>
<td>0.32</td>
</tr>
<tr>
<td>24 h $a^*$</td>
<td>1.41</td>
<td>1.41 (0 LV)</td>
<td>0</td>
<td>0.00</td>
</tr>
<tr>
<td>24 h $b^*$</td>
<td>1.32</td>
<td>1.32 (0 LV)</td>
<td>0</td>
<td>0.00</td>
</tr>
<tr>
<td>5 day $L^*$</td>
<td>2.13</td>
<td>1.87 (1 LV)</td>
<td>12.2</td>
<td>0.22</td>
</tr>
<tr>
<td>5 day $a^*$</td>
<td>1.27</td>
<td>1.27 (0 LV)</td>
<td>0</td>
<td>0.00</td>
</tr>
<tr>
<td>5 day $b^*$</td>
<td>1.12</td>
<td>1.12 (0 LV)</td>
<td>0</td>
<td>0.00</td>
</tr>
</tbody>
</table>
Using Raman spectra measured 5 days post mortem (Table 6.7) there was potential to predict only purge (18.3% reduction in RMSPE$_{cv}$, $R^2_{cv} = 0.33$; Fig 6.5).

Table 6.7. Chemometric analysis using Raman spectra measured 5 days PM to predict indicators of shear force values of ovine \textit{m. semimembranosus}.

<table>
<thead>
<tr>
<th>Trait</th>
<th>Null Model RMSEP$_{cv}$</th>
<th>Optimal RMSEP$_{cv}$ (Latent Variables)</th>
<th>Relative Reduction RMSEP$_{cv}$</th>
<th>$R^2_{cv}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>pHu</td>
<td>0.10</td>
<td>0.10 (0 LV)</td>
<td>0</td>
<td>0.00</td>
</tr>
<tr>
<td>Sarcomere Length</td>
<td>0.12</td>
<td>0.12 (0 LV)</td>
<td>0</td>
<td>0.00</td>
</tr>
<tr>
<td>5 Day PS</td>
<td>46.57</td>
<td>46.57 (0 LV)</td>
<td>0</td>
<td>0.00</td>
</tr>
<tr>
<td>Purge</td>
<td>1.15</td>
<td>0.94 (6 LV)</td>
<td>18.3</td>
<td>0.33</td>
</tr>
<tr>
<td>Cooking Loss</td>
<td>2.64</td>
<td>2.64 (0 LV)</td>
<td>0</td>
<td>0.00</td>
</tr>
<tr>
<td>Total Collagen</td>
<td>2.26</td>
<td>2.26 (0 LV)</td>
<td>0</td>
<td>0.00</td>
</tr>
<tr>
<td>Soluble Collagen</td>
<td>0.08</td>
<td>0.08 (0 LV)</td>
<td>0</td>
<td>0.00</td>
</tr>
<tr>
<td>5 day $L^*$</td>
<td>2.13</td>
<td>2.13 (0 LV)</td>
<td>0</td>
<td>0.00</td>
</tr>
<tr>
<td>5 day $a^*$</td>
<td>1.28</td>
<td>1.28 (0 LV)</td>
<td>0</td>
<td>0.00</td>
</tr>
<tr>
<td>5 day $b^*$</td>
<td>1.13</td>
<td>1.13 (0 LV)</td>
<td>0</td>
<td>0.00</td>
</tr>
</tbody>
</table>
Figure 6.5 The cross validated correlation between predicted purge values (%) using Raman spectra collected 5 days post mortem and observed values of ovine m. semimembranosus measured after 4 days ageing.

It is also interesting to note that there is also some ability to predict some colour parameters measured 24 h and 5 days post mortem. Data suggests there is potential to predict $L^*$ measured 24 h PM using Raman spectra measured pre rigor (12.0% reduction in RMSPE$_{cv}$, $R^2_{cv} = 0.27$; Table 6.5) and Raman spectra measured 24 h PM (20% reduction in RMSPE$_{cv}$, $R^2_{cv} = 0.32$; Table 6.6) as well as $L^*$ measured at 5 days PM using Raman spectra measured 24 h PM (12.2% reduction in RMSPE$_{cv}$, $R^2_{cv} = 0.22$).

Using HCW, GR Fat, Temp$_{pr}$, pH$_{pr}$ and pH$_{24}$ in a step-wise regression to determine the impact of meat quality indicators on pHu values demonstrated that of the indicators measured, pH$_{24}$ is the only significant trait in determining pHu ($R^2 = 0.27$, $P < 0.001$; Fig 6.6). Inclusion of Raman spectra measured pre-rigor with these indicators using either LAR or PLS gave no prediction for pHu ($R^2 = 0.00$, $P > 0.05$). However, it is interesting to note that pH$_{24}$ was not useful in this model suggesting that it is not a better value to predict pHu when the prediction model is cross validated.
Figure 6.6. The correlation between observed pH$_{24}$ and pHu values determined using a step-wise regression model ($R^2 = 0.27$, $P < 0.001$) with a 95% confidence interval (dotted line).

As illustrated by Figure 6.7, declining pHu values are related to significant increases with purge ($P = 0.004$). The result of the regression model indicated that for each unit increase in pHu value, purge decreases by $-0.029\%$ ($\pm 0.01$).

Figure 6.7. The relationship between observed pHu values and purge (%) of ovine $m.$ semimembranosus measured at 5 day post mortem ($R^2 = 0.10$) with a 95% confidence interval (dotted line).
6.3.2 Raman Spectra

As illustrated by Figure 6.8, there is little difference between signals present within the average unprocessed spectra measured 25 min, 24 h and 5 days PM. However, it also shows that spectra measured at 24 hours have the greatest intensities and spectra measured at 25 min have a larger contribution from fluorescence, as evident in the lower overall intensities which peak at 700 cm\(^{-1}\) causing lower overall intensities at larger wavenumbers between 1300 – 1650 cm\(^{-1}\).

![Figure 6.8. The average of all Raman spectra of ovine m. semimembranosus measured 25 min (black), 24 hours (dotted line) and at 5 days (grey) post mortem.](image)

A comparison of the average first integration and the total accumulation Raman spectra measured, using spectra collected at 25 min post slaughter as an example, indicates that the average of the first integration has substantially more background noise and fluorescence in comparison to the average of the total accumulation. However, as shown in Figure 6.9, spectra measured at 25 min post mortem have little Raman signals present.
Figure 6.9 The average first integration (grey) and the average of the total accumulation (black) for all Raman Spectra measured at 25 min post slaughter.

Background correction of Raman spectra collected at 24 h PM from SMs with the highest and lowest pHu values, suggests that differences are present in Raman signals between 563 – 720 cm\(^{-1}\) with clearer differences at 585 cm\(^{-1}\), as well as at 937 cm\(^{-1}\), 1042 cm\(^{-1}\), 1076 cm\(^{-1}\), 1124 cm\(^{-1}\), 1206 cm\(^{-1}\). Furthermore, smaller differences are evident at 1175 cm\(^{-1}\), 1134 cm\(^{-1}\), 1562 cm\(^{-1}\) and 1611 cm\(^{-1}\) (Fig 6.10).

Figure 6.10. The averaged background corrected Raman spectra collected 24 hours post mortem from the ovine m. semimembranosus with the 5 highest (5.90 – 6.16; black) and lowest (5.59 – 5.61; grey) pHu values.
Comparing the averaged background corrected spectra collected at 24 h PM from the samples with the lowest pH$_{24}$ and the lowest pHu values demonstrate that there are no major changes between 24 h and 5 days post mortem. As illustrated in Figure 6.11, there are minor changes at 582, 875, 1205, 1222, 1255, 1261, 1278, 1308, 1546 and 1561 cm$^{-1}$ (Fig 6.11).

**Figure 6.11** The average background corrected spectra collected at 24 hours post mortem from the ovine *m. semimembranosus* with the 5 lowest pH$_{24}$ values (5.44 – 5.48; grey) and the pHu values (5.59 – 5.61; black).
As illustrated in Figure 6.12, changes to the averaged and background corrected Raman spectra collected at 24 hours post mortem from the 5 SMs with the highest and lowest purge occur at numerous wavenumbers including 563 - 617 cm\(^{-1}\) with a distinct change at 580 cm\(^{-1}\), as well as 716 cm\(^{-1}\), 750 cm\(^{-1}\), 822 cm\(^{-1}\), 852 cm\(^{-1}\), 920 cm\(^{-1}\), 1044 cm\(^{-1}\), 1076 cm\(^{-1}\), 1308 cm\(^{-1}\), 1332 cm\(^{-1}\), 1448 cm\(^{-1}\), 1611 cm\(^{-1}\) and 1650 cm\(^{-1}\).

6.4 Discussion

6.4.1 Traditional Indicators of Meat Quality

Although the values of traditional indicators of tenderness measured (Table 6.2) were similar to those previously reported for lamb (Hopkins & Fogarty, 1998, Hopkins et al., 2013a, Fowler et al., 2014b), particle size measured at 5 days PM was the only indicator that was significant in determining shear force values (\(P < 0.05\); Fig 6.2). This is a reflection of the significance of particle size and increased proteolysis resulting in increased tenderness (Hopkins & Thompson, 2002a) as with each Newton decrease in shear force, particle size also decreased by 22 µm.

6.4.2 Prediction of Tenderness

Overall this study was unable to replicate the prediction of shear force values for fresh intact ovine m. semimembranosus using Raman Spectroscopy previously found (Table 4.1).
results gave no ability to predict shear force values, despite reducing noise in the spectra by increasing the total accumulation time of Raman spectroscopy measurements from 3 s to 15 s and by measuring at different times post mortem, including pre-rigor as proposed (section 4.5).

While the present study and the previous study conducted on fresh intact ovine (Chapter 4) have similar methodologies and sample handling, it must be acknowledged that the carcases sampled in the previous study were not subjected to electrical stimulation while carcases sampled in the present study were. Although reported improvements in tenderness have been inconsistent it has been found that electrical stimulation alters the rate of pH decline as carcases treated with electrical stimulation have been found to reach pHu earlier than non-stimulated carcases (Morton et al., 1999, Bond et al., 2004). A review by Hwang et al. (2003) summarises the effect, indicating that electrical stimulation results in a temperature dependant acceleration of glycolysis following an immediate fall in pH value. Furthermore, it has been suggested that as rigor occurs faster in electrical stimulated carcases, ageing commences at higher temperatures and therefore is more rapid.

While this does not alter the extent of glycolysis and pHu, it may alter the biochemical and biophysical properties during the development of rigor as physical disruption of the myofibril has been implicated as well as changes to the levels of ions, such as free calcium, and activity of proteolytic enzymes (Hwang et al., 2003). Consequently, the Raman spectra of the muscle would be altered when electrical stimulation is used as it reflects these biochemical and biophysical characteristics of the muscle. Yet it is difficult to determine what effects it would have, as the understanding of the mechanisms behind associated benefits of electrical stimulation on post mortem muscle biochemistry is limited due to differences in experimental design within literature, as species, muscle, voltage and time applied vary greatly between published studies. Thus, the poorer results for the prediction of shear force found by this study in comparison to Fowler et al. (2014b) may be linked to the changes in biochemistry with the use of electrical stimulation.

As the increase in total integration time by increasing the number of accumulations demonstrated a reduction in the noise and fluorescence in spectra giving more Raman spectral data (Fig 6.9), it is plausible that the lower accuracies of prediction in this study compared to those reported by Schmidt et al. (2013) may be a consequence of higher noise and less Raman signal. While in the present study 10 Raman spectra were measured
perpendicular to the muscle fibre over the largest face of the SM where the m. adductor was removed, Schmidt et al. (2013) measured 3 cm blocks of loin that had been quartered before 5 different positions were measured on the freshly cut surfaces with integration times of 5 and 4 s for the two sample groups.

Thus, Schmidt et al. (2013) collected 15 spectra per sample over a much smaller portion of muscle. Although the total integration time has been increased from 3 s to 15 s by increasing the number of 3 s accumulations from 1 to 3, longer integration times on smaller portions of the muscle may be required to achieve the correlations coefficients of 0.79 and 0.86, previously reported for the prediction of shear force values from two sample groups.

### 6.4.2 Prediction of pH values

Despite this study indicating there is no ability to predict shear force values, for the first time results suggest there is potential to predict pHu, pH\textsubscript{24}, lightness (L\textsuperscript{*}), and purge of intact lamb SM using Raman Spectroscopy ($R^2_{cv} = 0.22$ – 0.59, reduction in RMSEP\textsubscript{cv} = 12.2 – 30.0\%). Comparing these predictions using Raman spectra measured at different times post mortem showed that the best models for prediction of these indicators used Raman spectra measured at 24 hours post mortem (Table 6.6). This agrees with the hypothesis which suggested that poorer predictions using Raman spectra measured at 5 days post mortem were the result of myofibrillar degradation and changes to the biophysical and biochemical properties of the meat during ageing which causes diffusion and loss of the Raman signals (section 4.4.1). Given that spectra measured pre-rigor change rapidly due to the continuing cell metabolism, hence metabolites may only be temporarily detectable (Scheier et al., 2014), it is possible that a single pre-rigor measurement at 25 minutes post mortem is unable to provide enough information on the metabolic status of the muscle and anaerobic glycolysis to be informative on the progression of rigor and subsequent meat quality indicators. Consequently, further investigation into the changes of spectra in lamb SM throughout the progression of rigor may provide a more optimal time post mortem to measure pre-rigor spectra.

Of the prediction models using spectra collected 24 h post mortem, the prediction of pHu and pH\textsubscript{24} were the best models yielding cross-validated correlations between observed and predicted values ($R^2_{cv}$) of 0.59 and 0.48, respectively and reductions in prediction error of up to 30\% when using Raman spectra for the prediction. The reduction in prediction of pH
values at 24 h and 5 days using Raman spectra measured 24 h post mortem is unsurprising given that SMs with lower pH$_{24}$ values tended to have lower pHu values ($R^2 = 0.27$).

While this is the first study to report the prediction of pH values in fresh intact lamb so there is no existing literature to compare results, a previous study has demonstrated the ability of Raman Spectroscopy to determine deviations in the pH decline of pork during the development of PSE like qualities (Scheier et al., 2014). This was possible as Raman signals which reflected inosine monophosphate (IMP), lactate, glucose 6-phosphate, PCr, phosphoric acid and ATP concentrations present in samples changed during the progression into rigor. Hence, collecting lactate and pH reference values of porcine SMs measured and synthesising reference spectra of metabolites, Scheier et al. (2014) were able to qualitatively define the biochemical changes that occur during the pH decline to the on-set of rigor. Although the study conducted by Scheier et al. (2014) indicates the Raman signals which would be expected to change with the on-set of rigor, a small number of carcases (9) were sampled at increasing intervals between 50 minutes to 8.5 hours post mortem, which limits the application of this study’s findings to the quantitative prediction of pHu in lamb using Raman spectra measured 24 h PM.

Elucidating the underlying spectra for the prediction of the highest and lowest pHu values of lamb SM (Fig. 6.10) illustrates the complexity of the spectral changes, which makes it difficult to determine the biochemical characteristics which are reflected in the spectra as this is the first study to utilise Raman spectroscopy to predict pHu of lamb. While some spectral assignments can be made based on the research of Scheier et al. (2014), it must be stressed that pHu is a measure of the extent of pH decline while the rate of pH decline is dependent on the metabolic rates (Choe & Kim, 2014) which was measured by Scheier et al. (2014) in terms of metabolites present during the on-set of rigor. As porcine muscle contains more type IIB muscle fibres than lamb (Kerth, 2013) and therefore has different biochemistry characteristics including oxidative and glycolytic capacity, contraction speed and glycogen content (Choe et al., 2008), it can be expected that there would be differences in spectra evident in the pH decline of pork and lamb pHu measured at 24 hr post mortem which may limit the transfer of findings between these two studies.

Of the major changes in peak intensities present in spectra from samples with low and high pHu values, those that can be potentially identified using the previous study on pH decline in pork (Scheier et al., 2014), include the increases in intensity at 875 cm$^{-1}$, 937 cm$^{-1}$, 1042 cm$^{-1}$.
1334 cm\(^{-1}\), 1567 cm\(^{-1}\) and the numerous small changes within the region between 563 – 720 cm\(^{-1}\).

Overall these shifts suggest that SMs with low pHu values had increased concentrations of inorganic phosphate (875 cm\(^{-1}\), 970 cm\(^{-1}\) and 1042 cm\(^{-1}\)), adenine (1334 cm\(^{-1}\)), \(\alpha\) – helical proteins (937 cm\(^{-1}\)) and more significant changes to amino acid side chains (563 – 720 cm\(^{-1}\)) (Scheier et al., 2014). This may indicate that lamb SMs with lower pHu values has a more accelerated pH decline post mortem as higher levels of inorganic phosphate (England et al., 2013) and lactate (te Pas et al., 2004), as well as greater changes to \(\alpha\)-helical proteins such as actin and myosin (Huff Lonergan et al., 2010) and larger amounts of protein denaturation (Huff Lonergan & Lonergan, 2005) have all been associated with lower pHu values.

Although these Raman signals suggest increases in inorganic phosphate and \(\alpha\)-helical proteins are linked to low pHu, it is unclear what exactly biochemical pathways cause the relationships between these intensities and the free H\(^{+}\) that is measured by the pH reference measurement. Inorganic phosphate is a key element within many post mortem processes, including the breakdown of phosphocreatine, the synthesis and breakdown of glucose-6-phosphate and sugar phosphates, anaerobic glycolysis, formation and detachment of actin/myosin cross bridges and re-activation of myosin during muscular contraction (te Pas et al., 2004). Furthermore, there are many \(\alpha\)-helical proteins present in meat, including tropomyosin, myosin and actin, and the intensity of Raman signals relating to these \(\alpha\)-helical proteins is dependent on concentration as well as sample orientation (Pézolet et al., 1988). As a result, changes to the orientation of the collagen matrix (Nakamura et al., 2010), formation of more actomyosin cross bridges, changes to the orientation of myosin as the attached myosin heads pivot and shorten the sarcomere (te Pas et al., 2004) or higher concentrations of glycogen and glucose still within the cells as the muscle enters rigor may account for differences in the signal pertaining to \(\alpha\)-helical proteins. Consequently, it is difficult to determine which of these biochemical processes are vital in determining the increase in peak intensities measured in SMs with lower pHu at 24 h PM.
It is interesting to note that when spectra from the SMs with the lowest pH\textsubscript{24} are compared to spectra from the SMs with the lowest pH\textsubscript{u} are compared only minor changes are evident in the spectra (Fig 6.11). Tentative band assignments suggest that during the development of low pH\textsubscript{u} after 24 h there is a decrease in inorganic phosphate yet there is an increase in concentration of the acidic form of phosphate evident in the decreased intensity at 582 cm\textsuperscript{-1} and increased intensity at 875 cm\textsuperscript{-1} (Movasaghi \textit{et al.}, 2007, Scheier \textit{et al.}, 2014). Lower pH\textsubscript{u} values also demonstrate increased $\alpha$-helices (1308 cm\textsuperscript{-1}) and amide III (1278 cm\textsuperscript{-1}) protein bands as well as the breathing mode of nucleic acids including adenine (1255 cm\textsuperscript{-1}) and decreases in signals which have been related to COO$^-$ (1561 cm\textsuperscript{-1}) and NADH (1546 cm\textsuperscript{-1}) (Movasaghi \textit{et al.}, 2007). This suggests that between 24 hours and the development of pH\textsubscript{u}, the muscle is still undergoing anaerobic glycolysis and continued breakdown of ATP causing increased adenosine concentrations, decreased free NADH and changes to the structure of myofibrillar proteins as muscle contraction continues and actomyosin cross bridges continue to be made and broken (te Pas \textit{et al.}, 2004).

If it is hypothesised that increase of intensities at 1334 and 1567 cm\textsuperscript{-1} in spectra from SMs with low pH\textsubscript{u} values (Fig 6.10) are associated with higher concentrations of lactate as found by Scheier \textit{et al.} (2014), the decrease in COO$^-$ signal between low pH\textsubscript{24} and low pH\textsubscript{u} values is surprising given that increases in lactate are correlated with continued anaerobic glycolysis (te Pas \textit{et al.}, 2004, Savell \textit{et al.}, 2005). To assign changes to spectra during the pre-rigor period, Scheier \textit{et al.} (2014) collected reference Raman spectra of lactate that demonstrated it was characterised by peaks at 1334 cm\textsuperscript{-1} and 1567 cm\textsuperscript{-1}, which represent the CH group and COO$^-$ vibrations respectively. However, in comparing these reference signals to the Raman spectra collected from muscle, Scheier \textit{et al.} (2014) may have over simplified the assignments as their study suggests excellent agreement between spectra simulated from pure samples of metabolites and spectra measured from muscle, yet there is no consideration of the potential overlap in spectral contribution from other sources of CH group or COO$^-$ vibrations, which are likely to be present in complex biological samples such as intact porcine muscle. A review of Raman spectroscopic studies conducted on biological tissues that suggests that at approximately 1560 – 1570 cm\textsuperscript{-1} Raman signals from tryptophan, tyrosine, amide II secondary protein structures and porphyrin may also contribute to the vibrations of COO$^-$ (Movasaghi \textit{et al.}, 2007) and it is plausible that metabolites that are closely related or have chemical conformations similar to lactate, such as pyruvate and acetate would also contribute to these spectral regions.
Therefore, the decrease in COO$^-$ found in spectra characterising low pHu values may be a result of decreasing pyruvate. However, increasing concentrations of free H$^+$ with lower pHu values may result in free H$^+$ binding to other substrates, such as inorganic phosphate and lactate. If free H$^+$ binds to lactate creating lactic acid, the intensity of the COO$^-$ would also be reduced, yet it is unclear whether lactate converts to lactic acid as literature on early post mortem events is unclear as authors use these terms interchangeably despite the differences in chemical conformation.

The potential for overlap in spectral assignments is not only limited to the COO$^-$ signals by a CH$_2$ and CH$_3$ vibrations occur at 1333 – 1335 cm$^{-1}$ from a variety of sources which could be present in the muscle including hydroxyproline, a major component of collagen, nucleic acids and tryptophan (Movasaghi et al., 2007). Given that it is difficult to obtain Raman spectra with low variation (Beattie et al., 2004b) and no reference spectra or measurements were collected in this study, it is hard to justify strict assignment of bands, particularly since there were no SMs in the present study that deviate from the normal range pHu expected for lamb. Thus, more research is required to determine the chemical bonds that are contributing to the prediction of pHu and whether overlapping spectral assignments cause an over or under estimation for the prediction of meat quality traits resulting in lower predictability when cross validation methods are used.

Uncertainty of band assignments in Raman signals of complex samples also brings into question the validity of band assignments which have been completed in previous Raman spectroscopy studies aimed at predicting tenderness and sensory traits. Considering a broader number of Raman studies conducted on biological tissues, it is plausible that the spectral changes found by Beattie et al. (2008), Beattie et al. (2004a) and Schmidt et al. (2013) may relate to a broader number of biochemical characteristics of the meat. For example, it has been proposed that the intensity peak at approximately 930 – 940 cm$^{-1}$ of $\alpha$-helical protein structures is positively correlated with increasing tenderness (Schmidt et al., 2013). While Pedersen et al. (2003) and Scheier et al. (2014) have associated increases at this peak with pH and glycogen levels, as well as protein conformations.
Therefore, it is hypothesised that the correlations between tenderness and Raman spectra may be an indirect measurement of the relationships between anaerobic glycolysis and pH decline (Savell et al., 2005), pH decline, water holding capacity and tenderness (Tornberg, 1996, Huff Loneragan & Loneragan, 2005, Huff Loneragan et al., 2010) and the relationships between myofibrillar structure and water holding capacity, tenderness and colour (Hughes et al., 2014). Yet, this may be confounded by collagen content as the major components of collagen, such as tropocollagen, are α-helical proteins which would also contribute to this band. Consequently, increases in the band associated with α-helical proteins could indicate a higher pHu through more glycogen present in muscle and therefore a greater water holding capacity or an increase in collagen which has been associated with decreases in tenderness (Purslow, 2005). In another Raman spectroscopic study, Beattie et al. (2004a) propose that the changes in spectral region at approximately 1445 cm⁻¹ are related to differences in the hydrophobicity of the proteins within the myofibril of beef silverside as there was a positive correlation with juiciness, sensory determination of tenderness and consumer acceptability and the intensity of this peak. However, other band assignments for this region indicate that the CH₂ and CH₃ groups of both collagen and lipids contribute to the Raman signals of this wavenumber (Movasaghi et al., 2007). Therefore, it is plausible that the increases in juiciness, sensory tenderness and consumer acceptability could have been associated with increases in lipid content. As it is difficult to determine the exact biochemical and biophysical characteristics that are contributing the chemical bond vibrations and confounding relationships exist between meat quality traits, it may be a more useful approach to use Raman spectroscopy to predict a combination of meat quality traits of interest. Alternatively, Raman spectroscopy may provide a better prediction of meat sensory descriptors including tenderness, juiciness, flavour and overall liking as used in the Meat Standards Australia (MSA) grading systems (Thompson, 2002, Polkinghorne & Thompson, 2010) because these sensory properties are associated with more than one biochemical characteristic (Perry et al., 2001).
6.4.3 Prediction of Purge

While the prediction of pH$_{24}$ and pH$_u$ are the best models using spectra collected at 24 h, it is interesting that models also demonstrated some ability to predict purge ($R^2_{cv} = 0.42$ and a 21.7% reduction in RMSEP$_{cv}$). Given that there is a significant relationship between purge and pH$_u$ values ($P = 0.04$; Fig. 6.10), it is plausible Raman spectroscopy is predicting purge indirectly through effects of pH on metabolic processes or via metabolic substrates which are involved in the biochemical changes during the conversion of muscle to meat that determine the meat’s biophysical characteristics including pH. However, prediction of purge using Raman spectra measured 25 min ($R^2_{cv} = 0.32$) and 24 h PM ($R^2_{cv} = 0.42$) was more accurate than prediction using pH$_u$ alone ($R^2_{cv} = 0.10$).

The review on the mechanisms of water holding capacity by Huff Lonergan & Lonergan (2005) further supports this hypothesis, stating the links between the early post mortem events particularly the rate and extent of pH decline, are critical in determining the ability of meat to retain moisture. Indeed, a study conducted by Pedersen et al. (2003) demonstrated that Raman microscopy was able to predict variation in drip loss of pork *m. longissimus dorsi* (0.83% prediction error, $r = 0.95$, 3 LV) during the first 45 min post mortem. Pedersen et al. (2003) proposed that this finding was consistent with the relationship between water holding capacity, pH, glycogen and protein conformation as changes to the NH stretching regions (3140 cm$^{-1}$) and α-helical band (940 cm$^{-1}$) were evident in spectra. However, as with previous Raman spectroscopic studies of pH decline because the focus is on pork the transference of these findings to the current study is limited.

Pork muscles are characterised by a greater concentration of type IIB muscle fibres in comparison to lamb and beef. These type IIB muscle fibres are white, fast twitch fibres which are easily fatigued (Kerth, 2013) due to the cell metabolism, oxidative capacity, contraction speed, fibre size, ATPase activity, myoglobin and glycogen content. Overall this increases glycolytic potential (Choe et al., 2008, Choe & Kim, 2014), which is responsible for creating a faster rate of pH decline (te Pas et al., 2004). Consequently, the pH decline of pork carcases is usually completed in 6 – 12 hours post mortem with the on-set of rigor occurring at 4 – 6 hours post mortem (Savell et al., 2005) depending on chiller regime and fat thickness (Huff Lonergan et al., 2010). Hence, pork carcases are more susceptible to meat quality issues, such as pale soft and exudative (PSE) meat, as a result of higher temperatures at rigor and increased rates of pH decline in the first 15 – 30 minutes post mortem (Savell et al., 2005).
Increased purge and drip losses are associated with pH decline related quality issues, as evident in the study conducted by Melody et al. (2004) that found PSE affected carcases had up to 10% of weight lost due to high purge. Since lamb carcases undergo a slower rate of pH decline (te Pas et al., 2004) and are smaller carcases which consequently chill faster these same quality issues are not present. Therefore in comparison to lamb, deviations in water holding capacity and drip losses of pork may be more related to accelerated pH decline or onset of rigor at earlier times post mortem and therefore at higher temperatures, which can be detected by the metabolic state determined by Raman spectroscopy (Pedersen et al., 2003, Scheier et al., 2014).

Yet it is also important to acknowledge the differences between pH decline and pHu as while accelerated pH decline can cause higher purge, the ultimate pH of muscles with accelerated pH declines may not be below normal ranges (Hwang et al., 2004, Huff Lonergan & Lonergan, 2005). This is because pHu is a measure of the extent of pH decline and glycogen concentration of the muscle at slaughter (Choe & Kim, 2014) while pH decline is determined by cellular metabolism, oxidative capacity, contraction speed, fibre size, ATPase activity, myoglobin and glycogen content as well as an increased glycolytic potential (Choe et al., 2008, Choe & Kim, 2014). Therefore, the direct comparison of studies determining the early post mortem metabolic status of pork using Raman spectroscopy (Pedersen et al., 2003, Scheier et al., 2014) with the current study is further complicated as pH and temperatures in this study were measured at 25 min post-mortem and 24 hours post mortem as well as a final pHu measurement after 5 days and so it is not possible to determine the rate pH decline.

Like rapid pH decline, the development of low pHu has been related to high purge (Pearce et al., 2011). It is hypothesised that both accelerated pH decline and low pHu cause greater purge as there is a higher amount of protein denaturation, particularly if temperatures are higher at the development of pHu, as a result of cell structural changes which facilitate the movement of immobilised water through extracellular spaces (Huff Lonergan & Lonergan, 2005, Pearce et al., 2011). Once the pH of the muscle post mortem gets closer to isoelectric point of the major proteins, the repulsion of the structures within the myofibril is reduced causing them to pack closer together, reducing space (Huff Lonergan & Lonergan, 2005, Puolanne & Halonen, 2010).
In conjunction with these changes, formation of actin and myosin cross bridges and any shortening of the sarcomere as the muscle enters rigor results in longitudinal and lateral contractions, further reducing the space in the myofibril available for water (Tornberg, 1996). Consequently, immobilised water may be forced into extracellular compartments permitting it to be lost as purge (Pearce et al., 2011).

A tentative band assignment of spectra from SMs with the highest and lowest purge (Fig 6.12) suggests that differences between SMs with high and low purge are complex, however it does support the hypothesis that changes in purge are related metabolic processes in the conversion from muscle to meat and subsequent structural changes. Spectra obtained from SMs with high purge demonstrate an overall reduction in intensities at key wavenumbers including 716, 750, 822, 852, 870, 930, 1044, 1076, 1308, 1448, 1611 and 1650 cm\(^{-1}\). Furthermore, there are increases evident in the region between 500 – 700 cm\(^{-1}\) and in Raman signals at 716, 750, 1332, and 1567 cm\(^{-1}\).

Although these changes are complex and the impact of overlapping signals is unknown, the decreases in intensity at 580, 870, 890, 930, 1044, 1076, 1308 and 1332 cm\(^{-1}\) are similar to changes evident in spectra from high pHu values (Fig. 6.10) further agreeing with the correlation that indicates purge increase with increasing pHu (Fig. 6.7). Previous band assignments completed for changes in pHu identifies several of these changes suggesting that with increasing pHu and purge there is a reduction in inorganic phosphate (875 cm\(^{-1}\) and 1044 cm\(^{-1}\), \(\alpha\)-helical proteins (930 cm\(^{-1}\)), CH deformation signals (1448 cm\(^{-1}\)) and COO\(^{-}\) signals (Scheier et al., 2014) present in spectra collected at 24 hours post mortem. Raman spectra synthesised by Pedersen et al. (2003) for pure components present in meat provides further insight to spectral changes associated with variation in water holding capacity of meat suggesting that the peak evident at 852 cm\(^{-1}\) could be characteristic of \(\alpha\)-glucans, the pyrrolidine ring of hydroxyproline a main component of collagen or part of the lactate signal that was found to be split across three wavenumbers (850, 1330 and 1460 cm\(^{-1}\)). Furthermore, peaks at 940 cm\(^{-1}\), 1320 cm\(^{-1}\) and 1450 cm\(^{-1}\) were identified as \(\alpha\)-helical protein conformation, the CH bend and the symmetric methylene bend associated with myofibrillar proteins, while the peak at 820 cm\(^{-1}\) was determined to be a reflection of the phosphate group in ATP (Pedersen et al., 2003). Tentative band assignments on the remaining Raman spectral changes suggest that spectra from SMs with high purge may also be characterised by a
decrease in adenine (716 cm\(^{-1}\)), lactic acid (750 cm\(^{-1}\)), inorganic phosphate (580 cm\(^{-1}\) and 1076 cm\(^{-1}\)), phosphodiesters (822 cm\(^{-1}\)) and glycogen (852 cm\(^{-1}\)) (Movasaghi et al., 2007).

Overall these spectral changes agree with the conclusion of Pedersen et al. (2003) that the changes in Raman spectra are consistent with the relationship between water holding capacity and pH, glycogen concentration and protein conformations already established in existing literature. However, it is difficult to compare spectral assignments directly with Pedersen et al. (2003) as their interpretation is not detailed as there was few samples measured with Raman microscopy. Using the findings for high pH\(_u\) values (section 6.4.2) suggests that those samples with higher purge may have reached pH\(_u\) values earlier and therefore may enter rigor at slightly higher temperatures causing greater amounts of protein degradation (Huff Lonergan & Lonergan, 2005) as evident in the increased signals associated with amino acid side chains between 500 – 700 cm\(^{-1}\) (Scheier et al., 2014) and a lower overall intensity of signal due to increased diffuse scattering of excitation photons within the myofibril (Matousek & Stone, 2009). Although it could be considered that reaching pH\(_u\) earlier should demonstrate an increase for the Raman signals of inorganic phosphate, adenine and lactate as well as a decrease signals for glycogen (Scheier et al., 2014), the decreased intensities found in this study may be indicative of lower glycogen and ATP concentrations in the muscle at slaughter. Consequently, the rate of pH decline may not be different yet the extent of anaerobic glycolysis post mortem would be reduced resulting in higher pH\(_u\) (Choe et al., 2008) and potentially higher temperatures at rigor (Savell et al., 2005). However, as with the prediction pH\(_u\) values previously discussed, due to the lack of extreme pH values in this study and the ambiguity of spectral assignments as a result of the potentially overlapping bands, the exact biochemical pathways behind the variation in purge are unknown. Therefore, future research in Raman spectroscopy needs to address this.

### 6.4.4 Prediction of \(L^*\) Value

Data in this study also demonstrated that there is the potential to use Raman spectroscopy to predict \(L^*\) at 24 h and 5 days post mortem as models yielded cross validated correlations between predicted and observed values (\(R^2_{cv}\)) of 0.32 and 0.22, respectively (Table 6.6). This prediction using Raman spectra also gave reductions in the error of the prediction (\(RMSEP_{cv}\)) by 20% at 24 hours post mortem and by 12% at 5 days post mortem (Table 6.6).
The structural approach to understanding interactions between colour, water holding capacity and tenderness taken by Hughes et al. (2014) highlights the links between these structural changes related to the metabolic processes through the development of rigor, water holding capacity and colour. Therefore, the prediction of $L^*$ using Raman spectroscopy may be based on the same biochemical and biophysical characteristics established during early post mortem metabolism which determine purge and pH values.

Factors which alter the structure of the muscle, including shrinkage of the myofibril, interfilament spacing, development of extracellular spaces and protein denaturation affect water holding capacity as well as the reflectance of light (Hughes et al., 2014) and consequently lightness, which is measured by the $L^*$ value (Priolo et al., 2001). This occurs as the scattering of light within the microstructure effects how much light is transmitted into the depth of the muscle and how much is reflected. However, the reflectance of light is also a function of the abilities of different components within the meat to scatter entering light and their concentrations within the meat. Hence, lightness is also defined by other factors including myofibrillar size and density, striations of the muscle as well as amount and distribution of water, intramuscular fat and connective tissue (Hughes et al., 2014). The inter-relationships between these other factors may convolute the correlations between purge, pH values and $L^*$ in this study accounting for the differences in prediction correlations.

Given that the hand held device also uses the scattering of light photons to determine the biochemical and physical properties of meat (Schmidt et al., 2009) it could be hypothesised that $L^*$ values should have a higher correlation with Raman spectroscopic measurements than those found in this study. However, prediction accuracy of $L^*$ values using Raman spectroscopy could be reduced for samples with higher pH, darker meat, tighter myofibrillar structure or a denser myofibrillar mass as a result of lowered scattering properties. This is a result of more light traversing low scattering meat being lost by absorption due to the longer path light takes to pass through the meat interacting with more particles before being reflected at the surface (Hughes et al., 2014). Whilst a reduction in overall intensity would facilitate the prediction of darker meats due to the biophysical properties and increased absorption causing reduction in Raman signals present in spectra (McCreery, 2005), there would also be less information on the biochemical characteristics of the meat.
It is hypothesised that these factors which explain some variation in $L^*$ values may also explain some of the variation in prediction outcomes in Raman spectroscopic studies between species. If samples with lighter $L^*$ values have better scattering properties (Hughes et al., 2014) prediction of meat quality of pork using Raman spectra could be more accurate than darker meats such as beef and lamb, as more biochemical information will be contained in spectra. However, as beef has a larger myofibrillar size in comparison to lamb, more spectroscopic information will also be contained in spectra collected from beef as it will have greater light scattering properties. Furthermore, the reduction in absorption losses from lighter or less structurally dense samples could result in more information in spectra from deeper areas of the tissue as McCreery (2005) and Matousek & Stone (2009) suggest that optically dense samples have a shorter sampling depths.

Given that Pedersen et al. (2003) found that increasing baseline and fluorescence in reference spectra was caused by the trace components of glycogens and the high absorbance properties of myoglobin prevented them from obtaining a reference spectra, it is plausible that the muscle and muscle face used for Raman spectroscopic measurement also has an impact on the scattering properties. This may be a consequence of muscle fibre type and muscle type as glycogen content, capillary density, oxidative capacity and z-line width vary with muscle fibre type (te Pas et al., 2004) and the intra and inter muscular variation of muscle fibre type (Klont et al., 1998). Therefore, some muscles or muscles from some species may have a better ability to scatter the Raman signal providing a better prediction of meat quality traits. However, further research is needed to determine the impact of species and muscle differences on light scattering properties and subsequent Raman spectra.
6.5 Conclusion

Overall this research suggests that there is no potential to predict shear force values of fresh intact meat. Given this finding and the inconsistent results compared with previous research, it is evident that prediction of shear force values of fresh intact meat using Raman spectroscopy as tested in this work does not have the accuracy, precision or repeatability required by industry.

This study demonstrated the potential to use Raman spectra collected at 1 day post mortem to predict pHu, pH\textsubscript{24}, purge and \textit{L*} measured at 1 and 5 days post mortem on fresh intact lamb. Although it is not practical to use a spectroscopic technology to predict pH values given that a pH meter is a cheaper more robust option, there may be a benefit to industry from predicting purge loss using Raman spectra as the accuracy was improved compared to using pHu alone.

Comparison of the spectra revealed that samples with high and low purge were discriminated using a variety of Raman signals including those which characterise phosphate (875 cm\textsuperscript{1} and 1042 cm\textsuperscript{1}), lactate (1334 cm\textsuperscript{1} and 1567 cm\textsuperscript{1}), \(\alpha\) – helical proteins (937 cm\textsuperscript{1}) and the amino acid side chain vibrations (520 – 720 cm\textsuperscript{1}). However, these findings are limited to this study and further work is required to determine the repeatability of the prediction.
7. Changes to Raman Spectra and the prediction of eating quality indicators with the freezing and thawing of intact lamb *m. semimembranosus*

7.1 Introduction

Despite previous research indicating that Raman spectroscopy can predict sensory qualities and shear force values of beef (Beattie *et al.*, 2004a, Bauer *et al.*, 2013) as well as meat quality traits of pork (Beattie *et al.*, 2008, Scheier & Schmidt, 2013), studies conducted using lamb have demonstrated mixed results. While the study by Schmidt *et al.* (2013) suggested that there is good potential to predict shear force values of lamb *m. longissimus thoracis et lumborum* ($R^2 = 0.76$ and 0.86 from two sites), further research on the LL suggested that there is no potential ($R^2_{cv} = 0.06$; Table 3.2) to predict shear force values.

Although more recent research on lamb tenderness using Raman spectroscopy (Chapters 4 and 6) found predictions for shear force values using Raman spectra may be lower than others, it is difficult to determine the potential as experimental parameters and sample handling varies greatly between studies. While the studies conducted by Schmidt *et al.* (2013) use the same species, muscle and hand held Raman device, samples used by Schmidt *et al.* (2013) were frozen and thawed. As Raman spectroscopy is sensitive to the effects of freezing and thawing (Li-Chan *et al.*, 1994, Herrero, 2008b) the aim of this study was to determine the impact of freezing and thawing of intact lamb *m. semimembranosus* on the prediction of meat quality traits.
7.2 Materials and Methods

7.2.1 Samples

Over two consecutive days, 80 carcases were measured (45 on day 1 and 35 on day 2) from the same abattoir. Carcases were randomly selected from different consignments and were from unknown backgrounds, sex and age representing animals typically processed by the abattoir in order to obtain a spread in shear force values. Lambs were processed following standard commercial slaughter processes and were electrically stimulated pre-dressing with a mid- voltage unit (2000mA with variable voltage to maintain a constant current, for 25 s at 15 pulses/s, 500 microsecond pulse width, unipolar waveform) (Toohey et al., 2008).

At 24 h PM, hot carcase weight (HCW) and GR tissue depth (depth of the tissue over the 12th rib, 110 mm from the mid line) were recorded and the topsides (Product identification HAM 5077; Anonymous, 2005) were removed from the carcases. The cap muscle (m. gracilis) and m. adductor were removed to leave the m. semimembranosus (SM) that was the muscle of measurement.

7.2.2 Raman Spectroscopy

Raman spectroscopic measurements were conducted 24 hours, 5 and 8 days post mortem (PM).

Raman spectroscopy measurements conducted 24 hours post mortem on the m. semimembranosus after it had been removed from the carcase and the subcutaneous fat and silverskin had been removed. A 671nm hand held Raman spectroscopic device was used to conduct 10 spectral measurements on each SM on a freshly cut surface perpendicular to the muscle fibre (Fig. 5.1), at a room temperature of 16 °C. Spectra were recorded using 70mW of laser power and an integration time of 3 seconds and 5 accumulations (total measurement time 15 seconds). Once the 24 h PM Raman spectroscopic measurements were completed and sections to test the traditional indicators of tenderness had been excised, the remaining portion of SM was vacuum packed and held at 2.5 °C for 4 days.
At 5 days post mortem, SMs were removed from the vacuum packs, allowed to oxygenate or ‘bloom’ for 2 hours and a freshly cut surface was re-measured with the Raman hand held device, using the same parameters described for 1 day PM. After Raman spectroscopic scans at 5 days and the sampling for traditional indicators had been completed, the remaining SM was vacuum packed and frozen at -20 °C.

After being held frozen at -20 °C for 24 hours, SMs were thawed for 21 hours at 0.6 °C (8 days PM), samples were removed from the vacuum pack, allowed to bloom for 2 hours and a freshly cut surface was remeasured using the same spectral parameters as previously used for 24 h and 5 day PM Raman spectroscopic measurements.

7.2.3 Traditional Indicators

At 24 hours PM, after the SM was removed from the carcase, immediately prior to Raman spectroscopic measurements, pH (pH\textsubscript{24}), temperature (temp\textsubscript{24}) and colour were measured. pH\textsubscript{24} and temp\textsubscript{24} were measured using a using a TPS intermediate junction pH electrode with a BNC plug using buffers (pH 4.01 and pH 6.86) held at a room temperature of 16 °C. Colour readings were completed using a Minolta\textsuperscript{®} CR-400 Colourmeter (Milota Camera Co., Japan) under a D65 illuminant with an 8 mm aperture size, 10 degree observation angle and a closed cone that was calibrated using a white tile (Y = 92.8, X = 0.3160, Y = 0.3323) measured perpendicular to the muscle fibre.

Once Raman spectral measurements were completed, 1-2 g sections were excised for sarcomere length (SL) testing using the laser diffraction method (Bouton\textit{ et al.}, 1973c) and particle size (PS) analysis, as described by Karumendu\textit{ et al.} (2009).

After SMs were removed from the vacuum packs at 5 days PM, they were patted dry and weighed to determine purge. SMs were then allowed to bloom for 2 h before a fresh surface was cut and re-measured with the Colourmeter immediately prior to measurement with the Raman spectroscopic hand held device. After Raman spectroscopic scans were completed, further 1-2 g sections were excised for further PS and pHu determination using 2g of muscle homogenate in 10 ml of 5 mM iodoacetate/ 150 mM KCl (pH adjusted to 7.0), as described by (Dransfield\textit{ et al.}, 1992).
After being frozen and thawed (8 days PM), SMs were removed from vacuum packs, patted dry and weighed for freeze/thaw losses. Immediately prior to Raman spectroscopy measurements, SMs were allowed to bloom for 2 h and colour was measured again on a freshly cut surface. Once Raman spectra scans were completed, another 1 – 2 g section was removed for PS, a 25 g section for collagen determination and a ‘block’ to measure shear force (mean 65 g) were also excised.

Shear force was measured using the method previously described by Hopkins et al. (2011a). The average of 8 repetitions was used, except where the co-efficient of determination exceeded 24% then the median was reported. Shear force blocks were weighed prior to and after cooking to determine cooking losses.

7.2.4 Data Analysis

Raw spectra were prepared for chemometric analysis as previously described in section 3.2.4. Spectra were then analysed as previously described in Chapters 6 (section 6.2.4).

Background was removed from the spectra for band assignment by subtracting a 7th order polynomial through wavenumbers outlined in Table 7.1.

<table>
<thead>
<tr>
<th>Raman Spectra</th>
<th>Wavenumbers used for Baseline Correction (cm⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 h</td>
<td>528, 628, 767, 1140, 1374, 1529, 1744 and 1848</td>
</tr>
<tr>
<td>5 Day</td>
<td>538, 628, 767, 1139, 1368, 1524, 1744 and 1845</td>
</tr>
<tr>
<td>8 Day</td>
<td>542, 628, 765, 1139, 1366, 1524, 1744 and 1845</td>
</tr>
</tbody>
</table>

Prediction models using Raman spectroscopy for traditional indicators of meat quality were fitted using both Partial Least Squares methods, as outlined in section 6.2.4 and Least Angle Regression (LAR) (Efron et al., 2004). However, models to determine relationships existed between traditional indicators and shear force or pHu values were fitted using simple linear regression and simplified using stepwise regression.

7.3 Results

The summary statistics of the carcase traits (hot carcase weight (HCW) and GR Fat) as well as the traditional indicators of tenderness, including shear force values, PS, SL, purge, thaw and cooking losses, pH and colour, are outlined in Table 7.2.
Table 7.2 Mean, standard deviation (SD) and range for carcase and eating quality indicators measured.

<table>
<thead>
<tr>
<th>Trait</th>
<th>Mean</th>
<th>SD</th>
<th>Range (min, max)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HCW (kg)</td>
<td>24.1</td>
<td>3.2</td>
<td>18.5 – 34.9</td>
</tr>
<tr>
<td>GR Fat (mm)</td>
<td>13.9</td>
<td>3.2</td>
<td>8 – 22</td>
</tr>
<tr>
<td>Shear Force (N)</td>
<td>37.1</td>
<td>7.7</td>
<td>19.8 – 58.8</td>
</tr>
<tr>
<td>Cooking Loss (%)</td>
<td>23.9</td>
<td>2.6</td>
<td>16.6 – 31.7</td>
</tr>
<tr>
<td>Purge (%)</td>
<td>4.5</td>
<td>3.0</td>
<td>0.1 – 11.3</td>
</tr>
<tr>
<td>Freeze Losses (%)</td>
<td>2.7</td>
<td>1.5</td>
<td>0.1 – 5.8</td>
</tr>
<tr>
<td>pH</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24 h</td>
<td>5.7</td>
<td>0.2</td>
<td>5.5 – 6.12</td>
</tr>
<tr>
<td>pHu</td>
<td>5.8</td>
<td>0.2</td>
<td>5.57 – 6.35</td>
</tr>
<tr>
<td>Sarcomere Length (µm)</td>
<td>1.75</td>
<td>0.19</td>
<td>1.75 – 2.10</td>
</tr>
<tr>
<td>Particle Size (µm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 d</td>
<td>245</td>
<td>52.4</td>
<td>131 – 388</td>
</tr>
<tr>
<td>5 d</td>
<td>187</td>
<td>52.5</td>
<td>107 – 365</td>
</tr>
<tr>
<td>8 d</td>
<td>165</td>
<td>44.3</td>
<td>82 – 287</td>
</tr>
<tr>
<td>L*</td>
<td>37.7</td>
<td>1.80</td>
<td>34.4 – 43.1</td>
</tr>
<tr>
<td>Colour 1 day PM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a*</td>
<td>16.8</td>
<td>1.18</td>
<td>13.8 – 19.8</td>
</tr>
<tr>
<td>b*</td>
<td>-1.1</td>
<td>0.85</td>
<td>-3.5 – 1.0</td>
</tr>
<tr>
<td>L*</td>
<td>39.3</td>
<td>2.07</td>
<td>34.8 – 43.1</td>
</tr>
<tr>
<td>Colour 5 day PM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a*</td>
<td>16.1</td>
<td>1.26</td>
<td>13.1 – 19.1</td>
</tr>
<tr>
<td>b*</td>
<td>-1.5</td>
<td>0.81</td>
<td>-3.5 – 0.7</td>
</tr>
<tr>
<td>L*</td>
<td>36.0</td>
<td>1.96</td>
<td>32.0 – 40.6</td>
</tr>
<tr>
<td>Colour 8 day PM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a*</td>
<td>15.4</td>
<td>1.17</td>
<td>32.0 – 40.6</td>
</tr>
<tr>
<td>b*</td>
<td>-2.0</td>
<td>0.88</td>
<td>-3.6 – 1.3</td>
</tr>
</tbody>
</table>

Regression models to determine the relationships between shear force values and traditional indicators of meat quality demonstrated that the combination of the traits soluble collagen, cooking loss, freeze losses and sarcomere length gave a limited ability to predict shear force values. This model yielded an $R^2_{cv}$ of 0.11 and an optimal RMSPE of 7.2 N compared to the null RMSPE of 7.5, resulting in a 5% reduction in the error of the prediction using these traits for the prediction.

Of the individual traits to predict shear force values, freeze loss gave the best model yielding a 1% reduction in RMSPE (optimal RMSPE = 7.61 N, null RMSPE = 7.69), although this model has poor accuracy ($R^2_{cv} = 0.01$).

Models to predict pHu with traditional indicators of meat quality also using simple linear regression gave slightly better models when freeze loss and cooking loss were both used ($R^2_{cv} = 0.07$, 4.1% reduction in RMSPE) compared prediction of pHu values using freeze loss and cooking loss as individual traits ($R^2_{cv} = 0.05$, 3.4% reduction in RMSPE and $R^2_{cv} = 0.01$, 0.5% reduction in RMSPE, respectively). While the prediction of pHu using least angle regression (LAR) provided the most accurate model when HCW, GR fat and pH24 are used as covariates ($R^2_{cv} = 0.29$, 13.5% reduction in RMSPE). However, further analysis demonstrated a
significant \( (P = < 0.01) \) relationship only between pHu and pH\textsubscript{24} resulting in an \( R^2 \) of 0.35 (Fig 7.1). This \( R^2 \) is reduced to 0.16 if the three upper pHu values are removed.

Figure 7.1 The relationship between pHu and pH\textsubscript{24} values measured on lamb \textit{m. semimembranosus}.  
Using LAR including Raman spectroscopy to predict these traditional indicators of meat quality demonstrated that there is an ability to predict pHu values using Raman spectroscopy, HCW, GR fat and pH24 as model covariates. However, the prediction error is higher and the accuracy is lower when Raman spectra were included \((R^2_{cv} = 0.24, 12.7\% \text{ reduction in RMSPE})\). It is interesting to note that when Raman spectra were used in the LAR model, only the wavenumbers 590, 1362, 1760 and 1792 cm\(^{-1}\) were included as covariates in the model (Fig. 7.2).

The results of models fitted by PLS indicate that none of the traditional indicators of meat quality were able to be predicted using Raman spectra.
7.4 Discussion

Results found in this study indicate that there is no ability to predict shear force values using Raman spectroscopy measured from fresh or frozen and thawed intact muscle. This finding is inconsistent with the findings of Chapter 4, which suggested the prediction of shear force value measured from fresh intact SM using Raman spectroscopy was more precise than using a combination of cooking loss, pHu and PS (12.9% and -1.5% reductions in RMSEP respectively).

As there was no improvement to the prediction of shear force values when meat was frozen and thawed, this finding also disproves the previous hypothesis that the poorer accuracy of predictions found ($R^2_{cv} = 0.22$; Table 4.1) compared to those of Schmidt et al. (2013) ($R^2_{cv} = 0.76$ and 0.83 for two sampling groups) was the result of measuring fresh meat rather than meat which had been frozen and thawed (section 4.4.1). Given that in the previous study increasing the total accumulation time was found to increase the signal to noise ratio (Fig 6.9), it is plausible that the reduction in the prediction accuracies between the studies conducted on fresh and frozen meat are a consequence of differences in Raman spectroscopic parameters. Consequently, the integration times of 4 and 5 s for the 15 scans of each sample in the two groups measured by Schmidt et al. (2013) may have increased the signal to noise ratio compared to the present study which used an integration time of 3 s for 10 scans.

Furthermore, as Schmidt et al. (2013) quartered 3 cm² blocks of M. longissimus lumborum before Raman spectra were collected from 5 positions on three freshly cut surfaces, it is hypothesised that much more information over smaller blocks and on different faces provided greater information on the muscle facilitating a better prediction outcome but representing an impractical approach to measurement. As freezing and thawing disintegrates the myofibril, as water moves and forms ice crystals (Leygonie et al., 2012) the subsequent increase in space between myofibrils may allow for increased light scattering (Hughes et al., 2014) and therefore more Raman signal may be lost through diffusion after freezing and thawing. Hence, 10 spectra over a large surface area of muscle, such as the face of the SM where the M. adductor has been removed, may not provide the information required for an accurate prediction of meat quality indicators as found in this study. However, measurement of fresh intact meat may also be limited by the scattering properties of the muscle as denser myofibrils will return signals only from the surface tissues (Matousek & Stone, 2009).
Although there was no ability to predict shear force using Raman spectroscopy, this study demonstrated that the best prediction of shear force values used a combination of covariates including soluble collagen, cooking loss, freeze loss and sarcomere length ($R^2_{cv} = 0.11$, 5% reduction in RMSEP). This limited prediction is possible, as together this combination of traditional indicators reflects the myofibrillar density as a consequence of the behaviour and structural changes to the myofibril during rigor, freezing, thawing and cooking (Tornberg, 1996).

The development of rigor causes the myofibrils to shrink both laterally and longitudinally, partly due to a fall in pH and the creation of myosin and actin cross-bridges at the on-set of rigor (Tornberg, 1996, te Pas et al., 2004). As a consequence of this shrinkage, sarcomere lengths may shorten, decreasing the cross-sectional areas of the myofibril and increasing shear force values (Tornberg, 1996, te Pas et al., 2004) as well as increasing extracellular spaces around the myofibril facilitating the movement of fluids (Huff Lonergan et al., 2010). The movement of water through these extracellular spaces during freezing, thawing and cooking is responsible for fluid losses. If the rate of ice melting within these extracellular spaces exceeds the rate at which dehydrated fibres can reabsorb water during thawing, the excess is lost as freeze loss (Leygonie et al., 2012). During cooking, water held in intramyofibrillar spaces may contribute to cooking losses (Huff Lonergan & Lonergan, 2005) along with chemically bound water released when fats melt and proteins denature (Pearce et al., 2011, Leygonie et al., 2012) at cooking temperatures between 40 - 50°C. At approximately 60°C gelatinisation and denaturation of soluble collagen occurs, further shrinking the myofibrillar mass and increasing fluid losses caused by pressure exerted during thermal contraction (Weston et al., 2002).
However, the overall accuracy and precision of the prediction of shear force values using soluble collagen, cooking loss, freeze loss and sarcomere length is low as each indicator is the result of numerous interactions and therefore the prediction of shear force values are affected by many confounding variables. For example, the weight of the SM which effects the rate and time of freezing and thawing, affects the extent of exudate formation through the size and distribution of ice crystal formation on freezing as well as the reabsorption of water as ice crystals melt during thawing (Leygonie et al., 2012).

Models to predict pHu values demonstrated that there is no improvement to the prediction when using Raman spectra combined with HCW, GR fat and pH24 ($R^2_{cv} = 0.24$, 12.7% reduction in RMSPE) or HCW, GR fat and pH24 ($R^2_{cv} = 0.29$, 13.5% reduction in RMSPE) as pH24 alone yields the greatest correlation ($R^2 = 0.35$). However, inclusion of HCW and GR fat is likely to be indicative of the glycogen concentration within the muscles, as heavier carcases with greater GR fat depths are likely to have been on a higher plane of nutrition prior to slaughter and therefore higher glycogen concentrations within the muscle (Ferguson et al., 2008).

The relationship between pHu and pH24 values found in the present study also emphasises the impact of range on the prediction and correlation outcomes. The analysis indicates that when the three highest pHu values (6.35, 6.22 and 6.21; Figure 7.1) are included in the prediction there is a stronger positive correlation ($R^2 = 0.35$) than when they are excluded ($R^2 = 0.16$), reducing the range from 5.57 – 6.35 to 5.57 – 6.15. This is consistent with Davies & Fearn (2006) who suggest that the correlation co-efficient is not a robust interpretation of a relationship as it depends on the range.
It is interesting to note that while the use of Raman spectra measured at 24 h PM did not improve the prediction of pHu values, the least angle regression did identify four wavenumbers which it considered to be the most important for the prediction of pHu values. As illustrated in Figure 7.2, the LAR model identified only the intensities measured at 590, 1362, 1760 and 1792 cm\(^{-1}\) to include in the prediction. Although band assignments cannot be completed with any certainty, a tentative assignment suggests that some of these frequencies may relate to the chemical bond vibrations of inorganic phosphate (590 cm\(^{-1}\)), guanine as a free amino acid (1362 cm\(^{-1}\)) and the C=O stretch of cortisone (1760 cm\(^{-1}\)) (Movasaghi et al., 2007). However, future research needs to confirm these band assignments suggested by Movasaghi et al. (2007), identify the chemical bond at 1792 cm\(^{-1}\) and determine the biological pathways that determine their relevance to pHu values.

7.5 Conclusion

This study indicates that there was no ability to predict shear force values of meat which has been frozen and thawed using Raman spectroscopy disproving the hypothesis that freezing and thawing improved the predictions of shear force values. Therefore, it is clear the Raman spectroscopy as tested in this work is not able to predict of shear force values of intact lamb and is not worth pursuing further to predict shear force values of intact lamb.

Although there was no ability to predict shear force values, this study did demonstrate a limited ability to predict shear force values using traditional indicators including soluble collagen, cooking loss, thaw loss and sarcomere length demonstrating that the combination of traditional indicators yielded a better prediction of shear force values than that achieved by using Raman spectra.

This study also suggested that there is potential to predict pHu using the combination of carcass weight, GR Fat and pH\(_{24}\), yet there was no improvement to the prediction beyond using pH\(_{24}\) as an individual trait. Although there was no improvement to the prediction of pH\(_{24}\) using Raman spectroscopy, least angle regression did indicate that several wavenumbers including 590 cm\(^{-1}\), 1362 cm\(^{-1}\), 1760 cm\(^{-1}\) and 1792 cm\(^{-1}\), were informative for the prediction of pHu using Raman spectra.
8. Prediction of intramuscular fat content and major fatty acid groups of ovine *m. longissimus lumborum* using Raman Spectroscopy

8.1 Introduction

Due to the contribution of fat to meat sensory attributes (Thompson *et al.*, 2005b) and the nutritional value of the meat, much research has been conducted on measuring the intramuscular fat (IMF) and the fatty acid (FA) profile of lamb, particularly for those FAs which have health benefits, such as the omega-3 FAs including eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and docosapentaenoic acid (DPA) (Demirel *et al.*, 2006, Clayton, 2008, Wood *et al.*, 2008, Ponnampalam *et al.*, 2014a). However, traditionally the extraction and purification of FAs and IMF is costly, destructive, time intensive and requires large amount of chemicals as well as extensive sample preparation, as samples need to be freeze dried and homogenised prior to measurement (Folch *et al.*, 1957, Lepage & Roy, 1986, O'Fallon *et al.*, 2007). Consequently a technology which is rapid, non-destructive and capable of providing information on FAs and IMF from intact samples *in-situ* in online commercial situations is highly desirable for commercial lamb processors.

Raman Spectroscopy is one such potential technology as it is non-destructive, non-invasive, requires no sample preparation and is able to detect substances even if only small amounts are present (Das & Agrawal, 2011). These advantages of Raman Spectroscopy have not been overlooked and recent studies have shown the potential for Raman Spectroscopy to determine the species of origin of subcutaneous fat (Beattie *et al.*, 2007), measure and predict the fatty acid content and iodine value of pork adipose tissue (Olsen *et al.*, 2007, Olsen *et al.*, 2010, Lyndgaard *et al.*, 2011), determine whether pork has been affected by warmed over flavour (Brøndum *et al.*, 2000) and analyse fatty acid methyl esters (Beattie *et al.*, 2004b).
Schmidt et al. (2009) have further developed the technology by combining the lens optics, laser and Raman filter into one device using the principles of backscattering geometry and a fibre optics cable, creating a robust Raman hand held probe capable of online measurement. Although Schmidt et al. (2009) have overcome the limitations of using a bench top device and the probe has been shown to predict pH of porcine meat (Scheier et al., 2014), shear force of beef m. gluteus medius (Bauer et al., 2013) and tenderness of lamb (Schmidt et al., 2013), as yet no research has been conducted on the ability of a Raman hand held probe to predict the level of IMF and the major FA groups. Therefore, the aim of this research was to assess the ability of a Raman hand held probe to predict the level of IMF and the major FA groups of lamb m. longissimus lumborum (LL) which had not been frozen.

8.2 Materials and Methods

8.2.1 Samples

At 1 day post mortem (PM), the cranial portion of m. longissimus lumborum (LL; Product identification number HAM 4866; Anonymous, 2005) was collected from 80 lamb carcases over 4 consecutive days (20 per day) from the same abattoir. Samples were randomly selected from different consignments of unknown origins and therefore unknown backgrounds, age and gender, to represent the various animals typically processed by the abattoir, in order to obtain a spread in the levels of IMF and different FA composition. The same carcases were used for the experiments reported in Chapters 3 and 4. Sample weights of three LL portions were too small to provide adequate sample for FA reference analysis and thus these samples were not further considered.

8.2.2 Raman Spectroscopy

Raman Spectroscopic measurements were conducted at 24 h PM using a Raman hand held probe (Fig. 8.1; Schmidt et al., 2009) with a 70mW laser, an integration time of 3.45 s and 1 accumulation. Ten Raman scans were taken on each cranial portion at a room temperature of 16 °C on chilled LL samples. Once RS measurement was complete, the portion was frozen and held at -20°C until subsequent analysis of fatty acids.
8.2.3 Intramuscular Fat and Fatty Acid Analysis

The cranial portion of the LL was frozen at 24 h PM, freeze dried and homogenised prior to measurement. Reference measurements for intramuscular fat were conducted using a soxhlet method (J.AOAC, 1992).

Both a one-step extraction and methylation procedure adapted from Lepage & Roy (1986) and a two-step extraction methylation procedure adapted from Folch et al. (1957) were used to extract and methylate the fatty acid methyl esters (FAME).

The adapted one-step extraction methylation procedure used a chloroform: methanol (2:1 v/v) mixture and individual FAME were identified using an Agilent 6890 N gas chromatograph (GC) with a flame ionisation detector, as described by Clayton et al. (2012). However, the adapted two-step extraction and methylation procedure used a chloroform: methanol (2:1v/v) mixture which contained 10 mL/L (wt/vol) of butylated hydroxy toluene, as described by Ponnampalam et al. (2014b). The quality assurance method applied to this measurement is based on checks for 20% of samples, based on undertaking repeats.
8.2.4 Spectral Data Analysis

A measurement was taken of the sample without the laser on to determine the contribution of background noise, which was subtracted from the spectra before they were saved as raw data. Fat spectra were identified at time of measurement by the software that operates the Raman hand held probe and spectrograph and saves the Raman spectra (Schmidt et al., 2013). After measurement, fat spectra were further analysed using PCA with the lipid and protein spectral patterns as components to identify spectra which contained only Raman signals relating to lipids for chemometric analysis. Spectra for each sample were then averaged and reduced to wavenumber frequencies between 500 – 1800 cm⁻¹.

After measurement, spectra containing only signals from lipids were removed from those containing lipid, protein and other signals by conducting further PCA. These ‘only lipid’ spectra for each sample were then averaged and reduced from 300 – 2100 cm⁻¹ to contain only frequencies which included Raman signals (500 – 1800 cm⁻¹).

Models to predict IMF and the major FA groups were fitted using Partial Least Squares (PLS) with k- fold (k = 8) cross validation to determine the optimal number of latent variables to include. The number of latent variables used had the minimum average cross validated root mean square error of cross validation (optimal RMSECV) across 20 replicates. A null root mean square error of cross validation (RMSECV) for the observed IMF and major FA groups was also determined by predicting each trait, leaving one observation out at time. The RMSECV criterion was used to determine the best prediction model by assessing the reduction of the error of cross validation in comparison to the null model as a measure of precision (relative RMSECV reduction). Once fitted, prediction models were also cross-validated using the Leave One Out (LOO) approach to determine the correlation between the Raman spectra and average model covariate value ($R^2_{cv}$).

Spectra for band assignments were fitted to a 7th order polynomial using wavenumbers 523, 760, 982, 1139, 1382, 1522, 1712 and 1858 cm⁻¹ as no Raman signals were present at these frequencies. This background correction removed contributions of fluorescence and background noise from the spectra.
Key traits including IMF levels and concentrations of polyunsaturated fatty acids (PUFA), monounsaturated fatty acids (MUFA), total unsaturated fatty acids (USFA = MUFA + PUFA) and saturated fatty acids (SFA) were calculated as the sum of the FAs (mg/ 100 g meat) relevant to the specific category of the 27 individual FAs quantified by capillary gas chromatography (GC). Furthermore, the sum of these 27 individual FAs quantified by GC was calculated to determine total identified fatty acids. These categories were then used as model covariates. Several approaches were tried to account for the interactions between IMF and FA composition traits. These included incorporating IMF into the prediction using IMF amount (g/100g) as an additional model covariate and calculation of the FA group traits as percentage of IMF (Eq. 1).

\[
\text{FA}\% = \frac{\text{FA}_1 (\text{mg/100 muscle})}{\text{IMF}_1 (\text{mg/100 muscle})} \times 100/1 \quad \text{(Eq. 1)}
\]

**8.3 Results**

**8.3.1 Intramuscular Fat and Fatty Acid Composition**

Within this study, IMF levels ranged from 2.0 – 7.7 g/ 100g meat (mean 4.0 ± s.d. 1.1). Summary statistics for FA composition are described in Table 8.1. A considerable range was found for all key FA categories, although the two-step extraction methylation procedure had a higher efficiency of extraction. Consequently, amounts of all individual FA and major FA groups determined by using the two-step extraction procedure are also considerably higher in comparison to those determined by the one-step extraction.
Table 8.1. Summary statistics of lamb m. longissimus lumborum FA composition (mg /100g meat) determined using either a one-step or a two-step extraction methylation procedure.

<table>
<thead>
<tr>
<th>Fatty Acid (mg / 100g)</th>
<th>One Step Extraction Method</th>
<th>Two Step Extraction Method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (± s.d)</td>
<td>Range (min, max)</td>
</tr>
<tr>
<td>Individual Fatty Acids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14:0</td>
<td>50.1 ± 14.5</td>
<td>19.3 – 87.8</td>
</tr>
<tr>
<td>16:0</td>
<td>420.4 ± 86.1</td>
<td>239.2 – 682.3</td>
</tr>
<tr>
<td>18:0</td>
<td>283.9 ± 62.5</td>
<td>158.4 – 461.6</td>
</tr>
<tr>
<td>14:0 + 16:0</td>
<td>470.5 ± 97.1</td>
<td>285.5 – 762.1</td>
</tr>
<tr>
<td>18:1(∆ 9)</td>
<td>613.4 ± 156.6</td>
<td>185.9 – 962.3</td>
</tr>
<tr>
<td>18:2 n-6</td>
<td>64.2 ± 14.9</td>
<td>39.2 – 95.2</td>
</tr>
<tr>
<td>20:4 n-6</td>
<td>16.6 ± 3.9</td>
<td>10.0 – 28.1</td>
</tr>
<tr>
<td>Total n-6</td>
<td>86.3 ± 18.9</td>
<td>55.2 – 123.4</td>
</tr>
<tr>
<td>18:3 n-3 (ALA)</td>
<td>31.5 ± 6.6</td>
<td>19.8 – 49.1</td>
</tr>
<tr>
<td>20: 5 n-3 (EPA)</td>
<td>13.1 ± 2.4</td>
<td>7.0 – 18.7</td>
</tr>
<tr>
<td>22: 5 n-3 (DPA)</td>
<td>11.3 ± 1.6</td>
<td>7.4 – 15.1</td>
</tr>
<tr>
<td>22: 6 n-3 (DHA)</td>
<td>4.0 ± 1.1</td>
<td>1.9 – 6.7</td>
</tr>
<tr>
<td>EPA + DHA</td>
<td>17.1 ± 17.1</td>
<td>10.8 – 25.1</td>
</tr>
<tr>
<td>EPA + DHA + DPA</td>
<td>28.3 ± 4.4</td>
<td>18.9 – 39.4</td>
</tr>
<tr>
<td>Major Fatty Acid Groups</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Omega- 6</td>
<td>86.3 ± 18.9</td>
<td>55.2 – 123.4</td>
</tr>
<tr>
<td>Total Omega- 3</td>
<td>62.8 ± 8.8</td>
<td>46.1 – 82.7</td>
</tr>
<tr>
<td>PUFA</td>
<td>149.1 ± 24.4</td>
<td>104.9 – 205.3</td>
</tr>
<tr>
<td>MUFA</td>
<td>665.0 ± 164.8</td>
<td>211.2 – 1032.1</td>
</tr>
<tr>
<td>USFA</td>
<td>814.1 ± 159.5</td>
<td>381.3 – 1159.8</td>
</tr>
<tr>
<td>SFA</td>
<td>784.8 ± 156.8</td>
<td>455.0 – 1271.1</td>
</tr>
<tr>
<td>Omega-3: Omega- 6</td>
<td>1.4 ± 0.3</td>
<td>0.9 – 2.4</td>
</tr>
<tr>
<td>PUFA: SFA</td>
<td>0.12 ± 0.04</td>
<td>0.07 – 0.24</td>
</tr>
<tr>
<td>Total identified fatty acids (TIFA)</td>
<td>1598.9 ± 306.9</td>
<td>841.2 – 2430.9</td>
</tr>
<tr>
<td>Extraction Efficiency (%)</td>
<td>28.4 ± 5.5</td>
<td>17.7 – 45.4</td>
</tr>
</tbody>
</table>
Comparing the efficiency of the one-step and two-step extraction and methylation methods (Figure 8.2) illustrates the two-step extraction methylation had a higher efficiency of extraction (85% mean extraction compared with 28% for the one-step; Table 9.5). Yet both extraction methylation procedures had a reduction in the efficiency of the extraction as the total intramuscular fat amount increased.

![Figure 8.2. Efficiency of extraction (%) of a one-step (black; \( R^2 = 0.55 \)) and two-step (grey; \( R^2 = 0.38 \)) compared with intramuscular fat amounts (g/100g meat) from ovine m. longissimus lumborum.](image)

Further analysis demonstrates that the relationships between IMF levels and major FA groups (SFA, MUFA and PUFA) change between the extraction procedures. While there is a high correlation \((R^2 = 0.91 – 0.93)\) between IMF and major FA groups determined by the two-step procedure. Using the one-step procedure, there is a moderate correlation between IMF level and SFA \((R^2 = 0.6; \text{Fig 8.3})\) as well as IMF level and MUFA \((R^2 = 0.6; \text{Fig 8.4})\). However, there is a weak correlation between IMF and PUFA \((R^2 = 0.2; \text{Fig 8.5})\).
Figure 8.3. The correlation between saturated fatty acids (mg/100g meat) and intramuscular fat (g/100g meat) extracted using a one-step ($R^2 = 0.6$; black) and two-step ($R^2 = 0.91$; grey) methylation and extraction procedures from ovine *m. longissimus lumborum*.

Figure 8.4. The correlation between monounsaturated fatty acids (mg/100g meat) and intramuscular fat (g/100g meat) extracted using a one-step ($R^2 = 0.6$; black) and two-step ($R^2 = 0.92$; grey) methylation and extraction procedures from ovine *m. longissimus lumborum*. 
Figure 8.5. The correlation between polyunsaturated fatty acids (mg/100g meat) and intramuscular fat (g/100g meat) extracted using a one-step ($R^2 = 0.20$; black) and two-step ($R^2 = 0.93$; grey) methylation and extraction procedures from ovine m. longissimus lumborum.

Comparing the concentrations of major fatty acid groups extracted using the two-step procedure to IMF level (Fig. 8.6) highlights these relationships demonstrating that concentrations of MUFA and SFA were strongly correlated to increasing IMF levels ($R^2 = 0.91$ and 0.92, respectively), while PUFA remained constant despite increasing IMF levels ($R^2 = 0.15$).
8.3.2 Raman Spectroscopy

Spectra collected for this data set exhibit intensity peaks typical for the Raman spectra of fat. These key intensity peaks that are illustrated in Figure 8.7 and outlined in Table 8.2, include the C=O carbonyl stretch, the C=C olefinic stretch, the CH$_2$ methylene scissor twist and scissor deformations and the C-C in phase/out of phase aliphatic stretches.
Figure 8.7. The average intramuscular fat spectra, with no pre-processing, of lamb LL showing the intensity peaks that are indicative of fat.
Table 8.2. The wavenumber positions and band assignments of the main peaks in intensity for Raman spectra of adipose tissue (adapted from Olsen et al., (2007); Beattie et al., (2007); Socrates (2001)).

<table>
<thead>
<tr>
<th>Approx. Wavenumber (cm(^{-1}))</th>
<th>Assignment and Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1020 – 1130</td>
<td>C-C stretch</td>
</tr>
<tr>
<td>1270</td>
<td>= C – H deformations</td>
</tr>
<tr>
<td>1300</td>
<td>CH(_2) twist</td>
</tr>
<tr>
<td>1440</td>
<td>CH(_2) scissor</td>
</tr>
<tr>
<td>1500</td>
<td>CH(_2) scissor</td>
</tr>
<tr>
<td>1640 – 1660</td>
<td>C=C stretch</td>
</tr>
<tr>
<td>1655</td>
<td>HC=CH stretch</td>
</tr>
<tr>
<td>1740</td>
<td>C=O stretch</td>
</tr>
</tbody>
</table>

As illustrated in Figure 8.8, without pre-processing to remove the contributions of fluorescence and background noise to the spectra, Raman spectroscopy is unable to determine which wavenumber positions affect the prediction of IMF levels or major FA groups, such as PUFA.

![Average spectra of the 5 ovine m. longissimus lumborum samples with the highest (black; 432.28 – 477.25 mg/100g meat) and lowest (grey; 235.70 – 255.73 mg/100g meat) PUFA concentrations without pre-processing.](image)

Figure 8.8 Average spectra of the 5 ovine m. longissimus lumborum samples with the highest (black; 432.28 – 477.25 mg/100g meat) and lowest (grey; 235.70 – 255.73 mg/100g meat) PUFA concentrations without pre-processing.

Spectra which have had the contributions of fluorescence removed by fitting a 7\(^{th}\) order polynomial through wavenumbers that show no Raman signal (523, 760, 982, 1139, 1382,
1522, 1712, 1858 cm\(^{-1}\)) demonstrate that minor shifts in Raman peaks are evident between 830 – 914 cm\(^{-1}\) as well as larger intensity changes at key vibrations including those at approximately 1060 and 1070 cm\(^{-1}\) as well as 1260, 1295, 1430, and 1650 cm\(^{-1}\) (Fig. 8.9.).

Figure 8.9. Baseline corrected, normalised and averaged Raman spectra of the 5 samples with the most PUFA (dotted line; 432.28 – 477.25 mg/100g meat) compared to the 5 samples with the lowest PUFA (black; 235.70 – 255.73 mg/100g meat). Chemometric analysis using the Raman spectra to predict the major FA groups determined using both extraction methylation procedures are summarised in Table 8.3. The prediction of FA composition determined by the one-step extraction was highly variable with the cross validated correlation between predicted and observed values ranging from 0.00 (MUFA) to 0.57 (PUFA) and reductions in RMSECV between the null and optimal models ranging from -18.4% (EPA + DHA) to 84% (PUFA:SFA). While the models for the prediction of DHA (reduction in RMSECV = 47%, \(R^2 = 0.84, R^2_{cv} = 0.13\)) and PUFA (reduction in RMSECV = 35.3%, \(R^2 = 0.98, R^2_{cv} = 0.57\)) demonstrate some potential, these models have a high number of latent variables (10 and 12 LV, respectively).
Table 8.3. Summary of chemometric analysis using Raman spectroscopy to predict key fatty acid traits, including polyunsaturated (PUFA), monounsaturated (MUFA), total unsaturated (USFA), saturated (SFA), total FA amount determined by the methylation and extraction (Id. FA), omega-3 to omega-6 ratio and health claimable (DHA, DPA and DPA) FAs, of ovine m. longissimus lumborum determined by a one-step and a two-step FAME extraction methods.

<table>
<thead>
<tr>
<th>Fatty Acid Traits (mg/100g meat)</th>
<th>One-Step Extraction</th>
<th>Reduction in RMSECV</th>
<th>Two-Step Extraction</th>
<th>Reduction in RMSECV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R²</td>
<td>R²_cv</td>
<td>Null RMSECV</td>
<td>Optimal RMSECV (latent variables)</td>
</tr>
<tr>
<td>MUFA</td>
<td>0.01</td>
<td>0.00</td>
<td>165.86</td>
<td>165.52 (1 LV)</td>
</tr>
<tr>
<td>PUFAs</td>
<td>0.98</td>
<td>0.57</td>
<td>24.58</td>
<td>15.91 (12 LV)</td>
</tr>
<tr>
<td>USFA</td>
<td>0.00</td>
<td>0.01</td>
<td>160.45</td>
<td>162.54 (1 LV)</td>
</tr>
<tr>
<td>SFA</td>
<td>0.04</td>
<td>0.01</td>
<td>253.76</td>
<td>256.95 (1 LV)</td>
</tr>
<tr>
<td>PUFAs: SFA</td>
<td>0.19</td>
<td>0.11</td>
<td>0.38</td>
<td>0.06 (2 LV)</td>
</tr>
<tr>
<td>Id. FA</td>
<td>0.01</td>
<td>0.05</td>
<td>198.11</td>
<td>200.70 (1 LV)</td>
</tr>
<tr>
<td>n-3: n-6</td>
<td>0.51</td>
<td>0.26</td>
<td>0.15</td>
<td>0.13 (7 LV)</td>
</tr>
<tr>
<td>EPA</td>
<td>0.01</td>
<td>0.02</td>
<td>2.43</td>
<td>2.45 (1 LV)</td>
</tr>
<tr>
<td>DHA</td>
<td>0.84</td>
<td>0.13</td>
<td>2.00</td>
<td>1.06 (10 LV)</td>
</tr>
<tr>
<td>DPA</td>
<td>0.14</td>
<td>0.08</td>
<td>1.58</td>
<td>1.51 (2 LV)</td>
</tr>
<tr>
<td>EPA+ DHA</td>
<td>0.03</td>
<td>0.01</td>
<td>3.16</td>
<td>3.74 (2 LV)</td>
</tr>
</tbody>
</table>
Using Raman spectra to predict the FA composition as determined by the two step extraction method reduced the error of the prediction with reductions between the optimal and null RMSECV of between -14.3 (n-3: n-6) – 40.8% (TIFA). Correlations between the predicted and observed values ($R^2$) ranged between 0.04 (EPA + DHA) to 0.95 (EPA). However, cross validated correlations between the predicted and observed values ($R^2_{cv}$) were lower, ranging from 0.01 (USFA, SFA, EPA + DHA) to 0.24 (n3: n6 ratio).

Based on the greatest reduction in RMSECV and the highest coefficient of determination between cross validated predicted and observed values ($R^2_{cv}$), the most promising prediction model was observed for the prediction of PUFAs (Table 8.3, Fig. 8.10). This model yielded a 7.8% reduction in the error of the prediction when Raman spectra were used (optimal RMSECV = 46.57, null RMSECV = 50.50) and an $R^2_{cv} = 0.21$ ($R^2 = 0.93$). However, this model uses a high number of latent variables (11 LV). The prediction of MUFA determined using the two-step extraction method also shows potential as it yielded an optimal model with a 4.0% reduction in RMSECV, an $R^2 = 0.54$ and an $R^2_{cv} = 0.16$ (7 LV).

Figure 8.10 PUFA values in m. longissimus lumborum (mg/ 100g meat) predicted using Raman spectra compared to observed values (optimal RMSECV = 46.57, $R^2 = 0.93$, $R^2_{cv} = 0.21$).
Incorporating IMF as a covariate into predictive models with Raman spectra did not improve the prediction of the major FA groups determined by the two-step extraction procedure (Table 8.4).

Table 8.4. Coefficient of determination ($R^2$) values compared with the cross validated coefficient of determination between observed and predicted values ($R^2_{cv}$) for key fatty acid categories determined using the two-step procedure with and without inclusion of intramuscular fat amount (mg/100g) as a model covariate.

<table>
<thead>
<tr>
<th>Trait (mg/100g)</th>
<th>Coefficient of determination for Raman only models</th>
<th>Coefficient of determination for Raman + IMF models</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$R^2$</td>
<td>$R^2_{cv}$</td>
</tr>
<tr>
<td>IMF</td>
<td>0.08</td>
<td>0.01</td>
</tr>
<tr>
<td>MUFA</td>
<td>0.54</td>
<td>0.16</td>
</tr>
<tr>
<td>PUFA</td>
<td>0.82</td>
<td>0.20</td>
</tr>
<tr>
<td>SFA</td>
<td>0.08</td>
<td>0.01</td>
</tr>
<tr>
<td>PUFA: SFA</td>
<td>0.21</td>
<td>0.13</td>
</tr>
</tbody>
</table>

Adjusting the major FA groups determined by the two-step procedure to a percentage of IMF (Table 8.5) improved the prediction of SFA ($R^2 = 0.56$, $R^2_{cv} = 0.31$, 15.4% reduction in RMSECV). Furthermore, there was a greater reduction in the error of the prediction and latent variables for these models despite no improvement in the coefficients of determination between predicted and observed values.

Table 8.5. Summary chemometric analysis for the prediction of key fatty acid traits of ovine m. Longissimus thoracis, adjusted* and unadjusted for mean intramuscular fat levels.

<table>
<thead>
<tr>
<th>Major FA Groups</th>
<th>mg/100g meat</th>
<th>% of IMF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$R^2$</td>
<td>$R^2_{cv}$</td>
</tr>
<tr>
<td>MUFA</td>
<td>0.54</td>
<td>0.16</td>
</tr>
<tr>
<td>PUFA</td>
<td>0.82</td>
<td>0.20</td>
</tr>
<tr>
<td>USFA</td>
<td>0.08</td>
<td>0.01</td>
</tr>
<tr>
<td>SFA</td>
<td>0.08</td>
<td>0.01</td>
</tr>
</tbody>
</table>

* calculated using $FA\% = \left(\frac{FA}{IMF} \times \frac{mg}{100g}\right) \times 100$
8.4 Discussion

8.4.1 Intramuscular Fat and Fatty Acid Composition

The IMF level found in this study is consistent with the levels expected in retail lamb in Australia. Previous studies have reported IMF values for lamb loin (both *m. longissimus thoracis* and *m. longissimus lumborum*) in Australia of between 3.4 – 5.3% (Ponnampalam et al., 2001a, Ponnampalam et al., 2001b, Pannier et al., 2014).

FA composition data (Table 8.7) indicates that of the major FA groups (PUFA, MUFA and SFA), the FA composition varies between the one-step and two-step extraction methylation procedures used. Using the two-step extraction method the FA composition of lamb loin was comprised of mostly MUFA and SFA.

While the concentrations of SFA determined using the two-step extraction method (1568mg/100g) is within the 1200 – 1730mg/100g meat range that has been reported by other studies (Ponnampalam et al., 2001b, Williams, 2007, Ponnampalam et al., 2010, Guy et al., 2011, Ponnampalam et al., 2012a), it is double the amount of SFA determined using the one-step extraction method (Fig 8.3). This highlights the reduced extraction of FAs compared to the fat content determined by soxhlet using the one-step procedure as 28% of the total IMF measured was extracted using the one-step compared to 85% of total IMF measured when the two-step extraction methylation procedure was used (Table 8.1, Fig 8.2).
The difference in FAs extracted (Table 8.1) and prediction results (Table 8.3) of the two extraction procedures highlights the impact of varying reference analysis on the prediction of meat quality traits and the importance of comparing studies that have been conducted using the same reference methods. Methodological considerations are required for FA extraction and analysis based on the type of lipid extracted and the FAs being analysed (Clayton, 2014). This is a result of the charges of the FAs as triglycerides are neutral lipids whilst phospholipids contain negatively charged tails and are therefore polar. Consequently, acid catalysed methylation is commonly used to identify a number of common fatty acids in the triglycerides of feed and meat. However, base-catalysed methylation or a combination of base and acid methylations are used to identify a wider range of FAs (Christie, 2003, Clayton, 2014). Hence, the differences in extraction efficiency between the one-step and two-step procedures with increasing IMF may be a result of the difference in polarities required to extract a higher proportion of triglycerides in the muscle which occur as the IMF level increases (te Pas et al., 2004) or due to ‘flooding’ the GC column at higher IMF levels. Although, as there benefits in using the one-step procedure, including reduced toxicity of the solvents and reduced solvent volumes required (Clayton et al., 2012), further method development to determine whether the one-step procedure can be adapted for FA determination of commercial meat samples is warranted. Given the results of the two-step are comparable to several studies previously cited, the results determined by the two-step procedure are more likely to be indicative of the true FA composition of the meat.

It must be stressed that in order to obtain data which reflects the variability of FA composition of commercially produced lamb in Australia, samples in this study were of unknown background, age and sex sampled at a commercial abattoir. As previous studies have demonstrated the impact of breed (Demirel et al., 2006), genetics (Mortimer et al., 2014), diet (Bas & Morand-Fehr, 2000, Ponnampalam et al., 2012a), muscle sampled (Ponnampalam et al., 2010), sample location (Wood et al., 2008), age (Oriani et al., 2005), gender (Kosulwat et al., 2003), carcase weight and fat score (Kosulwat et al., 2003) and production system (Díaz et al., 2005) on FA composition it is not useful to directly compare the FA composition found by the current study with established literature where these factors have been controlled.
8.4.2 Prediction of Fatty Acid Composition

Overall, this study indicates that there is potential to predict the concentration of PUFA extracted using the two-step procedure using Raman spectroscopy. This model indicated the prediction using Raman spectra gave a 7.8% reduction in RMSECV compared to the null model and a promising coefficient of determination of 0.93 was found (Table 8.3, Fig 8.10). This prediction was found to be more accurate than that in intact salmon (Afseth et al., 2006) and beef (Moss et al., 2009).

Using a ball probe attached to a Raman spectroscopic instrument via a fibre optic cable, Afseth et al. (2006) found that multivariate correlations of $R = 0.67$ and 0.73 were achievable for prediction of the iodine value of the intact dark muscle from salmon. However, this was improved to $r = 0.86$, when the salmon samples were ground. Afseth et al. (2006) proposed the differences in multivariate correlation coefficients between ground and intact samples were the result of sampling differences that resulted in a mis-match between reference measurements and the spectra of intact samples, as spectral measurements and reference analysis were conducted on separate halves of the same portion of the fish. While the present study avoided these issues with sampling by conducting Raman spectral measurements on the same portion of muscle which was then freeze dried and ground for reference measurement and thus the accuracy of the prediction was improved. Although, the prediction of FA composition may be further improved by grinding the samples prior to RS scanning to remove the errors associated with measuring a complex heterogeneous sample, this would remove the advantages using Raman spectroscopy as a non-destructive tool for FA determination in commercial applications.

Similarly Moss et al. (2009) found that prediction coefficients were less accurate ($R^2 = 0.21 – 0.27$) than the current study despite also measuring intact muscle using Raman spectroscopy. However, Moss et al. (2009) co-added spectra which were measured over a matrix of 5 x 5 sample points, separated by 5 mm between each point using a bench top Raman device. While this method reduced the number of spectra allowing for more timely data analysis, it was suggested that this method of spectral collection was influenced by the amount of IMF and small surface area of sampling, which reduced the predictions found for MUFA and SFA. Therefore it was concluded that independently obtaining data of IMF over a larger surface
area of the muscle would be more fruitful in the prediction of FA composition which could account for the better predictions found in the present study.

While a limited ability to predict MUFA was also found in this study (4% reduction in RMSECV, $R^2 = 0.54$; Table 8.3), and SFA which had been adjusted for its strong correlation with IMF ($R^2 = 0.54$, $R^2_{cv} = 0.31$; Table 8.5) there was no potential to predict any other FA trait or IMF level. Although this study overcomes the previous limitations, being the first study to determine the potential of Raman spectra to predict the IMF level and FA composition of fresh intact ovine muscle in-situ using a hand held Raman spectroscopic device, no literature exists with which to compare the methods, chemometric analysis and thus the results of this study.

Other previous studies using Raman spectroscopy to predict the FA composition of porcine adipose tissue (Olsen et al., 2007, Olsen et al., 2010) have indicated that FA composition was able to be predicted with good accuracy (cross validated coefficients ranged from 0.95 – 0.98). However, measuring adipose tissue these studies avoided the need to discriminate between meat and fat spectra to remove contributions from overlapping protein vibrations on the lipid signals which can arise during the measurement of IMF levels in-situ. With previous work that determined the ability of Raman spectroscopy to predict the tenderness of intact ovine longissimus lumborum muscles from the same carcases (Chapter 3), the method to discriminate between lipid and protein spectra was already established. This was established using the hand held Raman device that had previously been developed to assess the quality of pork (Schmidt et al., 2009). This process uses PCA to identify lipid contributions to the spectra by comparing key vibrations in each spectra with saved components (Schmidt et al., 2010). As pork subcutaneous adipose tissue is higher in long chain PUFAs in comparison to lamb (Wood et al., 2008) and RS is sensitive to species differences in FA composition (Beattie et al., 2007), it is plausible that this process may have affected prediction models. Therefore, prediction outcomes may be improved by creating a PCA model for discrimination between protein and lipid spectra which uses species specific spectra as the saved components. Furthermore, this approach of discriminating between lipid and protein spectra does not distinguish between spectra containing only lipid signals and spectra containing mixed lipid and protein signals. Removing all spectra that contain mixed lipid and protein signals not only significantly reduces the number of spectra available for analysis but may also remove information on the lipids bound in membranes. Consequently, further
research is required to determine the best threshold to filter spectra with mixed lipid and protein signals.

Improving this discrimination between spectra could be achieved by quantifying the concentration of fat detected in the spectra. Using the surface area of the window of the handheld device and the depth of the laser focus combined with the intensities of the lipid peaks in the spectral data, it would be possible to calculate the percentage of the muscle area measured which includes contributions from lipids. Therefore, spectra with increasing contributions of protein bands could be saved separately facilitating a better distinction between ‘bad’ lipid spectra (e.g. noisy or highly fluorescent spectra containing only lipid signals) and spectra which have increasing contributions of proteins or increasing proportions of lipids. Furthermore, improving the discrimination of spectra containing mixed protein and lipid signals could also increase the potential to predict IMF amount of intact muscle.

**8.4.3 Fatty Acid Band Assignments**

As this is the first study assessing the Raman spectra of lipids in a complex matrix such as intact muscle, it is not possible to assign the Raman spectral bands of FA composition with any certainty. However, a tentative interpretation is plausible based on FA composition studies using data from Raman spectroscopy research on adipose tissues (Beattie et al., 2007, Lyndgaard et al., 2011). These studies confirm that peaks in the intensity counts found in this research (Fig. 8.7 and Table 8.2) are typical of lipids, including the chemical bond vibrations of the C=O carbonyl stretch (1750 cm\(^{-1}\)), the C=C and HC=CH stretches (1640 – 1650 cm\(^{-1}\)), CH\(_2\) twist and scissor (1300 and 1440 cm\(^{-1}\)) and the C-C in phase and out of phase vibrations (1020 – 1130 cm\(^{-1}\)). Although the signals are typical for FA composition of ruminant FAs (Beattie et al., 2007), changes in FA composition generates shifts in the wavenumbers and the intensity counts of these key vibrations that provide information on the individual FAs present.

As illustrated by Figure 8.9., spectra collected from samples with high levels of PUFA demonstrated a 20% increase in intensity, as well as a shift of +3 cm\(^{-1}\) for the peak representing the C=C at approximately 1650 cm\(^{-1}\). This is to be expected due to the chemical structure of FAs which have multiple unsaturated carbon – carbon bonds (Clayton, n.d.). Consequently, IMF containing higher amounts of PUFA also contains higher numbers of C=C bonds resulting in a positive correlation of this signal with increasing unsaturation.
Consequently, this intensity peak dominates the spectra of lipids which are known to be high in PUFAs such as salmon and pork adipose tissues (Afseth et al., 2006, Beattie et al., 2007, Olsen et al., 2007, Olsen et al., 2010, Lyndgaard et al., 2011).

The exact location of the C=C bond is also important as it indicates the conformation of the C=C bonds (Lyndgaard et al., 2011). Therefore the + 3 cm\(^{-1}\) shift evident in spectra from loins containing higher levels of PUFA (Fig 8.3.) may also suggest that the unsaturated FAs in these samples are comprised of more trans- bonds rather than cis-bonds, as the location of this band shifts to higher wavenumbers with increasing numbers of trans- bonds (Beattie et al., 2004b, Lyndgaard et al., 2011). This may be also linked to increasing PUFA and slight decreases in MUFA concentrations of these loins as shifts of this band to higher wavenumbers have also been associated with changes in composition from mono to polyunsaturated FAs in simple protein, fat and water emulsifications (Afseth et al., 2005).

As the PUFA concentration consists of contributions from the cis- and trans- bonds of linoleic acid (C18: 2 n-6) (Nelson & Cox, 2008), the increasing contribution of PUFA to the spectra may increase the contribution of trans- bonds. However, it is unlikely that it would be a significant shift, as overall there would still be a large contribution to this band from the concentration of oleic acid (C18:1(\(\Delta^9\))), which has a similar chemical conformation and is present in a relatively high concentration as it is one of the main FAs found in lamb (Table 8.1). This also highlights the difficulty in predicting FAs in complex mixtures as RS relies on structural differences, it is unable to discriminate between PUFAs and MUFAs which have similar chemical structures.

Although peak intensities and locations of bands are important in determining the chemical composition of IMF, previous Raman spectroscopic research on lipids suggests that ratios between key intensity peaks cannot be over looked (Bresson et al., 2005). One such example is the ratio of the C=C bond peak intensity to the C=O bond peak intensity at 1740 cm\(^{-1}\) (Table 8.2). While spectra collected (Fig 8.9.) indicate that there is little difference in the C=O peak intensity between samples of different PUFA concentrations, changes to the C=C peak height at 1650 cm\(^{-1}\) indicates that a difference in the C=C:C=O ratio is evident as PUFA levels increase. Whilst previous studies on pork (Olsen et al., 2007, Olsen et al., 2010) and salmon (Afseth et al., 2006) have used this ratio as an excellent indicator of the unsaturation of fats, this is not possible for this data set, since this ratio characterises the molal unsaturation rather than the mass unsaturation. This is due to the differences between mass
and molal unsaturation, as mass unsaturation depends on the number of double bonds as well as the number of carbon chains per gram of sample (Beattie et al., 2004b), which are not consistent between samples of this study due to the measurement of IMF rather than subcutaneous adipose tissue. Consequently, this ratio is a better indicator of iodine value rather than absolute amounts of unsaturated FAs. While a more accurate prediction of molal unsaturation may be achieved using Raman spectra, unlike pork manufacturing processes where iodine value has implications for fat firmness and product acceptability (Wood et al., 2004), the prediction of iodine value has no benefit to the sheep meat industry.

Determination of the degree of unsaturation of FAs using Raman spectra is not only limited to the number of C=C bonds present. As illustrated by Figure 8.9, an 18% increase in the intensity peak at 1260 cm$^{-1}$ that represents the C-H deformation (Table 8.2) is also evident in loins with higher amounts of PUFA. This agrees with the studies conducted by Afseth et al. (2006) and Lyndgaard et al. (2011) that have positively correlated increases in this peak with increasing concentrations of PUFA due to the CH deformation vibration which characterises the unsaturation of carbon- carbon chains. Given that this band decreases with increasing contributions from SFAs (Lyndgaard et al., 2011), particularly C16:0 (Beattie et al., 2004b), it is unsurprising that this band does not dominate the spectra as has previously been described by Lyndgaard et al. (2011) as lamb IMF contains more SFAs than pork adipose tissue (Wood et al., 2008).

It is interesting to note that while spectra from loins with higher levels of PUFA demonstrate increased intensities for the peaks relating to unsaturation, there is also an increase in the Raman intensity of peaks which characterise SFAs. This is evident with a 10% increase at the C-C out of phase vibration at 1059 cm$^{-1}$, an 8% increase in the CH$_2$ twist vibration at 1290 cm$^{-1}$ and a 15% increase and a $+3$ cm$^{-1}$ shift in the CH$_2$ scissor vibration at 1438 cm$^{-1}$ (Fig. 8.9). The increase of intensities at these wavenumbers suggests that the loins which are high in PUFA are also high in SFA as increasing intensity and decreasing width of these bands has been positively correlated with increasing levels of saturation (Beattie et al., 2004b, Afseth et al., 2006, Lyndgaard et al., 2011).
Although chain length elicits an effect on the height and width of peak intensities (Beattie et al., 2004b), it is unlikely that this is the cause of variation in spectra since lamb IMF is comprised of mostly medium chain FAs (carbon chain length between 12 – 20; Table 8.1) and Raman signals remain constant with carbon chain lengths between 13 - 24 (Beattie et al., 2004b). Temperature also has an effect on the Raman spectra as these bands broaden and decrease in intensities as FAs approach melting point (Beattie et al., 2004b, Lyndgaard et al., 2011). Although as Raman measurements were completed on loins at the same temperature it is unlikely that state of the lipids will account for the variation.

Despite findings from previous Raman spectroscopic research on simple lipids being applied to studies on porcine subcutaneous adipose tissue with excellent predictive power (Olsen et al., 2007, Olsen et al., 2010), it is unclear what impact the measurement of fat within the muscle has on the Raman spectra. Since the lipids of adipose tissues are mainly triglycerides (Christie, 2012) which consist of three FAs each in an ester linkage with a single glycerol (Nelson & Cox, 2008) by measuring a constant amount of adipose tissue, changes to the concentration of any one FA category would alter the overall proportion of all the FA categories. As a result of this chemical structure, it is possible to directly relate CH$_2$ group vibrations to the number of saturated bonds and the C=O bonds to the number of FA side chains present. Saturated and unsaturated FA side chains in triglycerides are distinguishable because each C=C bond present in unsaturated FA removes 2 positions in the chain where carbon can bond to hydrogen atoms (Beattie et al., 2004b). Therefore, at similar chain lengths SFA have more CH$_2$ groups compared to MUFA and PUFA.

Despite Afseth et al. (2006) proposing that in complex fat systems RS is unable to distinguish between MUFA and PUFA which have similar conformation when FAs are measured in the muscle of salmon, this may not be the case for the previous studies that have measured adipose tissue as it consists of only triglycerides. Due to the difference in the configuration of the C=C bonds in MUFA and PUFA, there are differences in the numbers of consecutive C-C bonds in the FA chain, as the C-C chain is ‘broken’ by C=C bonds. This means that while chemically similar FAs may contain the same number of C-C and C=C bonds, such as both α-linolenic acid (C18:2 n-3) and γ-linolenic acid (C18:3 n-6) that contain 14 C-C bonds and 3 C=C bonds, differences in the position of the C=C bonds along the chain results in consecutive chains of C-C bonds with different lengths. Thus, α-linolenic acid (C18:2 n-3) has consecutive chains of C-C bonds of 8, 2, 2 and 2, while γ-linolenic acid has consecutive chains of C-C bonds of 5, 2, 2 and 5.
Beattie *et al.* (2004b) refer to this as the “apparent” chain length because Raman spectra reflect the number of consecutive C-C bonds rather than the absolute number of C-C bonds. Thus, consecutive chains of C-C bonds between C=C bonds within the hydrocarbon tails of MUFA and PUFA are characterised in spectra as separate shorter C-C chains. Due to the chemical structure of triglycerides with mixed FA of similar chain lengths such as pork adipose tissue, increasing concentrations of PUFA would reduce these “apparent” chain lengths per FA chain reflected in spectra more than increasing MUFA concentrations. Consequently, discrimination and better predictions of both MUFA and PUFA could be possible for triglycerides measured in adipose tissue as decreasing chain lengths per FA chain would be evident in the comparison of the C-C vibration to the carboxyl group (C=O) vibration at approximately 1740 cm\(^{-1}\).

FAs in IMF are different to FAs in adipose tissue as they consist of triglycerides in adipocytes as well as phospholipids in the membranes of the myofibril (te Pas *et al.*, 2004). This difference in lipid fractions could account for the poorer prediction of SFA and MUFA as well as the decrease in prediction co-efficient of determination when cross-validated as found in this study, given phospholipids have a more complex chemical structure. Hence, the spectral assignments and predictions previously described for triglycerides (Olsen *et al.*, 2007, Olsen *et al.*, 2010, Lynggaard *et al.*, 2011) and simplified FA mixtures (Beattie *et al.*, 2004b, Afseth *et al.*, 2005) may not correlate with those of phospholipids deposited in red meat.

In contrast to the relatively simple structure of triglycerides, phospholipids consist of two FA chains covalently bonded to a glycerol molecule, a phosphate group and another molecule, which determines the phospholipid type (Nelson & Cox, 2008). For example, phosphatidyl choline has CH\(_2\) groups and C-C bonds in the choline molecule which forms part of its hydrophilic head. During excitation these bonds have the same vibration as the CH\(_2\) groups and C-C bonds present in the FA side chains. Consequently, Raman spectra from phospholipids have overlapping contributions from other chemical bonds in the hydrophilic head within the same wavenumbers used to determine FA composition (Wallach *et al.*, 1979). This is a unique limitation to the application of RS for IMF *in-situ* prediction within fresh intact muscle, as RS studies conducted on simplified phospholipids in laboratory conditions can use greater wavenumbers (e.g. 2900 – 3200 cm\(^{-1}\)) which partly avoids these overlaps (Larsson & Rand, 1973, Lis *et al.*, 1976, Bunow & Levin, 1977, Susi *et al.*, 1979).
However, the hand held Raman device used in this study has been designed to measure meat that gives a weak Raman signal so shorter measurement times and an excitation wavenumber to reduce the generation of fluorescence while maintaining the efficiency of detectors is required (Schmidt et al., 2009). Hence, the spectral range of this study is restricted to 500 – 2100 cm\(^{-1}\) and these larger wavenumbers have not been measured.

If overlap of the CH\(_2\) group and the C-C bond vibrations of the hydrophilic head of phospholipids are present when RS is conducted in-situ, they would contribute to predicted FA values. However, as they do not form part of the FA chains and are removed during the methylation and extraction of FAME for FA analysis, they are not accounted for in the observed FA values. As a result there would be a reduction in the coefficient of determination between predicted and observed FA values. Furthermore, it is plausible that the random selection of carcases sampled to obtain LLs with a range of IMF levels and FA compositions has resulted in varying contributions of phospholipids to the fat spectra, as increasing IMF levels increases the amount of triglycerides within the muscle, while phospholipids remain constant (te Pas et al., 2004). Consequently, LL portions with lower levels of IMF may be more affected by this spectral overlap effect of the CH\(_2\) and C-C vibrations compared to LL portions with higher levels of IMF. This may create an over or underestimation of the FA group concentrations causing reductions in the coefficients of predicted and observed values that occurred when models were cross validated (Table 8.3 and 8.4).

Based on this research, further study into the relationships between IMF, lipid fractions and FA composition in ruminants is warranted. It is likely that PUFA in lamb is mainly bound in the phospholipid fraction, similarly to pork, beef, rabbit and poultry (Hernández et al., 1998, te Pas et al., 2004), given that the proportion of phospholipids present in the muscle remains constant regardless of IMF levels, as did PUFA within this study (Fig 8.6). However, as SFA and MUFA were strongly correlated with increasing IMF (Fig 8.6) and adjustment for IMF levels significantly improved the predictive ability of SFA, there may also be a correlation between the quantity of triglycerides and FA composition in lamb LL. Therefore, a greater understanding of the interactions between proportions of lipid fractions, IMF levels and FA composition of ruminants is required. This information is also needed to provide better insight into the chemical structure of FAs in-situ within the myofibrillar matrix and the links between chemical bonds of lipids and FA which would provide a better approach for the prediction of FA composition using technologies such as RS.
However, there are larger implications of the correlations between IMF, lipid fractions and FA composition, as the polarity of the lipid fraction affects the extraction and recovery of FA (Clayton et al., 2012) as well as the oxidation and retail shelf life of meat products (Ponnampalam et al., 2001c).

8.5 Conclusion

Overall this study demonstrated that there is potential for Raman spectroscopy to predict the PUFA, MUFA major FA groups and SFA as a percentage of IMF. However, there is currently no opportunity to compare these results with other studies using Raman spectroscopy to predict major FA groups and IMF level in-situ within fresh intact muscle. Thus, validation of the accuracy and prediction errors found in this study is required.

Direct comparison of Raman spectra demonstrated that higher concentrations of PUFA were correlated with intensity peaks that correspond to the C=C bonds at 1650 cm\(^{-1}\) and the CH deformation at 1260 cm\(^{-1}\). Yet these spectra from LL portions with higher concentrations of PUFA also contained stronger signals for the CH\(_2\) group and C-C vibrations which are associated with increasing saturation. Therefore, it is difficult to determine the influence on the predictions from overlapping spectral signals that arise from the measurement of FA chains in the phospholipid and triglyceride fractions that are measured in IMF within fresh intact muscle in-situ. The impact of correlations between IMF levels, FA composition and lipid fraction proportions on the prediction of FA composition using Raman spectroscopy is yet to be determined and future research will need to address this.
9. General Discussion/ Conclusion

Overall, the highest correlation coefficients of the meat quality traits measured were the prediction of the major fatty acid groups, PUFA ($R^2 = 0.93$, 7.8% reduction in RMSECV; Table 8.3) as well as MUFA (4% reduction in RMSECV, $R^2 = 0.54$; Table 8.3), and SFA which had been adjusted for its strong correlation with IMF ($R^2 = 0.54$; Table 8.5). This is unsurprising given that fats have better scattering properties in comparison to proteins and therefore more Raman information is contained within spectra of lipids (Ozaki, 1999). However, spectral band assignments suggest there may be some overlap in Raman signals arising from the head groups of phospholipids, which may be causing a reduction in the accuracy when cross validation is used to determine the robustness of the prediction ($R^2_{cv} = 0.31 – 0.57$). As this is the first research investigating the potential to predict IMF level and major FA group concentration of intact muscle using a Raman spectroscopic hand held device, further research is required to determine the repeatability of these prediction accuracies and the impact of correlations between IMF levels and lipid fractions on the prediction of major FA group composition.

While the initial experiment to predict tenderness of *m. semimembranosus* suggested Raman spectroscopy was a better predictor of shear force values compared to the traditional indicators ($R^2_{cv} = 0.27$), this result was unable to be reproduced in later experiments. Inconsistent results between this research and previous studies was also found for the prediction of shear force values of intact lamb which had been frozen and thawed. Although research conducted for this research suggested that there was no ability to predict shear force values Schmidt *et al.* (2013) indicated that Raman spectroscopy was able to predict shear force values with accuracies of $R^2 = 0.79$ and $R^2 = 0.86$ for samples from two sites. Discrepancies between findings may be the result of differences in Raman spectroscopic parameters as Schmidt *et al.* (2013) measured Raman spectra from 5 positions on three freshly cut surfaces of quartered 3 cm² blocks of *m. longissimus lumborum*. Thus, more spectroscopic information was collected over a smaller muscle face, potentially reducing the signal to noise ratio and improving the prediction accuracy. Given that these experiments were conducted over multiple years, it is plausible that the inconsistent results may reflect a limitation in the long term robustness of the hand held Raman device indicating that in its current form, it may not be able to withstand the rigours of measuring a large number of
carcases within commercial situations. However, upgrading electrical components as technological advances continue could overcome this limitation.

Prediction of other meat quality traits using Raman spectroscopy demonstrated there was also an ability to predict pH$_{24}$ and purge using spectra measured pre-rigor ($R^2_{cv} = 0.27$ and 0.32, Table 6.5) and pHu, purge and L* values from spectra measured at 24 h post mortem ($R^2_{cv} = 0.22 – 0.59$, Table 6.6). Due to the correlations between colour, water holding capacity, structure pH decline and pHu (Hughes et al., 2014), it is hypothesised that Raman spectroscopy is able to predict meat quality indicators which relate to the early post mortem metabolic processes. Indeed, previous research on the prediction of pH early post mortem in pork suggests that Raman spectroscopy is able to determine variations in pH based on variations in inosine monophosphate (IMP), lactate, glucose 6-phosphate, creatine phosphate (PCr), phosphoric acid and ATP concentrations during the onset of rigor (Scheier & Schmidt, 2013). However, as pork contains more type IIB muscle fibres than lamb (Kerth, 2013) and therefore porcine muscle has a different oxidative and glycolytic capacity, contraction speed and glycogen content (Choe et al., 2008) further research is required to establish which biochemical processes contribute to the spectral changes associated with these predictions in lamb.

Overall Raman spectra collected during this research were informative on the biophysical and biochemical characteristics of the meat which relate to meat quality traits. Although the spectra are complex, a tentative interpretation of the underlying spectra suggests that lambs with lower pHu values have increased concentrations of phosphates, lactate, $\alpha$ – helical proteins and greater denaturation of amino acid side chains present in the muscle (Scheier et al., 2014), which may be indicative of a more accelerated rate of pH decline (Huff Lonergan et al., 2010, England et al., 2013). Furthermore, Raman spectra from samples with high purge demonstrate similar spectral changes to those associated with high pHu values suggesting that high purge and pHu are associated. Yet further band assignments suggest that increased purge is also associated with a decrease in adenine, lactic acid, inorganic phosphate, phosphodiesters and glycogen (Movasaghi et al., 2007) yet the biochemical pathways which contribute to these findings were not able to be identified. Given little research has been completed on the application of Raman spectroscopy to predict the meat quality traits of intact lamb, including reference spectra with higher concentrations of glycogen and myoglobin, the impact of spectral signals overlapping for compounds of similar chemical composition on the predictions found is unknown.
10. Implications for Industry

Despite the prediction of PUFA and SFA adjusted for IMF yielding the greatest coefficient of determination, as they were dramatically reduced during cross validation it is considered that these models are not robust enough to provide industry the level of accuracy required. Therefore, the Raman spectroscopic hand held device is best suited to predicting meat quality traits related to metabolic processes including pH, purge and $L^*$ values. Data presented in this thesis suggests it may be possible to identify carcases which deviate from the normal post mortem metabolic processes resulting in increased purge or low pHu values.

Using Raman spectroscopy to predict carcases susceptible to high purge early post mortem could be beneficial to sheep meat processors, providing them with a decision making tool to determine carcase suitability for chilled export markets. Yet there may be greater benefits from applying this technology to the beef industry where carcases are more susceptible to deviations in pH/temperature decline and pHu values. Consequently, it may be possible to determine beef carcases susceptible to heat shortening, pale soft and exudative (PSE) like characteristics as well as dark firm and dry (DFD) meat using Raman spectroscopy. However, further development and validation of the technology is required to ensure it is robust enough to provide predictions which are repeatable long term in the extreme conditions required for online measurement. Furthermore, future research needs to determine whether the findings of this thesis transfer to other species or muscles.
11. References


Bruker 2013. Opus© chemometric software for Raman and FTIR spectroscopy.


Food Standards Australia New Zealand. 2009. Intakes of trans fatty acids in New Zealand and Australia. review report.


Food Standards Australia New Zealand. 2009. Intakes of trans fatty acids in New Zealand and Australia. review report.


Holman, B. W. B., Ponnampalam, E. N., van de Ven, R. J., Kerr, M. G. & Hopkins, D. L. (2015b). Lamb meat colour values (HunterLab CIE and reflectance) are influenced by aperture size (5 mm v. 25 mm). *Meat Science, 100*, 202-208.


Media Cybernetics Inc 2011. Image-Pro Plus. Sliver Springs USA.


WiTec 2012. WiTec Project v2.10 chemometric software for Raman microscopy.


