FINAL REPORT

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1.0 EXECUTIVE SUMMARY

This milestone report outlines the progress made by higher degree research (HDR) student Bridgette Logan and her associated research project. She is working on a project called "Verification of grass-fed beef claims using Spectroscopic technologies" that is funded through the MLA NSW DPI Donor Company. This project focuses on using Raman Spectroscopy to identify and differentiate carcases from various production systems. The HDR student is financially supported by AMPC, who has provided a two-year stipend, and by the Graham Centre for Agricultural Innovation at Charles Sturt University, who have provided two years of tuition fees. Ms Logan is supervised by Prof. Leigh Schmidtke (Charles Sturt), Prof. John Mawson (Charles Sturt adjunct), Prof. David Hopkins (NSW Department of Primary Industries) and Dr. Stephanie Fowler (NSW Department of Primary Industries). Through her research the student has completed four statistical courses, presented at two international conferences, conducted two experiments sampling cattle from across Australia and published a research article in the Meat Science journal.

The current method of verifying the production system of beef is dependent on audits and reliant on producers following the requirements set by processors, which vary for individual grain and grass-fed brands. With different requirements set by different brands there is a lack of transparency on what constitutes grass or grain fed beef products as each allows different feedstuffs. Maintaining the current system of transparency through the supply chain for grass fed beef represents a significant cost to the industry in the form of auditing and an even greater potential cost if there is a failure in the auditing process. If the grass-fed claim was unable to be substantiated during a challenge, Australia risks losing market access to some markets which would result in a large economic loss.

Therefore, the need for a non-destructive on-site method to differentiate between production systems is evident. The aim of this post graduate students research was to test the viability of Raman Spectroscopy (RS) to accurately differentiate between production systems. Raman spectroscopy investigates molecular vibrations that can be used for functional group identification and compositional analysis. Testing of subcutaneous fat provides the opportunity to determine the fatty acid composition which reflects the diet cattle have been consuming as it is the primary depot of storage of fatty acids within the body and is sensitive to changes in the diet.

This study provided information on the feasibility of the joint application of Raman spectroscopy as an automated, non-destructive and rapid technique in the range of 600 -2000 cm^{-1} and pattern recognition of unsupervised (PCA) and supervised (PLS -DA) techniques to classify the production system of cattle between grain and grass-fed. While PCA was successful, this technique is better suited to preliminary analysis of spectra and testing of pre-processing techniques as PLS-DA provides more robust results. PLS-DA resulted in a model that was able to accurately predict grain (R^2 = 0.74) and grass-fed (R^2 = 0.74) carcases with a with a root mean square error prediction (RMSEP) of 0.28 and 0.15 respectively.

This investigation indicates that there is an ability to discriminate carcases from grass and grain-fed cattle using Raman spectra. Differences in the mean spectra from the carcases from grain-fed and grass-fed cattle are associated with intensities at wavelengths 1069 cm^{-1}, 1127 cm^{-1}, 1301 cm^{-1} and 1445 cm^{-1} which have been demonstrated to characterise the C-C and CH_2 vibrations of fatty acids. This was confirmed by the measurement of the fatty acid composition which demonstrated significant

differences in fatty acids including iso-C15:0, C15:0, C16:0, C17:0, C18:0 and C20:0. However, further research is needed as the increase in intensity at 1658 cm $^{-1}$ could not be explained by any phenomena. The effect of β- carotene was not able to be classified in the spectra, however β- carotene may be important for discriminating between samples from grass-fed cattle supplemented with various non-cereal feed sources.

Currently Raman Spectroscopy is a promising method for discrimination between grass and grain fed production systems and shows the potential to be used to predict the production system of origin for beef products on site. Further research including building a larger model with samples from across Northern and Southern production systems is being conducted by the student as she moves into her PhD. Expanding this research to investigate samples from various levels of grass and grain supplementation will prove difficult in the current climate but will enhance the quality of this research. Developing a more robust model will enable this technology to be adopted through processing facilities to be a rapid test for carcases to scientifically confirm the production system.

The HDR student was able to present her work at two international conferences. These conferences provided the opportunity for the student to develop public speaking experience and to receive feedback on her research including the discussion of new ideas for research. These conferences also fostered the opportunity for networking with experts in the field to develop connections to enhance future research. By conducting this project, the HDR student has enhanced their writing skills and has gained a greater understanding of what is required in research. This project has enabled the development of critical thinking and the ability to present information in a logical method.

2.0 INTRODUCTION

The student has completed two years of HDR research focused on the topic “Verification of grass-fed beef claims using Spectroscopic technologies”. Through this research the student has transitioned into a PhD program. The purpose of this project was to enable the HDR student to conduct her studies to advance her capacity for industry-based applied R&D, business development and innovation, and the introduction of new technologies in the industry.

The method that livestock are produced and raised is increasingly of concern to consumers. Consumers are becoming increasingly interested in products from pasture based or grass-fed beef production systems as they are perceived as low input production systems with improved animal health and welfare (Holman, van de Ven, Mao, Coombs, & Hopkins, 2017; Verbeke, Pérez-Cueto, Barcellos, Krystallis, & Grunert, 2010). Marketing has influenced consumers’ perceptions of grass-fed meat products and consumers have developed an inherent trust and willingness to pay for product identified with a grass-fed beef label.

There is currently no clear verification system to substantiate the claim of grain and grass-fed beef meat products in Australia. Guidelines, certification and auditing of grass-fed production systems varies depending on the brand and auditing body and despite vendor declarations consumers are not given a clear guarantee of the authenticity of grass-fed product claims. This auditing process varies greatly between brands as each ‘grass-fed’ brand has their own requirements including what feedstuffs are allowed. The confusion caused by these differences in the supply chain of what constitutes grass-fed cattle is highlighted by a Galaxy Survey (2014) and BIS Shrapnel (2014) which indicate that 43% of Australian consumers consider grass-fed beef to be produced from cattle which eat only grass throughout their lives and identify the production system as natural, yet

supplementation with various feed sources is allowed depending on the brand. In particular for lamb and beef there is a preference for grass based production systems, as there are beliefs and expectations that the grass-fed meat is related to a healthier, tastier and more natural meat, and based on these assumptions consumers have accepted a premium price for products that are raised in a grass-fed system (Font-i-Furnols & Guerrero, 2014).

To validate an objective measure of beef production system there is a requirement to develop a rapid measure to discriminate carcases of similar characteristics. Raman spectroscopy investigates molecular vibrations that can be used for functional group identification and compositional analysis (Li-Chan, 1996). Indeed, much research has been conducted to differentiate between species such as pork, mutton, goat, chicken, turkey, beef and horse (Al Ebrahim, Sowoidnich, & Kronfeldt., 2013; Boyaci et al., 2014; Ellis, Broadhurst, Clarke, & Goodacre, 2005; Sowoidnich & Kronfeldt, 2012). This species discrimination is possible as spectral data shows the differences in chemical composition (Beattie, Bell, Borggaard, Fearon, & Moss, 2007).

Cattle sourced from different production systems have a different chemical profile as a result of their diet. Grass-fed beef has been found to have higher levels of omega-3 PUFAs and consequently is seen by the general public as healthier (Enser, Hallett, Hewitt, Fursey, & Wood, 1996). When examining the fatty acid profile of grass and grain-fed beef there is a significant difference due to the diets regardless of the effects from the gender, age, breed, and geographical location of the animals (Daley, Abbott, Doyle, Nader, & Larson, 2010a; De La Fuente et al., 2009; De Smet, Raes, & Demeyer, 2004; Garcia et al., 2008). Grain feeding stimulates adipogenesis in beef cattle, whereas pasture feeding depresses the development of adipose tissues (Smith, Kawachi, Choi, Choi, Wu, & Sawyer, 2009b). Grain-fed cattle are usually fed on a high-concentrate diet that results in increased activity of adipose tissue stearoyl-CoA desaturase, which is responsible for the conversion of SFA to their delta (Δ) 9 desaturated counterparts (Smith, Gill, Lunt, & Brooks, 2009a). Grain feeding has also been identified to cause a decrease in ruminal pH from 6.4 to 5.6 (Fuentes, Calsamiglia, Cardozo, & Vlaeminck, 2009) which has an ongoing effect on the ruminal microorganisms involved in isomerization and hydrogenation of PUFA (Smith et al., 2009a). As a result, continued long-term grain feeding causes elevated adipose tissue stearoyl-CoA desaturase and decreased hydrogenation of PUFA leading to an increase in MUFA's. Given that the fatty acid profiles of beef vary with production system (De La Fuente et al., 2009; Van Elswyk & McNeill, 2014), an investigation into the use of Raman spectroscopy to differentiate between carcases from grass-fed and grain-fed cattle was conducted with the aim of developing a method to scientifically verify the production systems of beef carcases.

This research has had to overcome several limitations involved in conducting this research. The last two years have seen New South Wales and Queensland in drought, and this has hindered the ability for research to be conducted on grass-fed and grass supplemented cattle due to the lack of supply. This limitation in combination with technical difficulties have caused delays in the presentation of data from the second phase although the samples have been collected.

3.0 PROJECT OBJECTIVES

Support 1 recent graduate into a Masters level program to further develop their capacity for industry-based applied R&D, business development and innovation, and the introduction of new technologies in the industry.

4.0 METHODOLOGY

The methodology for sampling carcases is described in full in the attached Appendix 1. This research involved the sampling of cattle across two phases. Phase 1 involved 300 cattle from two production systems while Phase 2 included 910 samples from seven production systems across Australia. Phase two samples were collected in the same manner as Phase 1.

Phase 1 has been completed with the collection analysis of samples from 150 grain and grass-fed cattle at Teys Wagga and JBS Brooklyn abattoirs respectively, resulting in a total of 300 cattle sampled. The method of sampling and analysis is described below.

At 24 hrs the subcutaneous fat over the point end brisket was measured using a Raman Mira hand-held device (Metrohm®) in 3 positions on the navel end brisket using an integration time of 5 sec and 3 accumulations. Once Raman spectroscopic measurements were conducted, objective fat colour was measured using a Minolta® CR- 400 Colour meter (Minolta 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) with CIE Lab results recorded.

Once Raman spectroscopy and fat colour measurements were completed, a 30 g sub-sample of subcutaneous fat was removed from the measurement site and frozen at -20°C for transport. Further information including kill data such as body number, lot number, carcase weight, fat score and fat colour as well as background information as provided to the abattoir was also collected.

Prior to analysis for β- carotene content and fatty acid (FA) composition, samples were stored at -80°C before being freeze dried, and homogenised using a Foss KnifeTech® grinder for 15 s. β- carotene content was analysed using a method based on Yang, Larsen, and Tume (1992). In short, 1 g of the prepared subcutaneous tissue was saponified in 2 mL methanolic 20% potassium hydroxide (KOH), centrifuged and incubated at 65°C for 45 min, 6 mL of distilled water was then added, and the samples allowed to cool under running water. β -carotenes were extracted twice in 8 mL diethyl ether with 0.004% butylated hydroxytoluene (BHT) and the extracts washed with 16 mL of distilled water three times to remove any KOH. Sodium sulfate, dried at 100°C, was then added to remove any residual water from the extracts, prior to the extracts being filtered and evaporated to dryness under a stream of nitrogen. The residual sample was redissolved in 200 µL ethanol for grain fed samples and 500 µL ethanol for grass fed samples.

The β-carotene concentration was determined on an Agilent 1290 high performance liquid chromatography (HPLC) system, with methanol: water (99:1 v/v) as the mobile phase, using a flow rate of 0.6 mL/minute. An Agilent Zorbax Eclipse Plus C18 Rapid Resolution (2.1 x 50 mm) column with column guard was used. The β-carotene peak was measured at 450 nm using a photodiode array detector (PDA) and data were analysed using Agilent OpenLab software. A calibration curve of β-carotene pharmaceutical secondary standard (Sigma Aldrich, PHR129) was used to determine the β-carotene concentration.

Fatty acid concentrations were completed using a one-step extraction based on the method of (Lepage & Roy, 1986). Extraction of fatty acids was achieved by using 10 mL of chloroform/methanol mixture (2:1 v/v) added to the sample, shaken and centrifuged. Once extracted, an aliquot of 80 – 100 µl was evaporated to dryness under nitrogen gas. Once evaporated, the mixture was methylated using 2 mL of methanol/toluene mixture (4:1 v/v) containing C13:0 (4 µg/mL) and C19:0 (4 µg/mL) as internal standards, 200µL of acetyl chloride and 5 mL of a 6% potassium carbonate solution. Once extracted and methylated, fatty acids were identified from 80 µL of FAME using an Agilent 6890N gas chromatograph (GC) equipped with a SGE BPX70 analytical column.

Prior to statistical analysis, the 3 spectra per carcase were averaged and the wavelengths reduced to 600 – 1800cm^{-1} and continuum correction was then applied to correct for non-Raman background contributions. During this process, local minima points on each spectra are identified and connected by linear interpolation to make a set of continuum points c_i. The observed intensities x_i are then scaled to continuum corrected values by ratio:

$$\theta_i = \frac{x_i}{c_i}$$

Principal component analysis was then undertaken, and peaks of interest were identified numerically by taking second differences.

A two class (grass vs grain) partial least square-discriminant analysis (PLS-DA) was constructed using standard normal variates (SNV) scaling and mean centering (MC) corrected data. The number of latent variables (LV) chosen for modelling was determined by inspection of the eigenvalues and minima of the root mean square errors of calibration (RMSEC), cross validation (RMSECV) and prediction (RMSEP) for each LV. Cross validation of the model was achieved using random subsets of the calibration data with 10 data splits. To avoid overfitting of the data, a permutation test of the calibration data set was performed to asses overall model efficacy. The data was permuted 1000x and an empirical p-value for Q^2 and prediction efficacy determined. To determine overall predictive model accuracy the final model was tested against an independent test data set with prediction accuracy assessed using receiver operator curves and misclassifications. All PLS-DA statistical analysis was conducted utilising Matlab 2019a (The Mathworks Inc., Natick, Massachusetts, USA) and PLS Toolbox version 8.7.1 (Eigenvector Research Inc., Wenatchee, WA, USA).

Evaluation of the predictive model was conducted by determining the predictive uncertainty, at the set level, of the independent test set by calculating the root mean square error of prediction (RMSEP). RMSEP is a useful tool that describes the average difference between the measured and predicted values for each sample as it is calculated by summing all squared prediction errors during cross validation and provides the predictive ability of the model.

In a PLS-DA y values can be transformed into a class membership; this class membership can then be set as a discrimination threshold and the predicted samples will be assigned a class. The assigned class is compared to the true class membership and classified as a True Positive (TP), True Negative (TN), False Positive (FP) or a False Negative (FN). The total number of TP, TN, FP and FNs can be computed to create a confusion matrix (Brøndum, Munck, Henckel, Karlsson, Tornberg, & Engelsen, 2000) which summarises the predictive ability of the model

When evaluating the test data set against the calibration model it produced a precision value of 0.94 for grain and 1.00 for grass fed and resulted in a Matthews correlation coefficient of 0.94. The Matthews correlation coefficient (MCC) was calculated by:

$$MCC = \frac{TP \times TN - FP \times FN}{\sqrt{(TP + FP)(TP + FN)(TN + FP)(TN + FN)}}$$

By assessing the spectra and loadings from the latent variables key areas were established that reported a Variable Importance in Projection score over 1 and show the regions contributing to the classification between grass and grain.

Analysis for differences between the fatty acid composition, β-carotene and objective fat colour measures were completed using linear mixed effects models, deriving predicted means and standard errors and calculating least significant differences between means (at the P = 0.05) for the traits measured from the carcases of each feed type (grass and grain). To account for any batch effects, day of measurement was included as a fixed effect. PCA, objective colour, fatty acid and β-carotene statistical analyses were completed in R Core Software (R Core Team, 2017) using the 'emmeans' package (Lenth, Love, & Herve, 2017) and prospectr package (Stevens & Ramirez-Lopez, 2014).

Phase 2 has been completed incorporates the collection of samples from 1040 cattle from across extensive and intensive production systems in Northern and Southern Australia. From each of the following productions systems 130 samples were collected, with the exception of the Northern grass fed due to a lack of supply given the drought:

- **Southern long grain fed**
- **Northern long grain fed**
- **Southern short grain fed**
- **Northern short grain fed**
- **Southern grass fed**
- **Northern grass fed**
- **Southern supplemented grass fed**

Statistical analysis for Phase 2 is still being conducted by combining PCA and PLSDA techniques outlined above.

5.0 PROJECT OUTCOMES

5.1 Research Results

Differences were observed in the intensity of peaks at 1069 cm $^{-1}$, 1127 cm $^{-1}$, 1301 cm $^{-1}$, 1445 cm $^{-1}$ and 1658 cm $^{-1}$ (Fig. 3). The carcases from grain-fed cattle show a higher intensity at the wavelengths 1069 cm $^{-1}$, 1127 cm $^{-1}$, 1301 cm $^{-1}$ and 1445 cm $^{-1}$. Notably, grain-fed cattle did not have a higher intensity at 1658 cm $^{-1}$, as this peak was highest in the carcases from grass-fed cattle (Figure 1).

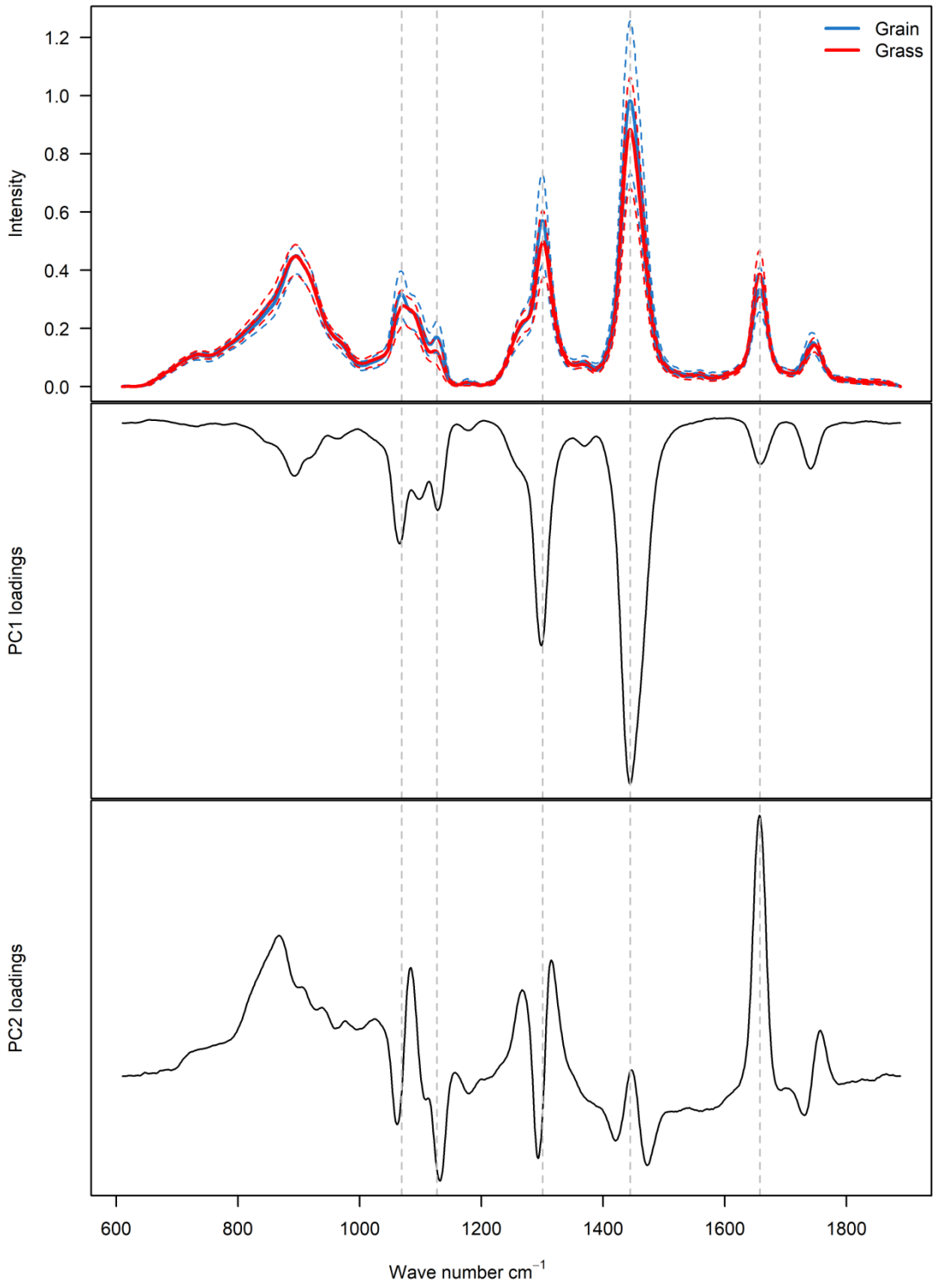

Figure 1. Raman spectra and loadings of the first two principal components collected from 24 h postmortem subcutaneous fat of 150 grass-fed (red) and 150 grain-fed (blue) beef cattle carcases. Dashed spectral lines show the 5% and 95% quantiles for each group. Vertical lines show the wavelengths of interest at local maxima identified by numerical differentiation of the spectra.

Carcases from grain-fed cattle had significantly higher saturated fatty acids (SFA) concentrations (11.1 g/100 g) compared to their grass-fed counterparts (8.3 g/100 g). This was due to differences in individual SFAs including C15:0, C16:0, C17:0, C18:0 and C20:0. While there was no significant differences detected in the total concentration of monounsaturated fatty acids (MUFA) in carcases from grass (12.1 g/100 g) and grain-fed cattle (13.6 g/100 g) in particular with individual fatty acids, most notably in C15:1n-5, C16:1n-7t, C17:1n-7, C18:1n-7t, C18:1n-9, C18:1n-9t, C20:1n-9, C20:1n-15 and C24:1n-9 (Table 1.). The carcases from grass-fed cattle were higher in concentration of omega-3 fatty acids (173.7 mg/100 g) compared to grain-fed (87.6 mg/100 g), this is mainly due to the differences in C18:3n-3, C20:3n-3, C20:4n-3, C20:5n-3 and in C22:5n-3. Carcases from grain-fed cattle (400.4 mg/100 g) exhibited a higher concentration of omega-6 fatty acids than carcases from grass-fed cattle (241.8 mg/100 g). Thus, there was a significant difference in the omega-6 to omega-3 ratio between the carcases from grass-fed and grain-fed cattle (1.5 mg/100 g and 5.1 mg/100 g, respectively). There was a significant difference in individual polyunsaturated fatty acids (PUFA) C16:3n-4, C16:2n-4 and C20:3n-9, although no difference was found between cattle from the grass and grain finishing system in the total of PUFA.

Table 1. Least square means (LSM) and standard errors (s.e.) of the subcutaneous fatty acid (FA) composition from carcases of 150 grass-fed and 150 grain-fed beef cattle.

	Fatty acid	Grain-Fed		Grass-Fed	
		LSM	s.e.	LSM	s.e.
SFA (mg/100g)	C10:0	31.1	2.22	24.3	2.22
	C12.0	25.2	3.54	24.6	3.54
	C14:0	837.4	67.48	682.7	67.41
	iso-C15:0	26.5a	2.02	49.7b	2.02
	anteiso-C15:0	30.9	4.19	45.3	4.19
	C15:0	148.7b	8.65	113.7a	8.63
	C16:0	6355.2b	256.09	4928.3a	255.46
	iso-C17:0	26.5a	2.02	49.7b	2.02
	anteiso-C17:0	168.2a	7.49	220.6b	7.46
	C17:0	385.4b	21.11	193.7a	21.06
	C18:0	2921.6b	117.28	1872.3a	116.88
	C20:0	18.9b	0.54	14.2a	0.54
	C21:0	39.6	1.89	37.3	1.89
	C22:0	14.7	8.86	2.8	8.74
	C23:0	0.1	0.04	0.1	0.04
	C24:0	1.5	0.30	2.3	0.30
MUFA (mg/100g)	C14:1n-5	365.9	44.28	430.7	44.23
	C15:1n-5	2.3b	0.26	0.4a	0.26
	C16:1n-7	1328.2	112.42	1557.0	112.26
	C16:1n-7t	21.0b	1.57	12.6a	1.57
	C17:1n-7	36.2a	0.83	42.1b	0.83
	C18:1n-7	474.3	29.18	392.2	29.12
	C18:1n-7t	850.9b	39.33	217.0a	39.07
	C18:1n-9	11286.3	441.03	9665.8	439.73
	C18:1n-9t	118.5b	7.98	52.5a	7.90
	C20:1n-9	81.2b	3.80	48.6a	3.80
	C20:1n-15	10.8b	0.66	6.5a	0.66
	C22:1n-9	2.4	0.39	1.6	0.39
	C24:1n-9	0.9b	0.08	0.6a	0.08
PUFA (mg/100g)	C16:2n-4	6.0a	0.30	7.9b	0.30
	C16:3n-4	3.2b	0.15	2.2a	0.15
	C18:2n-6	356.5b	16.6	203.5a	16.6
	C18:2n-6t	204.2	10.10	195.9	10.06
	C18:3n-3	51.1a	5.65	109.2b	5.65
	C18:3n-4	3.8	0.27	3.6	0.27
	C18:3n-6	6.2	0.54	6.1	0.54
	C18:4n-1	7.0	7.16	17.2	7.16
	C18:4n-3	16.1	2.26	19.8	2.26
	C20:2n-6	7.9b	0.21	4.5a	0.21
	C20:3n-3	3.3a	0.33	5.6b	0.33
	C20:3n-6	14.2	0.65	14.8	0.65
	C20:3n-9	3.0a	0.23	4.1b	0.23

	C20:4n-3	3.8a	1.08	11.9b	1.08
	C20:4n-6	10.0	0.29	9.4	0.29
	C20:5n-3	2.9a	0.61	7.1b	0.61
	C22:2n-6	0.9a	0.08	1.4b	0.08
	C22:4n-6	4.7b	0.64	1.2a	0.64
	C22:5n-3	9.0a	1.38	18.6b	1.38
	C22:5n-6	0.1	0.38	1.0	0.38
	C22:6n-3	1.3	0.25	1.4	0.25
	Cis 9 t11CLA	67.8	15.73	110.9	15.71
	Trans 10c12CLA	3.7	0.26	3.4	0.26
	Trans	1.2b	0.05	0.5a	0.05
Totals (mg/100g)	CLA	0.1	0.02	0.1	0.02
	Omega-3	87.6a	10.2	173.7b	10.2
	Omega-6	400.4b	16.91	241.8a	16.83
	Omega-6:omega-3	5.1b	0.51	1.5a	0.51
	PUFA	0.7	0.03	0.6	0.03
Totals (g/100g)	MUFA	13.6	0.57	12.1	0.57
	SFA	11.1b	0.43	8.3a	0.43

Different letters within rows indicate significance between means ($P < 0.05$).

Subcutaneous fat from carcases from grain-fed cattle was significantly lighter in colour than fat from carcases from grass-fed cattle, seen by the significant difference in L^* values (72.9 and 68.4 respectively). This trend was not seen in a^* values which showed the subcutaneous fat from carcases from grass-fed cattle to be redder (19.0) than the grain-fed (13.6). Although significant differences were found in the lightness and redness there was no difference detected for b^* values.

Predictive models utilising PLS-DA were successfully constructed to discriminate between grain-fed and grass-fed carcases with a coefficient of determination (R^2) of 0.74 with a RMSEP of 0.26 and 0.15 respectively (Table 2.). When evaluating the test data set against the calibration model it produced a Matthews correlation coefficient of 0.94. This model produced a 99 % accuracy of predicting the correct sample class in the current study (Table 3.). By assessing the spectra key areas were established with a VIP score over 1 (Figure 2). The key peaks contributing the most to the discrimination of classes are identifiable at 1066 cm^{-1}, 1130 cm^{-1}, 1301 cm^{-1}, 1440 cm^{-1} and 1658 cm^{-1}. These PLS-DA results agree with the peaks identified in the PCA.

Table 2. Model Statistics from a Partial Least Square Discriminant Analysis with 6 Latent Variables developed from Raman Spectra of the subcutaneous fat from 150 grain fed and 150 grass fed beef carcases.

	Grain	Grass
Sensitivity (Cal):	1.000	1.000
Specificity (Cal):	1.000	1.000
Sensitivity (CV):	0.978	0.954
Specificity (CV):	0.954	0.978
Sensitivity (Pred):	1.000	0.931
Specificity (Pred):	0.931	1.000
Class. Err (Cal):	0	0
Class. Err (CV):	0.0340649	0.0340649
Class. Err (Pred):	0.0344828	0.0344828
RMSEC:	0.152792	0.152792
RMSECV:	0.246568	0.246568
RMSEP:	0.257143	0.152792
Bias:	-5.55112e-17	0
CV Bias:	-0.0036973	-0.0036973
Pred Bias:	-0.00659322	0.00659322
R^2 Cal:	0.906612	0.906612
R^2 CV:	0.761956	0.761956
R^2 Pred:	0.736368	0.736368

Table 3. Confusion matrix for test set against the calibration and cross-validated data set for Raman spectra of beef subcutaneous fat.

Class	TPR	FPR	TNR	FNR	N	Err	P	F1
Grain	1.00000	0.0690	0.9310	0.0000	31	0.03333	0.9394	0.9688
Grass	0.9310	0.00000	1.00000	0.0690	29	0.03333	1.00000	0.9643

Abbreviations: TPR, true positive rate; FPR, false positive rate; TNR, true negative rate; FNR, false negative rate; N, number of test samples.

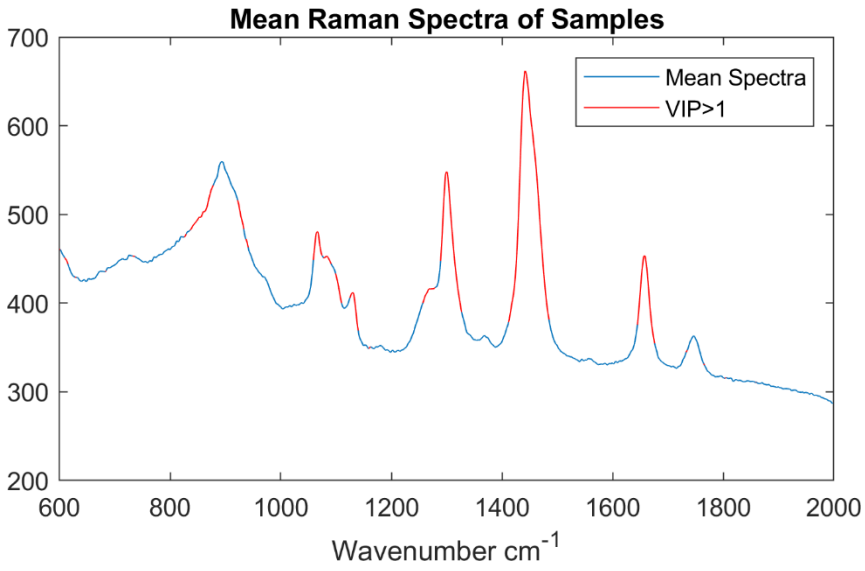

Figure 2. Mean Spectra overlaid with the Variable Importance in Projection (VIP; >1) calculated from the comprehensive PLS-DA models with Raman Spectra data.

Phase 2 results are still ongoing however spectral analysis of four production systems located within the Southern Production zones have been studied. Predictive models utilising PLS-DA were successfully constructed to discriminate between 100-day grain fed (Grain Long), 60-day grain fed (Grain Short), grass only (Grass) and grass supplemented (Grass Supplemented) carcases. This PLS-DA resulted in a coefficient of determination (R^2) with a root mean square error of prediction (RMSEP) for Grain Long (0.79; 0.21), Grain Short (0.53; 0.32), Grass (0.39; 0.32) and Grass Supplemented (0.55; 0.29) (Table 4.). This model produced an 87% accuracy of predicting the correct sample class in the current study (Table 5.).

Table 4. Model Statistics from a Partial Least Square Discriminant Analysis with 9 Latent Variables developed from Raman Spectra of the subcutaneous fat from beef carcases sourced from four production systems within Southern Australia.

	Grain Long	Grain Short	Grass	Grass Supplemented
Sensitivity (Cal):	0.990	0.950	0.948	0.972
Specificity (Cal):	0.993	0.924	0.935	0.983
Sensitivity (CV):	0.970	0.876	0.877	0.940
Specificity (CV):	0.978	0.906	0.914	0.966
Sensitivity (Pred):	1.000	0.900	0.684	0.870
Specificity (Pred):	0.986	0.873	0.890	0.949
Class. Err (Cal):	0.00825083	0.0628289	0.0585092	0.0224362
Class. Err (CV):	0.0257426	0.109039	0.104315	0.0470783
Class. Err (Pred):	0.00694444	0.11338	0.212773	0.0908584
RMSEC:	0.197242	0.260743	0.262307	0.228431
RMSECV:	0.223183	0.301799	0.299353	0.264433
RMSEP:	0.206313	0.316582	0.323904	0.291903
Bias:	-5.27356e-16	-6.66134e-16	8.32667e-16	3.88578e-16
CV Bias:	0.00263063	-0.00659197	0.00139074	0.0025706
Pred Bias:	-0.0106068	-0.00134034	0.0348663	-0.0229191
R^2 Cal:	0.792511	0.63498	0.620194	0.732001
R^2 CV:	0.737897	0.515948	0.511854	0.646023
R^2 Pred:	0.793102	0.529777	0.39116	0.545722

Table 5. Confusion matrix for test set against the calibration and cross-validated data set for Raman spectra of beef subcutaneous fat from Southern Australian production systems.

Class	TPR	FPR	TNR	FNR	N	Err	P	F1
Grain Long	0.93103	0.00000	1.00000	0.06897	29	0.01980	1.00000	0.96429
Grain Short	0.83333	0.05634	0.94366	0.16667	30	0.08911	0.86207	0.84746
Grass	0.89474	0.06098	0.93902	0.10526	19	0.06931	0.77273	0.82927
Grass Supplemented	0.82609	0.05128	0.94872	0.17391	23	0.07921	0.82609	0.82609

Abbreviations: TPR, true positive rate; FPR, false positive rate; TNR, true negative rate; FNR, false negative rate; N, number of test samples.

5.2 Post Graduate Student Outcomes

The student has advanced and continued her studies and research in the form of a PhD. The student has developed and enhanced their capacity for industry-based applied R&D and the introduction of new technologies in the industry. The student has successfully published her first research paper (Appendix 1) in Meat Science and has drafted an additional two papers with the intent to publish the findings from her research.

Through time spent working with the Oil and Feed Laboratories at NSW DPI, Wagga Wagga, the postgraduate research student has progressed their knowledge and understanding where she learnt the protocol for extracting β-carotene and fatty acids from subcutaneous fat. This provided an opportunity to develop her skills and abilities and facilitated a greater understanding of the methodology. The postgraduate student has completed four statistical courses to expand her knowledge of statistical analysis chemometrics in order to use them to understand the spectral data. In her two years of study she has attended three statistical workshops focusing on using the 'R Studio' program as well as learning how to use the 'Matlab' program for analysis. During time spent with her University supervisor at CSU Wagga Wagga, Prof. Leigh Schmidtke, she has advanced her understanding and capabilities in using Matlab. In an intense week-long workshop focused on using chemometric techniques to evaluate spectroscopic results, Bridgette Logan also had the opportunity to receive guidance and assistance from leading chemometrician, Doug Rutledge.

The post graduate student has also had the opportunity to present her research at two international conferences including the tenth International Conference on Advanced Vibrational Spectroscopy and the 65th International Congress of Meat Science and Technology (Appendix 2, 3, 4 and 5). During the International Congress of Meat Science and Technology there was the potential to attend a PhD course focusing on the Underlying mechanisms of meat quality and animal welfare as well as Raw fermented sausages from tradition to Innovation at the Max Rubner Institut and University of Hohenheim. These conferences provided an opportunity to gain a greater understanding of the research being conducted in meat science across the world.

These courses and experiences have encouraged fresh ideas for future work and has enabled the recognition of gaps in her knowledge.

6.0 DISCUSSION

This research has been the first to identify production system of origin using Raman Spectroscopy. Understanding this technology better will allow for uptake by processing companies and be able to be used in place or as an extra support for auditing of cattle production systems. The results for phase 1 are displayed below and a full paper has been published using this data (Appendix 1) with an additional two papers still being prepared based on these results in combination with additional information.

Raman spectra in combination with chemometric modelling were able to discriminate between carcases from cattle finished on grass and grain with the first 2 PCA components explaining 93% of variation in the spectra. This was due to distinct differences in the spectra at key intensities including 1069cm $^{-1}$, 1127 cm $^{-1}$, 1301 cm $^{-1}$, 1445 cm $^{-1}$ and 1658 cm $^{-1}$.

Predictive models utilising PLS-DA were successfully constructed to discriminate between grain-fed

and grass-fed carcases with a coefficient of determination (R^2) of 0.74 with a RMSEP of 0.26 and 0.15 respectively. The peak at 1066 cm $^{-1}$ has been identified as being a spectral feature of fatty acids including the *v*(C-C) trans conformation (Lakshmi, Kartha, Krishna, Solomon, Ullas, & Devi, 2002). The peak at 1130 cm $^{-1}$ is identifiable as the C-C skeletal stretch trans conformation (Notingher et al., 2004). The peak at 1301 cm $^{-1}$ has been hypothesised and observed in many formulas, the most common marker is as an indicator for lipids (Lakshmi et al., 2002) as well as triglycerides (Silveira, Sathaiah, Zangaro, Pacheco, Chavantes, & Pasqualucci, 2002). Typically, the chemical bond associated with this peak is C-H vibration and CH_2 twisting (Notingher et al., 2004). Through the PLS-DA model the peak at 1440 cm $^{-1}$ was highlighted and identified as having three main chemical structures including the CH_2 (Hanlon et al., 2000) and CH deformation (Krafft, Neudert, Simat, & Salzer, 2005) along with CH_2 in the bending formation (Koljenović, Schut, Vincent, Kros, & Puppels, 2005; Lakshmi et al., 2002). Polyunsaturated fatty acids (PUFAs) have been identified as the contributor to the spectral peak observed at 1658 cm^{-1} (Movasaghi, Rehman, & Rehman, 2007).

This investigation assessed the feasibility of using Raman spectroscopy to detect the known differences in the chemical composition of subcutaneous fat of carcases from cattle raised under different production systems. Grass fed cattle had a lower omega-6 to omega-3 ratio but did not differ from grain-fed cattle in terms of total polyunsaturated fatty acids. The peak at 1658 cm $^{-1}$ in grass fed cattle as more difficult to classify and requires further investigation of the bonds reflected in this peak. The increase in spectral signals at 1658cm^{-1} which demonstrated higher intensities in spectra from the fat of grass-fed cattle arose from the C=C bonds of the omega-3 fatty acids. However, the true origin of this peak is difficult to determine as the C=C bond from polyunsaturated fatty acids would also contribute to the increase in intensity at this peak. Previous research conducted by Afseth, Segtnan, Marquardt, and Wold (2005) has highlighted that the *cis* C – C bond is evident at approximately 1656cm^{-1}. The difference in this band may also arise from a greater number of *cis*- fatty acids present in the fat from grass fed beef, and although not significantly different, the fat from grass fed cattle showed a higher concentration of *cis*- CLA (110.9 mg/100 g) compared to grain fed cattle (67.8 mg/100g). This is likely given that grass fed ruminants have been shown to produce 2 -3 times more CLA than ruminants fed in confinement on a high grain diet due to a more favourable rumen pH (Daley, Abbott, Doyle, Nader, & Larson, 2010b).

Polyunsaturated fatty acids are mainly bound in the phospholipid membranes incorporated into the myofibril and do not significantly differ even within intramuscular fat deposits (Fowler, Ponnampalam, Schmidt, Wynn, & Hopkins, 2015) and have been found to hydrogenate and be broken down into saturated fatty acids in the rumen before deposition in the subcutaneous fat (Oyebade, Lifshitz, Lehrer, Jacoby, Portnick, & Moallem, 2019; Petri, Vahmani, Yang, Dugan, & McAllister, 2018). However, there was a difference in the concentrations of omega-6 and omega-3 fatty acids which is consistent with previous research on the fatty acid composition of grass and grain fed beef cattle carcases (Daley et al., 2010b).

Saturated fatty acids were significantly ($P < 0.05$) higher in grain-fed cattle (11.1 g/100g ± 0.43 s.e.) than grass-fed cattle (8.3 g/100g ± 0.43 s.e.) and were observed in the spectra as peaks at 1069 cm $^{-1}$, 1127 cm $^{-1}$, which have been associated with the measurement of grain fed beef carcases characterise the C-C bonds that constitute the long chain saturated fatty acids (Beattie, Bell, & Moss, 2004). The increases in individual saturated and monounsaturated fatty acids evident in the subcutaneous fat from grain fed carcases, particularly C15:0, C16:0, C17:0, C18:0, C20:0 C15:1n-5, C17:1n-7, C18:1n-9,

C20:1n-9, C20:1n-15 and C24:1n-9, C16:1n-7t, C18:1n-9t and C18:1n-7t also explain spectral differences evident in peaks at wavelengths 1301cm^{-1} and 1445cm^{-1} which reflect the CH_2 twist and scissor vibrations. This is consistent with previous research on fatty acid composition of grass and grain fed cattle which has demonstrated that grain fed cattle consistently yield higher concentrations of saturated and monounsaturated fatty acids (Daley et al., 2010b).

Currently β-carotene has not been able to be isolated in the spectra due to the intensity of the fat signals, they may be important for characterising cattle which are grazing grass and supplemented with grain from cattle which are in short term feedlot finishing systems. The large differences in β-carotene concentration have previously been utilised to distinguish between carcases and are measurable in large quantities indicating more research to understand the impact of β-carotene on the spectra would be beneficial to further research, Thus, reference measurements of β-carotene are also required to determine where the spectral signals are likely to occur and how the strong Raman signals of fat affects the spectra of β-carotene as proposed in the research proposal.

Southern production systems analysis provided an insight into the various levels of feeding regime located within production systems. Using a model developed on a calibration and test data set excluding the phase 1 data resulted in a model that was able to discriminate all the Grain Long samples. This model does have some shortfalls as it is not able to predict every class with a high degree of accuracy as the grass and grass supplemented results showed some misclassification of samples as Grain Short. This research is preliminary and further investigation will yield results that can be linked to the fatty acid data to understand the differences in the samples that were misclassified. This research is showing the potential to discriminate between production systems in Australia using Raman Spectroscopy.

7.0 CONCLUSIONS/RECOMMENDATIONS

The student has been approved to transition into a PhD program to expand her research and explore various avenues of utilising Raman spectroscopy for the meat industry. As such there will be a continuation of research into an additional year, allowing for advancement of knowledge and development of industry connections and deeper understanding.

Phase 1 has highlighted that Raman spectroscopy is able to discriminate between carcases of grass and grain-fed beef cattle which we believe is due to the fatty acid composition changes that are being detected. Phase 2 samples have been collected from seven production systems to provide a sample from the common production systems in Australia, although the fatty acid and β-carotene analysis is still under way. This research will be analysed and published in a scientific journal and used as a part of the HDR student's thesis. These phase 2 samples provide crucial information that can be used to test the model developed in phase 1. This study has identified gaps in knowledge including investigating the influence of fat depth and location of sampling with the Raman spectroscopy device. Currently this research has focused on cattle and in the future, there is a need to examine the potential for Raman Spectroscopy to be utilised to assess the effect of grain feeding on lamb.

Overall this project has enabled a HDR student to advance her research and complete sampling for her experiments that demonstrates that Raman Spectroscopy is a promising technology that can be utilised in the future for the verification of grass and grain-fed beef products produced in Australia.

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9.0 APPENDICES

9.1 Appendix 1. Preliminary investigation into the use of Raman Spectroscopy for the verification of Australian grass and grain-fed beef (submitted in a separate document)

9.2 **Appendix 2.** 10th International Conference of Vibrational Spectroscopy Abstract

Verification of the Production System of Beef Products using Spectroscopic Technologies

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Keywords: Food security and quality, Raman Spectroscopy, Beef, grass-fed vs grain-fed

The system by which meat products have been produced is becoming an increasing concern to consumers [1] and the interest in products from pasture based or grass-fed beef production systems is growing as they are perceived as low-input production systems with improved animal health and welfare, providing a wholesome product to consumers[2,3] . Australian grass and grain-fed beef products attract premium prices at sale and several beef processor's now market beef underwritten by production system claims. There is currently no clear verification system to substantiate the claim of grain and grass-fed beef meat products in Australia. Guidelines, certification and auditing of grass-fed production systems varies depending on the brand and auditing body and despite vendor declarations consumers are not given a clear guarantee of the authenticity of grass-fed product claims. This investigation assessed the feasibility of using Raman spectroscopy to detect the known differences in the chemical composition of subcutaneous fat of carcases from cattle raised under different production systems (e.g. grass vs grain).

The Raman spectra and fatty acid profile were measured on 150 grass and 150 grain-fed cattle. Saturated fatty acids were significantly ($P < 0.05$) higher in grain-fed cattle (11.1 g/100 g ± 0.43 s.e.) than grass-fed cattle (8.3 g/100 g ± 0.43 s.e.) and differences were observed in the spectra as peaks at 1069 cm $^{-1}$, 1127 cm $^{-1}$, 1301 cm $^{-1}$ and 1445 cm $^{-1}$ (Figure 1.). Grass fed cattle had a lower omega-6 to omega-3 ratio, but did not differ from grain-fed cattle in terms of total polyunsaturated fatty acids. The peak at 1658 cm $^{-1}$ in grass fed cattle is more difficult to classify and requires further investigation of the bonds reflected in this peak. Grass-fed and grain-fed cattle are able to be successfully differentiated through the use of Raman spectroscopy.

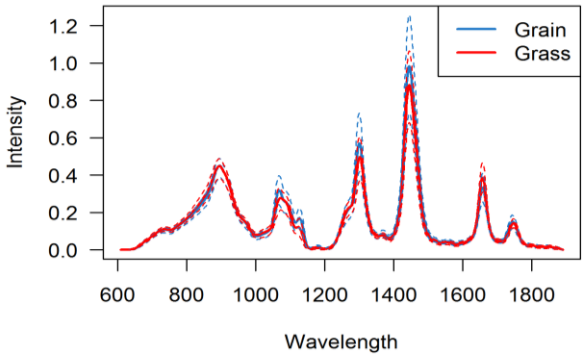

Figure 1. Mean Raman spectra collected from the subcutaneous fat of 150 grass-fed (red) and 150 grain-fed (blue) beef cattle carcases where the dashed lines show the 5 – 95% quantiles.

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9.3 Appendix 3. 65th International Congress of Meat Science and Technology Abstract (submitted as a separate document)

9.4 Appendix 4. 65th International Congress of Meat Science and Technology (submitted as a separate document)