Department of Primary Industries
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Final report

Verification of grass and grain fed beef using spectroscopic technologies

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Abstract

The interest in products from grass fed beef production systems is increasing as they are perceived by some as low input with improved animal welfare and producing healthier beef. While Australia is currently a leading exporter of grass-fed beef as it is seen as “clean and green”, to maintain this position it is vital production systems are transparent and underpinned by clear cues for production systems and raising claims which can be translated into consumer descriptions. Therefore, this project aimed to provide the beef industry, through processors, a scientific based method for the verification of production system of origin.

To this end, 1940 beef carcases from grass-fed, supplemented grass-fed, short-term and long-term grain-fed production systems in New South Wales and Queensland were measured using a Raman spectroscopic device and feeding system was substantiated via fatty acid composition. Overall, it was demonstrated Raman spectroscopy is a robust tool with 86% of grass-fed cattle correctly classified in combined north/south validation models. Yet, accuracy was improved by up to 23% when models were separated based on region. Therefore, this project provides the evidence required to demonstrate the potential for Raman spectroscopy to verify production system of origin and the initial calibration and validation models which could be utilised in commercialisation.

Executive summary

Background

Australia is currently a leading exporter of grass-fed beef as it is accepted by some as being "clean and green" with a high standard of animal welfare, low environmental impact and a wholesome source of health beneficial fatty acids. Yet, to maintain this reputation it is vital that Australia has a transparent beef supply chain, which is underpinned by clear and trustworthy guidelines, auditing processes and quality assurance procedures that can substantiate labelling claims of the production systems used for raising and finishing cattle.

Current guidelines, certification and auditing of grass-fed production systems vary depending on the brand and auditing body and despite vendor declarations consumers are not given a clear guarantee of the authenticity of grass-fed products. Thus, processors require a more scientific approach to verification and certification to ensure clear cues for production system and raising claims can be translated into consumer descriptions.

Objectives

This project aimed to provide the beef industry with a scientific based verification method for grass-fed and grain fed beef products, using Raman spectroscopy.

Given Raman spectroscopic hand-held devices are already commercially available, this project focused on the application of the technology, development of the method for verification and validation of the method over multiple seasons.

With accuracies over 80% found across models created in the preliminary phases and accuracies of up to 96% in validation models, this project has successfully delivered these two outcomes and the method is now ready for commercialisation.

Methodology

Carcases from 1940 beef cattle produced in grass-fed, supplemented grass-fed, short-term and long-term grain-fed production systems in New South Wales and Victoria (southern) and Queensland (northern) were measured using a Raman spectroscopic device and sampled to determine fatty acid composition over 3 phases.

After differentiation was possible and spectral differences were related to fatty acid composition in phase 1, calibration models were created to classify carcases based on production system of origin and characterise spectra based on differences in fatty acid composition. These models were subsequently validated on independent data collected in phase 3.

Results/key findings

Validation of models demonstrated that 86% of carcases were correctly classified into production system of origin when both southern and northern cattle were combined into one model. While 96% of carcases from northern production systems were correctly classified when carcases were separated into northern and southern models, only 70% of southern carcases were correctly classified. Initial data indicates these reduced accuracies may be the result of similarities between omega fatty acids found in supplemented grass-fed carcases and short -term grain-fed.

Benefits to industry

This research provides the evidence required to underpin the use of Raman spectroscopy for objectively verifying grass fed beef to maintain market access while reducing the cost of auditing to the supply chain.

As Raman spectroscopy had sufficient sensitivity to classify carcases from grass fed, grass supplemented, short-term grain fed and long-term grain fed production systems in northern and southern production systems, this research also supports the use of Raman spectroscopy to verify brands which are often based on unique regions and feeding systems.

Future research and recommendations

Further research is required to determine the sources of variation noted in the spectra collected from carcases of short-term grain fed cattle and determine the impact of highly variable spectra on the calibration of southern models.

As this research demonstrated similarities in the fatty acid composition of short-term grain fed and supplemented grass-fed beef carcases, further research is required to assess the impact on grass supplements on the fatty acid content of beef to ensure the grass-fed industry can meet both the demand and consumer expectations of health beneficial fatty acids.

Further research is also required to incorporate carcases from cattle in Western Australia and South Australia in the calibration models to ensure models represent the national production system and determine if there is an east/west regional difference.

Table of contents

1. Background

The method in which meat products have been raised and produced is becoming an increasing concern to consumers (Realini et al., 2013) and the interest in products from pasture based or grass-fed beef production systems is growing as they are perceived by some as low-input production systems with improved animal health and welfare, providing a wholesome product to consumers (Verbeke & Ward, 2006; Holman, van de Ven, Mao, Coombs & Hopkins, 2017). Australia is currently a leading exporter of meats due to our global reputation as a "clean and green" producer able to meet the expectations of consumers around the world. However, to maintain this position in these key global markets which are highly competitive, it is vital that Australia has transparent production systems which are underpinned by clear and trustworthy guidelines, auditing processes and quality assurance procedures which can substantiate any claims of the production systems used for raising the cattle. Indeed, MLA research (Project Dandelion, 2014) has shown that for trade customers, product specifications which include production system are meaningful and guide purchase decisions. However, these customers must have confidence in the labelling of meat products as in most consumer goods categories and fast-moving consumer goods organisations, the product specifications do not usually translate into consumer descriptions or language.

There is currently no clear verification system to substantiate the claim of grass-fed products which is placing our competitiveness in high valued markets at risk given that food fraud is an issue which is increasingly becoming a concern to consumers globally (Realini et al., 2013) and there is confusion in the supply chain regarding the raising claims of grass-fed and grain-fed products which is not limited to consumers (Project Dandelion, 2014). Guidelines, certification and auditing of grass-fed production system 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 products. The confusion in the supply chain of what constitutes grass-fed cattle is highlighted by the 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. Consequently, a more scientific approach to product verification and certification is required to assure consumers, maintain competiveness in global markets and provide a clear cue for production system and raising claims which can be translated into consumer descriptions.

Raman spectroscopy is a technology which is suitable for the differentiation of grass and grain- fed meat products given that it is rapid, non-invasive, non-destructive and capable of providing information on the chemical composition of matter (Li-Chan, 1996). Indeed, much research has been conducted to differentiate between species such as pork, chicken, turkey, mutton, goat, beef and horse (Ellis, Broadhurst, Clarke & Goodacre, 2005; Sowoidnich & Kronfeldt, 2012; Boyaci et al., 2014). This species differentiation was possible with a high accuracy of R^2 equal to 0.993 as spectral data obtained reflects the differences in fat composition (Beattie, Bell, Borggaard, Fearon & Moss, 2007). Further applications of Raman spectroscopy in food science have demonstrated that authentication of wine characteristics including grape cultivar, provenience and ageing times is possible using Raman spectroscopy (predictive capability of 86%) as spectral information was sensitive enough to discriminate between organogenetic and compositional differences among the wines (Mandrile, Zeppa, Giovannozzi & Rossi, 2016).

Given that the fatty acid profiles of beef and sheep varies with production system (Van Elswyk & McNeill, 2014; Clayton, Wilkins, Refshauge & Friend, 2015), it may be possible to use Raman spectroscopy to differentiate between carcases from grass-fed, grain-fed and supplemented cattle

and sheep. However, there is currently no research which has addressed this opportunity. Therefore, the aim of the research conducted was to develop and validate a method to use Raman spectroscopy to verify the production systems of beef.

2. Objectives

The objective of the project was to provide the beef industry with a scientific based verification method for grass-fed and grain fed beef products.

As Raman spectroscopic hand-held devices are already commercially available, this project focused on the application of the technology, development of the method for verification and validation of the method over multiple seasons.

With high accuracies found across models created in the preliminary phases and high accuracy of validation models, this project has successfully delivered these two outcomes and the method is now ready for commercialisation.

3. Methodology

3.1 Phase 1

Phase 1 was completed with the collection and analysis of samples from 150 grain and grass-fed cattle from two abattoirs, resulting in a total of 300 cattle sampled. At 24 hrs the subcutaneous fat over the point end brisket was measured using a Mira hand-held device (Metrohm®) in 3 positions on the navel end brisket (Fig 1) using an integration time of 5 seconds 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.

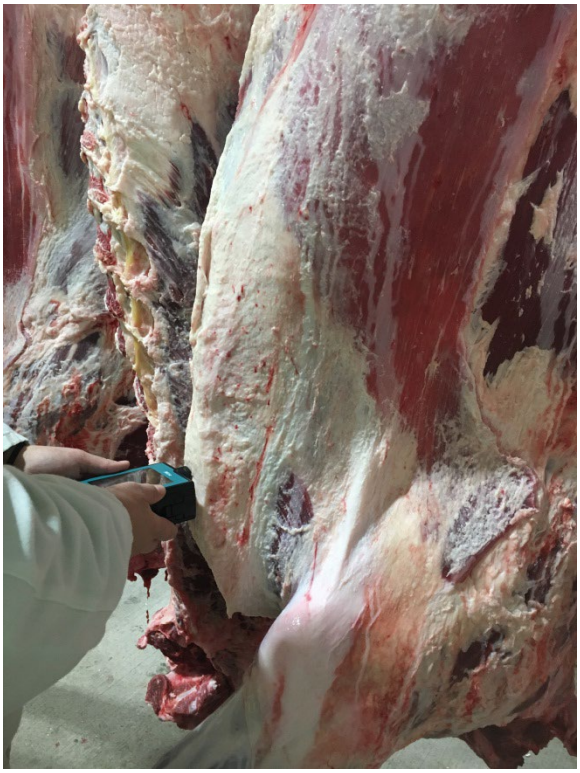

Figure 1. Measurement of the subcutaneous fat near the navel end of the brisket from a grain fed carcase during phase 1.

Once Raman spectroscopy and fat colour measurements were completed, a 30g 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 15s. β- carotene content was analysed using a method based on Yang, Larsen & Tume (1992). In short, 1g of the prepared subcutaneous tissue was saponified in 2 mL methanolic 20% potassium hydroxide (KOH), centrifuged and incubated at 65°C for 45min, 6 mL 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 distilled water three times to remove any KOH. Sodium sulphate, 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 was 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 10mL of chloroform/methanol mixture (2:1 v/v) added to the sample, shaken and centrifuged. Once extracted, an aliquot of 80 - 100µl was evaporated to dryness under nitrogen gas. Once evaporated, the mixture was methylated using 2mL 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 average and the wavelengths reduced to 600 – 1800cm^{-1}, 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 components analysis was then completed and peaks of interest were identified numerically by taking second differences.

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. All 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).

3.2 Phase 2

After the successful completion of phase 1, data for phase 2 was collected from cattle produced in the following systems: 100-day grain fed (n = 260), 70-day grain fed (n = 260), grass fed (n = 260) and grass supplemented fed (n= 260) production systems from both northern and southern grass-fed systems (total = 1040).

At 24 hrs the subcutaneous fat over the point end brisket was measured using a Mira hand-held device (Metrohm®) in 3 positions on the navel end brisket (Fig 1) using an integration time of 5 seconds 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 30g 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.

In preparation for analysis for β- carotene content and fatty acid (FA) composition, samples have been stored at -80°C before being freeze dried and homogenised using a Foss KnifeTech® grinder for 15s.

β- carotene content is analysed using a method based on Yang et al. (1992). In short, 1g of the prepared subcutaneous tissue is saponified in 2 mL methanolic 20% potassium hydroxide (KOH), centrifuged and incubated at 65°C for 45min, 6 mL distilled water is added and the samples are cooled under running water. β -carotenes are then extracted twice in 8 mL diethyl ether with 0.004% butylated hydroxytoluene (BHT) and the extracts washed with 16 mL distilled water three times to remove any KOH. Sodium sulphate, dried at 100^{o}C, is added to remove any residual water from the extracts, prior to the extracts being filtered and evaporated to dryness under a stream of nitrogen and the residual sample redissolved in 200 µL ethanol for grain fed samples and 500 µL ethanol for grass fed samples. The β-carotene concentration is 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 was 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.

Similarly, fatty acid concentrations will be completed using the methods established in phase 1 using a one-step extraction based on the method of (Lepage & Roy, 1986). Extraction of fatty acids is achieved by using 10mL of chloroform/methanol mixture (2:1 v/v) added to the sample, shaken and centrifuged. Once extracted, an aliquot of 80 - 100µl is evaporated to dryness under nitrogen gas. Once evaporated, the mixture is methylated using 2mL 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 are identified from 80 µL of FAME using an Agilent 6890N gas chromatograph (GC) equipped with a SGE BPX70 analytical column.

Spectral data from each carcase was averaged before partial least squares discrimination analysis (PLS-DA) and principal component analysis (PCA) was undertaken to establish whether significant spectral differences are evident between production systems. The predictive accuracy of the model to classify the classes was assessed against an independent test data set with the prediction accuracy, misclassifications and receiver operator curves reported. Sensitivity, specificity, coefficient of determination (R^2), root mean square error of prediction (RMSEP), number of misclassifications and accuracy were calculated by the model. Class error for the model was calculated by:

Class Error = 1 – (sensitivity + specificity) / 2

A confusion table was developed using the test datasets assigned class compared to the true class of the sample. All spectra modelling statistical analysis was performed utilising Matlab and PLS Toolbox version 8.7.1 (Eigenvector Research Inc., Wenatchee, WA, USA).

3.3 Phase 3

After the successful completion of phases 1 and 2, data collection for phase 3 was completed with 600 samples collected from Southern grain and grass-fed cattle from two Southern abattoirs as well as Northern grain and grass-fed cattle from two Northern abattoirs.

As with previous phases, spectral data was collected at 24 hrs the subcutaneous fat over the point end brisket using a Mira hand-held device (Metrohm®) in 3 positions on the navel end (Fig 1) using an integration time of 5 seconds 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.

Similarly, fatty acid concentrations were completed using the methods established in phases 1 and 2 using a one-step extraction based on the method of (Lepage & Roy, 1986). Extraction of fatty acids is achieved by using 10mL of chloroform/methanol mixture (2:1 v/v) added to the sample, shaken and centrifuged. Once extracted, an aliquot of 80 - 100µl is evaporated to dryness under nitrogen gas. Once evaporated, the mixture is methylated using 2mL 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 are identified from 80 µL of FAME using an Agilent 6890N gas chromatograph (GC) equipped with a SGE BPX70 analytical column.

Phase 2 data was analysed using the method as described by Logan, Hopkins, Schmidtke & Fowler (2022) whereby spectra were averaged by carcase and reduced to the range of between 600 – 2000cm^{-1} before background noise was removed and the triplicate spectra for each sample were averaged. Standard normal variate (SNV) and mean centring were then applied as pre-processing techniques and principal components analysis (PCA) was completed, to provide the eigenvalues and percentage of explained variance which were used to assess the optimum number of components (PC).

Following this exploratory analysis, 2 class partial least squares discrimination analysis (PLS-DA) was completed to determine the potential for spectral models created in phase 2 to discriminate between production systems of the samples collected in phase 3. To this end, phase 2 data was divided in a 70:30 split to calibration: validation whilst preserving the class proportion for grain and grass-fed cattle in each model. The number of latent variables (LV) were selected by investigating the eigenvalues and consideration of the root means square errors of calibration and cross validation, Q2Y, R2, misclassification and area under receiver operator curves (AUROC) values. Permutation testing of each calibration data set was undertaken with 1000 iterations and empirical p-values determined from the permuted prediction of class outcomes. Each PLS-DA model constructed was used to predict the sample class of the independent test samples for the data sets and AUROC curves, confusion tables and confusion matrices used to determine the model performance where;

TPR = True Positive Rate
FPR = False Positive Rate
TNR = True Negative Rate
FNR = False Negative Rate
N = number of samples

Model Error or Misclassification Error rate
= average of false positive rate and false negative rate for class,
= 1 - (sensitivity + specificity)/2
P = Precision (positive predictive value) = TPR/ (TPR + FPR)
Negative predictive value = TNR/ (TNR + FNR)
F1Score = 2*TPR/ (2*TPR + FPR + FNR)
Accuracy = (TPR + TNR)/ (TPR + TNR + FPR + FNR)
Sensitivity = TPR/ (TPR + FNR)
Specificity = TNR/ (TNR + FPR)
Mathews Correlation Coefficient (MCC) = TPR * TNR – FPR * FNR/ (sqrt ((TPR + FPR) * (TPR + FNR) * (TNR + FPR) * (TNR + FNR))

Models were tested by cross-validating the calibration data using random subsets with 10 data splits and 5 iterations. The specificity, sensitivity, accuracy, number of misclassifications and predictive error classes were produced from the test data set. Important spectral regions for each model were determined using variable importance to projections scores >1.0.

Models assessed included phase 2 all grain versus grass (Phase 2 All), phase 2 north grain versus grass (Phase 2 North), phase 2 south grain versus grass (Phase 2 South) and phase 2 south long grain versus grass (Phase 2 long grain/grass).

These models were then applied to phase 3 spectra to assess the best option for classifying beef carcases based on region of origin including a combined model which aimed to predict the production system of origin from cattle produced in both northern and southern Australian supply chains (Phase 3 All) as well as individual models for predicting production system of origin from only northern (Phase 3 North) and only southern (Phase 3 South) supply chains.

All PLS_DA models were constructed using PLS_Toolbox version 9.1 (Eigenvector Inc, Manson, WA) and Matlab version 9.12 R2022a (The Mathworks Inc, MA).

4. Results

4.1 Phase 1

Modelling of data in Phase 1 demonstrated Raman spectra 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 $^{-1}$ (Fig 2).

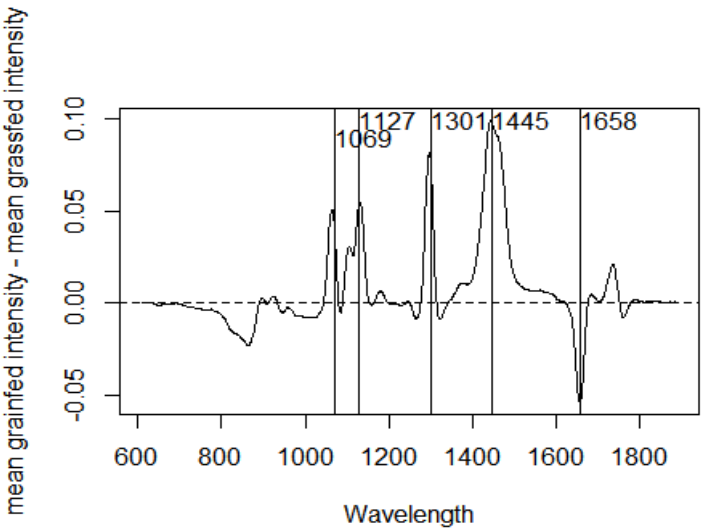

Figure 2. Differences of intensities from spectra collected from carcases of grass and grain fed cattle.

Analysis of the fatty acid composition highlighted significant differences in the fatty acid composition between grain and grass-fed carcases which contribute to these spectral differences. As demonstrated by Figure 3, grain fed carcases had significantly higher concentrations of saturated fatty acids present in subcutaneous fat compared to carcases from grass fed cattle (11.1 g/100g and 8.3g/100g, respectively). This is due to increases in individual fatty acids including C15:0, C16:0, C17:0, C18:0 and C20:0, which agrees with the spectra as the increases in peaks at 1069cm $^{-1}$ and 1127 cm $^{-1}$ (Table 1) 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). However, it is likely that the increases in individual monounsaturated fatty acids including C15:1n-5, C17:1n-7, C18:1n-9, C20:1n-9, C20:1n-15 and C24:1n-9 also contribute to this increase in the spectra as some spectral overlap is expected given that the monounsaturated fatty acids contain only one C=C bond and therefore also include long C-C chains which will contribute to the C-C vibrations at these wavelengths.

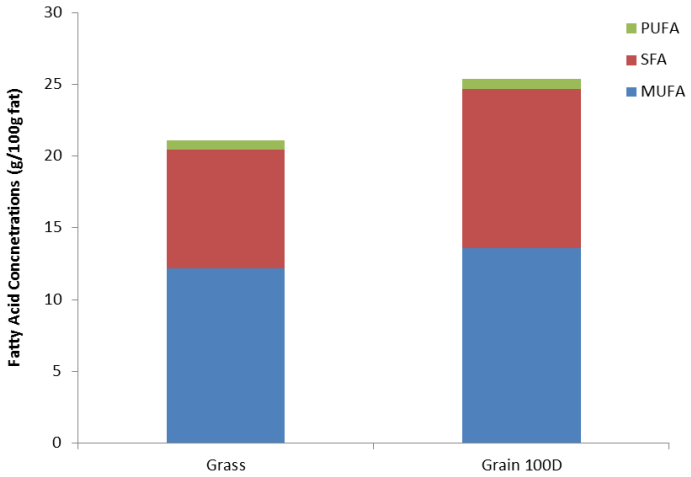

Figure 3. Concentrations of saturated (SFA), monounsaturated (MUFA) and polyunsaturated fatty acids measured in the subcutaneous fat from grass and grain fed beef cattle carcases.

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

	Fatty acid	Grain Fed Carcases		Grass Fed Carcases	
		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	26.5_a	2.02	49.7_b	2.02
	anteiso-C15:0	30.9	4.19	45.3	4.19
	C15:0	148.7_b	8.65	113.7_a	8.63
	C16:0	6355.2_b	256.09	4928.3_a	255.46
	iso-C17:0	26.5_a	2.02	49.7_b	2.02
	anteiso-C17:0	168.2_a	7.49	220.6_b	7.46
	C17:0	385.4_b	21.11	193.7_a	21.06
	C18:0	2921.6_b	117.28	1872.3_a	116.88
	C20:0	18.9_b	0.54	14.2_a	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	2.3_b	0.26	0.4_a	0.26
	C16:1n-7	1328.2	112.42	1557.0	112.26
	C16:1n-7t	21.0_b	1.57	12.6_a	1.57
	C17:1n-7	36.2_a	0.83	42.1_b	0.83
	C18:1n-7	474.3	29.18	392.2	29.12
	C18:1n-7t	850.9_b	39.33	217.0_a	39.07
	C18:1n-9	11286.3	441.03	9665.8	439.73
	C18:1n-9t	118.5_b	7.98	52.5_a	7.90
	C20:1n-9	81.2_b	3.80	48.6_a	3.80
	C20:1n-15	10.8_b	0.66	6.5_a	0.66
	C22:1n-9	2.4	0.39	1.6	0.39
	C24:1n-9	0.9_b	0.08	0.6_a	0.08
PUFA (mg/100g)	C16:2n-4	6.0_a	0.30	7.9_b	0.30
	C16:3n-4	3.2_b	0.15	2.2_a	0.15
	C18:2n-6	356.5_b	16.6	203.5_a	16.6
	C18:2n-6t	204.2	10.10	195.9	10.06
	C18:3n-3	51.1_a	5.65	109.2_b	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	7.9_b	0.21	4.5_a	0.21
	C20:3n-3	3.3_a	0.33	5.6_b	0.33
	C20:3n-6	14.2	0.65	14.8	0.65
	C20:3n-9	3.0_a	0.23	4.1_b	0.23
	C20:4n-3	3.8_a	1.08	11.9_b	1.08
	C20:4n-6	10.0	0.29	9.4	0.29
	C20:5n-3	2.9_a	0.61	7.1_b	0.61
	C22:2n-6	0.9_a	0.08	1.4_b	0.08
	C22:4n-6	4.7_b	0.64	1.2_a	0.64
	C22:5n-3	9.0_a	1.38	18.6_b	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	1.2_b	0.05	0.5_a	0.05
	CLA	0.1	0.02	0.1	0.02
Totals (mg/100g)	Omega-3	87.6_a	10.2	173.7_b	10.2
	Omega-6	400.4_b	16.91	241.8_a	16.83
	Omega-6: omega-3	5.1_b	0.51	1.5_a	0.51
Totals (g/100g)	PUFA	0.7	0.03	0.6	0.03
	MUFA	13.6	0.57	12.1	0.57
	SFA	11.1_b	0.43	8.3_a	0.43

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

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 (Table 1) also explain spectral differences evident in peaks at wavelengths 1301cm^{-1} and 1445cm^{-1} which reflect the CH_2 twist and scissor vibrations. These vibrations arise from the long chains of carbon and hydrogen atoms which are not interrupted by C=C double bonds. 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, Abbott, Doyle, Nader & Larson, 2010).

It is unsurprising that the concentrations of total polyunsaturated fatty acids did not significantly differ between carcases from grass and grain fed cattle (Figure 3) given that they 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). However, there was a difference in the concentrations of omega-6 and omega-3 fatty acids (Figure 4; Table 1) which is consistent with previous research on the fatty acid composition of grass and grain fed beef cattle carcases (Daley et al., 2010).

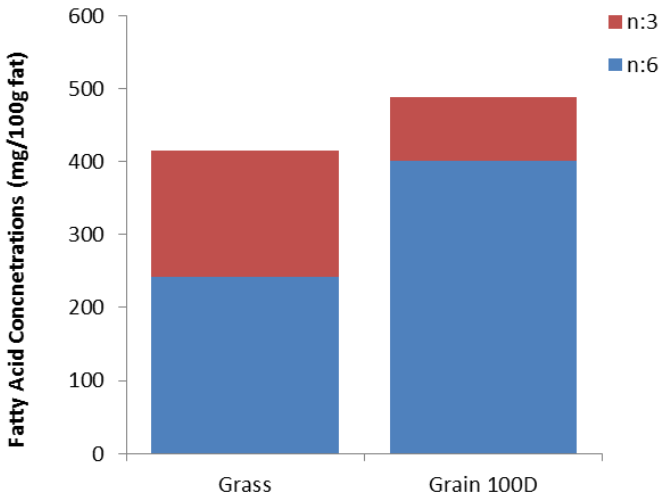

Figure 4. Concentrations of Omega-6 and Omega-3 fatty acids measured in carcases from grass and grain fed beef carcases.

Consequently, it can be expected that 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. Yet the origin of this peak remains unclear as it is expected all

polyunsaturated fatty acids would contribute to this signal. Yet difference in this band may arise from a greater number of cis- fatty acids present in the fat from grass fed beef, although not a significantly different between the fat of grass and grain fed carcases, the fat from grass fed cattle tended to have a higher concentration of cis- CLA (110.9 mg/100g) compared to grain fed cattle (67.8 mg/100g). Previous research conducted by Afseth, Segtnan, Marquardt & Wold (2005) has highlighted that the cis carbon – carbon bond is evident at approximately 1656cm^{-1}. 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 et al., 2010). However, as these signals may also be due to the higher concentrations of omega-3 fatty acids, this will need to be confirmed by measuring reference spectra of purified fatty acids.

As expected β-carotene concentrations were significantly higher in grass fed carcases as highlighted by Figure 5. Although the signals for β-carotene are currently not evident 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. 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.

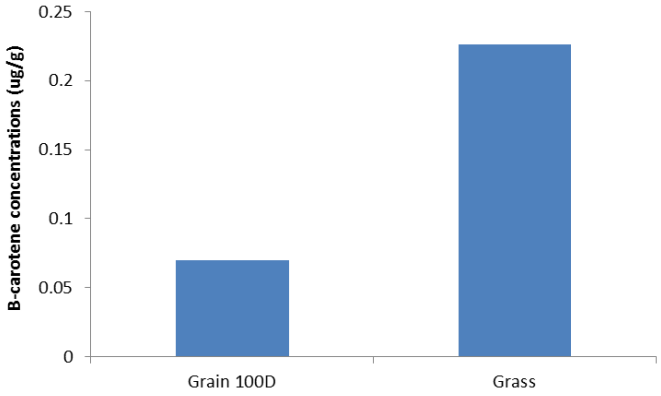

Figure 5. β-carotene concentrations of subcutaneous fat from carcases of grass and grain fed cattle.

4.2 Phase 2

PCA modelling and plotting the scores of spectral data collected in Phase 2 has shown clustering and produced a separation of samples, which accounts for 74% of the variation in spectra. Separation of carcases from southern long-term grain fed production systems from southern grass and grass supplemented carcases was evident from the score plot, however the separation of carcases from southern short-term grain fed production systems was not so clear (Fig 6.).

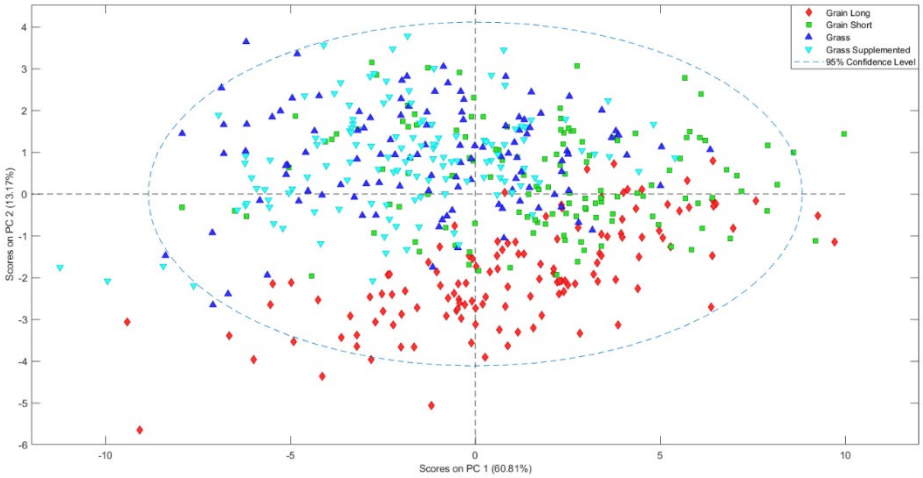

Figure 6. Score plot of principal components (PCs) 1 and 2 from a Principal Components Analysis (PCA) of Raman spectra from the subcutaneous fat from cattle produced in differing production systems.

The PCA loadings indicate that the clustering is based on key peaks at 1301 and 1440 cm^{-1} (PC 1) as well as 1658 cm^{-1} (PC 2). These peaks agree with the research from phase 1 which indicated key chemical bonds associated with saturated fatty acids and polyunsaturated fatty acids are responsible for the differences observed (Logan, Hopkins, Schmidtke, Morris & Fowler, 2020). However, the lack of clustering of short-term grain fed samples, suggests the short term grain fed diet may not give sufficient time on feed to alter the fatty acid profile of the carcases when compared to the fatty acid composition of grass and grass supplemented cattle. However, given the complex nature of the Raman spectra, futher data analysis is required before the principle investigators can reach a solid conclusion.

An alternate approach to classify carcases based on production system using PLS-DA models demonstrated an ability to classify samples with calibration model accuracies of 94% for carcases from grass fed and 96% for other production system types as there was 4 misclassifications for each class, except for grain fed cattle which had 6. The confusion matrix for these models (Table 2), highlights the high accuracies of the classification rates as an overall accuracy of 87% was achieved, with accuracies of 83 – 93% for each of the individual feed types. Full model results are given in Table 3.

Table 2. The number of carcases classified using a PLS-DA model based on Raman spectra collected from beef subcutaneous fat from Southern Australian production systems, where the total number used in the test set is given after each production system.

	Grain Long (29)	**Grain Short (30)**	**Grass (19)**	**Grass Supplemented (23)**
Predicted as Grain Long	27	0	0	0
Predicted as Grain Short	2	25	1	1
Predicted as Grass	0	2	17	3
Predicted as Grass Supplemented	0	3	1	19

Table 3. 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

The largest number of misclassifications are present in the short grain and grass supplemented classes. In the test data, 5 carcases from the short-term grain fed class were misclassified (2 were incorrectly classified as grass only and 3 were classified incorrectly as grass supplemented), while 3 from the grass supplemented were identified as grass only and 1 was predicted as short-term grain. Although the prediction of grass only by the grass supplemented is likely due to the similarities in diet, it poses no challenge to industry under current branding and labelling regulations. However, the misclassifications of short-term grain fed carcases as grass fed carcases and grass supplemented carcases as short-term grain fed carcases suggests that these diets may result in similar fatty acid compositions. Further analysis including the full fatty acid composition will address this and determine whether the spectra, fatty acid composition of misclassified carcases or modelling is the cause of the misclassification. Although carcases from grass and grass supplemented production systems were expected to have overlap, the classifications for these groups highlight the sensitivity of the technology in being sensitive enough to detect small changes in feeding regime.

The average Raman spectra collected from beef cattle produced in grass, grass supplemented and short- and long-term grain fed production systems are given in Fig 7. These spectra illustrate the differences which are evident at wavelengths including 1069, 1125, 1300, 1445 and 1650 cm^{-1}. Although cattle from short- term grain fed and grass supplemented production systems have been included in this phase, these differences agree with those found in phase 1. This indicates differences in saturated fatty acids and the omega3: omega 6 ratio are responsible for the differentiation of production systems as spectra collected from grain fed carcases demonstrated higher intensities at wavelengths which represent the CH^2 and C-C bonds (Logan et al., 2020).

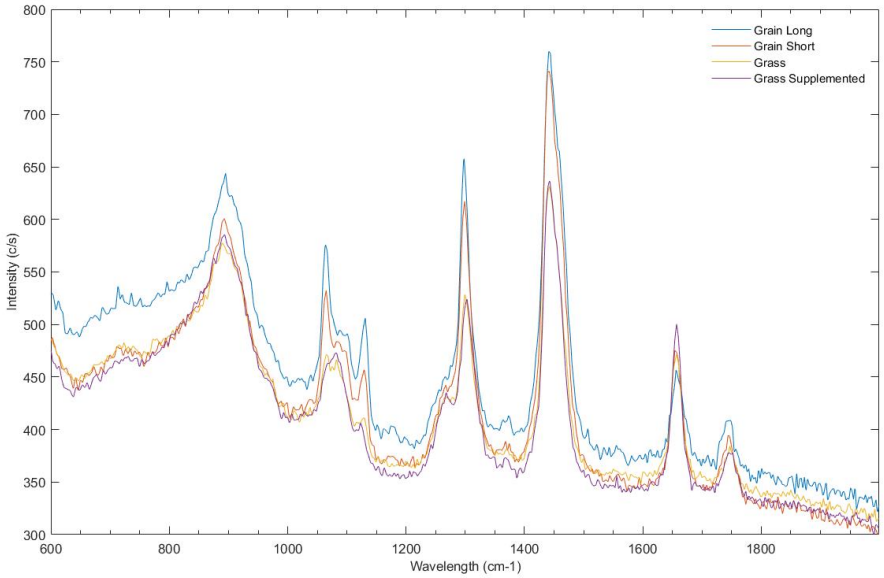

Figure 7. Raman spectra of subcutaneous fat from carcases of cattle from grain fed (long and short) and grass fed (grass only and grass supplemented) production systems.

While spectral patterns are similar for grass and grain fed cattle at most of these intensities, the peak at 1658cm^{-1} and spectral features around 1069cm^{-1} indicate some similarities are present in spectra collected from short term grain fed cattle and grass supplemented cattle. This suggests the cis fatty acids and ratio of omega 3 and 6 fatty acids may be affected by supplementing grass fed cattle (Olsen et al., 2008).

Further PLS-DA modelling completed on respective data collected in northern Australian production systems and both northern and southern production systems combined is given by Logan et al. (2022). In short, analysis demonstrated the northern models were more accurate with 95% of samples correctly classified in a two-class model with 12 carcases misclassified, including 7 carcases from grain fed cattle incorrectly classified as grass fed and 5 carcases from grass fed cattle incorrectly classified as grain fed cattle.

While the two-class model is most beneficial to industry, an eight-class model which combined northern and southern data to predict individual feed classes including both northern and southern grass fed, grass supplemented, long grain and short grain were trialled. However, these models yielded variable predictive accuracies. This is evident in the F1 score given the prediction of carcases produced in southern long-term grain fed systems gave F1 score of 87%, while the lowest accuracy was 57% observed for carcases produced in northern long-term grain fed systems. Separation of data based on region resulting in two four-class models yielded increases of up to 23% and thus it was recommended to create separate models based on region of production.

4.3 Phase 3

Prior to the completion of validating the models, models were coded again and reanalysed to ensure model accuracy. Spectra collected throughout phase 2 which underpin this model are given in figures 8 and 9. From these figures it is evident, spectra collected from grain fed cattle have greater variation than those from grass fed cattle, as highlighted by the standard deviation of the mean spectra for the group.

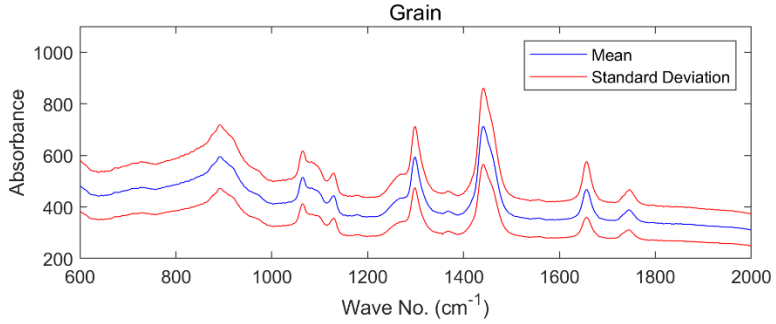

Figure 8. The mean spectra and standard deviation collected from cattle fed on grain diets for 70d and 100d prior to slaughter.

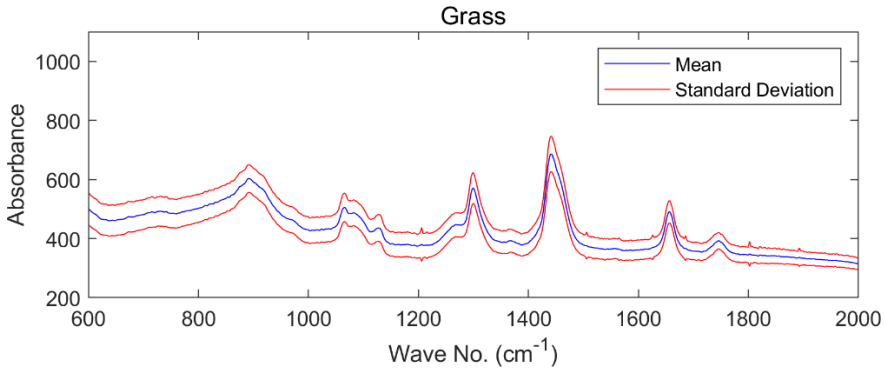

Figure 9. The mean spectra and standard deviation collected from cattle fed on grass and grass supplemented diets prior to slaughter.

When spectra from each of the feed groups which make up the grain fed samples, including both long and short term fed cattle from both northern and southern production systems (Figs 10, 11, 12 and 13) are compared, it is clear the variation in spectra was from the “south grain short” i.e., 70-day grain fed cattle from southern Australia.

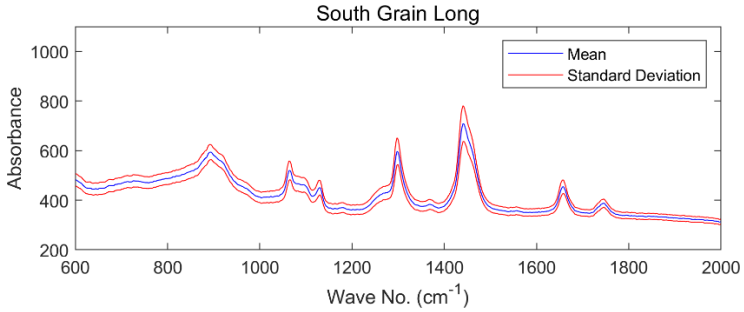

Figure 10. The mean spectra and standard deviation collected from carcases from long fed grain production systems (100d grain fed) in southern Australia.

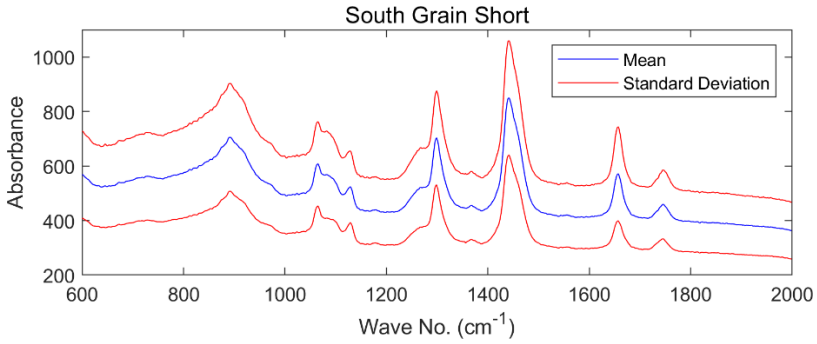

Figure 11. The mean spectra and standard deviation collected from carcases of short term fed grain production systems (70d grain fed) in southern Australia.

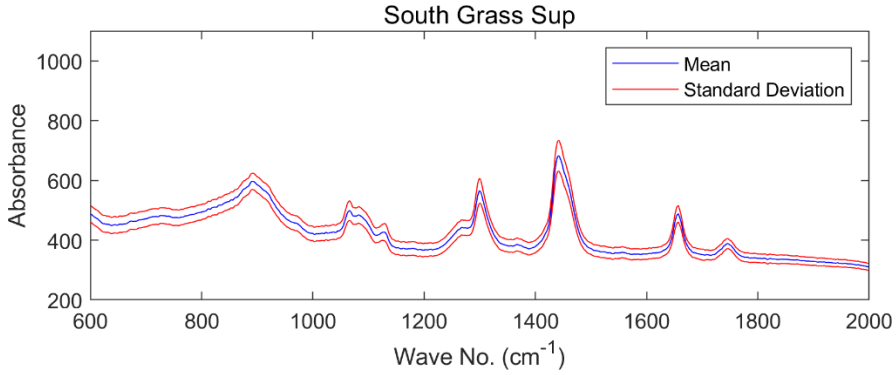

Figure 12. The mean spectra and standard deviation collected from carcases of grass supplemented production systems in southern Australia.

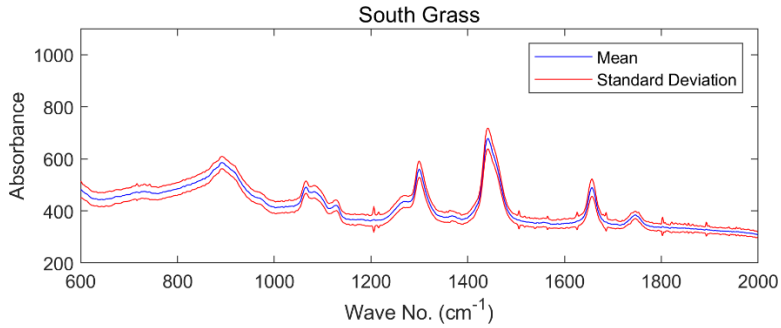

Figure 13. The mean spectra and standard deviation collected from carcases of grass production systems in southern Australia.

The confusion table from the PLS-DA model which combined all data to predict “grass” which included samples from both grass and grass supplemented cattle, and “grain” which included samples from both 70d grain fed and 100d grain fed cattle (Table 4), highlights an increased error in the prediction of grass samples. This is noted as 15 of the 296 “grass” samples were predicted as “grain”, which resulted in a higher false negative rate, as shown by the confusion matrix given in Table 5. Overall, the error of the class prediction for the independent test set was 12.8%.

Table 4. Confusion table for the prediction of production system of origin using the combined north/south and grass/grain calibration data.

	Actual Class	
	Grain	Grass
Predicted as Grain	141	15
Predicted as Grass	4	129
Predicted as Unassigned	0	0

Table 5. Confusion matrix for the prediction of production system of origin using the combined north/south and grass/grain calibration data.

Class:	TPR	FPR	TNR	FNR	N	Err	P	F1
Grain	0.972	0104	0.896	0.028	145	0.066	0.904	0.937
Grass	0.898	0.028	0.972	0.104	144	0.066	0.970	0.931

TPR = True Positive Rate
FPR = False Positive Rate
TNR = True Negative Rate
FNR = False Negative Rate
N = number of samples
Err = Misclassification Error rate
= average of false positive rate and false negative rate for class,
= 1 - (sensitivity + specificity)/2.
P = Precision = TP/ (TP + FP)
F1Score = 2*TP/ (2*TP + FP + FN)

Permutation testing results for this model indicate the excellent prediction of sample class is robust (Fig 14). The 8% error found is modest and demonstrates the model is useful in discriminating with acceptable accuracy (92%), sensitivity and specificity. However, these findings do also suggest separate models for data derived from samples in the North and Southern production systems will produce a more robust predictive outcome.

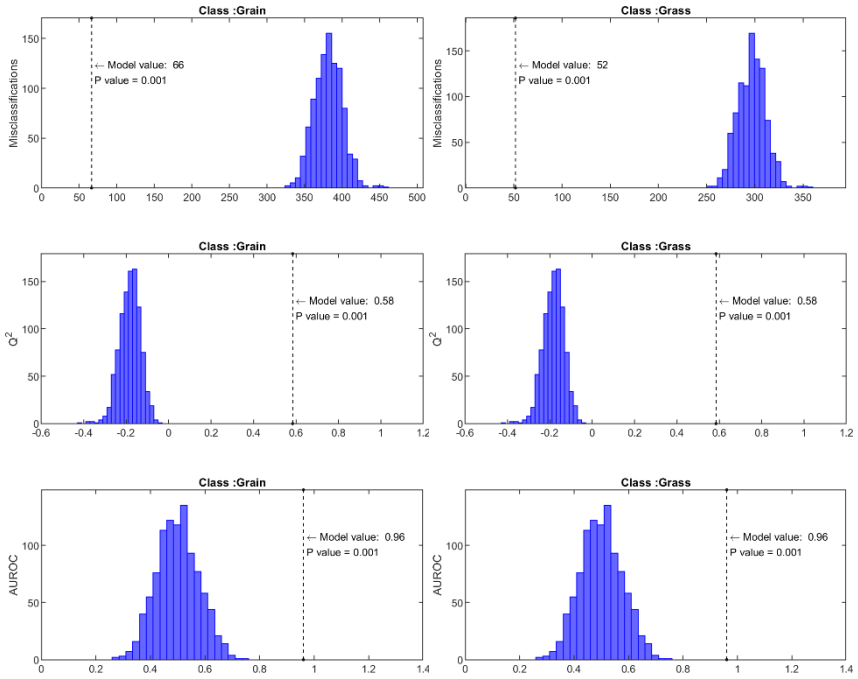

Figure 14. PLS-DA permutation results for Phase 2 samples (North and South) showing a distribution of misclassifications, Q2 and AUROC values for permuted samples compared to the true model values sown as a dotted line. Empirical p-values for the true model compared to the permuted results are indicated.

4.3.1 Validation of models using all data

The confusion table using this phase 2 model to predict the production system of origin for samples collected in phase 3 is given in Table 6, while the confusion matrix is given in Table 7.

Table 6. Confusion table for the prediction of production system of origin using the combined north/south and grass/grain phase 3 validation data.

	Actual Class	
	Grain	Grass
Predicted as Grain	251	62
Predicted as Grass	27	288

Table 7. Confusion matrix for the prediction of production system of origin using the combined north/south and grass/grain phase 3 validation data.

Class:	TP	FP	TN	FN	N	Err	P	F1	Acc	Neg. Predict. Value	Sensitivity	Specificity
Grain	0.903	0.177	0.823	0.097	278	0.142	0.802	0.849	0.863	0.895	0.903	0.823
Grass	0.822	0.097	0.902	0.177	350	0.141	0.914	0.866	0.863	0.836	0.823	0.903

A considerably higher predictive error is apparent for samples from both grain and grass-fed production systems when applying the phase two PLS-DA to the phase 3 samples, as the error for the independent test data set used for validation of the model was 14% versus 8% for the calibration model. Sample variation in both the calibration and phase 3 validation datasets will contribute to the prediction errors, although it appears that a slightly higher error and lower precision occurs for grain

fed samples. A lower precision for prediction of grain fed samples may be indicative of inclusion of grain samples of short duration into the predictive model given the variability noted in the spectra. This may suggest the period of adaptation to grain or the shorter duration of feeding may influence the spectral characteristics which could more closely resemble spectra from carcases of grass supplemented cattle. However, as shown by the area under receiver operator characteristics curves (AUROC curves) in Fig 15, the phase 2 model was still capable of separating carcases from grass-fed and grain-fed cattle given the high AUC (0.9479) which suggests there is a 94.7% chance the model is able to distinguish between grass and grain fed beef carcases.

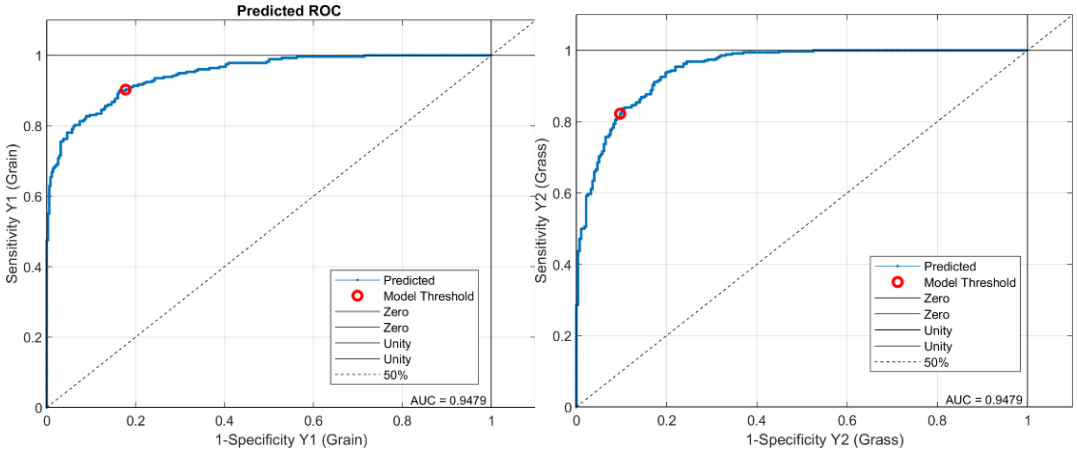

Figure 15. Area under receiver operator characteristics curves (AUROC curves) for prediction of sample class using a PLS-DA model from combined Phase 2 data applied to combined Phase 3 data.

4.3.2 Validation of models using Northern data only

Models created using data only collected in Northern Australia is robust as shown by the permutation testing (Fig 16) as permuted data was significantly different to the true model (*P*<0.001).

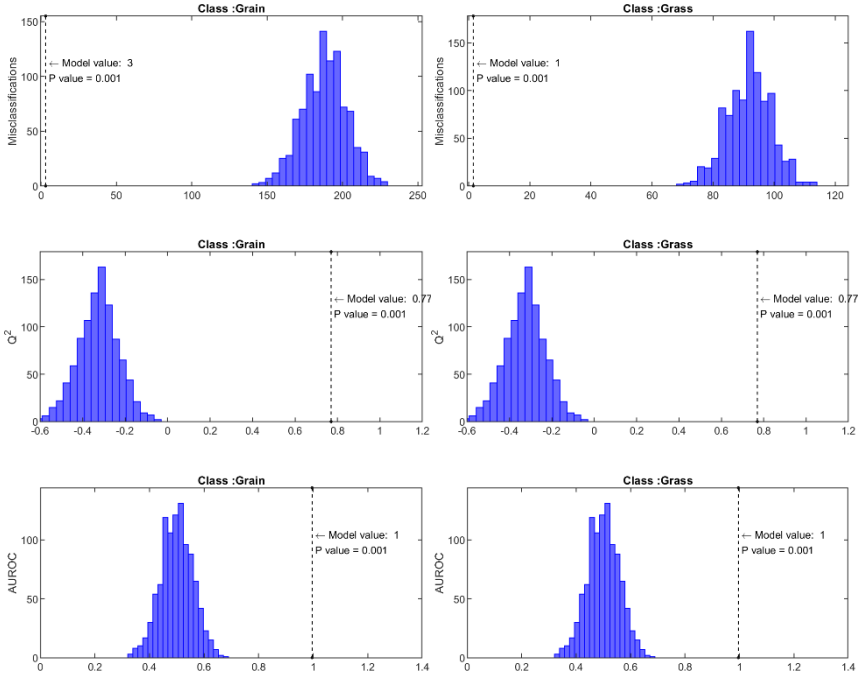

Figure 16. PLS-DA permutation results for Phase 3 samples collected in the north showing a distribution of misclassifications, Q2 and AUROC values for permuted samples compared to the true model values sown as a dotted line. Empirical p-values for the true model compared to the permuted results are indicated.

Application of this model to predict the production system of origin for the phase 3 samples collected as an independent data set from northern Australia is given in the confusion table and matrix, tables 5 and 6, respectively. Overall, these tables indicate the models derived in phase 2 were able to classify carcases in phase 3 accurately and precisely, yielding an error of 4.3% and an accuracy of 95.7%. These results show its capacity for application given they were only slightly poorer in their predictive ability when compared to the 2.5% error and 97.5% accuracy which was yielded by the calibration model for carcases from northern Australia. Therefore, the models developed in phase 2 are robust and capable of classifying samples from the same region over time with varying seasons. The strength of the validation is further illustrated in the AUROC curves (Fig 17) which highlight the high model threshold with an AUC of 0.9956.

Table 8. Confusion table for the prediction of production system of origin using the northern grass/grain phase 3 validation data.

	Actual Class	
	Grain	Grass
Predicted as Grain	77	1
Predicted as Grass	2	38

Table 9. Confusion matrix for the prediction of production system of origin using the northern grass/grain phase 3 validation data.

Class:	TP	FP	TN	FN	N	Err	P	F1	Acc	Neg. Predict. Value	Sensitivity	Specificity
Grain	0.975	0.026	0.974	0.025	79	0.025	0.987	0.981	0.975	0.975	0.975	0.974
Grass	0.974	0.025	0.974	0.025	39	0.025	0.950	0.859	0.975	0.975	0.975	0.975

Matthew's Correlation Coefficient = 0.95

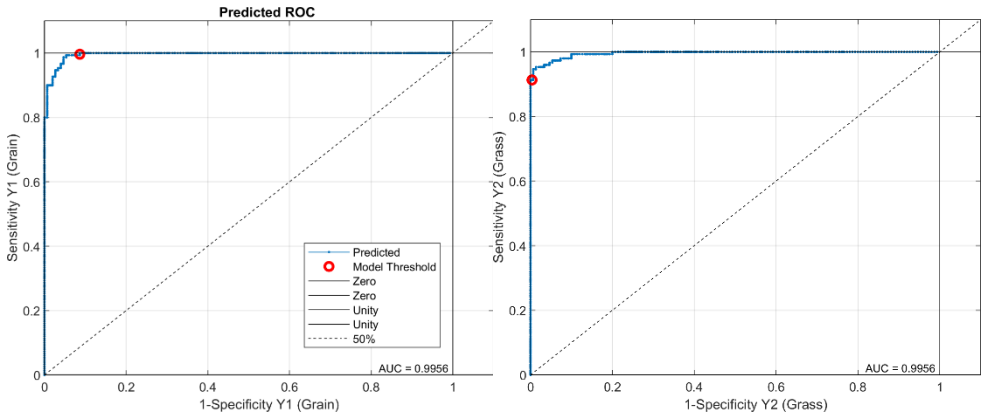

Figure 17. Area under receiver operator characteristics curves (AUROC curves) for prediction of sample class using a PLS-DA model from northern Phase 2 data applied to northern Phase 3 data.

4.3.3 Validation of models using Southern data only

As shown by Figure 18, the model is robust as the permuted data was significantly different from the true model.

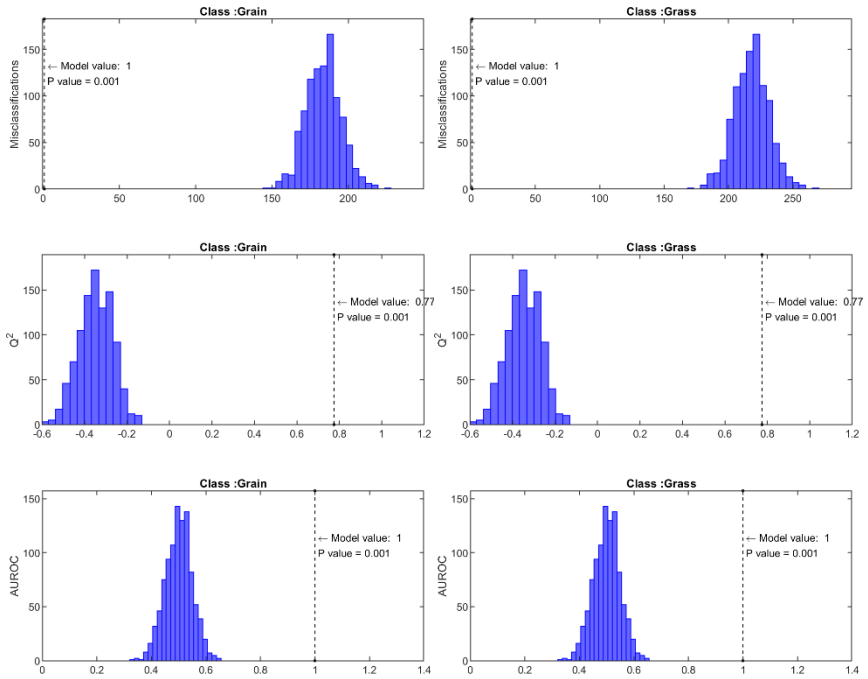

Figure 18. PLS-DA permutation results for Phase 3 samples collected in the south showing a distribution of misclassifications, Q2 and AUROC values for permuted samples compared to the

true model values sown as a dotted line. Empirical p-values for the true model compared to the permuted results are indicated.

As evident in the confusion table (Table 10) and confusion matrix (Table 11), this model was much lower performing when compared to both the northern and combined models with a predictive error of 30.2% and an accuracy of 70.8%. Similar to the combined model, inclusion of the short grain fed carcases which had a greater spectral variation is contributing to the lower accuracy and higher errors found. This was confirmed by the increase in model performance when the short grain fed carcases were removed from the models as demonstrated in Tables 12 and 13.

Table 10. Confusion table for the prediction of production system of origin using the southern grass/grain phase 3 validation data.

	Actual Class	
	Grain	Grass
Predicted as Grain	96	67
Predicted as Grass	32	133

Table 11. Confusion matrix for the prediction of production system of origin using the southern grass/grain phase 3 validation data.

Class:	TPR	FPR	TNR	FNR	N	Err	P	F1	Acc	Neg. Predict. Value	Sensitivity	Specificity
Grain	0.750	0.335	0.665	0.25	128	0.302	0.589	0.660	0.708	0.727	0.750	0.665
Grass	0.665	0.250	0.750	0.335	200	0.302	0.806	0.729	0.708	0.691	0.665	0.936

Matthew's Correlation Coefficient = 0.405

Table 12. Confusion table for the prediction of production system of origin using the southern grass/grain phase 3 validation data with short term grain fed carcases removed.

	Actual Class	
	Grain	Grass
Predicted as Grain	126	2
Predicted as Grass	2	198

Table 13. Confusion matrix for the prediction of production system of origin using the southern grass/grain phase 3 validation data with short term grain fed carcases removed.

Class:	TPR	FPR	TNR	FNR	N	Err	P	F1	Acc	Neg. Predict. Value	Sensitivity	Specificity
Grain	0.984	0.010	0.990	0.016	128	0.012	0.984	0.984	0.987	0.984	0.9684	0.990
Grass	0.990	0.016	0.984	0.010	200	0.012	0.990	0.990	0.987	0.990	0.990	0.984

Matthew's Correlation Coefficient = 0.974

4.3.4 Summary of Validation Models

Overall, a summary of the validation models (Table 14), demonstrates the models based on region (north/south with short grain removed) outperformed the combined models with precisions of 99 and 96 compared to 86 and errors of 4.3% and 1.2% compared to 14%. Furthermore, Table 15 also highlights the impact of the spectral variation of south grain samples given their removal increases the precision of the model from 70 to 99 while reducing the error from 30% to 1.2%.

Table 14. Summary of the predictive performance of validation PLS-DA models.

Calibration Model	Error %	Precision (Positive Prediction Value)	F1 %	Accuracy %	Negative Predictive Value	Sensitivity	Specificity	Matthews Correlation Coefficient	N (total)
Phase 2 combined	14	86	86	86	87	86	86	0.73	628
Phase 2 North	4.3	96	96	96	96	96	96	0.92	300
Phase 2 South	30	70	70	71	71	71	71	0.42	328
Phase 2 South with Short Grain Removed	1.2	99	99	99	99	99	99	0.97	328

Given that these models are a validation of the models created in phase 2, VIP score plots given in Figure 19 highlight the same spectral regions as models previously reported. These include the C-C stretch including the out of phase aliphatic, liquid aliphatic and in phase aliphatic stretches at 1050-1150cm^{-1}, the CH wagging at 1300-1380 cm^{-1}, the CH_2 methylene scissor deformation at 1450-1500 cm^{-1} and the C=C cis and trans olefinic stretches between 1650-1700 cm^{-1} (Bresson, Marssi & Khelifa, 2005; Beattie et al., 2007).

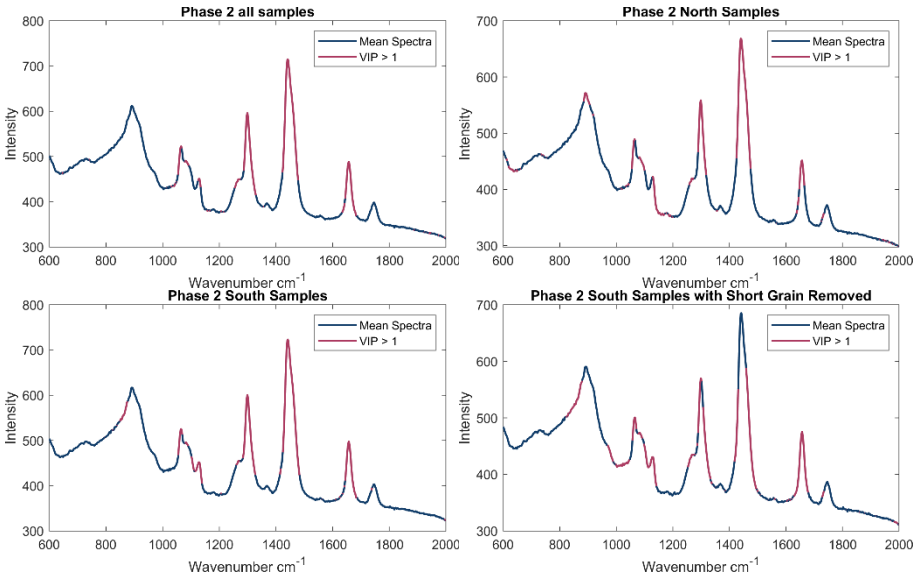

Figure 19. Mean spectra of calibration data identifying regions (in red) with VIP>1 for PLS-DA predictive models (grain versus grass).

4.3.5 Fatty Acid Composition

Predicted means for the fatty acids given in Table 15, highlights the main differences between feed groups in the validation of models were attributed to the n-6 and n-3 fatty acids as well as MUFA which were all significantly between grass and grain fed cattle. This is due to differences in individual fatty acids including C15:1n-5, asteisoC17:0, C18:1n-7t, C18:1n-9t, C20:1n-5, C18:3n-3, C18:3n-4, C20:3n-3, C20:5n-3 and C22:5n-3.

Table 15. Predicted means and the standard errors of the fatty acid composition of carcases from grain and grass production systems in northern and southern Australia measured in Phase 3.

		North Grain		North Grass		South Grain		South Grass	
	Fatty Acid	LSM	s. e.	LSM	s. e.	LSM	s. e.	LSM	s. e.
	C10:0	88.9a	1.9	99.2b	1.9	285.3c	2.0	90.3a	1.6
	C12:0	72.5c	1.3	78.6d	1.3	44.7a	1.4	65.5b	1.2
	C14:0	2943.7d	39.6	2496.5c	39.6	1954.6a	42.9	2138.5b	34.3
	isoC15:0	79.4a	3.6	240.5c	3.6	81.4a	3.9	187.2b	3.1
	anteisoC15:0	106.3b	3.5	192.1d	3.5	86.6a	3.8	157.0c	3.0
SFA	C16:0	2070.9d	173.3	17006.5c	173.3	14934.2a	187.6	16294.8b	150.1
	C17:0	823.2b	13.2	645.7a	13.2	786.6b	14.3	627.3a	11.5
(mg/100g)	anteisoC17:0	545.2b	8.1	717.1c	8.1	468.9a	8.8	692.2c	7.0
	C18:0	7193.5b	123.8	6866.4b	123.8	6136.1a	134.1	6072.9a	107.2
	C20:0	43.3a	1.2	72.6b	1.2	43.2a	1.3	47.7a	1.1
	C22:0	5.6b	0.3	12.8d	0.3	3.0a	0.3	10.3c	0.3
	C23:0	4.9b	0.3	11.5d	0.3	0.1a	0.3	9.0c	0.2
	C24:0	6.1b	0.3	12.2d	0.3	0.4a	0.3	10.4c	0.2
	C14:1n-5	1271.1b	31.1	1331.2b	31.1	811.8a	33.64	1334.2b	26.9
	C15:1n-5	19.2a	0.7	40.1c	0.7	18.7a	0.7	31.8b	0.6
	C15:1n-6	92.7a	3.3	211.2b	3.3	-	-	170.6c	2.8
	C16:1n-5	306.9b	7.7	301.0b	7.7	1740.4a	8.3	323.2b	6.6
	C16:1n-7	4360.0b	80.4	4792.8c	80.4	3288.4a	87.1	5232.4d	69.66
	C16:1n-7t	75.6c	1.6	37.0a	1.6	46.7b	1.8	50.9b	1.4
	C16:1n-9	199.2a	5.5	186.5a	5.5	-	-	278.8b	4.8
	C16:2n-4	19.1d	0.3	17.5c	0.4	7.5b	0.4	0.2a	0.3
	C16:3n-4	16.2d	0.3	10.5c	0.4	4.8b	0.3	0.0a	0.2
MUFA	C17:1n-7	65.7a	1.9	149.6d	1.9	84.6b	2.0	107.5c	1.6
	C18:1n-7	1468.2b	33.4	1365.3b	33.4	1204.8a	36.2	1810.2c	29.0
(mg/100g)	C18:1n-7t	3743.6c	69.0	1546.23a	69.0	2362.7b	74.7	1584.5a	59.7
	C18:1n-9	31457.7c	256.4	28601.6b	256.4	26199.9a	277.6	33173.1d	222.1
	C18:1n-9t	370.3c	6.6	201.5a	6.6	256.2b	7.1	218.8a	5.7
	C20:1n-12	13.3a	0.6	21.2b	0.6	59.0c	0.6	21.1b	0.5
	C20:1n-15	41.7	1.3	48.6	1.3	41.8	1.4	59.4	1.2
	C20:1n-5	17.69b	0.6	14.9a	0.6	-	-	18.4b	0.5
	C20:1n-7	19.0b	0.6	27.0c	0.6	14.8a	0.7	27.3a	0.5
	C20:1n-9	233.8c	4.7	168.2a	4.7	214.7b	5.1	213.9b	4.1
	C22:1n-9	7.9bc	0.4	7.2ab	0.4	6.3a	0.5	9.1c	0.4
	C24:1n-9	4.2b	0.2	4.8b	0.2	0.1a	0.2	6.5c	0.2
	C18:2n-6	1360.9d	17.7	527.8a	17.7	774.1c	19.2	669.4b	15.3
	C18:2n-6t	52.4a	5.1	70.0ab	5.1	559.6c	5.5	71.4b	4.4
	C18:3n-3	167.6a	7.5	320.9b	7.5	173.3a	8.1	359.2c	6.5
	C18:3n-4	6.4b	0.3	9.4c	0.3	3.5a	0.3	9.7c	0.3
	C18:3n-6	26.4b	0.6	30.5c	0.6	11.6a	0.7	39.3d	0.5
	C18:4n-3	9.3a	2.0	14.6ab	2.0	17.5b	2.2	94.1c	1.8
PUFA	C20:2n-6	29.7c	0.5	26.1b	0.5	17.5a	0.6	28.9c	0.4
	C20:3n-3	8.5a	0.4	17.2b	0.4	8.9a	0.5	20.0c	0.4
(mg/100g)	C20:3n-6	55.0d	0.9	36.3b	0.9	29.0a	1.0	43.2c	0.8
	C20:4n-3	3.7a	0.3	5.3b	0.3	4.6ab	0.3	7.8c	0.3
	C20:4n-6	32.4b	0.8	31.2b	0.8	19.3a	0.8	37.3c	0.7
	C20:5n-3	7.2b	0.6	16.2c	0.6	1.5a	0.6	23.1d	0.5
	C21:5n-3	4.4d	0.2	3.5c	0.2	0.2a	0.2	1.1b	0.7
	C22:2n-6	3.3b	0.1	3.3b	0.1	0.1a	0.1	0.3a	0.1
	C22:4n-6	20.0d	0.4	13.41b	0.4	7.5a	0.4	15.42c	0.4

	C22:5n-3	25.7a	2.2	67.6b	2.2	27.7a	2.4	84.4c	1.9
	C22:5n-6	3.5b	0.2	3.1b	0.2	0.2a	0.2	6.2c	0.2
	C22:6n-3	3.6b	0.3	7.6c	0.3	0.1a	0.3	10.7d	0.25
CLA (mg/100g)	c9c11CLA	79.5a	3.2	253.4c	3.2	-	-	104.5b	2.7
	c9t10CLA	164.8b	6.0	134.9a	6.0	154.0ab	6.5	221.52c	5.2
Trans (mg/100g)	t10c12CLA	65.2c	5.5	528.3b	5.5	46.2a	5.9	20.6d	4.7
	t911CLA	337.4a	12.5	99.4b	12.5	-	-	553.4a	10.8
Totals (g/100g)	SFA Total	33.0b	0.3	28.8b	0.3	25.2b	0.3	26.8a	0.2
	MUFA	39.6c	0.3	37.3a	0.3	32.1b	0.4	42.8a	0.3
	n-3 PUFA	0.2b	0.01	0.5b	0.01	0.2a	0.01	0.6c	0.01
	n-6 PUFA	1.5d	0.02	0.7a	0.02	0.9c	0.02	0.8b	0.02
	Ratio n6: n3	6.7c	0.1	1.5a	0.1	4.1b	0.1	1.7a	0.1

Although SFAs were not significantly different between south grain, north grain and north grass, they were significantly different between carcases from these three production systems and south grass-fed beef carcases. This is in contrast to previous phases which suggested SFAs accounted for a significant difference in fatty acid composition between all production systems (Logan et al., 2022). Given diets high in carbohydrates beyond the need for maintenance and growth stimulate lipogenesis in the liver and adipose tissues which leads to high levels of triglycerides (Schumacher, DelCurto-Wyffels, Thomson & Boles, 2022), it is possible that northern grass fed cattle were finished on higher carbohydrate pastures when compared to their southern counterparts resulting in an increased SFA composition and therefore reducing the model accuracy and increasing error when all data from northern and southern regions were included in models.

As previously reported results have demonstrated the greatest differences in fatty acid composition between carcases from different production systems were observed in the omega-6 to omega-3 ratio due to differences in the total omega-6 fatty acids (Logan et al., 2022). Based on the total omega-6 measured in phase 3, it is plausible the variation and misclassification of southern grass and grain fed carcases when the short-term grain fed was included is due to the total n-6. While the means are significantly different, there is little practical difference with the confidence interval ranging from 0.81 – 0.87mg/100g (mean 0.84mg/100g) for carcases from southern grass-fed systems and 0.82 – 0.90mg/100g (mean 0.86mg/100g) for carcases from southern grain fed systems. Yet phase 2 data from carcases included in the calibration model yielded confidence intervals of 0.43 – 0.73mg/100g (mean 0.58mg/100g) for carcases from grass fed, 0.74 – 1.04mg/100g (mean 0.89mg/100g) for carcases from long term grain fed cattle and 0.74 – 1.00mg/100g (mean 0.87mg/100g) for carcases from short term grain fed. Consequently, the total n-6 content of grass-fed carcases in phase 3 more closely represented that of carcases from grain fed cattle then grass-fed carcases resulting in a higher rate of misclassification of grass-fed carcases as grain.

Yet the total omega-3 fatty acids may also contribute, given the ratio of omega-6 to omega-3 fatty acids is mostly driven by increased omega-3 fatty acids in grass fed beef (Daley et al., 2010). Indeed, results previously reported in phase 2 demonstrate that the omega-3 concentration of carcases from southern supplemented grass finished production systems (0.22mg/100g) is not significantly different from that of carcases from southern short-term grain fed systems (0.22mg/100g), while carcases from southern grass-fed systems yielded twice the concentration (0.43mg/100g). Given that in the grain/grass classification model, short term grain was included as part of the grain classification and grass supplement was included in the grass classification, the model may consider carcases in phase 3 from grain fed production systems which had a mean omega-3 concentration of 0.23mg/100g supplemented grass. Therefore, models may have classified them as grass also contributing to the higher rate of misclassifications.

Significant differences between carcases from northern and southern production systems were evident for individual fatty acids including C18:0 and C20:0 as well as C22:2n-6, which are likely to account for the increased accuracy when PLS-DA models are completed based on region of production and suggest that Raman spectroscopy is a technology suitable for verifying brands which are often based on a combination of a specific feed and region.

5. Conclusion

Overall, this project demonstrated the Raman spectroscopic hand-held device is an accurate and robust tool for the verification of production systems of origin. This is highlighted by the successful discrimination between samples from grass and grain fed cattle over several years including a variety of seasonal conditions with samples collected during years of drought, above average rainfall and floods.

While discrimination between grass and grain fed carcases was possible using PCA in the preliminary investigation undertaken in phase 1, the most robust calibration models created in phase 2 was achieved with PLS-DA when regional differences were accounted for with individual models created for both northern and southern regions of production. These PLS-DA models proved to be robust and reliable as noted by the high accuracy and low errors found during the validation phase with an 86% accuracy for combined north and south models and a 96% accuracy for the prediction of grass-fed beef from northern systems. However, validation models for southern production regions were less accurate at 70% as greater variation occurred in spectra collected from carcases of short-term grain fed cattle.

Analysis of the spectra and fatty acid data revealed that the predictive ability of models was based on the saturated fatty acid content of the subcutaneous fat as well as the ratio of omega 6 and omega 3 fatty acids, which were evident in the spectra at 1050-1150cm^{-1}, 1300-1380 cm^{-1}, 1450-1500 cm^{-1} and between 1650-1700 cm^{-1}. Further analysis of the fatty acid data also revealed the concentration of omega 6 and omega 3 fatty acids were similar in carcases from southern short- term grain fed and supplemented grass-fed production systems which may have caused the variation which was associated with an increased error and decreased accuracy. Thus, further research is required to investigate the differences in fatty acids between short term grain fed cattle and supplemented grass-fed cattle to ensure feeding practices meet the demand for grass fed beef whilst maintaining a similar fatty acid profile to grass fed beef.

5.1 Key findings

1. Raman spectroscopy is a robust and reliable tool for verifying production system of origin for beef carcases with an overall accuracy of 86% for combined northern and southern validation models.
2. Predictive abilities were improved by up to 23% by creating separate models for northern and southern production systems.
3. Models for southern Australian production systems were the least accurate and had the highest error which is likely due to similarities in the fatty acid composition of carcases from supplemented grass fed and short-term grain fed cattle.

5.2 Benefits to industry

This research provides the evidence required to underpin the use of Raman spectroscopy to objectively verify grass fed beef and maintain market access while reducing the cost of auditing to the supply chain.

As Raman spectroscopy had sufficient sensitivity to classify carcases from grass fed, grass supplemented, short-term grain fed and long-term grain fed production systems in northern and southern production systems, this research also supports the use of Raman spectroscopy to verify brands which are often based on a region and feeding system.

6. Future research and recommendations

While the high accuracies and low errors of models warrants commercialisation, further research is required to determine the sources of variation noted in the spectra collected from carcases of short-term grain fed cattle and determine the impact of highly variable spectra on the calibration of southern models.

As this research demonstrated similarities in the fatty acid composition of short-term grain fed and supplemented grass-fed beef carcases, further research is required to assess the impact on grass supplements on the fatty acid content of beef to ensure the grass-fed industry can meet both the demand and consumer expectations on the concentrations of health beneficial fatty acids.

While both northern and southern production systems have been measured, this has focused on Eastern Australian states measuring carcases of cattle from Queensland, New South Wales and Victoria. Therefore, further research is required to incorporate carcases from cattle in Western Australia and South Australia in the calibration models to ensure models represent the national production system and determine if there is an east/west regional difference.

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