Final Report

AMPC
AUSTRALIAN MEAT PROCESSOR CORPORATION

Real-time identification of red meat provenance and quality attributes

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1.0 Executive Summary

The red meat supply chain comprises a complex network that attempts to transfer a range of products from production to consumption in a safe and secure way. Consequently, there are opportunities across the supply chain where vulnerabilities may be exploited and instances of food fraud and issues relating to food safety and quality can occur. Equally, there is an increasing need for the red meat supply chain to provide evidence that supports the credentials associated with its products. Rapid Evaporative Ionisation Mass Spectrometry (REIMS) is a recently developed ambient ionisation-mass spectrometry technique that is being evaluated in international markets and has demonstrated capacity relating to provenance, food quality and food safety credentials thereby providing opportunity for red meat product verification. Prior to evaluating the applicability of REIMS to red meat supply chains, priority investigation areas were identified through alignment of published studies in global agricultural systems with provenance, quality and safety attributes of interest (e.g. breed, marble score, pathogen presence) to the red meat industry. A review on the application of REIMS to the Australian red meat industry was published (https://www.mdpi.com/2218-1989/11/3/171) and was used to inform the objective and design of proof-of-concept studies.

All food products have a history and food provenance is about understanding this history and knowing about the events in the supply chain that have contributed to it. The applicability of REIMS to the classification of provenance-aligned attributes including production system, breed, brands, and combinations of breed and production system were evaluated as part of the study. A total of 192 *longissimus thoracis* samples comprising 123 grain-fed (59 Angus, 51 Wagyu, and 13 unknown) and 69 grass-fed, were sourced at retail (originating from at least 18 processing establishments) for analysis. All samples were subjected to REIMS analysis and then subsets were used to develop classification models for provenance-aligned attributes such as production system, breed, and brands using principal component analysis (PCA) and linear discriminant analysis (LDA) analysis. REIMS generates spectral profiles across the *m/z* 100-1200 range with models able to be constructed using the entire or parts of the spectral profile. All models were cross-validated, and LDA analysis of the *m/z* 600-900 range was shown to produce correct classification rates exceeding 90% for all provenance models confirming the potential of REIMS for real-time verification of red meat product attributes.

Meat quality and the subsequent sensory experience it achieves are key drivers of a consumer's ongoing purchasing decision. Marbling is a key indicator of quality and is associated with tenderness, juiciness and flavour. In addition, knowledge that a product has remain chilled and not frozen during its distribution or confirming the period of time elapsed since slaughter may be important in resolving market access issues. In the meat quality study, a total of 216 Angus and Wagyu loin steak samples comprising marble scores (MB) 2, 3 and 4 for Angus and 3, 5, 7 for Wagyu were sourced from an Australian export registered abattoir. Samples were stored at -0.5 °C for a period of up to 12 weeks and subjected to meat quality and REIMS analysis at weeks 1, 2, 4, 6, 8 and 12. The resulting REIMS spectral profiles were used to develop classification models to identify Angus and Wagyu products of varying marble scores and aged from between 1 and 12 weeks using linear discriminant analysis (LDA) analysis.

Test classification rates for the Angus models exceeded 90% regardless of whether marble score alone or marble score plus age was considered. The Wagyu model incorporating marble score and age was the best performing at 93.54%. A breed-independent model incorporating marble score and age performed well, producing a test result of 90.93%. In addition to models for the prediction of marbling scores, models that classify Angus, Wagyu, and grass-fed samples into their respective groups based on whether the product was fresh or frozen were also constructed. Testing of these models gave results of 84.57% to 87.54% depending on the spectral range used. Importantly, fresh product was never classified as frozen product, and vice versa.

Australian red meat processors verify hygiene performance and meet regulatory requirements for bacterial pathogens through a myriad of testing programs. REIMS was assessed for its ability to detect and classify the microorganisms *E. coli*, *Salmonella* and *Enterococci* which are relevant to trade, hygiene, and human health. In addition to assessing REIMS across food safety applications, there was also opportunity during the project to trial the RADIAN direct mass detector system. A total of 180 *E. coli*, *Salmonella* and *Enterococci* isolated from beef cattle-associated samples were included in the study. The REIMS and RADIAN systems were both able to develop classification models for bacterial genus, *E. coli* serogroups, *Salmonella* serovars, and *Enterococcus* speciation. All models were cross-validated with models generated using spectra profiles from REIMS resulting in correct classification rates for all models exceeding 93.33%. By comparison, models developed using spectra from the RADIAN system achieved correct classification rates exceeding 81.25%, albeit from a smaller subset of samples. The transition from using REIMS to assess beef samples for provenance and quality attributes to food safety applications proved challenging with large concentrations of bacteria and novel sampling approaches required. Transformative food safety applications would be required to detect very low concentrations of bacteria preferably without the need for prior growth of the organisms to have occurred. In contrast, the RADIAN system is likely to have strong applicability for food safety applications in red meat supply chains due to its simple non-destructive sampling approach and ability to detect bacteria of interest at much lower concentrations. The relatively straight forward analysis workflow that could be navigated by personnel with limited training makes RADIAN a suitable candidate for use in red meat processing environments.

This study represents the first description of the use of REIMS on Australian foods and confirmed its capacity to accurately classify provenance, meat quality and food safety attributes of importance to the industry and its customers. REIMS has the potential to sample and classify hundreds of products each day with a time to result of less than one minute. Furthermore, a single REIMS spectrum can be used across multiple classification models enabling the verification of several product attributes from a single test thereby providing advantages over technologies which are constrained to a specific attribute. Whilst it would be ideal to consider REIMS as a technology that can be deployed into processing plants, the need for skilled operators, high capital cost, and its destructive sampling approach perhaps makes it more applicable to commercial laboratories capable of offering a product verification service that augments a processing plant's quality assurance program for provenance and meat quality attributes. Further investigation of this type of service offering for the industry is warranted. Conversely, the applicability of REIMS to food safety applications that enable practice change via rapid detection of organisms of interest during red meat processing is unlikely at present due to the issues associated with sampling small concentrations of bacteria. In this context, the RADIAN system is of interest, and it may be possible to identify

opportunities where the RADIAN system could be used to simultaneously assess beef products in-line to confirm an attribute (e.g. Wagyu) and identify any food safety implications (e.g. the presence of STEC) using a single sample. In summary, ambient mass spectrometry systems continue to demonstrate promise for rapid verification of product attributes in the red meat industry. In a proof-of-concept setting, the REIMS and RADIAN (food safety) systems were able to demonstrate their utility for classification of provenance, quality and food safety attributes of high relevance to the red meat industry. As processing plants, and red meat supply chains more broadly, continue their evolution towards an environment of continuous assurance, there will be increasing focus on systems which can biologically verify the credentials of red meat products and work in conjunction with digital traceability and export systems. The potential to apply ambient mass spectrometry systems across several key areas in red meat supply chains bodes well for the continued development and deployment of models which support the objective classification of red meat products.

2.0 Introduction

The red meat supply chain comprises a complex network that attempts to transfer a range of products from production to consumption in a safe and secure way. As a consequence there are points within the supply chain where fragmentation exists and vulnerabilities may be exploited. There is ever increasing demand to de-fragment these systems and ensure that Australian red meat products enter domestic or export markets with the desired quality, safety and provenance attributes. Novel technologies that can be incorporated across the red meat supply chain will be central facilitators in the evolution of the meat plant of the future. This study will explore the capabilities of rapid evaporative ionisation mass spectrometry (REIMS) profiling with regard to a range of product attributes relating to provenance as well as food safety and quality. REIMS technology is being utilised in international markets in response to recent food fraud episodes in red meat and seafood supply chains and has demonstrated capacity relating to species identification, location of production, production system, and slaughter and processing methods. Additionally, REIMS has demonstrated taxonomic specificity across the bacterial spectrum which may permit its use in real-time assessment of food quality and safety attributes. This primary phase of the project will review the application of REIMS and will identify areas for greatest return on investment. Subsequent to the primary phase, a proof of concept project will be conducted that will explore the use of REIMS in up to three application areas relating to provenance, quality and safety.

3.0 Project Objectives

The objectives of the study include:

1. Review and rank the application areas for REIMS in Australian red meat supply chains. Recommend three application areas for further investigation.
2. Development, application, and evaluation of the application of REIMS in a provenance aligned area (e.g. breed differentiation, production system, geography of location).
3. Development, application, and evaluation of REIMS in a food quality area (e.g. substitute for aspects of MSA grading, rapid prediction of terminal storage life for vacuum packaged red meat products).
4. Development, application, and evaluation of REIMS in a food safety related activity (e.g. pathogen detection).

4.0 Project Outcomes

The project objectives of AMPC project 2019-1065 have been successfully completed. The project reviewed potential application areas for REIMS in the red meat industry and subsequently used the findings of the review to inform experimental design for investigating the applicability of REIMS to the verification of provenance, quality, and safety attributes of red meat systems. The outcomes of these investigations are detailed in a peer-reviewed publication and a series of milestone reports as shown below. This final report is a compilation of a series of reports and publications produced as a result of the project's successful completion.

- Review article - Barlow, R.S.; Fitzgerald, A.G.; Hughes, J.M.; McMillan, K.E.; Moore, S.C.; Sikes, A.L.; Tobin, A.B.; Watkins, P.J. Rapid Evaporative Ionization Mass Spectrometry: A Review on Its Application to the Red Meat Industry with an Australian Context. *Metabolites* **2021**, *11*, 171. https://doi.org/10.3390/metabo11030171.
- Milestone report – REIMS provenance applications: Real-time identification of red meat provenance and quality attributes.
- Milestone report – REIMS quality applications: Real-time identification of red meat provenance and quality attributes.
- Milestone report – REIMS food safety applications: Real-time identification of red meat provenance and quality attributes.

5.0 Conclusions / Recommendations

This study represents the first description of the use of REIMS on Australian foods and confirmed its capacity to accurately classify provenance, meat quality and food safety attributes of importance to the industry and its customers. REIMS has demonstrated capacity to sample and classify hundreds of products each day with a time to result of less than one minute. Furthermore, a single REIMS spectrum can be used across multiple classification models enabling the verification of several product attributes from a single test thereby providing advantages over technologies which are constrained to a specific attribute. Whilst it would be ideal to consider REIMS as a technology that can be deployed into processing plants, the need for skilled operators, high capital cost, and its destructive sampling approach perhaps makes it more applicable to commercial laboratories capable of offering a product verification service that augments a processing plant's quality assurance program for provenance and meat quality attributes. Further investigation of this type of service offering for the industry is warranted. Conversely, the applicability of REIMS to food safety applications that enable practice change via rapid detection of organisms of interest during red meat processing is unlikely at present due to the issues associated with sampling small concentrations of bacteria. In this context, the RADIAN system is of interest, and it may be possible to identify opportunities where the RADIAN system could be used to simultaneously assess beef products in-line to confirm an attribute (e.g. Wagyu) and identify any food safety implications (e.g. the presence of STEC) using a single sample. Further investigation of the RADIAN as an in-line verification tool for the red meat industry should be commenced. In summary, ambient mass spectrometry systems continue to demonstrate promise for rapid verification of product attributes in the red meat industry. In a proof-of-concept setting, the REIMS and RADIAN (food safety) systems were able to demonstrate their utility for classification of provenance, quality and food safety attributes of high relevance to the red meat industry. As processing plants, and red meat supply chains more broadly, continue their evolution towards an environment of continuous assurance, there will be increasing focus of systems which can biologically verify the credentials of red meat products and work in conjunction with digital traceability and export systems. The potential to apply ambient mass spectrometry systems across several key areas in red meat supply chains bodes well for the continued development and deployment of models which support the objective classification of red meat products.

6.0 Milestone report: REIMS provenance applications

6.1 Executive summary

The red meat supply chain comprises a complex network that attempts to transfer a range of products from production to consumption in a safe and secure way. As a consequence, there are opportunities across the supply chain where vulnerabilities may be exploited and instances of food fraud and issues relating to food safety and quality can occur. Rapid Evaporative Ionisation Mass Spectrometry (REIMS) is a recently developed ambient ionisation-mass spectrometry technique that is being evaluated in international markets in response to recent food fraud episodes in red meat and seafood supply chains and has demonstrated capacity relating to species identification, location of production, production system identification, and slaughter and processing methods.

This project aims to review the application areas for REIMS as they pertain to Australia's red meat industry and to subsequently carry out a series of proof of concept studies for attributes aligned with provenance, food safety, and food quality. A total of 192 *longissimus thoracis* or loin steak samples comprising 123 grain-fed and 69 grass-fed, were sourced at retail (originating from at least 18 processing establishments) for analysis. Of the 123 grain-fed products collected, 59 were Angus, 51 were Wagyu and 13 of unknown breed. All samples were subjected to REIMS analysis and then subsets were used to develop the following classification models using principal component analysis (PCA) and linear discriminant analysis (LDA) analysis.

- Production system - Grass v Grain
- Breed – Angus v Wagyu
- Supply chain – Angus brands
- Supply chain – Wagyu brands
- Supply chain – Grass brands
- Breed/production systems – Grass v Angus v Wagyu

All models were cross-validated, and LDA analysis of the *m/z* 600-900 range was shown to produce correct classification rates exceeding 90% for all models confirming the potential of REIMS for real-time verification of red meat product attributes. Loading plots were generated for all dimensions of all models which permits the preliminary identification of molecules and ions which drive the separation of groups within models. Accurate identification of these compounds can be achieved using high-resolution metabolomics approaches, and although identification of specific compounds is not needed for the modelling of beef spectral profiles for compliance purposes, accurate identification of specific molecules could enable the development of rapid analytical devices.

This report is the first description of the use of REIMS on Australian foods and the development of classification models for production system, breed, and supply chains demonstrating its capacity to accurately identify red meat attributes of importance to the industry and its customers. It is necessary to recognise that the models developed as part of this proof of concept study require further development to capture the effect of additional factors such as seasonality that may impact REIMS spectral profiles due to variations in pasture and grain quality and availability.

Nevertheless, this study has confirmed that REIMS shows a high potential for the real-time identification of provenance aligned attributes, which are of importance to the red meat industry. Subsequent discussions with industry participants should identify focused use cases and provide input for optimisation of modelling to further refine and improve the accuracy of REIMS application.

6.2 Introduction

The red meat supply chain comprises a complex network that attempts to transfer a range of products from production to consumption in a safe and secure way. As a consequence, there are opportunities across the supply chain where fragmentation can arise and vulnerabilities may be exploited. There is ever-increasing demand to de-fragment these systems and ensure that Australian red meat products enter domestic or export markets with the desired quality, safety, and provenance attributes. Novel technologies that can be incorporated across the red meat supply chain will be central facilitators in the evolution of the meat plant of the future. Rapid Evaporative Ionisation Mass Spectrometry (REIMS) is a recently developed ambient ionisation-mass spectrometry technique that is being evaluated in international markets in response to recent food fraud episodes in red meat and seafood supply chains and has demonstrated capacity relating to species identification, location of production, production system, and slaughter and processing methods. The REIMS System (Waters Corporation, USA) combines an electrosurgical knife (iKnife), with a quadrupole time-of-flight mass spectrometer to generate unique mass spectral ‘fingerprints’ within seconds that can be used to assess key attributes and differences of, and between, samples of interest. Furthermore, the iKnife is connected to the mass spectrometer by a length of tubing which facilitates REIMS biggest advantage by enabling sampling to occur remotely from the mass spectrometer within seconds. This advantage increases potential for REIMS to be used in processing facilities where the mass spectrometer unit can be kept in a clean area while an operator uses the iKnife within the processing plant as required.

The provenance of a food item or ingredient refers to the origin or source from which it comes, and the history of subsequent operations (supply chain) [1]. The ability to communicate the provenance of a product to consumers is of increasing importance in global trade and Australia’s red meat industry have embraced this desire and continually look to communicate information relating to geographical origin, production system, breed, animal health and sustainability as part of their broader commitment to ‘end to end’ traceability systems. The ability to verify product attribute claims will therefore be an integral component of traceability systems and therefore, technologies such as REIMS, will be key in linking the physical and digital attributes of products as they move through a supply chain.

In order to use the spectral ‘fingerprints’ generated by REIMS to predict product attributes of interest, classification models must be developed. Data analysis typically occurs through the application of unsupervised or supervised methods of analysis. Unsupervised approaches such as principal component analysis (PCA) make no assumptions in relation to the data, and it is used as a visualisation technique to identify patterns within a dataset [2]. Supervised methods such as linear discriminant analysis (LDA) are tools used for classification, dimension reduction and data visualisation and depend on accepting that the identity applied to each grouping is correct. The aim of this study is to

utilise supervised and unsupervised data analysis methods to determine the applicability of REIMS as a tool for verifying provenace attributes of red meat products.

6.3 Materials and Methods

6.3.1 Sample collection

A total of 192 loin muscle samples were collected for analysis. Samples were collected from retail establishments including butcher shops, wholesalers, and supermarkets. All samples were sourced from the *longissimus* muscle and were presented at retail as scotch fillet or porterhouse. Vacuum packaged primals were opened and two 25 mm steaks were removed and transported to the laboratory for analysis. REIMS sampling occurred 24-72 h after collection and samples were stored at 4°C whilst in the laboratory. The name and location of the retail establishment were recorded and where possible details of the production system (grass or grain), breed (Angus or Wagyu), establishment number of the processing plant, and brand were also collected.

6.3.2 REIMS

REIMS analysis was conducted using an electrosurgical knife (iKnife, Waters, UK) combined with a Xevo G2 qToF mass spectrometer (Waters, UK). The iKnife was powered by an Erbe VIO 50C generator (Erbe Medical, UK) set at 25 W power in dry-cutting mode. Each sample was cut with the iKnife for a period of 3-5 s per cut. A total of 10 technical replicates were performed for each sample with a delay of at least 5 s between each cut. Spectral 'fingerprints' were acquired between the mass range *m/z* 100-1200 in negative ionisation mode using a scan rate of 0.5 s per scan. Leucine enkephalin (Waters, UK) was used as a lockmass by dissolving it in MS-grade isopropanol (Fisher Scientific, USA) at a concentration of 0.1 ng/µl and infusing it into the mass spectrometer at a rate of 150 µl/min. Following the completion of each sample, carbonised sample was scraped from the iKnife and subsequently wiped with a tissue dampened with isopropanol.

6.3.3 Data analysis

REIMS data was processed and analysed using the Abstract Model Builder (AMX) [Beta] version 1.0.2159.0 (Waters Research Centre, Hungary). For each sample, mass spectra were loaded, and individual cuts identified. Pre-processing was used to remove the background signal, correct burn ends, apply a lockmass correction (leu enk 554.2615) and normalise. To avoid 'over-fitting' models, the number of PCA dimensions were set to 20 and when LDA analysis was performed, the LDA dimensions were set to maximum which equals the number of classification groups minus one. The intensity limit for all models was set at 10 000 and binning of data was done at a scale of *m/z* 0.1.

REIMS spectral profiles typically exhibit a concentration of signal in the *m/z* 100-500 and 600-900 range which relate to the detection of fatty acids and glycerophospholipids, respectively. To understand the relative contribution of these spectral ranges to the overall classification models, PCA and LDA analysis was conducted for all samples using the

spectral ranges *m/z* 100-500, 600-900 and 100-1200. Cross-validation of all models was performed using either the full group out or 20% out approach with outlier calls based on a standard deviation multiplier of 5. Full group out cross-validation was conducted with models containing >1000 spectral profiles with each classification randomly assigned across five groups prior to validation. The 20% approach to cross-validation was used for all other models.

6.4 Model development

A total of 192 beef samples comprising 123 grain-fed and 69 grass-fed products were collected. The grain-fed products could be further differentiated by breed with 59 Angus, 51 Wagyu and 13 unknown breed samples included. The entire sample set represents product from at least 18 processing establishments across Queensland, New South Wales, Victoria and Tasmania. All samples were subjected to REIMS analysis and then subsets were used to develop the following classification models:

- Production system - Grass v Grain
- Breed – Angus v Wagyu
- Supply chain – Angus brands
- Supply chain – Wagyu brands
- Supply chain – Grass brands
- Breed/production systems – Grass v Angus v Wagyu

6.4.1 Production system – grass v grain

The Australian red meat industry maintains a series of independently audited quality assurance programs aimed at guaranteeing the safety and integrity of beef. The Pasturefed Cattle Assurance System (PCAS) and the National Feedlot Accreditation Scheme (NFAS) are prominent Australian based programs that amongst other things, ensure animals are lifetime pasture fed (PCAS) or the welfare and environmental sustainability of feedlots and the animals within them are continuously improved (NFAS). Consumers trust programs such as PCAS and NFAS to assist in delivering grass-fed and grain-fed products to market at which point several personal preferences determine the type of product a particular consumer will seek out. REIMS was evaluated for its ability to correctly classify 123 grain-fed and 69 grass-fed beef products. A PCA plot of the ions identified in the *m/z* 600-900 range (Fig. 1) demonstrates reasonable separation of the production system classes and resulted in a correct classification rate of 78.34% (Table 1) with outliers included. The use of LDA provided enhanced classification outcomes with all three LDA models producing a minimum correct classification rate of 88.03%. Slight differences were observed between the LDA models depending on the *m/z* range included in the analysis with the *m/z* 600-900 range giving the highest correct classification score of 93.65%.

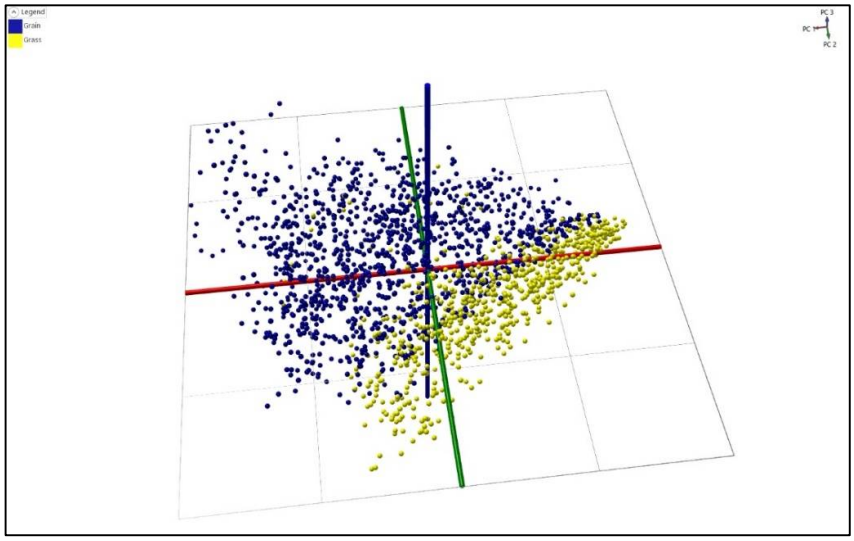

Fig. 1. PCA plot of the *m/z* 600-900 range of grass-fed (yellow) and grain-fed (blue) samples following REIMS analysis.

Table 1. Cross-validation scores for PCA and LDA classification models for grass-fed and grain-fed beef samples for the spectral ranges *m/z* 100-1200, 100-500, and 600-900.

Spectral range (m/z)	***100-1200***	***100-500***	***600-900***	***100-1200***	***100-500***	***600-900***
Model type	PCA	PCA	PCA	LDA	LDA	LDA
Number of spectra	1921	1921	1921	1921	1921	1921
Number of passes	1357	1327	1505	1738	1691	1799
Number of failures	541	567	393	183	228	122
Number of outliers	23	27	23	0	2	0
Correct classification - excluding outliers	71.50%	70.06%	79.29%	90.47%	88.12%	93.65%
Correct classification - including outliers	70.64%	69.08%	78.34%	90.47%	88.03%	93.65%

Furthermore, loading plots provide an opportunity to determine the compounds or ions which drive the separation of the two classes: grass-fed and grain-fed. The loading plot for the first dimension of the *m/z* 100-1200 LDA model is shown in Fig. 2. Ions or compounds with greater positive magnitude (top of plot) are associated with grain-fed product. Similarly, ions or compounds with the greatest negative magnitude (bottom of plot) are associated with grass-fed product. Table 2 shows the top 15 positive and negative loadings for the first dimension of the *m/z* 100-1200 LDA model. It is important to note that REIMS does not allow collection of MS and MS/MS data at the same time which limits the ability to accurately identify the compounds associated with the highest loadings. These compounds could be identified using other high-resolution mass spectrometry approaches would could lead to the development of rapid analytical tools. However, identification of specific compounds is not needed for the modelling of beef spectral profiles for compliance purposes and consequently REIMS is a technology that could be used for real-time verification of grass-fed or grain-fed production systems.

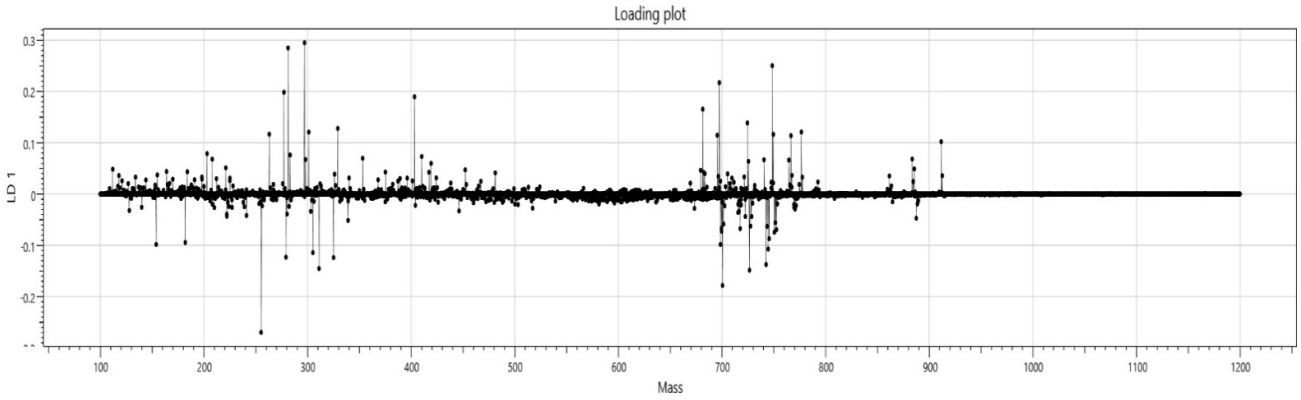

Fig. 2. Loading plot for the first dimension of the *m/z* 100-1200 LDA model for grass-fed (negative) and grain-fed (positive) products.

Table 2. Top 15 positive and negative loadings from LD1 of the *m/z* 100-1200 LDA model for grass-fed and grain-fed products.

LD1 positive	Loading	LD1 negative	Loading
297.15	0.294961	255.25	-0.26989
281.25	0.284838	700.55	-0.17832
748.55	0.250175	726.55	-0.1485
697.45	0.217149	311.15	-0.14533
277.25	0.198444	742.55	-0.13752
403.25	0.189533	325.15	-0.12397
681.45	0.165327	279.25	-0.12312
724.55	0.138663	305.25	-0.11427
329.25	0.127953	744.55	-0.10719
776.55	0.120849	698.55	-0.09837
301.25	0.120685	154.05	-0.09836
263.15	0.116683	182.05	-0.09444
749.55	0.116271	745.55	-0.08707
695.45	0.11459	750.55	-0.07459
766.55	0.113765	699.55	-0.07265

6.4.2 Breed – Angus v Wagyu

The trend for retailers and restaurants to identify the type of beef they are selling or serving on a menu continues to develop. There are strong opportunities for premium breed-specific Australian beef products such as Angus and Wagyu in key markets, particularly in Asia. The Australian Wagyu Association and Angus Australia have developed breed verification programs designed to support breeders and processors to uphold market integrity. These programs are underpinned by genomic testing and whilst DNA testing is, and will remain the gold standard, it is unlikely to emerge as a true real-time verification tool. REIMS was used to generate spectral profiles on 59 grain-fed

Angus and 51 grain-fed Wagyu products with subsequent PCA and LDA analysis used to determine the correct classification rates of the models developed. The PCA plot of the *m/z* 600-900 range is shown in Fig. 3, with separation of Angus and Wagyu samples evident. Table 3 lists the cross-validation scores for PCA and LDA classification models for grass-fed and grain-fed beef samples for the spectral ranges *m/z* 100-1200, 100-500, and 600-900. Classification rates were improved through the use of LDA models with the *m/z* 600-900 LDA model producing a correct classification rate of 92.28%. This finding was slightly higher than the correct classification rates produced by the *m/z* 100-1200 and 100-500 LDA models and has been observed previously when analysing REIMS data [3].

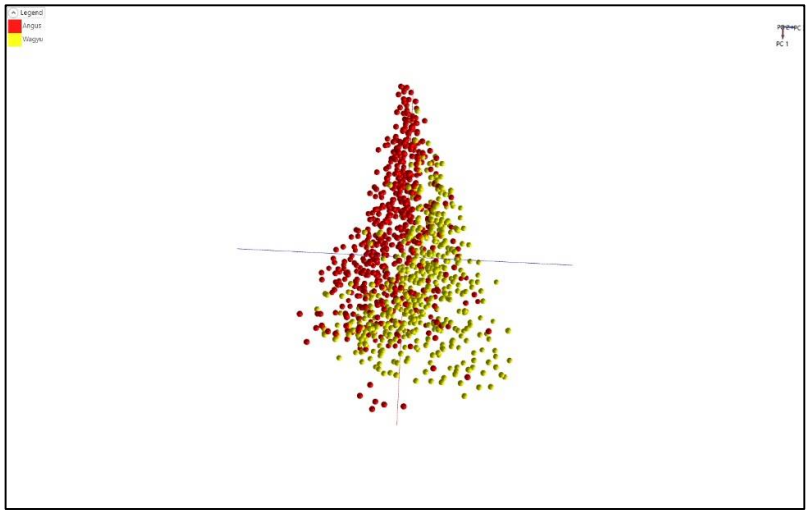

Fig. 3. PCA plot of the *m/z* 600-900 range of Angus(red) and Wagyu (yellow) samples following REIMS analysis.

Table 3. Cross-validation scores for PCA and LDA classification models for Angus and Wagyu beef samples for the spectral ranges *m/z* 100-1200, 100-500, and 600-900.

Spectral range (m/z)	***100-1200***	***100-500***	***600-900***	***100-1200***	***100-500***	***600-900***
Model type	PCA	PCA	PCA	LDA	LDA	LDA
Number of spectra	1101	1101	1101	1101	1101	1101
Number of passes	746	729	810	983	988	1016
Number of failures	335	358	274	118	110	85
Number of outliers	20	14	17	0	3	0
Correct classification - excluding outliers	69.01%	67.07%	74.72%	89.28%	89.98%	92.28%
Correct classification - including outliers	67.76%	66.21%	73.57%	89.28%	89.74%	92.28%

The loading plot for the first dimension of the *m/z* 600-900 LDA model for Angus and Wagyu products is shown in Fig. 4. Several pronounced differences can be observed between the breed-specific products and the relative loadings of the top 15 discriminatory ions or compounds as shown in Table 4. Once again, the

ions and compounds have not been identified, and whilst they are not critical for classifying Wagyu and Angus products by breed, they may be important to other attributes such as flavour, however investigation of such compounds is outside the scope of this project. Although not investigated as part of this study, it should be recognised that a number of the Wagyu products sourced for this study are likely to be F1 Wagyu and could actually be Wagyu crossed with Angus. Further model development and biomarker identification may provide opportunities for rapid determination of Wagyu crossbreeds (i.e. fullblood, purebred or F1).

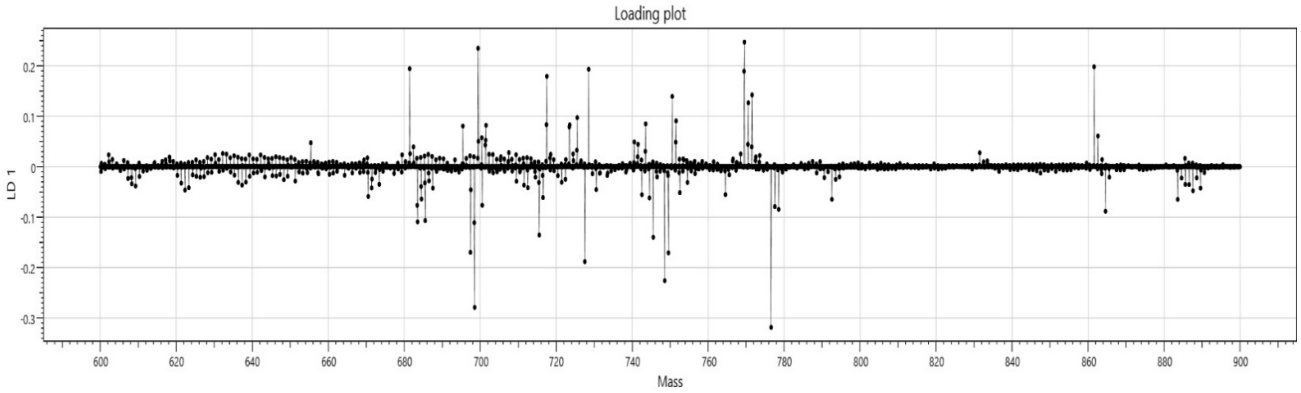

Fig. 4. Loading plot for the first dimension of the *m/z* 600-900 LDA model for Angus and Wagyu products.

Table 4. Top 15 positive and negative loadings from LD1 of the *m/z* 100-1200 LDA model for grass-fed and grain-fed products.

LD1 positive	Loading	LD1 negative	Loading
769.55	0.246957	776.55	-0.318449027
699.45	0.234594	698.55	-0.278798739
861.55	0.198049	748.55	-0.225922442
681.45	0.194235	727.55	-0.188093571
728.55	0.193154	749.55	-0.170613063
769.45	0.189156	697.45	-0.169676859
717.55	0.179132	745.55	-0.139780942
771.55	0.142246	715.55	-0.135391684
750.55	0.139283	698.45	-0.110903808
770.55	0.126615	683.55	-0.109253604
725.55	0.097154	685.55	-0.106729922
751.55	0.090684	864.55	-0.088225953
743.55	0.084892	778.55	-0.084528558
717.45	0.083271	777.55	-0.079092494
723.55	0.08273	683.45	-0.076471352

6.4.3 Supply chains – brand differentiation

Participants within the Australian red meat industry invest significant amounts of money in the pursuit of continually improving their product offering. This is particularly relevant for Wagyu and Angus beef products where genetic improvement and the use of specialised feeding regimes produce product characteristics that are repeatedly sought by consumers. The premiumisation of any product brings with it increased risk of fraud and substitution. Brands are exploring digital and biological approaches that maintain the integrity of the product being produced, thereby assuring 'end-to-end' traceability. It is expected that digital solutions will mitigate many of the issues that affect product integrity, however, investigations that follow food safety incidents, product complaints, and substitution events may all likely benefit from technologies that can rapidly differentiate products from different supply chains. As part of this study, we evaluated the ability of REIMS to distinguish branded Angus, Wagyu, and grass-fed beef products.

6.4.3.1 Supply chain – Angus brands

A total of 47 Angus products representing four brands that has been sourced on multiple occasions were included in the models. The *m/z* 100-500 LDA model is shown in Fig. 5 and separation of the four brands is clear. Cross-validation of all PCA and LDA models resulted in correct classification rates ranging from 67.09 to 68.79% for PCA models and 94.27 to 96.60% for LDA models (Table 5), with the *m/z* 100-500 LDA model achieving the highest classification rate.

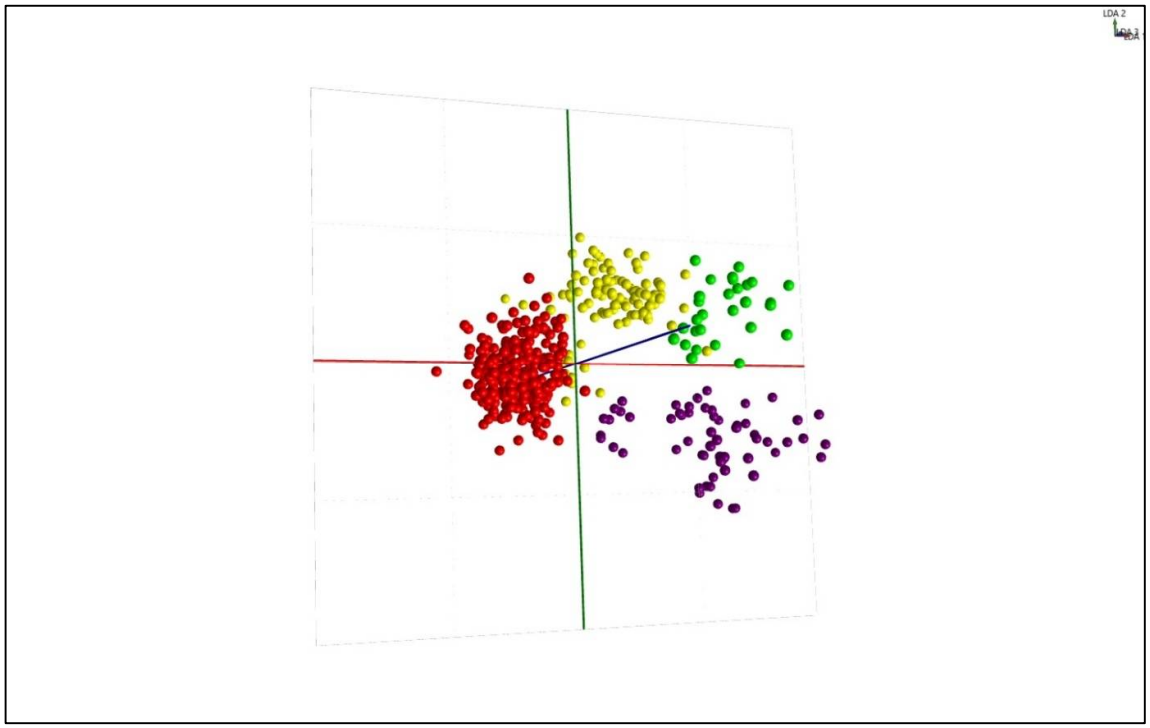

Fig. 5. LDA plot of the *m/z* 100-500 range of four branded Angus products following REIMS analysis. NB: brands have been de-identified but are represented by individual colours.

Table 5. Cross-validation scores for PCA and LDA classification models for branded Angus products for the spectral ranges *m/z* 100-1200, 100-500, and 600-900.

Spectral range (m/z)	***100-1200***	***100-500***	***600-900***	***100-1200***	***100-500***	***600-900***
Model type	PCA	PCA	PCA	LDA	LDA	LDA
Number of spectra	471	471	471	471	471	471
Number of passes	324	316	323	450	455	444
Number of failures	142	147	141	20	16	27
Number of outliers	5	8	7	1	0	0
Correct classification - excluding outliers	69.53%	68.25%	69.61%	95.74%	96.60%	94.27%
Correct classification - including outliers	68.79%	67.09%	68.58%	95.54%	96.60%	94.27%

6.4.3.2 Supply chain – Wagyu brands

Branded Wagyu products that were collected on three or more occasions were selected for inclusion in the Wagyu brand model. The *m/z* 600-900 LDA model (Fig. 6) achieved the highest correct classification rate of 94.14% and was notably better than the LDA models for *m/z* 100-500 or 100-1200 which were 85.36% and 86.19%, respectively. LDA analysis resulted in higher classification rates than PCA analysis with correct classification rates ranging from 46.03 to 50.21% for the three PCA models (Table 6).

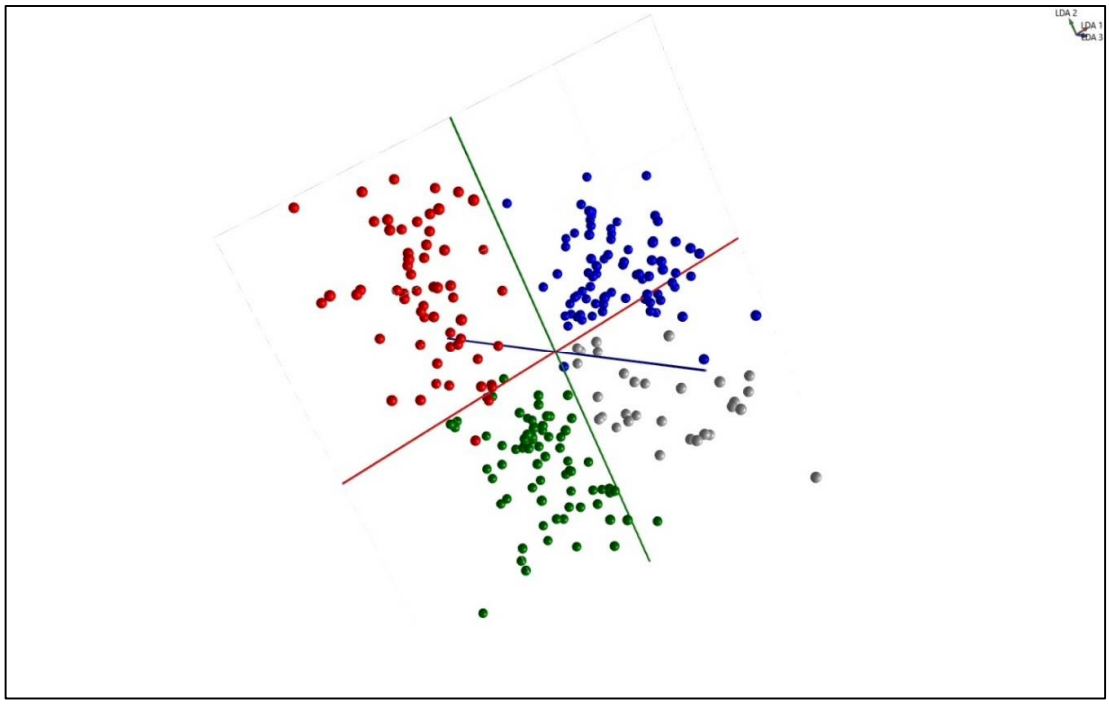

Fig. 6. LDA plot of the *m/z* 600-900 range of branded Wagyu products from four different brands following REIMS analysis. NB: brands have been de-identified but are represented by individual colours.

Table 6. Cross-validation scores for PCA and LDA classification models for branded Wagyu products for the spectral ranges *m/z* 100-1200, 100-500, and 600-900.

Spectral range (m/z)	***100-1200***	***100-500***	***600-900***	***100-1200***	***100-500***	***600-900***
Model type	PCA	PCA	PCA	LDA	LDA	LDA
Number of spectra	239	239	239	239	239	239
Number of passes	114	110	120	206	204	225
Number of failures	124	125	109	33	35	13
Number of outliers	1	4	10	0	0	1
Correct classification - excluding outliers	47.90%	46.81%	52.40%	86.19%	85.36%	94.54%
Correct classification - including outliers	47.70%	46.03%	50.21%	86.19%	85.36%	94.14%

Australia imports limited amounts of Japanese Wagyu into Australia, mainly for use in high-end Japanese and Korean restaurants. REIMS was used to analyse Japanese Kagoshima A5 Wagyu and an LDA model constructed using the *m/z* 600-900 range (Fig. 7). The model clearly differentiates the Japanese Wagyu from the Australian Wagyu brands. Opportunities to sample and evaluate beef products from other countries are often limited, however, REIMS has demonstrated potential to differentiate product at country and brand level and further investigations in the space are therefore warranted.

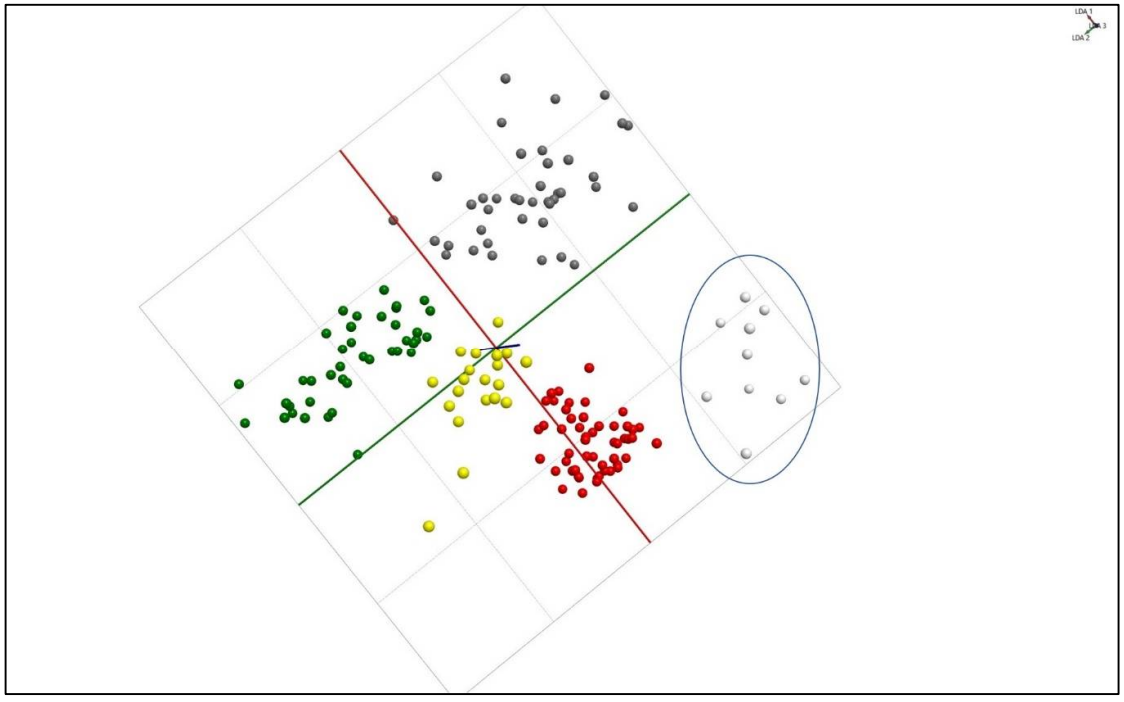

Fig. 7. LDA plot of the *m/z* 600-900 range from four different Australian Wagyu and one Japanese Wagyu brand following REIMS analysis. NB: Australian brands have been de-identified but are represented by individual colours. Spectral profiles from the Japanese Wagyu brand are circled.

6.4.3.3 Supply chains – Grass-fed brands

Forty-two grass-fed beef products representing three Australian brands were analysed by REIMS and the resulting spectral profiles used to construct PCA and LDA models. The *m/z* 600-900 LDA model is shown in Fig. 8 and it achieved the highest correct classification rate of 93.33% (Table 7). Correct classification rates for the three PCA models ranged from 66.02 to 70.24%, with the *m/z* 100-500 and 100-1200 LDA models achieving correct classification rates of 90.71% and 90.95%, respectively.

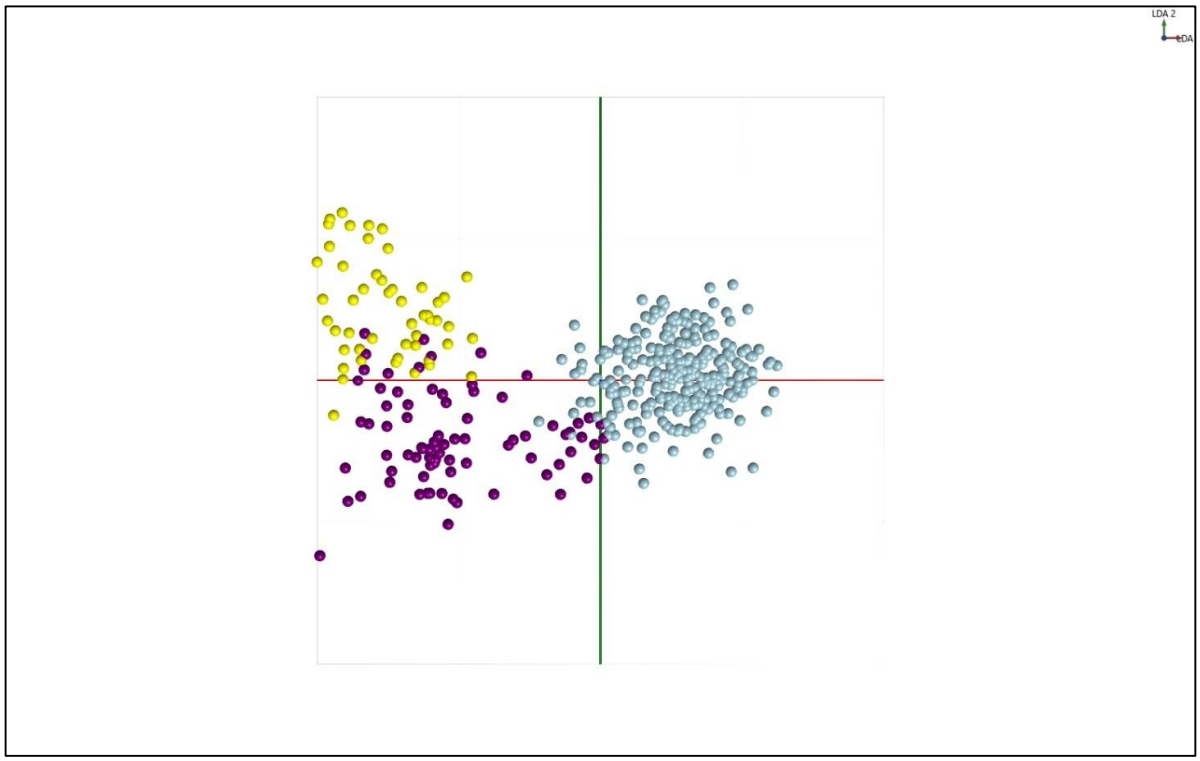

Fig. 8. LDA plot of the *m/z* 600-900 range from three different grass-fed beef brands following REIMS analysis. NB: brands have been de-identified but are represented by individual colours.

Table 7. Cross-validation scores for PCA and LDA classification models for branded grass-fed beef products for the spectral ranges *m/z* 100-1200, 100-500, and 600-900.

Spectral range (m/z)	***100-1200***	***100-500***	***600-900***	***100-1200***	***100-500***	***600-900***
Model type	PCA	PCA	PCA	LDA	LDA	LDA
Number of spectra	420	420	420	420	420	420
Number of passes	286	295	274	382	381	392
Number of failures	133	125	141	38	39	28
Number of outliers	1	0	5	0	0	0
Correct classification - excluding outliers	68.26%	70.24%	66.02%	90.95%	90.71%	93.33%
Correct classification - including outliers	68.10%	70.24%	66.02%	90.95%	90.71%	93.33%

The brands shown to the left-hand side of Fig. 8 were expected to be difficult to separate as the animals are produced on pastures with close proximity and are therefore likely to be comparable in terms of lipid content. The groups are separated by the second dimension of the LDA model and the loading plot and respective loadings are shown in Fig. 9 and Table 8 below. The resolution at which the spectral profiles produced by REIMS analysis can separate beef cattle that have minimal production system differences needs further investigation. These findings suggest that there is opportunity to separate animals at low spatial scale and furthermore, there may be opportunity to identify specific compounds that are indicative of the types of pastures that prevail in certain geographies.

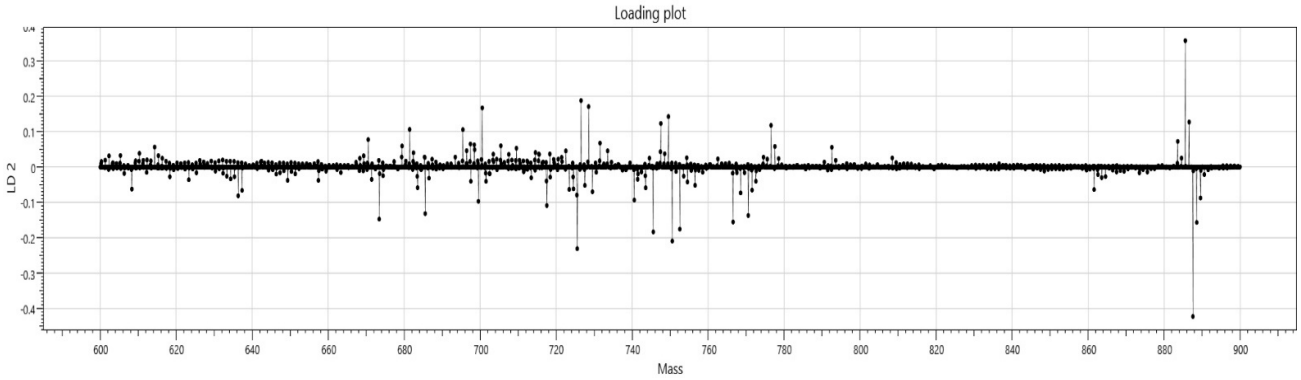

Fig. 9. Loading plot for the second dimension of the *m/z* 600-900 LDA model for branded grass-fed products.

Table 8. Top 15 positive and negative loadings from LD2 of the *m/z* 600-900 LDA model for branded grass-fed products.

LD2 positive	Loading	LD2 negative	Loading
885.55	0.356927	887.55	-0.42258
726.55	0.187577	725.55	-0.23085
728.55	0.170635	750.55	-0.20941
700.55	0.166999	745.55	-0.18351
749.55	0.142444	752.55	-0.17552
886.55	0.126992	888.55	-0.15675
747.55	0.12282	766.55	-0.1555
776.55	0.117575	673.45	-0.14724
681.45	0.105948	770.55	-0.13706
695.45	0.105356	685.55	-0.13177
670.55	0.077315	717.55	-0.1089
883.55	0.072215	699.55	-0.09698
731.55	0.067333	740.55	-0.09354
697.45	0.064879	889.55	-0.08761
698.45	0.062438	636.35	-0.08136

6.4.4 Breed and production systems – grass v Angus v Wagyu

Beef provenance and quality are rarely described using a single attribute and are instead a complex phenotype of multiple factors often measured against different criteria using a range of verification tools. The potential for REIMS spectral profiles to be used for combinations of attributes were investigated through the construction of PCA and LDA models that combined production system (grass-fed or grain-fed) and breed (Angus and Wagyu). The *m/z* 600-900 LDA model shown below (Fig. 10) results in a correct classification rate of 91.12%. The remaining LDA models produced correct classification rates of 82.69 to 83.81%, with the PCA models resulting in correct classification rates of 57.96% to 67.56% (Table 9).

Although the three classifications being explored in the model show separation, there is some crossover at the boundary between grass-fed and Angus and between Angus and Wagyu. As previously mentioned, breed type is rarely disclosed in the branding of grass-fed beef products with greater focus placed on the region of production. It is likely that the grass-fed products contain a proportion of Angus or other European breed genetic content which may, in part, describe a reason for the crossover. Similarly, Angus are a predominant breed for crossbreeding F1 Wagyu and could explain the crossover between the Angus and Wagyu groups. Further investigation and identification of the underlying drivers of the separation are required before a combinatorial model could be used for verification purposes.

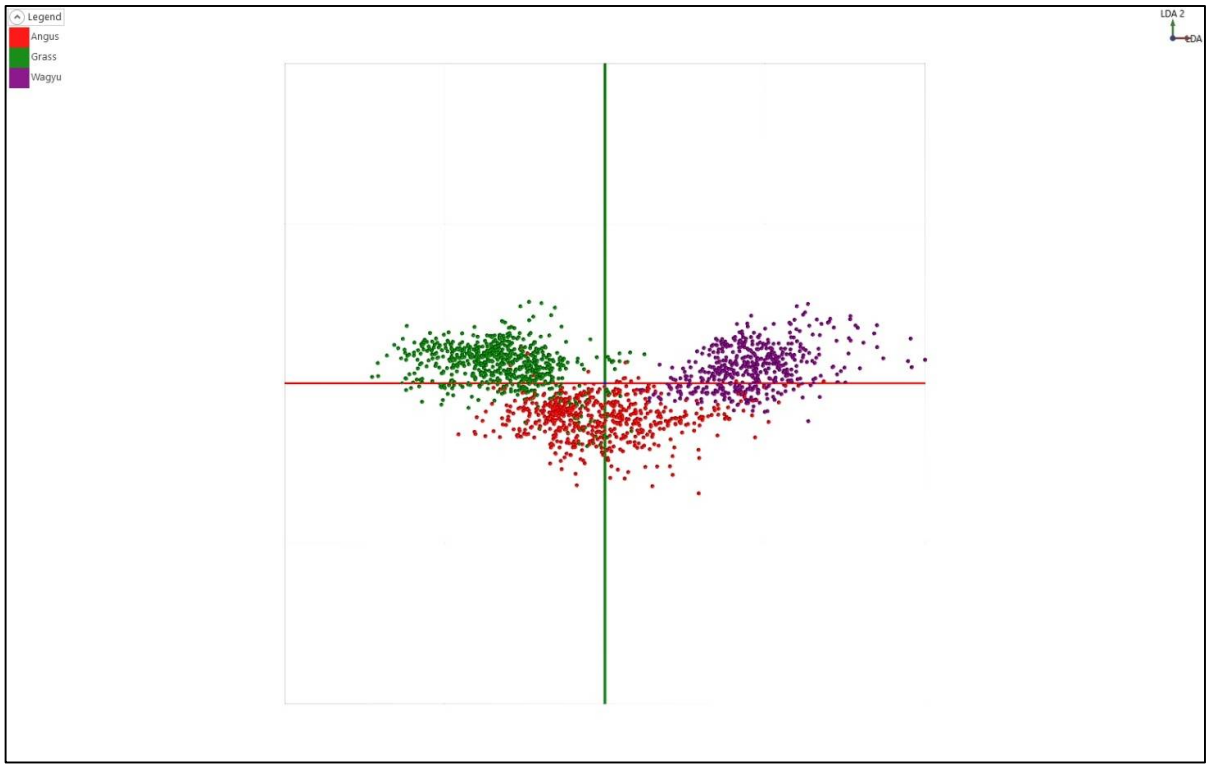

Fig. 10. LDA plot of the *m/z* 600-900 range from REIMS analysis of grass-fed cattle (green) and grain-fed Angus (red) and Wagyu (purple).

Table 9. Cross-validation scores for PCA and LDA classification models for grass-fed, Angus or Wagyu beef products for the spectral ranges *m/z* 100-1200, 100-500, and 600-900.

Spectral range (m/z)	***100-1200***	***100-500***	***600-900***	***100-1200***	***100-500***	***600-900***
Model type	PCA	PCA	PCA	LDA	LDA	LDA
Number of spectra	1791	1791	1791	1791	1791	1791
Number of passes	1069	1038	1210	1501	1481	1632
Number of failures	705	728	563	283	302	159
Number of outliers	17	25	18	7	8	0
Correct classification - excluding outliers	60.26%	58.78%	68.25%	84.14%	83.06%	91.12%
Correct classification - including outliers	59.69%	57.96%	67.56%	83.81%	82.69%	91.12%

6.5 Conclusion

REIMS is a recently emerged technology that has shown high potential in provenance, quality, and safety applications. Furthermore, its ability to conduct real-time in-situ analysis provides opportunity for its deployment into food processing facilities. The installation of a REIMS system at CSIRO represents the first deployment of REIMS into Australia for use in food systems with the red meat industry conducting the first proof of concept study. The purpose of this study was to evaluate the ability of REIMS to generate spectral profiles which could be used to develop proof of concept models for verification of the provenance of Australian beef products. Classification models for production system, breed, and brands were developed and each demonstrated capacity to accurately identify attributes of red meat of importance to the red meat industry and its customers. In particular, the identity of Angus or Wagyu cattle were highlighted, with a distinct segregation of Australian country of origin from Japanese Wagyu. Meat processors should also be aware that this technique clearly segregated out different brands in Australia and could have an appealing approach for distinguishing the fatty acids and lipid profiles responsible for unique flavour signatures associated with specific brands. Additionally, the ability to correctly classify samples using a dual-attribute model (e.g. breed and production system) was confirmed, though further investigation of the molecules or ions driving the differentiation is required to ensure that the model reflects both attributes of interest.

It is necessary to recognise that the models developed as part of this proof-of-concept study require further development with factors such as seasonality likely to have an impact as varying weather impacts the types of pastures and grains available for grass-fed and grain-fed animals, respectively. Nevertheless, this study has confirmed that REIMS shows high potential for the real-time identification of provenance aligned attributes of importance to the red meat industry. Subsequent discussions with industry participants should identify focused use cases to further showcase the potential of REIMS.

7.0 Milestone report: REIMS quality applications

7.1 Executive summary

The red meat supply chain comprises a complex network that attempts to transfer a range of products from production to consumption in a safe and secure way. Consequently, there are opportunities across the supply chain where vulnerabilities may be exploited and instances of food fraud and issues relating to food safety and quality can occur. Rapid Evaporative Ionisation Mass Spectrometry (REIMS) is a recently developed ambient ionisation-mass spectrometry technique that is being evaluated in international markets in response to recent food fraud episodes in red meat and seafood supply chains and has demonstrated capacity relating to species identification, location of production, production system identification, and slaughter and processing methods.

This project aims to review the application areas for REIMS as they pertain to Australia's red meat industry and to subsequently carry out a series of proof-of-concept studies for attributes aligned with provenance, food safety, and food quality. This milestone focussed on the application of REIMS for classification of meat quality aligned attributes of importance to the red meat industry. A total of 216 Angus and Wagyu *longissimus lumborum* samples comprising marble scores (MB) 2, 3 and 4 for Angus and 3, 5, 7 for Wagyu were sourced from an Australian export registered abattoir. Samples were stored at -0.5 °C for a period of up to 12 weeks and subjected to meat quality and REIMS analysis at weeks 1, 2, 4, 6, 8 and 12. The resulting REIMS spectral profiles were used to develop the following classification models using linear discriminant analysis (LDA) analysis.

- Angus MB static – ability to predict Angus MB score at any point across 12 weeks
- Angus MB dynamic – ability to predict Angus MB score and weeks aged at any point across 12 weeks
- Wagyu MB static – ability to predict Wagyu MB score at any point across 12 weeks
- Wagyu MB dynamic – ability to predict Wagyu MB score and weeks aged at any point across 12 weeks
- MB static – ability to predict MB score regardless of breed at any point across 12 weeks
- MB dynamic – ability to predict MB score and weeks aged at any point across 12 weeks regardless of breed

All models were cross-validated and subsequently tested using at least 20% of the samples. The spectral range of *m/z* 100-500 produced models that resulted in the highest classification rates except for Angus MB dynamic where *m/z* 100-1200 was superior. Test classification rates for the Angus models were 90.14% (static) and 90.00% (dynamic). The results for the Wagyu MB static model were lower at 82.99%, however the Wagyu MB dynamic model was the best performing at 93.54%. The result for the MB static model was 79.97%, however this result appeared to be affected by the number of weeks the product was aged. The construction of a model using product from the first two weeks of sampling resulted in a test classification score of 98.23%. The breed-independent MB dynamic model performed well, producing a test result of 90.93% Objective meat quality traits such as intramuscular fat percentage (IMF%), colour, texture, and cook loss were measured throughout the study and it was hypothesised that IMF% would provide an objective basis for model development. The measured IMF% did not align with graded marbling scores and attempts to construct models using re-categorised samples based on IMF% were unsuccessful, with the highest test classification score being 42.36%. As this is a proof-of-concept study, it is anticipated that future studies which comprise greater numbers of samples will permit the construction of models that perform equally well against subjective (marble grading scores) and objective (e.g. IMF%) measures.

In addition to models for the prediction of marbling scores, models that classify Angus, Wagyu, and grass-fed samples into their respective groups based on whether the product was fresh or frozen were also constructed. Testing of these models gave results of 84.57% to 87.54% depending on the spectral range used. Importantly, fresh product was never classified as frozen product, and vice versa. Incorrect classifications occurred at the boundary of the product clusters and likely relates to the use of Angus genetics in Wagyu breeding programs and the high potential that some of the grass-fed products derived from Angus animals.

This study continues to report the first description of the use of REIMS on Australian foods and the development of classification models for provenance, quality and safety, demonstrating its capacity to accurately identify red meat attributes of importance to the industry and its customers. It is necessary to recognise that the models developed as part of this proof-of-concept study require further development to capture the breadth of product offerings. Nevertheless, this study has confirmed that REIMS shows a high potential for the real-time identification of quality aligned attributes, which are of importance to the red meat industry. Furthermore, it suggests that REIMS has applicability as an objective grading tool for the beef industry and there is high potential that it may be suitable for the grading of pre-chill carcases which would provide substantial benefit and value to the industry. Subsequent discussions with industry participants should identify focused use cases and provide input for optimisation of modelling to further refine and improve the accuracy of REIMS applications.

7.2 Introduction

The red meat supply chain comprises a complex network that attempts to transfer a range of products from production to consumption in a safe and secure way. As a consequence, there are opportunities across the supply chain where fragmentation can arise, and vulnerabilities may be exploited. There is ever-increasing demand to de-fragment these systems and ensure that Australian red meat products enter domestic or export markets with the desired quality, safety, and provenance attributes.

Novel technologies that can be incorporated across the red meat supply chain will be central facilitators in the evolution of the meat plant of the future. Rapid Evaporative Ionisation Mass Spectrometry (REIMS) is a recently developed ambient ionisation-mass spectrometry technique that is being evaluated in international markets in response to recent food fraud episodes in red meat and seafood supply chains and has demonstrated capacity relating to species identification, location and type of production system, food quality, and slaughter and processing methods. The REIMS System (Waters Corporation, USA) combines an electrosurgical knife (iKnife), with a quadrupole time-of-flight mass spectrometer to generate unique mass spectral ‘fingerprints’ within seconds that can be used to assess key attributes and differences of, and between, samples of interest. Furthermore, the iKnife is connected to the mass spectrometer by a length of tubing which facilitates REIMS biggest advantage by enabling sampling to occur remotely from the mass spectrometer within seconds. This advantage increases potential for REIMS to be used in processing facilities where the mass spectrometer unit can be kept in a clean area while an operator uses the iKnife within the processing plant as required.

Beef marbling is characterised by the flecks and streaks of intramuscular fat that exist within the lean sections of a meat cut. Beef marbling is seen as a key indicator of quality by consumers and purchasing decisions are aided by the association of marbling with tenderness, juiciness and flavour. Globally, beef producing nations implement beef

grading systems that utilise beef marbling scores as the key or a major contributor to the final beef grade (Figure 11). Two beef grading systems exist within Australia; the AUS-MEAT and Meat Standards Australia (MSA) grading systems. AUS-MEAT grading focuses on marbling whereas MSA incorporates several additional attributes such as carcase weight, fat and meat colour and pH (among others) to provide an eating quality assessment. As Figure 11 suggests, AUS-MEAT grades marbling across a scale of 0-9 in increments of one, whereas MSA grades it across a scale of 100-1190 in increments of 10. Regardless of the grading system used, assessment of marbling score is a subjective process completed by highly trained personnel. The subjective nature of the process may allow for variability to occur in grading evaluations and, as a result, present research is exploring the use of objective carcase measurements (OCM, e.g.ALMtech) of marbling scores so that Australian beef producers and exporters can provide verifiable evidence of the claims being made about Australian beef products. Equally, beef producers and exporters are interested in supply chain events occuring during distribution and retail. Opportunities for previously frozen products to be thawed and subsequently sold as fresh, chilled product are present and may impact the sensory experience of the consumer resulting in a decreased desire to re-purchase an Australian beef product. Similarly, several countries importing Australian beef place limitations on the length of time chilled product can be in the market and therefore systems that can objectively determine the period of time since slaughter may be useful in resolving market access issues that could arise.

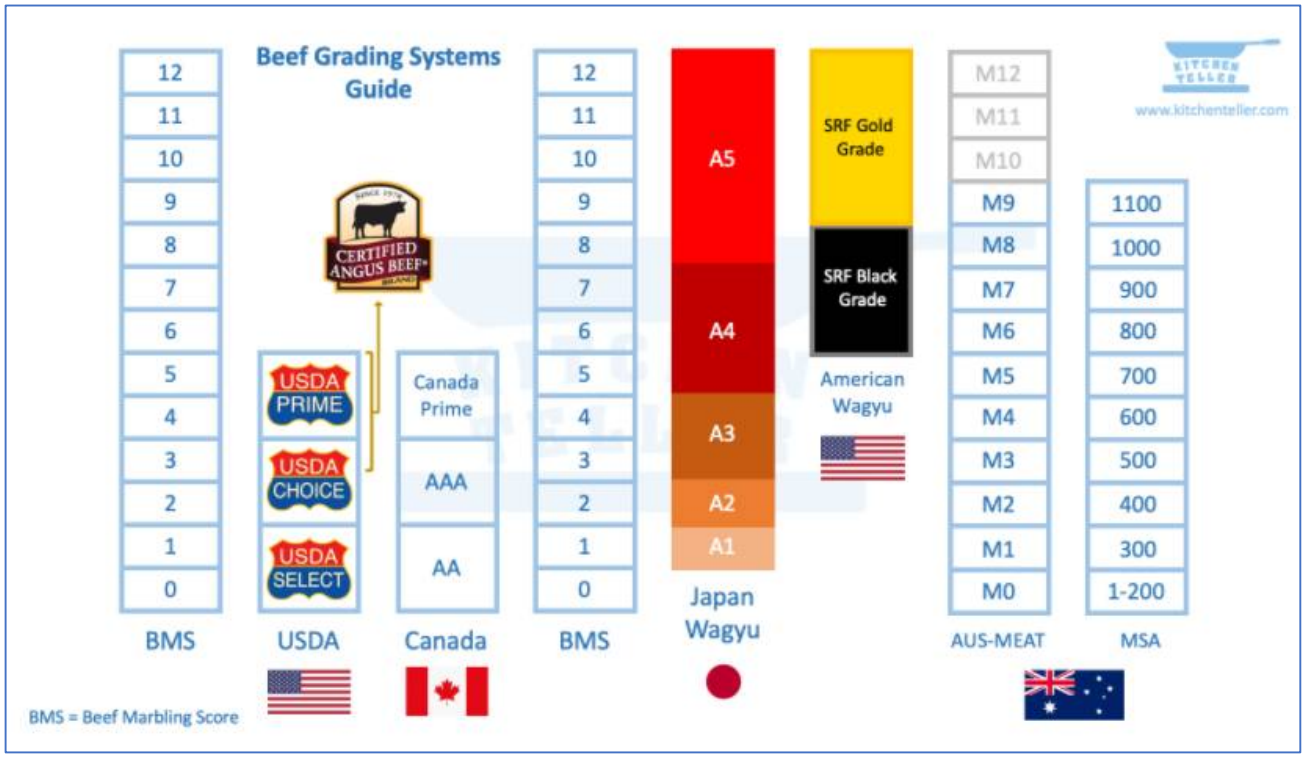

Figure 11. A comparative guide to beef marbling score systems in USA, Canada, Japan and Australia [4].

In order to use the spectral 'fingerprints' generated by REIMS to predict product attributes of interest, classification models must be developed. Data analysis typically occurs through the application of unsupervised or supervised

methods of analysis. Unsupervised approaches such as principal component analysis (PCA) make no assumptions in relation to the data, and it is used as a visualisation technique to identify patterns within a dataset [5]. Supervised methods such as linear discriminant analysis (LDA) are tools used for classification, dimension reduction and data visualisation and depend on accepting that the identity applied to each grouping is correct. The aim of this study is to utilise supervised and unsupervised data analysis methods to determine the applicability of REIMS as a tool for verifying quality attributes of red meat products.

7.3 Materials and Methods

7.3.1 Sample collection

Two sets of samples were used for REIMS analysis and subsequent model development. The first set of samples were collected directly from an export registered processing plant and used to develop Angus and Wagyu marbling score models, Angus and Wagyu marbling score and storage trials, breed-independent marbling score models, and breed-independent marbling and storage time models. The details of the samples collected are described in Section 7.3.1.1. An independent set of samples, previously collected as part of the 'Provenance application' phase of this project was used to generate the fresh v frozen models. The details and storage conditions of these samples are detailed in Section 6.3.1.

7.3.1.1 Storage trial

Striploins (*longissimus lumborum*) were collected from an Australian export registered abattoir between the 21st September 2021 and the 7th December 2021 for use in the storage trial. Paired striploins from 6 animals were collected for each timepoint and were selected based on breed (Angus or Wagyu) and marbling score (MB 2, 3 and 4 for Angus and 3, 5 and 7 for Wagyu), resulting in 36 animals in total (see Appendix A1 for the trial design). Marbling score was determined by comparison to the AUS-MEAT marbling standards during routine chiller assessment by the abattoir's grader. The loins were packed in styrofoam eskies on wet ice and an iButton temperature logger was added to the cartons to monitor temperature during transport. Upon arrival to the Coopers Plains site, the loins were stored at -0.5 °C overnight before sample preparation the following morning. The paired striploins were aligned according to side (left or right) and orientation (anterior/posterior) and were sliced into six equally sized portions, resulting in 216 samples (36 animals x 6 portions). Each portion was allocated to one of the six timepoints (Week 1, 2, 4, 8 and 12) before being weighed, vacuum packaged and stored at -0.5 °C for the designated storage time.

7.3.1.2 Fresh v frozen

A total of 50 duplicate loin muscle samples to those collection in Section 6.3.1 were collected for analysis comprising 17 Wagyu, 16 Angus and 17 grass-fed products. Samples were collected from retail establishments including butcher shops, wholesalers, and supermarkets. All samples were sourced from the *longissimus* muscle and were presented at retail as scotch fillet or porterhouse. Vacuum packaged primals were opened and two 25 mm steaks were removed and transported to the laboratory for analysis. Upon arrival at the laboratory, one steak from each sample was placed at -20 °C for at least six months. The remaining steak was stored at 4 °C until REIMS analysis

was conducted. All REIMS sampling occurred within 24-72 h of sample collection. The name and location of the retail establishment, the establishment number of the processing plant and any brand information were recorded. In this milestone, the frozen steaks were defrosted in a 4 °C chiller overnight and analysed using REIMS. The data from fresh and defrost samples was used to determine if REIMS can be used to classify fresh and frozen Wagyu, Angus and grass-fed products.

7.3.2 Microbiological analysis

Samples were tested microbiologically for total viable counts (TVC), lactic acid bacteria counts (LAB) and *Escherichia coli*/coliforms. A composite sample was prepared for each sample by aseptically taking two 10 cm^2 adipose cores and two 10 cm^2 lean meat cores (total of 40 cm^2). The cores were placed in a stomacher bag and a 100 mL aliquot of 0.85% saline was added to each and were subsequently stomached for 30 s. Decimal dilution series were prepared in 0.85% saline and 1 mL of appropriate dilution was plated onto APC (aerobic plate count Petrifilm) for TVC and 1 mL onto *E. coli*/coliform Petrifilm plates for *E. coli*/coliforms. APC Petrifilm were incubated at 25±1 °C for 72±3 h and *E. coli*/coliforms Petrifilm were incubated at 35±1 °C for 24±2 h. Decimal dilution series were also prepared in MRS broth (Oxoid) for LAB counts and 1 mL of each dilution was plated onto APC (aerobic plate count petrifilm). LAB petrifilm were incubated anaerobically at 25±1 °C for 120±3 h. Microbial counts were converted to $\log_{10}$CFU/cm^2 for each sample and the mean count determined. For the purposes of generating the mean counts, samples with counts below the limit of detection (LOD) were arbitrarily assigned a count equal to the limit of detection. The LOD's for TVC, *E. coli*/coliforms and LAB were 0.40, 0.40 and 1.40 $\log_{10}$CFU/cm_2, respectively.

7.3.3 REIMS

REIMS analysis was conducted using an electrosurgical knife (iKnife, Waters, UK) combined with a Xevo G2 qToF mass spectrometer (Waters, UK). The iKnife was powered by an Erbe VIO 50C generator (Erbe Medical, UK) set at 25 W power in dry-cutting mode. Each sample was cut with the iKnife for a period of 3–5 s per cut. A total of 10 technical replicates were performed for each sample with a delay of at least 5 s between each cut. Spectral 'fingerprints' were acquired between the mass range *m/z* 100–1200 in negative ionisation mode using a scan rate of 0.5 s per scan. Leucine enkephalin (leu enk, Waters, UK) was used as a lockmass by dissolving in MS-grade isopropanol (Fisher Scientific, USA) at a concentration of 0.1 ng/µl and infusing into the mass spectrometer at a rate of 150 µl/min. Following the completion of each sample, carbonised sample was scraped from the iKnife and subsequently wiped with a tissue dampened with isopropanol.

7.3.4 Data analysis

7.3.4.1 REIMS spectral profiles

REIMS data was processed and analysed using the Abstract Model Builder (AMX) Version 1.0.2159.0 (Waters Research Centre, Hungary). For each sample, mass spectra were loaded, and individual cuts identified. Pre-processing was used to remove the background signal, correct burn ends, apply a lockmass correction (leu enk 554.2615) and normalise. The number of PCA dimensions were set to 100 and when LDA analysis was performed, the LDA dimensions were set to maximum which equals the number of classification groups minus one. The intensity limit for all models was set at 100 000 and binning of data was done at a scale of *m/z* 0.1.

REIMS spectral profiles typically exhibit a concentration of signal in the *m/z* 100–500 and 600–900 range which relates to the detection of fatty acids and glycerophospholipids, respectively. To understand the relative contribution of these spectral ranges to the overall classification models, PCA and LDA analysis was conducted for all samples using the spectral ranges *m/z* 100–500, 600–900 and 100–1200. Cross-validation of all models was performed using the 'full group out' approach with outlier calls based on a standard deviation multiplier of 8. Testing of all models was performed using either the '20% out' or 'leave all at once'. When the 'leave all at once' test validation was used, all samples from randomly selected animals were removed until the total number of samples removed exceeded 20%. Models were reconstructed using the remaining samples and subsequently tested using the removed samples. The process was repeated six times for each model with each animal being removed at least once during testing. For '20% out' testing, 20% of samples were randomly selected, the model reconstructed using remaining samples, and the removed samples then used to test the model. This process was repeated times with all samples being used as a test exactly once.

7.3.4.2 Meat quality traits

An initial quality checking of the datasets was carried out: 1) to examine the boxplot distributions of all meat quality and FAME attributes within the breed and individual storage time point for obvious errors and outliers, 2) to replace the outliers with the imputed median values of the groups that the outliers belong to. The clean data from 12 meat quality and 20 FAME traits were then combined for further analysis. To illustrate the relationships among all traits, especially between the marbling score and the rest of the traits, the pair-wise Pearson's correlations were calculated for all traits within each breed, individual time points, as well as across time points. Please note that the marbling score was treated as a continuous trait in the calculation. The R program (window's version 4.0.2) was used for the imputation and calculation of correlations.

7.3.5 pH measurements, drip loss and total moisture loss

The pH of the samples was measured using a TPS WP-80 pH meter with a polypropylene spear-type gel electrode (IJ 44) and temperature probe (TPS Pty Ltd, Brisbane, QLD, Australia). Calibration was performed using pH 4.00 and pH 7.00 buffers equilibrated to the sample temperature (10 °C). Drip loss was calculated as the percentage difference between the original weight of the sample and the post-storage weight. Total moisture loss was calculated as the sum of the drip loss and cook loss.

7.3.6 Cook loss and texture measurement

All samples were cut into 5 x 10 x 3 cm (l x w x d) sized pieces and cooked in a 75 °C water bath for 41 min to an internal temperature of 72 °C. After cooking, samples were immediately cooled by plunging into an ice bath for 20 min. The cook loss was calculated as the difference in weight between raw and cooked samples, presented as a percentage of the initial weight. Samples were stored overnight at 4 °C prior to texture analysis.

Texture measurements were carried out using a Lloyd LS 2.5 with a 500 N load cell (Lloyd Instruments, West Sussex, United Kingdom) and a modified Warner-Bratzler shear device [6]. The samples were cut into rectangular shapes (15 mm x 6.7 mm, giving a cross-sectional area of 1 cm^2) and at least 25 mm long to enable secure clamping of the sample into the holder. A straight blade with a thickness of 0.64 mm was attached to an overhead clamp and pulled up through

the muscle fibres, perpendicular to the fibre direction, at a speed of 100 mm/min. The maximum peak force (PF) was determined using Nexygen Plus V3.0 software (Lloyd Instruments, West Sussex, United Kingdom). At least six measurements were made on each sample and the mean recorded.

7.3.7 Colour measurement

Objective colour measurements were made on the inside cut surface of 25 mm thick steaks after blooming for 60 ± 10 min at 10 °C. A Hunterlab Miniscan EZ 45/0 LAV (light source A, observer angle 10°, 25 mm viewed area) was used to measure L* (lightness), a* (redness) and b* (yellowness) attributes in triplicate. The instrument was calibrated using white and black calibration tiles, as supplied with the instrument (Novasys group Pty Ltd, Ferntree Gully, VIC, Australia), at the same temperature as the samples (~10 °C).

7.3.8 Lipid analysis

7.3.8.1 Sample preparation

Pieces of loin were trimmed of external fat and connective tissue, diced, and then vacuum packaged. Samples were stored at -80 °C and defrosted overnight at 4 °C prior to analysis. The diced loin samples were minced in an Oskar food processor and subsamples used to analyse lipid characteristics.

7.3.8.2 Estimated intramuscular fat (IMF%)

IMF was estimated by oven moisture as per Thornton *et al* [7]. Approximately 15 g minced sample was weighed into tared moisture tins, in duplicate, and placed in a laboratory oven set at 105 °C for 16 h. Samples were removed, cooled in a desiccator, and re-weighed. The moisture content was calculated and used to estimate IMF % (w/w) using the following equation:

$$\%\,\text{Fat in boneless beef} = 95.6 - [(\%\,\text{moisture}) \times 1.24]$$

7.3.8.3 Thiobarbituric acid reactive substances (TBARS)

Lipid stability was determined by the thiobarbituric acid reactive substances (TBARS) assay as per Witte *et al* [8], with modifications. A sample of 2 ± 0.05 g was weighed into scintillation vials and homogenized on ice in 6 mL of chilled TCA solution (7.5% trichloroacetic acid, 0.1% propyl gallate and 0.1% EDTA) using an Ultra Turrax for 30 s at 13 000 rpm. The homogenate was filtered and rinsed with an additional 2 mL of TCA solution. Aliquots (2.5 mL) of the filtrate were transferred, in duplicate, to test tubes, diluted with 2.5 mL distilled water and reacted for 16 h with 5 mL of 0.02 M thiobarbituric acid solution at room temperature, in a dark cupboard. An aliquot of sample (200 µL) was added to a microplate well, in duplicate, and the samples were read at 532 nm using an EnSpire Multimode Plate reader (Part number: 2300-0000, PerkinElmer Pty Ltd, Glen Waverley, Australia) and the TBARS value as mg/kg malondialdehyde (MDA) equivalents was determined against a standard curve prepared from 1,1,3,3-tetraethoxypropane.

7.3.8.4 Lipid extraction

Lipids were extracted by the method of Folch *et al* [9]. Minced sample (1 ± 0.05 g) was weighed, in duplicate, into 15 mL scintillation vials and 10 mL of extraction solution (2:1 chloroform: methanol) was added. The sample was homogenised with an Ultra Turrax before another 10 mL of extraction solution was added. The samples were left at room temperature for 2 h. Samples were filtered through filter paper (Filtech, grade 165) into 50 mL falcon tubes. The filter residue was washed three times with a small volume of solvent to give ca. 20 mL of extract. An aliquot of 0.73 % NaCl (4 mL) was added to the tube prior to shaking. The tubes were spun at 1000 g in a centrifuge for 1 min to separate out the phases and the top layer was removed and discarded. The bottom layer was transferred into a tared 15 ml scintillation vial and evaporated under nitrogen to a constant weight. The resulting residue was dissolved in toluene to give a lipid concentration of 20 mg/mL and stored at -20 °C until analysis.

7.3.8.5 Fatty acid methyl ester analysis

Fatty acid methyl esters (FAMEs) were prepared as follows. One mL of the lipid extract in toluene (20 mg/mL lipid) was transferred to a 12 mL vial and 3 mL of 2% H_2SO_4 in methanol was added. The vial was capped with a Teflon-lined lid, vortexed, and placed into an 80 °C water bath for 1 h, with shaking approximately every 10–15 min. Vials were removed and cooled under running tap water before the addition of 2 mL of hexane, containing 0.5 mg/mL C19:0 internal standard, and 3 mL of Milli-Q water. The vials were shaken (approximately 30 s) and transferred to 15 mL falcon tubes before centrifugation at 1000 g for 1 min. The organic layer was transferred to a 12 mL scintillation vial and evaporated under nitrogen before reconstitution with 1 mL of hexane. The FAMEs were analysed on a Shimadzu GC-2010 gas chromatograph, fitted with a flame ionisation detector (FID) and Supelco SP-2560 capillary column (100 m x 0.25 mm, df 0.2 µm). Instrument settings are shown below in Table 10. FAMEs were identified by comparison to standard FAME mixtures (Supelco, Bellefonte, PA, USA) and quantified using the C19:0 internal standard and calculated retention factors. Results are reported as mg FAME per 100 g of fresh sample.

Table 10: Instrument conditions used for FAME analysis.

Oven	140 °C (5 min), 4 °C/min to 240 °C (15 min)
Injector	250 °C
FID	260 °C
Carrier gas	Helium, 20 cm/s
Injection	1 µL, 1:100 split

7.4 Results and Discussion

7.4.1 Storage trial – microbiology

Mean TVC values for the Angus and Wagyu beef samples stored for 12 weeks are shown in Figure 12. The TVC for all six sets of samples generally increased over the testing period. Mean TVC for the Angus beef samples ranged from 0.99 $log_{10}CFU/cm^2$ at week 1 to a maximum of 6.11 $log_{10}CFU/cm^2$ at week 12, while those for the Wagyu beef samples ranged from 0.96 $log_{10}CFU/cm^2$ at week 1 to a maximum of 5.94 $log_{10}CFU/cm^2$ at week 12. As expected, all counts remained <7.00 $log_{10}CFU/cm^2$ for the entire 12-week storage trial.

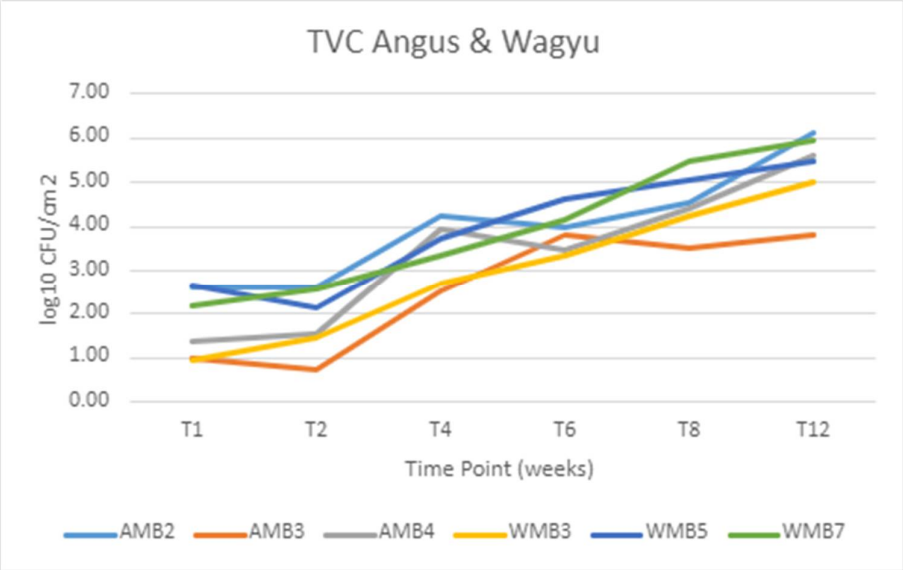

Figure 12: Mean TVC values ($log_{10}CFU/cm^2$) for both Angus and Wagyu beef stored for 12 weeks (AMB – Angus marble scored beef sample, WMB – Wagyu marble scored beef sample).

Mean LAB values for the Angus and Wagyu beef samples stored for 12 weeks are shown in Figure 13. The LAB generally increased over time for all samples and ranged from <1.40 $log_{10}CFU/cm^2$ at week 1 to a maximum of 6.49 $log_{10}CFU/cm^2$ at week 12 for the Angus samples and from 1.40 $log_{10}CFU/cm^2$ at week 1 to a maximum of 5.83 $log_{10}CFU/cm^2$ at week 12 for the Wagyu samples. All counts remained < 7.00 $log_{10}CFU/cm^2$ for the entire 12-week storage trial.

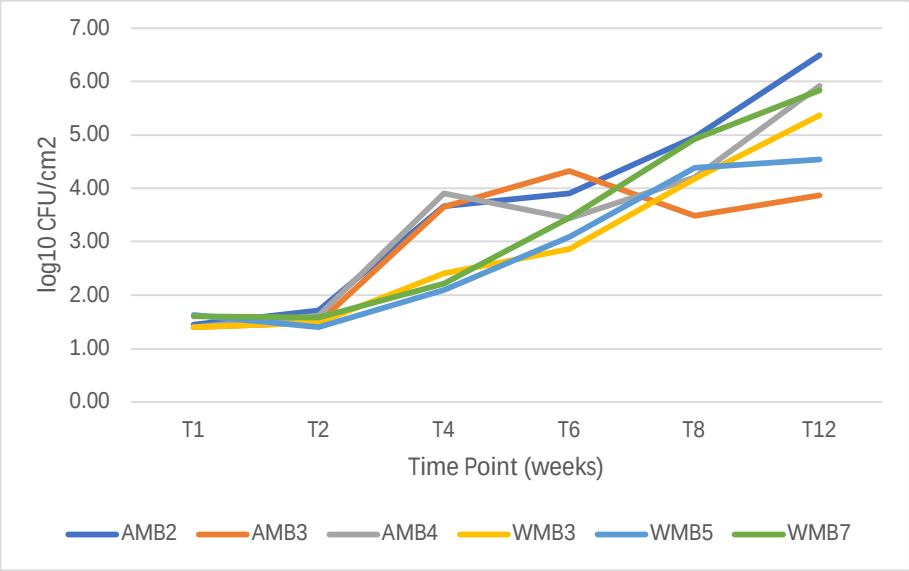

Figure 13: Mean LAB values ($log_{10}CFU/cm^2$) for both Angus and Wagyu beef stored for 12 weeks (AMB – Angus marble scored beef sample, WMB – Wagyu marble scored beef sample).

Overall, both the Angus and Wagyu samples performed as expected microbiologically. The LAB closely mirrored the respective TVC for the duration of the trial, both in general trends and approximate numbers, indicating the LAB formed the majority of the microflora present. Both the TVC and LAB counts for all samples at the end of the 12-week trial were below 7.00 $log_{10}CFU/cm^2$, implying that the vacuum packaged environment was being maintained as expected.

7.4.2 Storage trial – meat quality

During the storage trial, all products were assessed for several meat quality attributes including pH, colour, drip loss, cook loss, total moisture loss and texture. Summary snapshots for each attribute are provided below. Attempts to build classification models for meat quality data is described in Section 7.4.7. It was assumed that a relatively simple relationship would exist between the REIMS data and the measured meat quality markers. On the contrary, simple relationships could not be identified at this time as, on one hand, REIMS is a large complex suite of data requiring a great deal of mining to generate information but, on the other, the measured quality markers are a much smaller (simpler) dataset. Further investigation is needed to tease out these relationships.

7.4.2.1 pH

The pH of all products at start of the storage trial ranged from 5.30 to 5.56 and increased slightly over the course of the 12 weeks. The pH of any sample ranged from 5.37 to 5.81, with only four samples having pH >5.70. The slight increase in pH over time is typical of chilled, vacuum packaged product and the majority of samples with pH <5.70 supports the microbiological data where LAB counts comprised the majority of the TVC.

7.4.2.2 Colour

Mean colour parameters (L*, a*, b*) for Angus and Wagyu samples of different marble scores stored for 12 weeks are shown in Table 11. All colour parameters are typical of vacuum-packed beef, with no differences between breed, marble score or time of storage.

Table 11: Mean lightness (L*), redness (a*) and yellowness (b*) results, ± standard deviation, for both Angus and Wagyu samples during storage (1, 2, 4, 6, 8 and 12 weeks).

Attribute	Breed	MB	T1	T2	T4	T6	T8	T12
L*	Angus	4	41.74 ± 2.45	43.62 ± 1.75	42.12 ± 3.36	42.15 ± 2.8	42.92 ± 2.18	42.88 ± 3.89
		3	42.37 ± 2.13	44.39 ± 1.34	43.11 ± 2.07	39.07 ± 1.73	44.38 ± 1.65	39.57 ± 2.24
		2	42.09 ± 2.87	43.67 ± 0.83	43.17 ± 2.37	42.03 ± 2.09	41.20 ± 3.14	43.63 ± 1.73
	Wagyu	7	40.10 ± 3.34	40.89 ± 1.49	40.53 ± 3.96	42.66 ± 4.87	40.32 ± 2.02	41.80 ± 5.01
		5	41.49 ± 2.67	43.90 ± 1.11	42.83 ± 0.94	45.37 ± 4.12	45.07 ± 2.46	42.56 ± 2.15
		3	38.28 ± 1.39	40.81 ± 1.25	40.33 ± 2.01	41.17 ± 2.24	38.20 ± 2.32	41.28 ± 1.9
a*	Angus	4	31.64 ± 1.75	30.86 ± 0.99	36.60 ± 1.38	31.36 ± 1.04	32.09 ± 0.77	30.63 ± 1.94
		3	32.15 ± 1.34	32.85 ± 1.01	31.16 ± 1.11	35.93 ± 0.82	30.5 ± 1.1	35.28 ± 1.59
		2	30.88 ± 0.83	30.85 ± 0.78	30.33 ± 1.55	34.59 ± 1.05	36.34 ± 2.05	31.52 ± 1.02
	Wagyu	7	32.85 ± 1.49	32.91 ± 0.65	31.75 ± 1.13	30.81 ± 0.39	31.12 ± 0.93	30.37 ± 1.93
		5	32.43 ± 1.11	31.63 ± 1.19	32.25 ± 1.25	30.20 ± 0.98	30.50 ± 1.19	31.73 ± 1.27
		3	34.24 ± 1.25	32.59 ± 2.26	32.66 ± 1.1	32.21 ± 1.03	32.63 ± 0.92	30.12 ± 0.92
b*	Angus	4	25.00 ± 1.39	24.20 ± 0.64	31.05 ± 1.58	24.45 ± 0.59	25.94 ± 0.98	24.83 ± 1.65
		3	25.41 ± 1.08	27.04 ± 0.82	24.24 ± 0.95	30.94 ± 0.96	23.83 ± 1.19	30.78 ± 1.78
		2	24.33 ± 0.95	23.94 ± 0.75	23.40 ± 1.64	29.06 ± 0.95	31.14 ± 2.09	25.36 ± 0.98
	Wagyu	7	26.27 ± 1.65	25.79 ± 0.94	24.35 ± 1.15	23.69 ± 0.51	24.48 ± 0.83	23.23 ± 1.6
		5	25.03 ± 1.07	24.65 ± 1.47	24.79 ± 1.44	23.19 ± 0.67	23.52 ± 1.48	24.27 ± 1.27
		3	27.37 ± 1.39	25.20 ± 2.04	25.78 ± 1.37	24.87 ± 1.17	25.50 ± 0.91	22.65 ± 1.02

7.4.2.3 Drip loss, cook loss and total moisture loss

As expected, drip loss for all products trended upwards during storage for all beef products regardless of breed or marble score (Table 12). The largest average drip losses were observed in Angus products, Angus MB2 (weeks 6, 8 and 12) and Angus MB3 (week 6), with drip losses exceeding 3%. Drip losses of 3.0-3.5% were recorded in a previous study of vacuum-packed beef stored for 12 weeks [10]. Drip losses from Wagyu products did not exceed 2.5% at any stage of the trial regardless of marble score or weeks stored.

Cook losses were typical for both Angus and Wagyu, ranging from approximately 19–26% (Table 12). No differences were observed between breed, marble score or storage time, however, the cook loss of Angus products stored for 12 weeks tended to be higher than Wagyu products. This trend was similar for the total moisture loss.

Table 12: Mean drip, cook loss and total moisture loss, ± standard deviation, for both Angus and Wagyu samples during storage (1, 2, 4, 6, 8 and 12 weeks)

Attribute	*Breed*	*MB*	*T1*	*T2*	*T4*	*T6*	*T8*	*T12*
Drip %	Angus	4	1.17 ± 0.69	1.23 ± 0.46	1.55 ± 0.82	2.44 ± 0.66	1.28 ± 0.37	1.72 ± 0.29
		3	1.56 ± 0.84	1.99 ± 0.54	2.72 ± 0.82	3.03 ± 1.0	2.70 ± 0.71	1.85 ± 0.76
		2	0.47 ± 0.22	0.93 ± 0.51	2.95 ± 1.5	3.45 ± 0.72	4.53 ± 2.3	3.85 ± 0.93
	Wagyu	7	0.82 ± 0.53	0.86 ± 0.28	1.31 ± 0.77	1.63 ± 1.06	1.93 ± 1.2	1.95 ± 1.4
		5	0.67 ± 0.37	0.95 ± 0.45	0.71 ± 0.37	1.05 ± 0.43	1.11 ± 0.25	1.09 ± 0.25
		3	0.66 ± 0.15	0.83 ± 0.26	1.12 ± 0.41	1.28 ± 0.54	1.97 ± 0.82	2.47 ± 0.98
Cook loss %	Angus	4	22.23 ± 2.51	23.30 ± 1.72	21.81 ± 2.74	21.62 ± 1.42	23.93 ± 1.55	26.20 ± 2.98
		3	22.42 ± 2.32	22.53 ± 1.26	20.76 ± 1.08	22.85 ± 1.86	20.17 ± 2.1	22.91 ± 1.22
		2	23.16 ± 1.51	26.26 ± 2.06	23.61 ± 2.55	25.13 ± 1.38	22.84 ± 2.43	23.62 ± 1.98
	Wagyu	7	21.06 ± 2.23	19.44 ± 1.63	21.06 ± 2.23	21.51 ± 2.37	22.10 ± 2.08	21.74 ± 2.1
		5	18.96 ± 1.77	20.30 ± 2.02	19.36 ± 2.33	21.26 ± 0.99	20.24 ± 0.84	19.72 ± 2.38
		3	23.43 ± 1.92	21.81 ± 1.73	21.05 ± 0.68	20.04 ± 1.88	21.69 ± 1.24	19.88 ± 1.6
Total Moisture Loss %	Angus	4	23.40 ± 2.76	24.53 ± 1.99	23.37 ± 3.06	24.06 ± 1.94	25.21 ± 1.58	27.92 ± 2.91
		3	23.99 ± 2.15	24.52 ± 1.29	23.48 ± 1.04	25.88 ± 1.58	22.87 ± 1.87	24.86 ± 1.49
		2	23.63 ± 1.64	27.09 ± 2.36	26.56 ± 2.55	28.58 ± 2.04	27.37 ± 3.89	27.47 ± 1.8
	Wagyu	7	21.88 ± 2.67	20.29 ± 1.76	22.37 ± 1.96	23.13 ± 3.07	24.03 ± 2.89	23.44 ± 3.47
		5	19.63 ± 1.73	21.25 ± 1.73	20.07 ± 2.57	22.70 ± 0.62	21.35 ± 0.91	20.81 ± 2.41
		3	24.10 ± 1.89	22.64 ± 1.74	22.18 ± 0.87	21.32 ± 1.93	23.66 ± 1.33	22.35 ± 1.23

7.4.2.4 Texture

Figures 14 and 15 highlight the classical increase in tenderness (reduction in peak force) with storage (ageing) of vacuum-packed beef, with approximately 30% reduction in the peak force values at 12 weeks for both breeds. Within a breed, marble score had no impact on tenderness at any storage time. It has previously been reported that the cut-off peak force value for consumer acceptability for tender meat is approximately 40 N [11], so the Angus and Wagyu products would be ranked as tender at one week storage and as very tender at 12 weeks storage, with peak force values ranging from 25–32 N.

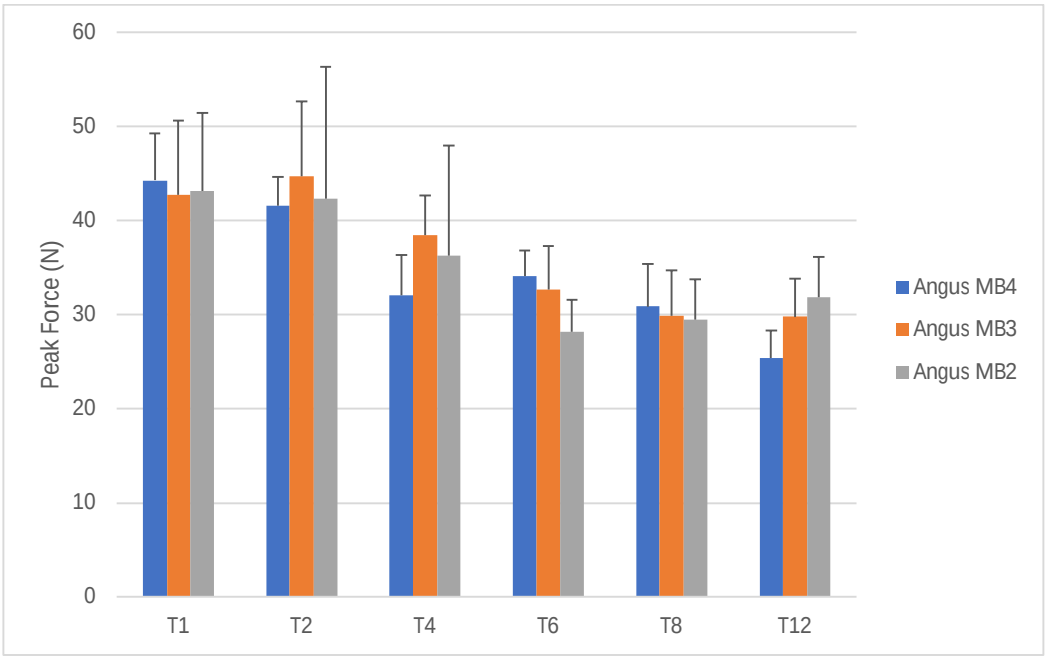

Figure 14: Mean peak force measurements ± standard deviation, for the Angus samples during storage (1, 2, 4, 6, 8 and 12 weeks)

8.0

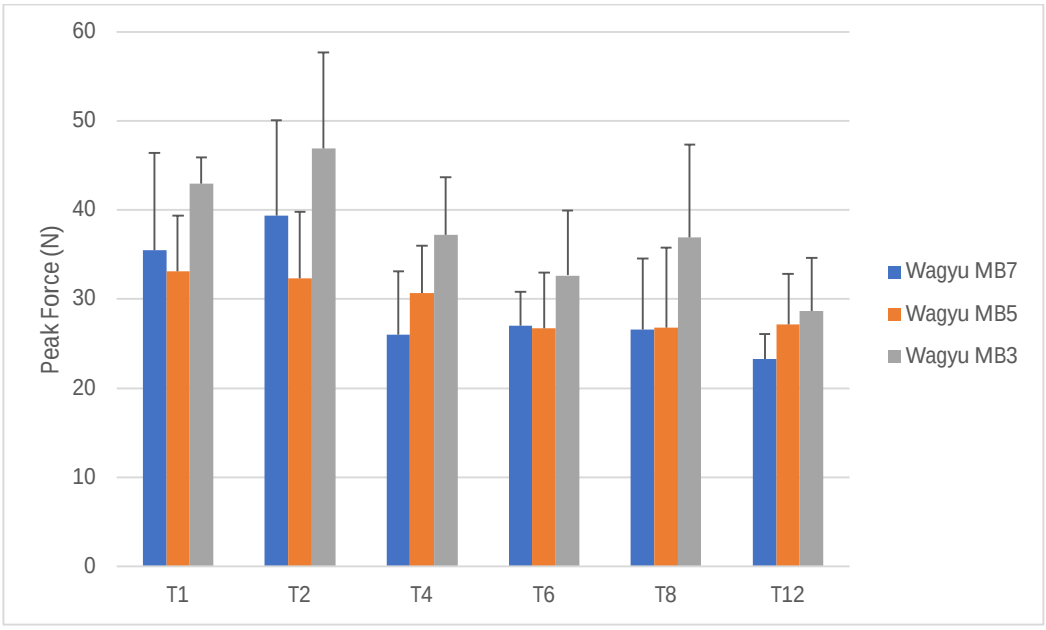

Figure 15: Mean peak force measurements ± standard deviation, for the Wagyu samples during storage (1, 2, 4, 6, 8 and 12 weeks)

7.4.3 Model development – Marble score: static

The Australian red meat industry grades beef according to the AUS-MEAT beef quality grading system with product achieving scores between 0 and 9. Higher marbling scores are associated with increased eating quality and sensory panels report improvements in tenderness, juiciness, and flavour with increasing marble scores. Consequently, beef with elevated marbling scores is sought by consumers looking for an enhanced sensory experience and they are generally willing to pay premium prices to obtain such products. Consumers therefore have an expectation that repeat purchase of beef with the same marble score (e.g. Wagyu MB5) will provide the same sensory experience. Despite this, grading of marble score in Australian processing plants is generally achieved via subjective assessment which introduces opportunity for variability and error. REIMS was evaluated for its ability to correctly classify the 108 Angus samples into MB2, MB3 or MB4 and 108 Wagyu samples into MB3, MB5 and MB7. Models were developed for the spectral ranges *m/z* 100–1200, 100–500 and 600–900. Models for Angus and Wagyu products were developed separately to determine classification rates within breeds. A final model comprising all 216 samples was developed to determine the classification rates independent of breed.

7.4.3.1 Angus MB static

PCA-LDA modelling of REIMS spectral profiles demonstrated good separation of the three Angus marble groups (Figure 16), as determined by the processor. Cross-validation of the models produced for *m/z* 100–1200, 100–500 and 600–900 resulted in correct classification rates ranging from 74.63 to 92.04% (Table 13), with the *m/z* 100–500 model giving the highest classification rate. Testing of the models saw small reductions in overall correct classification rates with *m/z* 100–500 continuing to produce the best result at 90.14%.

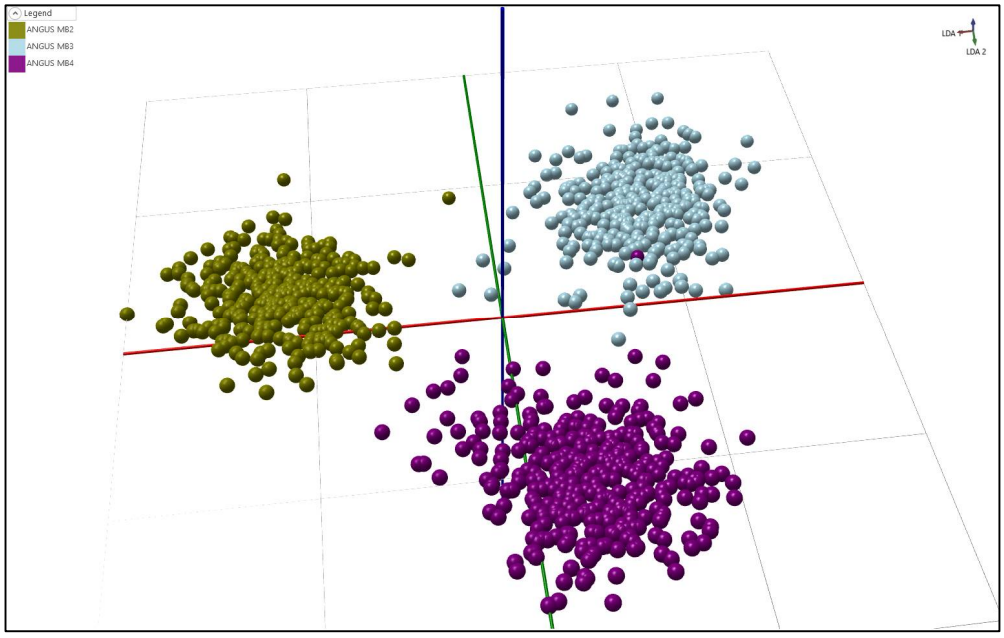

Figure 16. PCA-LDA plot of the *m/z* 100–500 range of Angus MB2, MB3 and MB4 samples following REIMS analysis.

Table 13. Cross-validation scores for PCA-LDA classification models for Angus MB2, MB3 and MB4 samples for the spectral ranges *m/z* 100–1200, 100–500, and 600–900.

Model	*Spectra*	*Run*	*Spectra*	*Passes*	*Failures*	*Outliers*	*Correct classification rate*
Angus MB static	100-1200	Full group out	1080	970	110	0	89.81%
Angus MB static	100-500	Full group out	1080	994	86	0	92.04%
Angus MB static	600-900	Full group out	1080	806	274	0	74.63%
Angus MB static	100-1200	1	240	221	19	0	92.08%
Angus MB static	100-1200	2	240	209	31	0	87.08%
Angus MB static	100-1200	3	240	183	57	0	76.25%
Angus MB static	100-1200	4	240	204	36	0	85.00%
Angus MB static	100-1200	5	240	218	22	0	90.83%
Angus MB static	100-1200	6	240	206	34	0	85.83%
Angus MB static	100-1200	Overall	1440	1241	199	0	86.18%
Angus MB static	100-500	1	240	222	18	0	92.50%
Angus MB static	100-500	2	240	227	13	0	94.58%
Angus MB static	100-500	3	240	202	37	1	84.17%
Angus MB static	100-500	4	240	213	27	0	88.75%
Angus MB static	100-500	5	240	237	3	0	98.75%
Angus MB static	100-500	6	240	197	43	0	82.08%
Angus MB static	100-500	Overall	1440	1298	141	1	90.14%
Angus MB static	600-900	1	240	207	33	0	86.25%
Angus MB static	600-900	2	240	147	93	0	61.25%
Angus MB static	600-900	3	240	119	121	0	49.58%
Angus MB static	600-900	4	240	132	108	0	55.00%
Angus MB static	600-900	5	240	175	65	0	72.92%
Angus MB static	600-900	6	240	195	45	0	81.25%
Angus MB static	600-900	Overall	1440	975	465	0	67.71%

7.4.3.2 Wagyu MB static

PCA-LDA modelling of REIMS spectral profiles demonstrated good separation of the three Wagyu marble groups (Figure 17). Cross-validation of the models produced for *m/z* 100–1200, 100–500 and 600–900 resulted in correct classification rates ranging from 69.54 to 87.96% (Table 14) with the *m/z* 100–500 model giving the highest classification rate. The correct classification rates were lower than those observed for Angus samples and are predominantly related to the misclassification of MB3 and MB5 samples. Testing of the models saw small reductions in overall correct classification rates, with *m/z* 100–500 continuing to produce the best result at 87.96%.

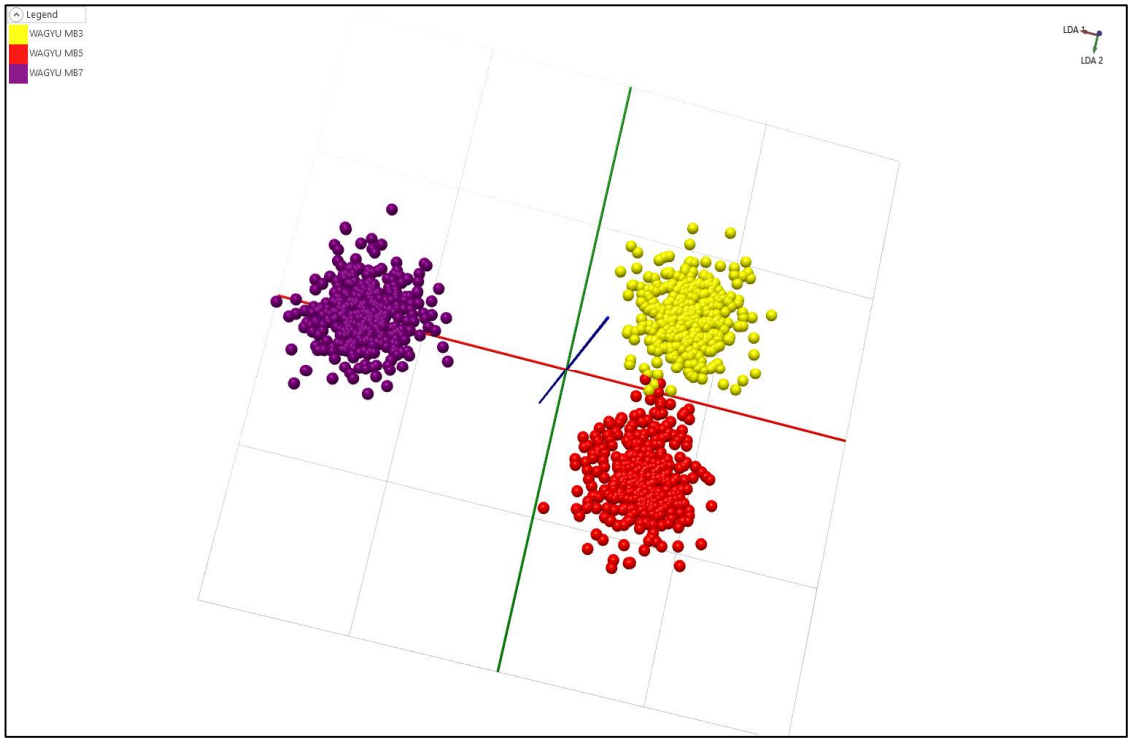

Figure 17. PCA-LDA plot of the *m/z* 100–500 range of Wagyu MB3, MB5 and MB7 samples following REIMS analysis.

Table 14. Cross-validation scores for PCA-LDA classification models for Wagyu MB3, MB5 and MB7 samples for the spectral ranges *m/z* 100–1200, 100–500, and 600–900.

Model	*Spectra*	*Run*	*Spectra*	*Passes*	*Failures*	*Outliers*	*Correct classification rate*
Wagyu MB static	100-1200	Full group out	1080	803	268	9	74.35%
Wagyu MB static	100-500	Full group out	1080	950	130	0	87.96%
Wagyu MB static	600-900	Full group out	1080	751	328	1	69.54%
Wagyu MB static	100-1200	1	240	141	99	0	58.75%
Wagyu MB static	100-1200	2	240	205	35	0	85.42%
Wagyu MB static	100-1200	3	240	185	37	18	77.08%
Wagyu MB static	100-1200	4	240	163	68	9	67.92%
Wagyu MB static	100-1200	5	240	171	68	1	71.25%
Wagyu MB static	100-1200	6	240	182	58	0	75.83%
Wagyu MB static	100-1200	Overall	1440	1047	365	28	72.71%
Wagyu MB static	100-500	1	240	194	46	0	80.83%
Wagyu MB static	100-500	2	240	215	25	0	89.58%
Wagyu MB static	100-500	3	240	170	70	0	70.83%
Wagyu MB static	100-500	4	240	228	12	0	95.00%

Model	Spectra	Run	Spectra	Passes	Failures	Outliers	Correct classification rate
Wagyu MB static	100-500	5	240	180	60	0	75.00%
Wagyu MB static	100-500	6	240	208	32	0	86.67%
Wagyu MB static	100-500	Overall	1440	1195	245	0	82.99%
Wagyu MB static	600-900	1	240	126	114	0	52.50%
Wagyu MB static	600-900	2	240	211	29	0	87.92%
Wagyu MB static	600-900	3	240	195	44	1	81.25%
Wagyu MB static	600-900	4	240	129	111	0	53.75%
Wagyu MB static	600-900	5	240	169	71	0	70.42%
Wagyu MB static	600-900	6	240	157	83	0	65.42%
Wagyu MB static	600-900	Overall	1440	987	452	1	68.54%

7.4.3.3 Combined Angus and Wagyu MB static

PCA-LDA modelling of REIMS spectral profiles demonstrated reasonable separation of the five marble groups, though MB3 and MB5 did show an amount of overlap between the groups (Figure 18). Cross-validation of the models produced for *m/z* 100–1200, 100–500 and 600–900 resulted in correct classification rates ranging from 70.14 to 82.96% (Table 15), with the *m/z* 100–500 model giving the highest classification rate. Testing of the models saw small reductions in overall correct classification rates with *m/z* 100–500 continuing to produce the best result at 79.97%.

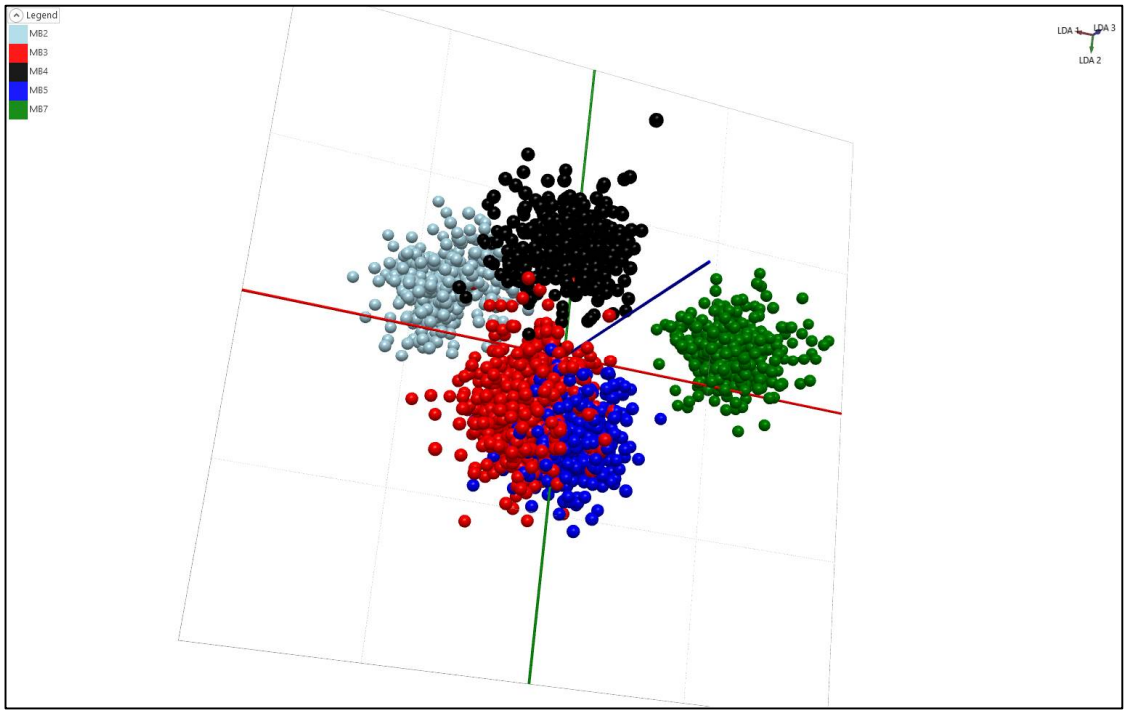

Figure 18. PCA-LDA plot of the *m/z* 100–500 range of MB2, MB3, MB4, MB5, and MB7 samples following REIMS analysis.

Table 15. Cross-validation scores for PCA-LDA classification models for MB2, MB3, MB4, MB5, and MB7 samples for the spectral ranges *m/z* 100–1200, 100–500, and 600–900.

Model	*Spectra*	*Run*	*Spectra*	*Passes*	*Failures*	*Outliers*	*Correct classification rate*
MB Static	100-1200	Full group out	2160	1723	402	35	79.77%
MB Static	100-500	Full group out	2160	1792	368	0	82.96%
MB Static	600-900	Full group out	2160	1515	643	2	70.14%
MB Static	100-1200	1	480	351	129	0	73.13%
MB Static	100-1200	2	480	388	92	0	80.83%
MB Static	100-1200	3	480	333	147	0	69.38%
MB Static	100-1200	4	480	372	108	0	77.50%
MB Static	100-1200	5	480	385	95	0	80.21%
MB Static	100-1200	6	480	367	113	0	76.46%
MB Static	100-1200	Overall	2880	2196	684	0	76.25%
MB Static	100-500	1	480	342	138	0	71.25%
MB Static	100-500	2	480	391	89	0	81.46%
MB Static	100-500	3	480	374	106	0	77.92%
MB Static	100-500	4	480	407	73	0	84.79%
MB Static	100-500	5	480	391	89	0	81.46%
MB Static	100-500	6	480	398	82	0	82.92%
MB Static	100-500	Overall	2880	2303	577	0	79.97%
MB Static	600-900	1	480	322	158	0	67.08%
MB Static	600-900	2	480	325	155	0	67.71%
MB Static	600-900	3	480	274	206	0	57.08%
MB Static	600-900	4	480	292	187	1	60.83%
MB Static	600-900	5	480	346	134	0	72.08%
MB Static	600-900	6	480	332	148	0	69.17%
MB Static	600-900	Overall	2880	1891	988	1	65.66%

Further examination of the validation results, in particular understanding patterns that may exist for samples that repeatedly caused failures, determined that failures were more likely to be caused by samples that had been stored for four weeks or more. Chilled, vacuum packed storage of beef (i.e. ageing) is expected to result in changes to the product over time. As the static models presented above do not consider time of storage, it is plausible to suggest that changes occurring during storage are affecting the REIMS spectra observed and hence providing additional challenges to the supervised machine learning approach being applied here. To test this assumption an additional MB static model was developed utilising spectra collected from beef products that were aged for no more than two weeks. The model developed focussed on the *m/z* 100–500 spectral range which has consistently provided higher classification rates for the models presented thus far (Figure 19).

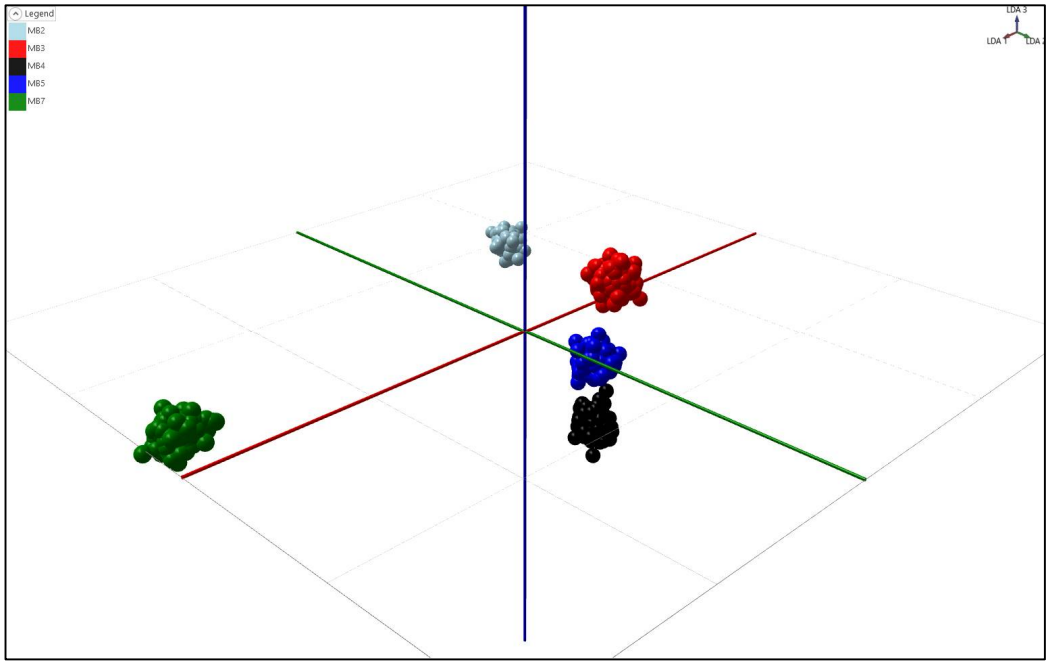

Figure 19. PCA-LDA plot of the *m/z* 100–500 range of MB2, MB3, MB4, MB5, and MB7 samples aged for ≤2 weeks following REIMS analysis.

The model produced shows clear separation of the five marble scores and produced a correct classification rate of 99.31% (Table 16). Testing of the model with >20% of animals removed from the model construction resulted in an overall classification rate of 98.23%. These findings support the assumption that time of storage was impacting on the ability of the models to correctly classify samples but more importantly it suggests that REIMS has good potential for use as an objective marbling assessment tool. Furthermore, it may be possible to conduct lipidomic assessment (i.e. REIMS) on pre-chill carcases which could support pre-chill grading of carcases and facilitate better planning of boning room runs to most accurately meet customer order specifications and ultimately ensuring maximum value from the products.

Table 16. Cross-validation scores for PCA-LDA classification model for MB2, MB3, MB4, MB5, and MB7 samples for the spectral range *m/z* 100–1200.

Model	*Spectra*	*Run*	*Spectra*	*Passes*	*Failures*	*Outliers*	*Correct classification rate*
MB Static	100-500	Full group out	720	715	1	4	99.31%
MB Static	100-500	1	160	156	1	3	97.50%
MB Static	100-500	2	160	160	0	0	100.00%
MB Static	100-500	3	160	149	4	7	93.13%
MB Static	100-500	4	160	160	0	0	100.00%
MB Static	100-500	5	160	158	0	2	98.75%
MB Static	100-500	6	160	160	0	0	100.00%
MB Static	100-500	Overall	960	943	5	12	98.23%

7.4.4 Model development – Marble score: dynamic

Australian beef exporters access a range of global markets, some of which are prescriptive about the period of time by which Australian product must be sold or sent to an alternate distribution pathway (e.g. frozen). On occasion, it may be necessary for Australian exporters to provide evidence of the age of their product over and above the statements made on any accompanying packaging. REIMS was evaluated for its potential to verify the marble score of Angus and Wagyu beef products and the number of weeks chilled storage the product had received. Models for Angus and Wagyu products were developed separately to determine classification rates within breeds across storage time. A final model comprising all 216 samples was developed to determine the classification rates for marble score and storage time alone.

7.4.4.1 Angus MB dynamic

PCA-LDA modelling of REIMS spectral profiles from Angus resulted in samples grouping by marble score and time stored (Figure 20). Cross-validation of the models produced for *m/z* 100–1200, 100–500 and 600–900 resulted in correct classification rates ranging from 88.89 to 93.61% (Table 17), with the *m/z* 100–1200 model giving the highest classification rate. Testing of the models saw small reductions in overall correct classification rates with *m/z* 100–500 and 100–1200 models both giving correct classification rates of 90.00%.

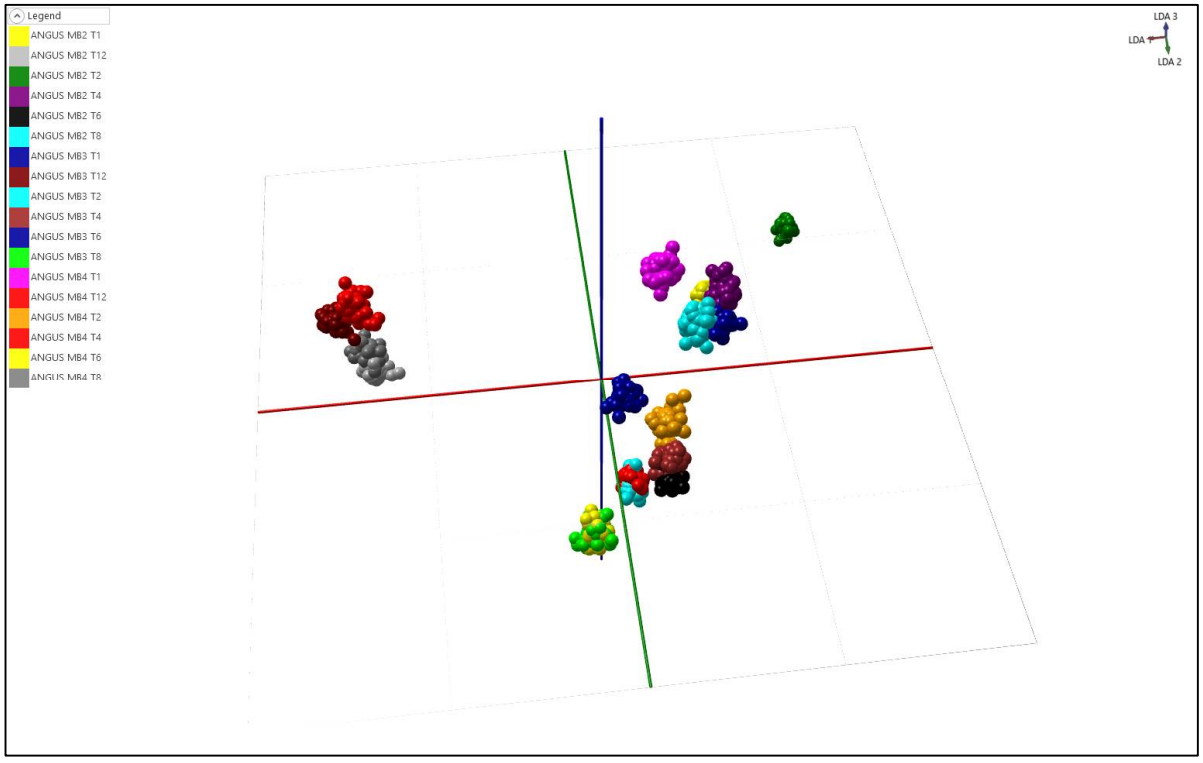

Figure 20. PCA-LDA plot of the *m/z* 100–1200 REIMS spectral range of Angus MB2, MB3 and MB4 samples stored for up to 12 weeks.

Table 17. Cross-validation and test scores for PCA-LDA classification models for Angus MB2, MB3 and MB4 samples stored for up to 12 weeks for the spectral ranges *m/z* 100–1200, 100–500, and 600–900.

Model	*Spectra*	*Run*	*Spectra*	*Passes*	*Failures*	*Outliers*	*Correct classification rate*
Angus MB dynamic	100-1200	Full group out	1080	1011	44	25	93.61%
Angus MB dynamic	100-500	Full group out	1080	1004	53	23	92.96%
Angus MB dynamic	600-900	Full group out	1080	960	110	10	88.89%
Angus MB dynamic	100-1200	1	240	223	12	5	92.92%
Angus MB dynamic	100-1200	2	240	230	1	9	95.83%
Angus MB dynamic	100-1200	3	240	206	14	20	85.83%
Angus MB dynamic	100-1200	4	240	207	27	6	86.25%
Angus MB dynamic	100-1200	5	240	217	12	11	90.42%
Angus MB dynamic	100-1200	6	240	213	20	7	88.75%
Angus MB dynamic	100-1200	Overall	1440	1296	86	58	90.00%
Angus MB dynamic	100-500	1	240	211	18	11	87.92%
Angus MB dynamic	100-500	2	240	236	2	2	98.33%
Angus MB dynamic	100-500	3	240	212	16	12	88.33%
Angus MB dynamic	100-500	4	240	209	28	3	87.08%
Angus MB dynamic	100-500	5	240	218	15	7	90.83%
Angus MB dynamic	100-500	6	240	210	26	4	87.50%
Angus MB dynamic	100-500	Overall	1440	1296	105	39	90.00%
Angus MB dynamic	600-900	1	240	226	12	2	94.17%
Angus MB dynamic	600-900	2	240	208	27	5	86.67%
Angus MB dynamic	600-900	3	240	178	55	7	74.17%
Angus MB dynamic	600-900	4	240	201	37	2	83.75%
Angus MB dynamic	600-900	5	240	212	26	2	88.33%
Angus MB dynamic	600-900	6	240	206	33	1	85.83%
Angus MB dynamic	600-900	Overall	1440	1231	190	19	85.49%

7.4.4.2 Wagyu MB dynamic

PCA-LDA modelling of REIMS spectral profiles from Wagyu resulted in samples grouping by marble score and time stored (Figure 21). Cross-validation of the models produced for *m/z* 100–1200, 100–500 and 600–900 resulted in correct classification rates ranging from 81.94 to 95.83% (Table 18), with the *m/z* 100–500 model giving the highest classification rate. Testing of the models saw small reductions in overall correct classification rates with *m/z* 100–500 giving the highest correct classification rates of 93.54%.

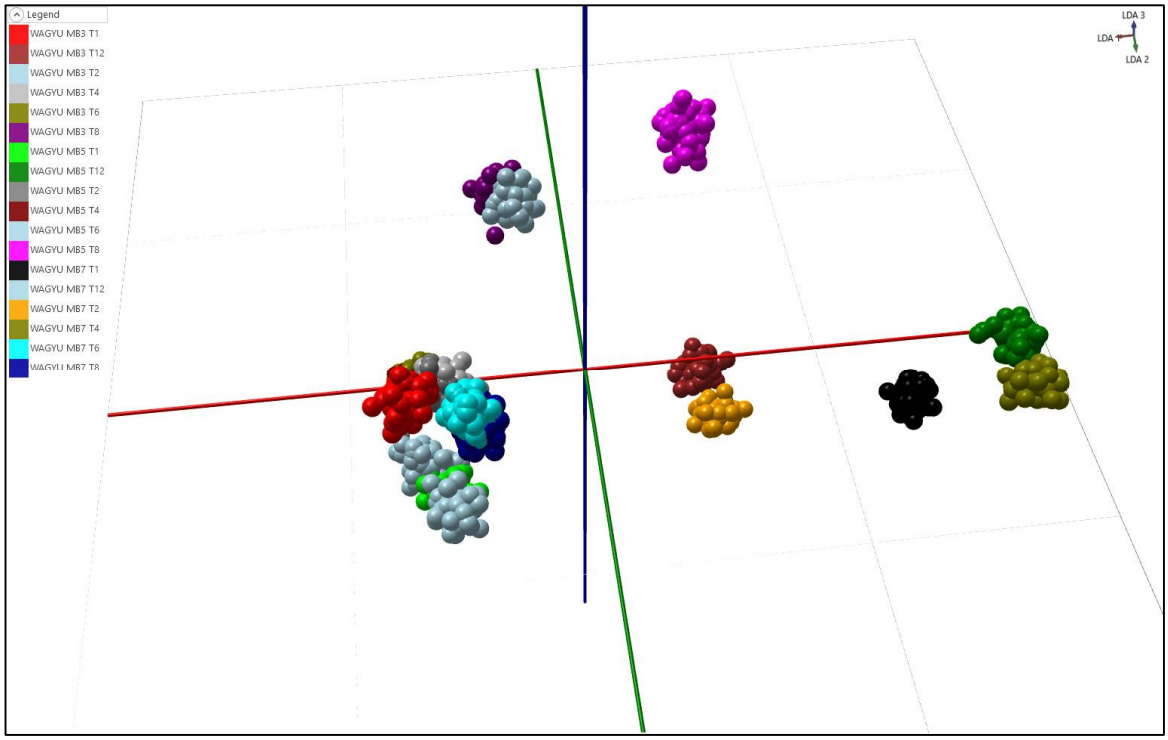

Figure 21. PCA-LDA plot of the *m/z* 100–500 REIMS spectral range of Wagyu MB3, MB5 and MB7 samples stored for up to 12 weeks.

Table 18. Cross-validation and test scores for PCA-LDA classification models for Wagyu MB3, MB5 and MB7 samples stored for up to 12 weeks for the spectral ranges *m/z* 100–1200, 100–500, and 600–900.

Model	*Spectra*	*Run*	*Spectra*	*Passes*	*Failures*	*Outliers*	*Correct classification rate*
Wagyu MB dynamic	100-1200	Full group out	1080	983	82	15	91.02%
Wagyu MB dynamic	100-500	Full group out	1080	1035	39	6	95.83%
Wagyu MB dynamic	600-900	Full group out	1080	885	186	9	81.94%
Wagyu MB dynamic	100-1200	1	240	189	42	9	78.75%
Wagyu MB dynamic	100-1200	2	240	218	17	5	90.83%
Wagyu MB dynamic	100-1200	3	240	181	19	40	75.42%
Wagyu MB dynamic	100-1200	4	240	200	20	20	83.33%
Wagyu MB dynamic	100-1200	5	240	211	26	3	87.92%
Wagyu MB dynamic	100-1200	6	240	222	13	5	92.50%
Wagyu MB dynamic	100-1200	Overall	1440	1221	137	82	84.79%
Wagyu MB dynamic	100-500	1	240	224	9	7	93.33%
Wagyu MB dynamic	100-500	2	240	230	4	6	95.83%
Wagyu MB dynamic	100-500	3	240	216	9	15	90.00%

Model	Spectra	Run	Spectra	Passes	Failures	Outliers	Correct classification rate
Wagyu MB dynamic	100-500	4	240	228	4	8	95.00%
Wagyu MB dynamic	100-500	5	240	217	22	1	90.42%
Wagyu MB dynamic	100-500	6	240	232	6	2	96.67%
Wagyu MB dynamic	100-500	Overall	1440	1347	54	39	93.54%
Wagyu MB dynamic	600-900	1	240	153	80	7	63.75%
Wagyu MB dynamic	600-900	2	240	223	17	0	92.92%
Wagyu MB dynamic	600-900	3	240	184	34	22	76.67%
Wagyu MB dynamic	600-900	4	240	175	53	12	72.92%
Wagyu MB dynamic	600-900	5	240	206	34	0	85.83%
Wagyu MB dynamic	600-900	6	240	189	48	3	78.75%
Wagyu MB dynamic	600-900	Overall	1440	1130	266	44	78.47%

7.4.4.3 Combined Angus and Wagyu MB dynamic

PCA-LDA modelling of REIMS spectral profiles demonstrated clustering of samples with the same marble score and storage time (Figure 22). Cross-validation of the models produced for *m/z* 100–1200, 100–500 and 600–900 resulted in correct classification rates ranging from 81.30 to 90.93% (Table 19), with the *m/z* 100–500 model giving the highest classification rate. Testing of the models saw small reductions in overall correct classification rates with *m/z* 100–500 continuing to produce the best result at 89.72%.

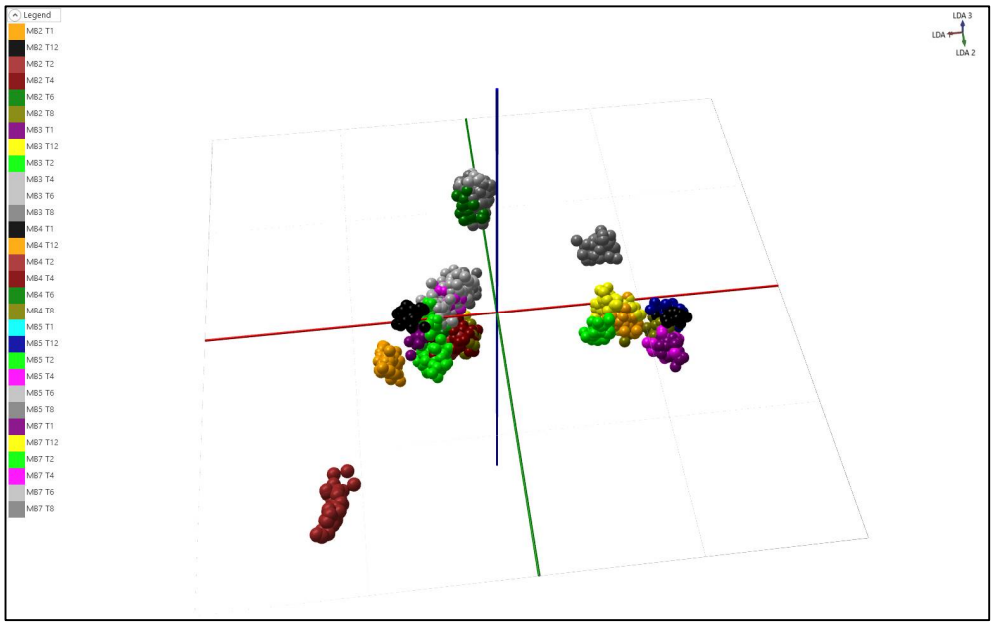

Figure 22. PCA-LDA plot of the *m/z* 100–500 REIMS spectral range of MB2, MB3, MB4, MB5 and MB7 samples stored for up to 12 weeks.

Table 19. Cross-validation and test scores for PCA-LDA classification models for MB2, MB3, MB4, MB5, and MB7 samples stored for up to 12 weeks for the spectral ranges *m/z* 100–1200, 100–500, and 600–900.

Model	Spectra	Run	Spectra	Passes	Failures	Outliers	Correct classification rate
MB Dynamic	100-1200	Full group out	2160	1793	178	189	83.01%
MB Dynamic	100-500	Full group out	2160	1964	182	14	90.93%
MB Dynamic	600-900	Full group out	2160	1756	394	10	81.30%
MB Dynamic	100-1200	1	480	399	79	2	83.13%
MB Dynamic	100-1200	2	480	460	18	2	95.83%
MB Dynamic	100-1200	3	480	383	64	33	79.79%
MB Dynamic	100-1200	4	480	417	55	8	86.88%
MB Dynamic	100-1200	5	480	430	40	10	89.58%
MB Dynamic	100-1200	6	480	435	44	1	90.63%
MB Dynamic	100-1200	Overall	2880	2524	300	56	87.64%
MB Dynamic	100-500	1	480	425	54	1	88.54%
MB Dynamic	100-500	2	480	464	16	0	96.67%
MB Dynamic	100-500	3	480	419	45	16	87.29%
MB Dynamic	100-500	4	480	424	54	2	88.33%
MB Dynamic	100-500	5	480	418	57	5	87.08%
MB Dynamic	100-500	6	480	434	43	3	90.42%
MB Dynamic	100-500	Overall	2880	2584	269	27	89.72%
MB Dynamic	600-900	1	480	368	109	3	76.67%
MB Dynamic	600-900	2	480	397	81	2	82.71%
MB Dynamic	600-900	3	480	341	138	1	71.04%
MB Dynamic	600-900	4	480	370	103	7	77.08%
MB Dynamic	600-900	5	480	405	75	0	84.38%
MB Dynamic	600-900	6	480	383	97	0	79.79%
MB Dynamic	600-900	Overall	2880	2264	603	13	78.61%

7.4.5 Model development – fresh v frozen product

PCA-LDA modelling of REIMS spectral profiles demonstrated distinct clustering of fresh samples away from the frozen samples. Within the broader fresh or frozen sample clusters, there was further clustering of Wagyu, Angus and grass-fed samples, with Angus sharing borders with either Wagyu or grass-fed samples (Figure 23). This relationship between the three groups had been previously observed and is thought to represent the use of Angus animals in the production of F1 Wagyu animals and the likelihood that Angus genetics and traits are present in some grass-fed production systems. Cross-validation of the models produced for *m/z* 100–1200, 100–500 and 600–900 resulted in correct classification rates ranging from 83.73 to 85.13% (Table 20), with the *m/z* 100–1200 model giving the highest classification rate. Testing of the models saw small improvements in overall correct classification rates with *m/z* 100–1200 continuing to produce the best result at 87.54%. Despite the validation and testing of the models revealing several incorrect classifications, frozen product was never identified as fresh, or vice-versa. Incorrect

classifications always occurred with samples that modelled nearest the boundaries between Angus and Wagyu or Angus and grass-fed.

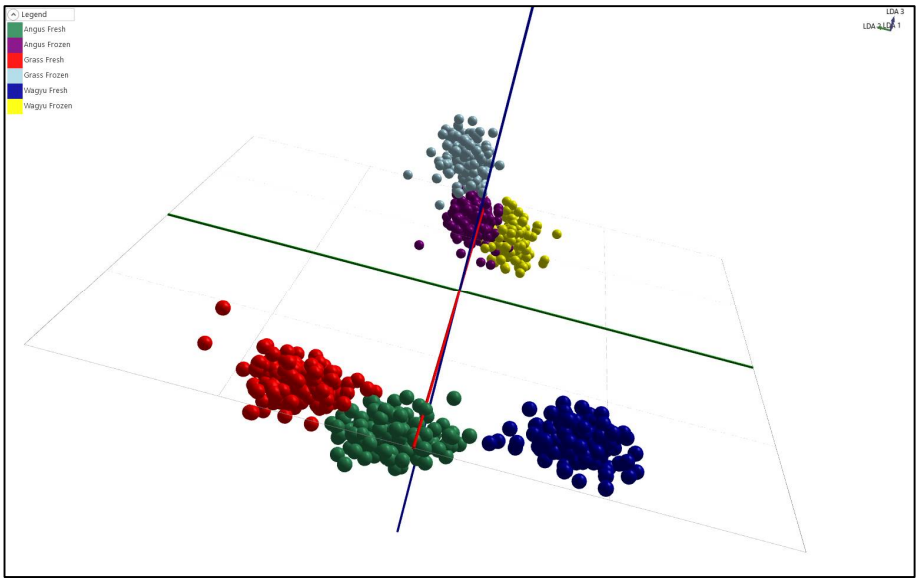

Figure 23. PCA-LDA plot of the *m/z* 100–500 REIMS spectral range for fresh and frozen Wagyu, Angus and grass-fed samples collected at retail.

Table 20. Cross-validation and test scores for PCA-LDA classification models for fresh and frozen Wagyu, Angus and grass-fed samples collected at retail.

Model	***Spectra***	***Run***	***Spectra***	***Passes***	***Failures***	***Outliers***	***Correct classification rate***
Fresh Frozen	100-1200	Full group out	1002	853	145	4	85.13%
Fresh Frozen	100-500	Full group out	1002	845	156	1	84.33%
Fresh Frozen	600-900	Full group out	1002	839	162	1	83.73%
Fresh Frozen	100-1200	1	210	196	13	1	93.33%
Fresh Frozen	100-1200	2	210	184	25	1	87.62%
Fresh Frozen	100-1200	3	191	154	36	1	80.63%
Fresh Frozen	100-1200	4	201	161	37	3	80.10%
Fresh Frozen	100-1200	5	190	165	24	1	86.84%
Fresh Frozen	100-1200	6	210	201	7	2	95.71%
Fresh Frozen	100-1200	Overall	1212	1061	142	9	87.54%
Fresh Frozen	100-500	1	210	182	26	2	86.67%
Fresh Frozen	100-500	2	210	183	27	0	87.14%

Model	Spectra	Run	Spectra	Passes	Failures	Outliers	Correct classification rate
Fresh Frozen	100-500	3	191	157	33	1	82.20%
Fresh Frozen	100-500	4	201	158	43	0	78.61%
Fresh Frozen	100-500	5	190	157	33	0	82.63%
Fresh Frozen	100-500	6	210	200	9	1	95.24%
Fresh Frozen	100-500	Overall	1212	1037	183	4	85.56%
Fresh Frozen	600-900	1	210	183	25	2	87.14%
Fresh Frozen	600-900	2	210	189	20	1	90.00%
Fresh Frozen	600-900	3	191	141	50	0	73.82%
Fresh Frozen	600-900	4	201	158	43	0	78.61%
Fresh Frozen	600-900	5	190	177	13	0	93.16%
Fresh Frozen	600-900	6	210	177	32	1	84.29%
Fresh Frozen	600-900	Overall	1212	1025	183	4	84.57%

7.4.6 Lipid analysis

7.4.6.1 Lipid stability

Lipid oxidation (as measured by TBARS) for Angus and Wagyu products over the storage period are shown in Figures 24 and 25. Overall, lipid oxidation tended to slightly increase with storage time for both breeds, with maximum values of 0.140 and 0.128 mg/kg MDA equivalents for Angus and Wagyu samples, respectively. The presence of TBARS in beef at concentrations of 0.6–2.0 mg/kg has been linked to the detection of oxidised or rancid flavours, negatively affecting eating quality [12]. Despite the trend to higher concentrations of TBARS over time in this study, the recorded levels are well below these reported thresholds at which there is a negative impact on flavour. This finding is consistent with the steady rise in TVC and LAB counts over time, and indicates intact packaging and a stable storage environment.

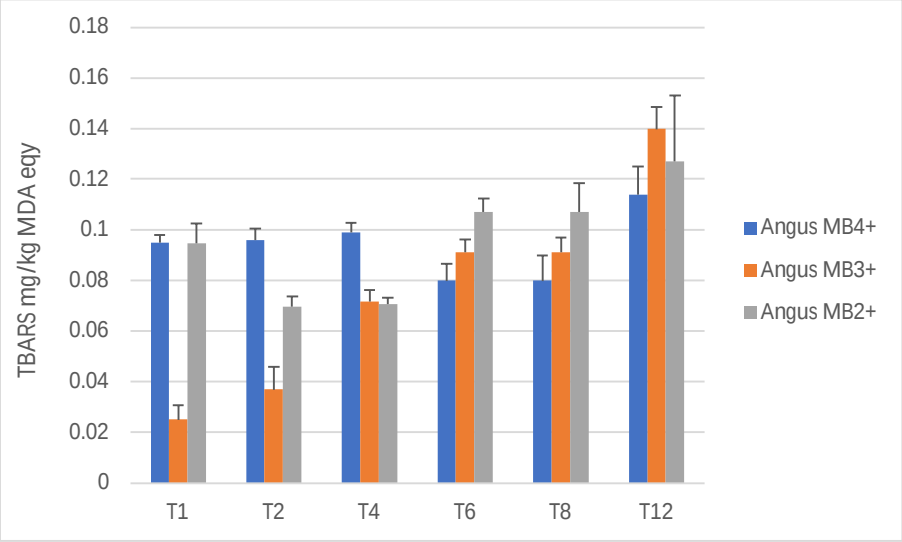

Figure 24: TBARS values ± standard deviation for Angus samples during storage (1, 2, 4, 6, 8 and 12 weeks).

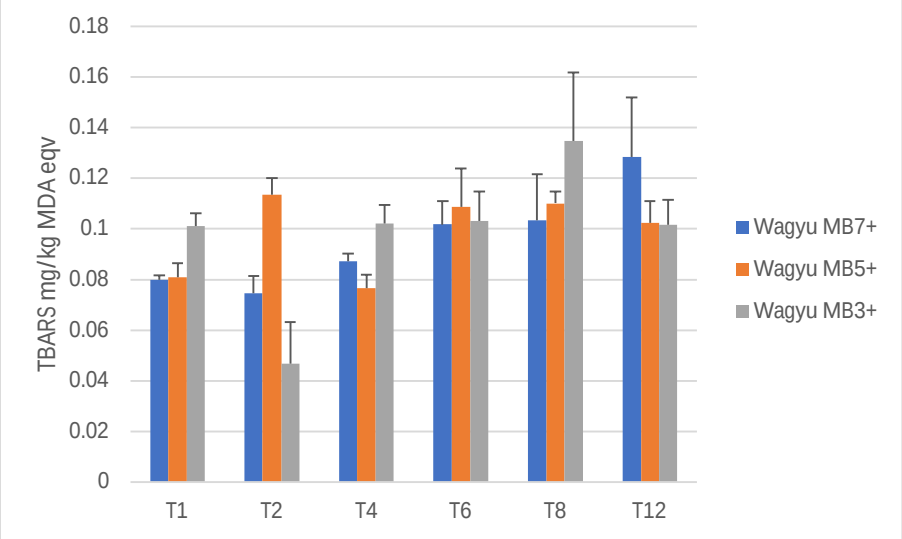

Figure 25: TBARS values ± standard deviation for Wagyu samples during storage (1, 2, 4, 6, 8 and 12 weeks).

7.4.6.2 FAME analysis

The mean, combined timepoint FAME results for each breed/MB combination, reported as mg FAME/100g fresh sample, and the estimated IMF % are reported in Table 21. These FAME data are an estimation of the intramuscular fatty acid content of the analysed samples. In this study, Oleic acid (C18:1 *cis* 9), a monounsaturated fatty acid, was the most abundant across all samples with the saturated fatty acids, palmitic (C16:0) and stearic acids (C:18:0) the next most, which is in agreement with literature [13]. Therefore, the expectation is that these FAMEs, and the sums of the saturated (SFA) and monounsaturated (MUFA) classes for which they make up the bulk of, would correspond well to the assigned MB score and the IMF %. The Angus samples all showed increases in oleic, palmitic, stearic acids, SFA and MUFA with the rise in assigned marbling score and IMF %. Further, positive correlations between these attributes and assigned marbling score between 0.514 – 0.569 (See appendix A2) was determined. Surprisingly, this trend was not fully reciprocated for the Wagyu samples, with no clear correlations between any FAMEs and marbling score (Appendix A3), however, levels of oleic acid and total MUFA did trend in accordance with the estimated IMF % determinations, whereby the highest levels of these fatty acids were found in the highest IMF samples (MB5 Wagyu est. IMF 18.16 %). While marbling score is often a reliable indicator of IMF %, possible reasons for this discrepancy are discussed further in section 7.4.7. Overall, the Wagyu samples had higher proportions of oleic acid and MUFA, when compared to the Angus samples, which displayed a tendency to slightly higher proportions of SFA. As lipid classes are a major contributor to the mass spectral data collected by REIMS analysis, and the subsequent model building, this difference in the fatty acid profile between breed may contribute to the separation of Angus and Wagyu in the presented classification models, however further data analysis will be required to determine this.

Table 21: Mean fatty acid methyl ester (FAME) results, reported as mg/100g fresh sample, and estimated intra-muscular fat content (IMF) as % w/w for the Angus and Wagyu samples. Results represent the combined mean from all timepoints for each breed/MB configuration (n = 72 per configuration).[#]

FAME	*Common name*	*Angus MB4*	*Angus MB3*	*Angus MB2*	*Wagyu MB7*	*Wagyu MB5*	*Wagyu MB3*
C12:0	Lauric	6	5	3	6	8	5
C14:0	Myristic	279	241	156	281	393	257
C14:1	Myristoleic	45	50	30	72	106	90
C15:0		42	38	27	33	43	31
C16:0	Palmitic	2360	2109	1390	2172	3337	2189
C16:1	Palmitoleic	224	227	129	343	453	358
C17:0		115	105	76	79	121	74
C17:1		68	69	47	74	95	72
C18:0	Stearic	1255	1039	735	965	1513	862
C18:1 cis 9	Oleic	2969	2784	1788	3367	5143	3543
C18:2	Linoleic	202	170	138	235	257	189
C20:0	Arachidic	8	6	4	6	9	5
C18:3	α-Linolenic	25	23	17	13	21	12
cis 9, trans-11 C18:2	Rumenic	25	30	18	28	50	31
C20:4	Arachidonic	21	21	23	34	24	26
C20:5	EPA	4	4	6	1	1	1
C22:6	DHA	1	1	1	ND	ND	ND
SFA		4076	3558	2401	3942	6067	3904
MUFA		3790	3541	2306	4168	6272	4330
PUFA		278	244	206	303	326	245
Estimated IMF		12.55 %	12.49 %	8.85 %	12.96 %	18.16 %	13.53 %

[#] SFA = saturated fatty acids, MUFA = monounsaturated fatty acids & PUFA = polyunsaturated fatty acids.

7.4.7 Model development – objective measurements

Studies have demonstrated that the percentage of intramuscular fat (IMF%) can be correlated to marbling score [14]. However, the extent to which these values are correlated does appear to be variable [15] and consideration to additional marbling attributes such as fleck fineness and the area evaluated is suggested when attempting to understand the relationship [16]. All meat quality and lipid analysis measurements obtained throughout this study were evaluated for their correlation to marbling score (Appendix A2 and A3). A mean positive correlation of 0.52 was observed between IMF% and marbling score for Angus products, however no clear correlation was observed for Wagyu products nor were any other single attributes shown to be strongly correlated to marbling score for Angus or Wagyu products. Reasons for the lack of correlation between IMF% and marbling score were considered and may relate to several factors including variability of IMF% within portions of the samples being analysed, inconsistency of sample preparation, particularly for highly marbled cuts where coarse seams of IMF may have been excluded, or the high degree of variability caused by a relatively low number dataset, as is present in this proof-of-concept study.

Therefore, to determine if REIMS could be used to model objective measurements, the graded marbling score of each product was ignored and Angus and Wagyu were ranked based on their measured IMF% and arbitrarily assigned to three equal groups for each breed. PCA-LDA modelling of REIMS spectral profiles was conducted using the highest performing spectral ranges identified in Sections 7.4.3 and 7.4.4. The models developed did not produce

the tightly bound clusters observed previously when graded marbling score was used for Angus or Wagyu (Figure 26) products. In addition, there was no improvement to model performance when weeks stored was considered with Angus cross-validation results improving from 36.81 to 42.36% and Wagyu improving from 32.99 to 36.94% (Table 22). The results indicate that the models generated from grouping samples based on the IMF% values generated in this study perform poorly. Notwithstanding, there is a need to explore the use of REIMS spectra for the modelling of IMF% such that the models provide a point of calibration between subjectively graded marbling scores and IMF%.

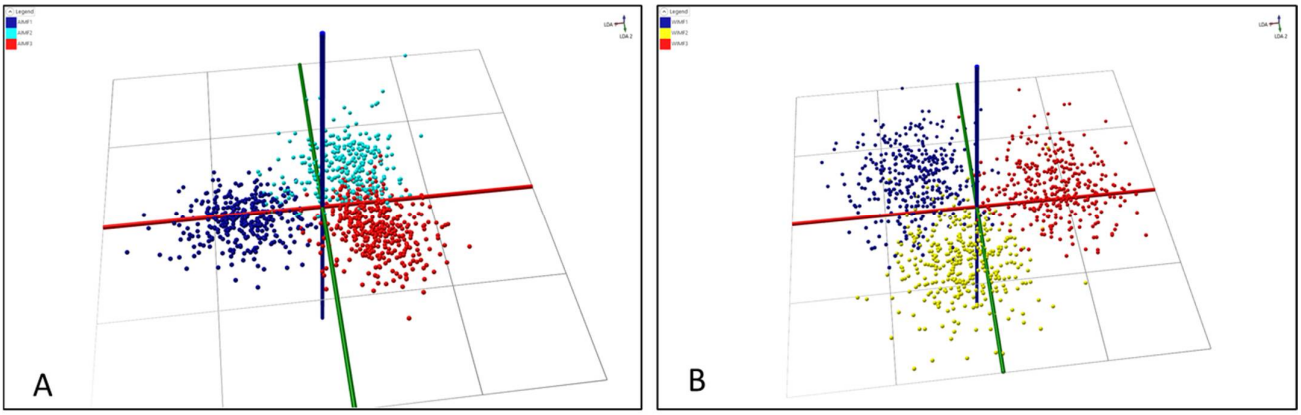

Figure 26. PCA-LDA plot of the *m/z* 100–500 REIMS spectral range for Angus (A) and Wagyu (B) products reassigned into groups defined by IMF%.

Table 22. Cross-validation and test scores for PCA-LDA classification models for Angus (A) and Wagyu (B) products reassigned into groups defined by IMF%.

Model	*Spectra*	*Run*	*Spectra*	*Passes*	*Failures*	*Outliers*	*Correct classification rate*
Angus MB static	100-500	Full group out	1080	465	615	0	43.06%
Angus MB static	100-500	1	240	130	110	0	54.17%
Angus MB static	100-500	2	240	102	137	1	42.50%
Angus MB static	100-500	3	240	56	184	0	23.33%
Angus MB static	100-500	4	240	97	143	0	40.42%
Angus MB static	100-500	5	240	63	176	1	26.25%
Angus MB static	100-500	6	240	82	158	0	34.17%
Angus MB static	100-500	Overall	1440	530	908	2	36.81%
Angus MB dynamic	100-1200	Full group out	1080	471	596	13	43.61%
Angus MB dynamic	100-1200	1	240	137	98	5	57.08%
Angus MB dynamic	100-1200	2	240	158	80	2	65.83%
Angus MB dynamic	100-1200	3	240	44	191	5	18.33%
Angus MB dynamic	100-1200	4	240	119	119	2	49.58%
Angus MB dynamic	100-1200	5	240	53	181	6	22.08%

Angus MB dynamic	100-1200	6	240	99	139	2	41.25%
Angus MB dynamic	100-1200	Overall	1440	610	808	22	42.36%
Wagyu MB static	100-500	Full group out	1080	280	800	0	25.93%
Wagyu MB static	100-500	1	240	40	200	0	16.67%
Wagyu MB static	100-500	2	240	78	162	0	32.50%
Wagyu MB static	100-500	3	240	93	147	0	38.75%
Wagyu MB static	100-500	4	240	71	169	0	29.58%
Wagyu MB static	100-500	5	240	59	181	0	24.58%
Wagyu MB static	100-500	6	240	134	106	0	55.83%
Wagyu MB static	100-500	Overall	1440	475	965	0	32.99%
Wagyu MB dynamic	100-500	Full group out	1080	369	711	0	34.17%
Wagyu MB dynamic	100-500	1	240	52	187	1	21.67%
Wagyu MB dynamic	100-500	2	240	76	164	0	31.67%
Wagyu MB dynamic	100-500	3	240	102	128	10	42.50%
Wagyu MB dynamic	100-500	4	240	64	171	5	26.67%
Wagyu MB dynamic	100-500	5	240	106	134	0	44.17%
Wagyu MB dynamic	100-500	6	240	132	108	0	55.00%
Wagyu MB dynamic	100-500	Overall	1440	532	892	16	36.94%

7.5 Conclusion

REIMS is a recently emerged technology that has shown high potential in provenance, quality, and safety applications. Furthermore, its ability to conduct real-time in-situ analysis provides opportunity for its deployment into food processing facilities. The installation of a REIMS system at CSIRO represents the first deployment of REIMS into Australia for use in food systems with the red meat industry conducting the first proof of concept study. The purpose of this study was to evaluate the ability of REIMS to generate spectral profiles which could be used to develop proof of concept models for verification of the quality of Australian beef products.

Based on the data from this trial classification models for graded marble scores of Angus and Wagyu animals as well as independent of breed (i.e. marble score only) were developed. Furthermore, models were developed such that marbling score and breed could be identified at any point of the distribution pathway or to identify the age, breed and marbling at any point within 12 weeks of slaughter. Models were also developed that accurately classified Angus, Wagyu, and grass-fed products that had been chilled or frozen. Each model demonstrated capacity to identify quality attributes of red meat that were important to the industry and its customers. It was anticipated that intramuscular fat % could also have been used in conjunction with REIMS spectral profiles to develop classification models, but this was not the case as there was no correlation between graded marbling score and the measured IMF% and it is anticipated that larger studies will offset the variability observed and REIMS models will be developed which can provide an interface between graded marbling score and IMF%. Nevertheless, this study has confirmed that REIMS shows high potential for the real-time identification of quality aligned attributes of importance to the red meat industry. Indeed, it is proposed that the findings be explored further with the potential for REIMS to have application in the objective grading of pre-chill carcases for marbling score. Subsequent discussions with industry participants should identify focused use cases to further showcase the potential of REIMS.

8.0 Milestone report: REIMS food safety applications

8.1 Executive summary

The red meat supply chain comprises a complex network that attempts to transfer a range of products from production to consumption in a safe and secure way. Australian red meat processors verify hygiene performance and meet regulatory requirements for bacterial pathogen presence via participation in the national carcase microbiological monitoring program. Microbiological testing is complex, laborious, and costly, often occurring at the end of production and processing where there are limited opportunities to deploy interventions which may offset food safety risks. Rapid Evaporative Ionisation Mass Spectrometry (REIMS) is a recently developed ambient ionisation mass spectrometry technique that has demonstrated capacity in verifying red meat product provenance and credentials.

This project aims to conduct proof of concept testing of the REIMS system for attributes of relevance to the Australian red meat industry including provenance, quality, and food safety. This milestone focussed on the application of REIMS to food safety applications which include the detection and classification of the microorganisms *E. coli*, *Salmonella* and *Enterococci* which are relevant to trade, hygiene, and human health. In addition to assessing REIMS across food safety applications, there was also opportunity during the project to trial the RADIAN direct mass detector system. A total of 180 *E. coli*, *Salmonella* and *Enterococci* isolated from beef cattle-associated samples were included in the study. The *E. coli* group was comprised of predominant Shiga toxin-producing *E. coli* serogroups including O157, O111 and O26. The *Salmonella* group consisted of commonly identified serovars from cattle including Typhimurium, Anatum, Bovismorbificans and Saintpaul, and similarly the *Enterococci* group included the species of *faecium*, *faecalis* and *hirae* which are routinely used to assess development of antimicrobial resistance in red meat production systems.

REIMS spectral profiles were generated using a modified protocol that utilised a Direct REIMS inlet assembly coupled with a cell line electrode sampler. RADIAN spectral profiles were generated using a non-destructive sampling approach followed by analysis as per the manufacturer's directions. The resulting spectral profiles were used to develop the following REIMS or RADIAN-based classification models using linear discriminant analysis (LDA). REIMS classification models were generated from the total isolate pool with RADIAN classification models being generated from a subset of 89 isolates.

- Bacterial genus – *E. coli* v *Salmonella* v *Enterococcus*
- *Salmonella* serovars – Anatum v Typhimurium v Bovismorbificans v Saintpaul
- *E. coli* serogroups – generic *E. coli* v O157 v O111 v O26
- *Enterococcus* species – *faecium* v *faecalis* v *hirae*

The REIMS and RADIAN systems were both able to develop classification models for bacterial genus, *E. coli* serogroups, *Salmonella* serovars, and *Enterococcus* speciation. All models were cross-validated with models generated using spectra profiles from REIMS resulting in correct classification rates for all models exceeding

93.33%. By comparison, models developed using spectra from the RADIAN system achieved correct classification rates exceeding 81.25%. It is important to note that the RADIAN system was loaned to CSIRO for a small portion of this study and therefore the number of samples that contributed to each model was lower than the corresponding REIMS-based model. Similarly, there was limited opportunity to refine the RADIAN workflow to optimise the outputs.

The transition from using REIMS to assess beef samples for provenance and quality attributes to food safety applications proved challenging. Spectra generated using the manufacturer's recommendations (i.e. bipolar forceps) were inconsistent and of poor quality. The use of the prototype DRIA and cell line electrode facilitated the generation of spectra from which the models presented in this report were developed. However, sampling was conducted on a relatively high concentration of bacterial cells to achieve sufficient signal strength. Ultimately, this would limit the application of REIMS to food safety applications, particularly as food safety testing responds to a need to analyse lower concentrations of bacterial cells in greater detail. Conversely, the RADIAN system is likely to have strong applicability for food safety applications in red meat supply chains due to its simple non-destructive sampling approach and ability to detect lower concentrations of bacterial cells. When combined with a relatively straight forward analysis workflow that could be navigated by personnel with limited training, RADIAN is a suitable candidate for use in red meat processing environments. Furthermore, it is possible to identify opportunities where the RADIAN system could be used to simultaneously assess beef products in-line to confirm an attribute (e.g. Wagyu) and identify any food safety implications (e.g. the presence of STEC) using a single sample.

Ambient mass spectrometry systems continue to demonstrate promise for provenance, attribute and food safety applications in the red meat industry. In a proof of concept setting, both the REIMS and RADIAN systems were able to demonstrate their utility for classification of microorganisms of high relevance to the red meat industry. However, when practicality is considered, it is not possible to suggest that the REIMS system could create practice change within the red meat industry that would provide opportunity to proactively identify risks and to deploy interventions that ensure the safety of final products. On the other hand, the RADIAN system demonstrated substantial promise for food safety applications in red meat processing environments and therefore a more thorough assessment of the RADIAN system is encouraged.

8.2. Introduction

In the Australian red meat industry, processors wishing to export meat and meat products conduct microbiology testing on products to determine hygienic performance and to detect pathogenic organisms. To verify hygiene performance, abattoirs participate in the national carcase microbiological monitoring program by testing for spoilage indicators including generic *E. coli* [17]. Additionally, processors that produce manufacturing beef for select markets such as the USA and Canada must test for pathogenic *E. coli* including serotypes O111, O157 and O26. Most recently, the US Department of Agriculture's (USDA) Food Safety Inspection Service (FSIS) has released a notice of its intention to include *Salmonella* serovars as adulterants of specific poultry products [18]. These testing regimes come at a significant cost to industry with pathogenic *E. coli* testing requiring a test and hold of meat products and a negative testing outcome resulting in product not being able to enter commerce. Current methods of microbial testing are not only labour intensive but can take up to a week to complete therefore incurring significant costs to industry.

Incorporation of novel technologies to detect indicator and pathogenic organisms in real-time could allow for rapid and sensitive testing to occur on the processing floor. Insights to the potential for contamination to be occurring would provide opportunity for risk mitigation interventions to be deployed to ensure all products can enter commerce as anticipated. Rapid evaporative ionisation mass spectrometry (REIMS) and the RADIAN Direct mass detector system are recently developed ambient ionisation mass spectrometry technologies which can generate a unique mass spectral "fingerprint" of bacterial cells in a matter of seconds. Bacterial cell walls are lipid rich with the configurations of these lipids shown to be species dependent. REIMS captures a wealth of spectra relating to the presence of lipids and metabolites with several studies demonstrating the capacity of REIMS to conduct bacterial sub-species classification, sense virulence molecules, and identify antimicrobial resistance [19-23]. The RADIAN system is newly introduced has advantages relating to its small footprint, lower initial costs and the ability to conduct non-destructive analysis.

Novel ambient mass spectrometry approaches have not been trialled for food safety applications in Australia and therefore the aim of this study was to utilise supervised and unsupervised data analysis methods to determine the applicability of REIMS and RADIAN as tools for detecting and differentiating bacterial pathogens and spoilage organisms in real-time.

8.2 Materials and Methods

8.2.1 REIMS sample preparation

A total of 180 *Salmonella*, *E. coli*, and *Enterococcus* isolates were selected from the CSIRO culture collection and grown over night at 37°C on tryptone soya agar (TSA; Oxoid). The isolates were all previously recovered from red meat industry samples and had been confirmed to species, serovar or serotype using MALDI BioTyper or sequence data. The 60 *Salmonella* consisted of four serovars, made up of 15 *S.* Anatum, 15 *S.* Bovismorbificans, 15 *S.* Typhimurium and 15 *S.* Saintpaul. The 60 *E. coli* consisted of four serogroups, made up of 15 generic *E. coli*, 15 *E. coli* O111, 15 *E. coli* O157 and 15 *E. coli* O26. The 60 *Enterococcus* consisted of four subspecies, made up of 15 *E. faecalis*, 15 *E faecium*, 15 *E. hirae* and 15 *E. mundtii*.

Test samples were prepared by placing a single colony of each isolate into 50ml of buffered peptone water (BPW; Oxoid) and growing overnight at 37°C. Broths were allowed to reach stationary phase (~8.70 $\log_{10}$CFU/mL) and were then centrifuged at 10,000 rpm for 10 mins at room temperature. The supernatant was poured off, and the pellet allowed to dry briefly by inverting on a paper towel. The resulting pellets were subsequently analysed by REIMS.

8.2.2 REIMS analysis

REIMS analysis was conducted using a direct REIMS inlet assembly (DRIA) (Waters, Budapest) combined with a Xevo G2 ToF mass spectrometer (Waters, UK). The DRIA was powered by an Erbe VIO 50C generator (Erbe Medical, UK) set at 25 W power in dry-cutting mode. Each sample was burnt with a cell line electrode for a period of 3-5 s per cut. Each sample was done in duplicate and a total of 3 technical replicates were performed for each

sample with a delay of at least 5 s between each burn. Spectral 'fingerprints' were acquired between the mass range *m/z* 100-1200 in negative ionisation mode using a scan rate of 0.5 s per scan. Leucine enkephalin (Waters, UK) was used as a lockmass by dissolving it in MS-grade isopropanol (Fisher Scientific, USA) at a concentration of 0.1 ng/µl and infusing it into the mass spectrometer at a rate of 150 µl/min. Following the completion of each sample the cell line electrode was dipped in ethanol, dried, and any carbonised material was scraped off.

8.2.3 REIMS data analysis

REIMS data was processed and analysed using the Abstract Model Builder (AMX) [Beta] version 1.0.2159.0 (Waters Research Centre, Hungary). For each sample, mass spectra were loaded, and individual burns identified. Pre-processing was used to remove the background signal, correct burn ends, apply a lockmass correction (leu enk 554.2615) and normalise. To avoid 'over-fitting' models, the LDA dimensions were set to maximum which equals the number of classification groups minus one. The intensity limit for all models was set at 10 000 and binning of data was done at a scale of *m/z* 0.1.

REIMS spectral profiles typically exhibit a concentration of signal in the *m/z* 100-500 and 600-900 range which relate to the detection of fatty acids and glycerophospholipids, respectively. To understand the relative contribution of these spectral ranges to the overall classification models, LDA analysis was conducted for all samples using the spectral ranges *m/z* 100-500, 600-900 and 100-1200. Cross-validation of all models was performed using the 20% out approach with outlier calls based on a standard deviation multiplier of 5.

8.2.4 RADIAN sample preparation

A subset of 89 isolates comprising 29 *Salmonella*, 30 *E. coli*, and 30 *Enterococcus* were selected from the group of isolates used for the REIMS analysis. The 29 *Salmonella* consisted of three serovars, made up of 10 *S.* Anatum, 10 *S.* Bovismorbificans and nine *S.* Typhimurium. The 30 *E. coli* consisted of three serogroups, made up of six *E. coli* O111, seven *E. coli* O157, seven *E. coli* O26, and 10 generic E. coli. The 30 *Enterococcus* isolates consisted of three subspecies, made up of 10 *E. faecalis*, 10 *E. faecium* and 10 *E. hirae*. Isolates were sub-cultured on TSA and grown overnight at 37°C. Test samples were prepared by removing a colony of each isolate from the overnight plates and suspending it in 1ml of milliQ water. Samples were prepared in duplicate and were vortexed for 10 s immediately prior to RADIAN analysis.

8.2.5 RADIAN analysis

Radian analysis was conducted with the RADIAN ASAP Direct mass detector system (Waters, US). A new glass rod was used for each sample. Prior to sampling, the RADIAN was used to clean ("bake out") the glass rod. Before each analysis the tube was briefly vortexed. The glass rod was then dipped and swirled into the suspension for 15 seconds. The glass rod was immediately placed in the RADIAN ASAP sample loader and inserted into the machine to begin ionisation. For each sample six burns were acquired, three from each duplicate, using the same sample glass rod.

8.2.6 RADIAN data analysis

Radian data was processed and analysed using LiveID 2.0 software (Waters). Pre-processing of the Radian raw data was carried out by normalising the data according to the total ion current (TIC) and defining the peak detection threshold by manually adjusting the scale to remove the background spectra for each sample. PCA and LDA models were built in Live ID by using the spectral range m/z 100-1200.

8.2.7 Bacterial mixtures

Primary cultures of *E. coli* O157, *Salmonella* Typhimurium and *Enterococcus faecium* were individually prepared by inoculating four representative colonies of each into 1.5L of BPW. The three cultures were incubated at 37°C and allowed to reach stationary phase. A 3 ml aliquot of culture was removed from each bottle and serial diluted to 1:10, 1:100 and 1:1000 in sterile 0.85% saline for co-culturing (spiking) the primary inoculum at different ratios.

Binary cultures were made for the following six combinations of bacteria

- *Salmonella* and *E. coli*
- *Salmonella* and *Enterococcus*
- *E. coli* and *Enterococcus*
- *E. coli* and *Salmonella*
- *Enterococcus* and *E. coli*
- *Enterococcus* and *Salmonella*

The following ratios of each combination were prepared

1:1 (25ml :25ml); 1:100 (49.5ml + 500µl of 1/10 of co-culture); 1:1000 (49.5ml + 500 µl of 1/100 of co-culture); and 1:10000 (49.5ml + 500µl of 1/1000 of co-culture). REIMS analysis was conducted as described in 4.2 REIMS analysis. The four proof of concept models constructed in AMX model builder were used for live recognition of microbial classifications using the AMX recognition software.

8.3 Results and Discussion

8.3.1 REIMS model development

A total of 180 bacterial samples comprising 60 *Salmonella*, 60 *E. coli* and 60 *Enterococcus* were subjected to REIMS analysis and then subsets were used to develop the following classification models:

- Bacterial Genus – *Enterococcus* v *E. coli* v *Salmonella*
- *Salmonella* serovars – *S.* Anatum v *S.* Bovismorbificans v *S.* Saintpaul v S. Typhimurium
- *E. coli* serogroups – Generic *E. coli* v *E. coli* O111 v *E. coli* O157 v *E. coli* O26

Enterococcus species – *E. faecium* v *E. faecalis* v E. hirae V E. mundtii

8.3.2 *E. coli* v *Enterococcus* v *Salmonella*

REIMS was evaluated for its ability to correctly classify 60 *E. coli*, 60 *Enterococcus* and 60 *Salmonella*. LDA plots for the spectral ranges *m/z* 100-1200, 100-500, and 600-900 were constructed with each spectral range able to separate the three groups of organisms. Cross-validation resulted in correct classification rates exceeding 99% for all of the models with *m/z* 600-900 producing the highest correct classification rate of 99.91%. The *m/z* 600-900 model is shown in Figure 27 with the cross-validation results for all three models shown in Table 23.

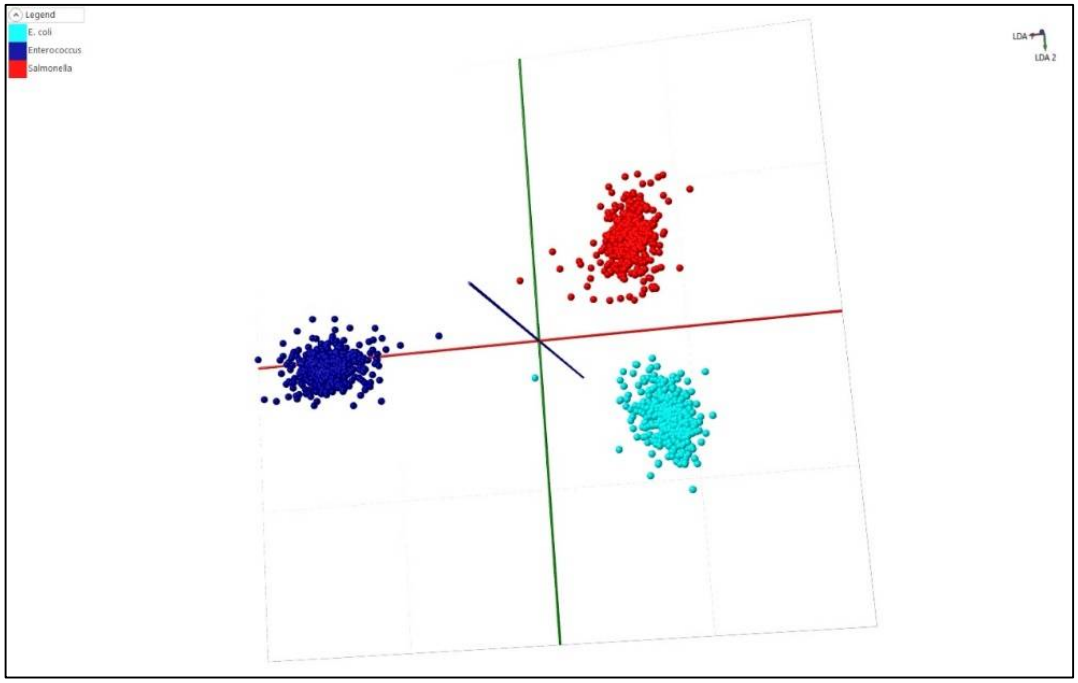

Figure 27. LDA plot of the *m/z* 600-900 range of *E. coli* (light blue), *Enterococcus* (purple) and *Salmonella* (red) samples following REIMS analysis.

Table 23. Cross-validation scores for LDA classification models for *E. coli, Enterococcus* and *Salmonella* samples for the spectral ranges *m/z* 100-1200, 100-500, and 600-900.

Spectral range (m/z)	***100-1200***	***100-500***	***600-900***
Model type	LDA	LDA	LDA
Number of spectra	1080	1080	1080
Number of passes	1072	1075	1079
Number of failures	3	3	0
Number of outliers	5	2	1
Correct classification - excluding outliers	99.72%	99.72%	100.00%
Correct classification - including outliers	99.26%	99.54%	99.91%

Further assessment of the LDA plots for all spectral ranges confirmed the pronounced separation of *Enterococcus* spectra from those of *E. coli* and *Salmonella*. *Enterococcus* are Gram-positive organisms whereas *E. coli* and *Salmonella* are Gram-negative organisms. Unlike, Gram-negative organisms, Gram-positive organisms do not have an outer lipid membrane, with this physiological difference likely to be the basis of the observed differences. The loading plot for the first dimension of the *m/z* 600-900 model is shown in Figure 28. Molecules with positive scores are highly associated with Gram-positive organisms (*Enterococcus*) with negative scores associated with Gram-negative organisms (*E. coli* and *Salmonella*).

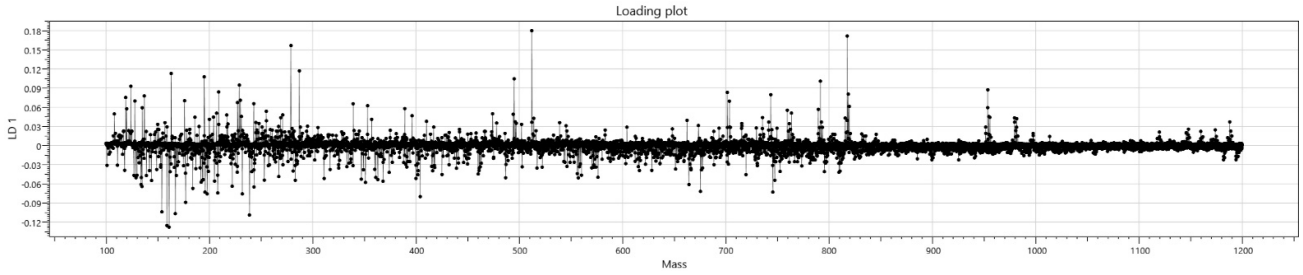

Fig. 28. Loading plot for the first dimension (LD1) of the *m/z* 600-900 model for *E. coli, Enterococcus* and *Salmonella*

8.3.3 *S.* Anatum v *S.* Bovismorbificans v *S.* Saintpaul v *S.* Typhimurium

REIMS was evaluated for its ability to correctly classify 15 *S.* Anatum, 15 *S.* Bovismorbificans*, 15 S.* Saintpaul and 15 *S.* Typhimurium isolates. A LDA plot of the spectra generated across the *m/z* 100-1200 range (Fig. 29) demonstrates separation of the four serotype classes and resulted in the highest correct classification rate of 93.33% (Table 24) with outliers included. LDA plots of the spectra acrossthe *m/z* 100-500 and *m/z* 600-900 range also demonstrated reasonable separation and resulted in a correct classification rate of 91.67% and 81.67% respectively (Table 24).

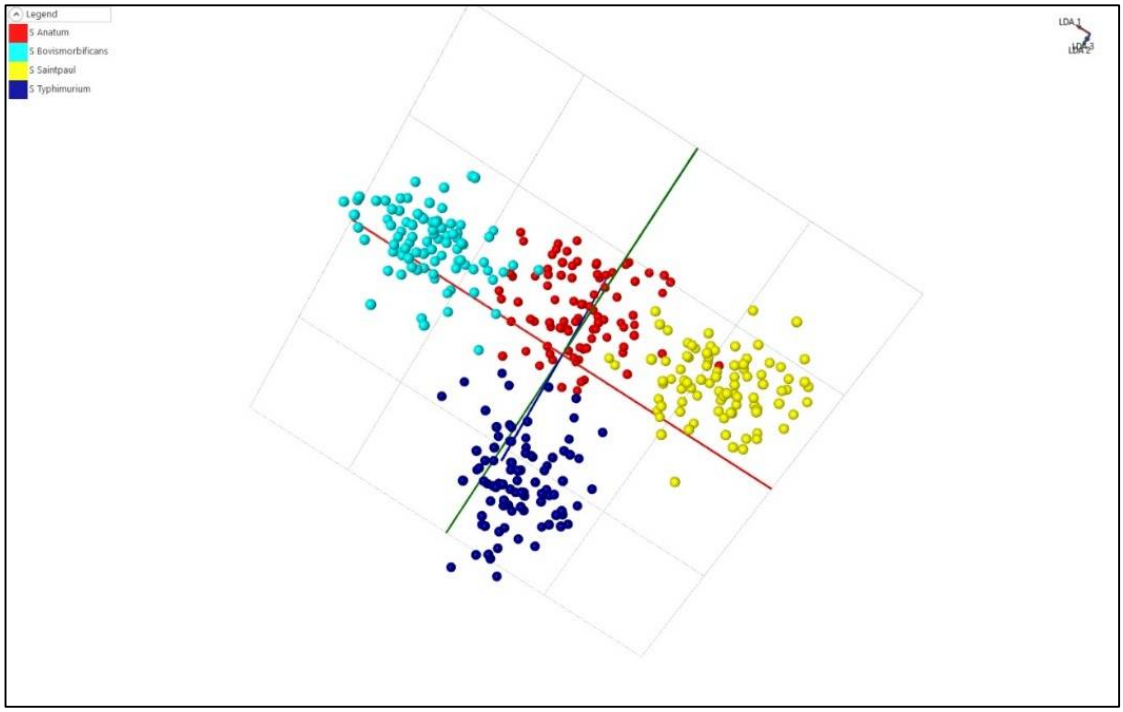

Figure 29. LDA plot of the *m/z* 100-1200 range of *S.* Anatum (red), *S.* Bovismorbificans (light blue), *S.* Saintpaul (yellow) and *S.* Typhimurium (purple) samples following REIMS analysis.

Table 24. Cross-validation scores for LDA classification models for *Salmonella* serotyping for the spectral ranges *m/z* 100-1200, 100-500, and 600-900.

Spectral range (m/z)	***100-1200***	***100-500***	***600-900***
Model type	LDA	LDA	LDA
Number of spectra	360	360	360
Number of passes	336	330	294
Number of failures	24	30	66
Number of outliers	0	0	0
Correct classification - excluding outliers	93.33%	91.67%	81.67%
Correct classification - including outliers	93.33%	91.67%	81.67%

8.3.4 *E. coli* serogroups – Generic *E. coli* v O111 v O157 v O26

Shiga toxin-producing *E. coli* (STEC) are of regulatory importance to the Australian red meat industry. Presently there are seven serogroups of STEC listed as adulterants of manufacturing beef entering North America. STEC of serogroups O157, O26 and O111 typically comprise >95% of STEC isolated from manufacturing beef samples in Australia. REIMS was evaluated for its ability to correctly classify 15 Generic *E. coli*, 15 *E. coli* O111, 15 *E. coli* O157 and 15 *E. coli* O26 isolates. The models for each of the spectral ranges performed well with correct classification

rates exceeding 99% (Table 25). A LDA plot of the spectra generated for the *m/z* 100-1200 range is shown in Figure 30.

Table 25. Cross-validation scores for LDA classification models for *E. coli* serogroups for the spectral ranges *m/z* 100-1200, 100-500, and 600-900.

Spectral range (m/z)	***100-1200***	***100-500***	***600-900***
Model type	LDA	LDA	LDA
Number of spectra	360	360	360
Number of passes	358	358	357
Number of failures	1	2	3
Number of outliers	1	0	0
Correct classification - excluding outliers	99.72%	99.44%	99.17%
Correct classification - including outliers	99.44%	99.44%	99.17%

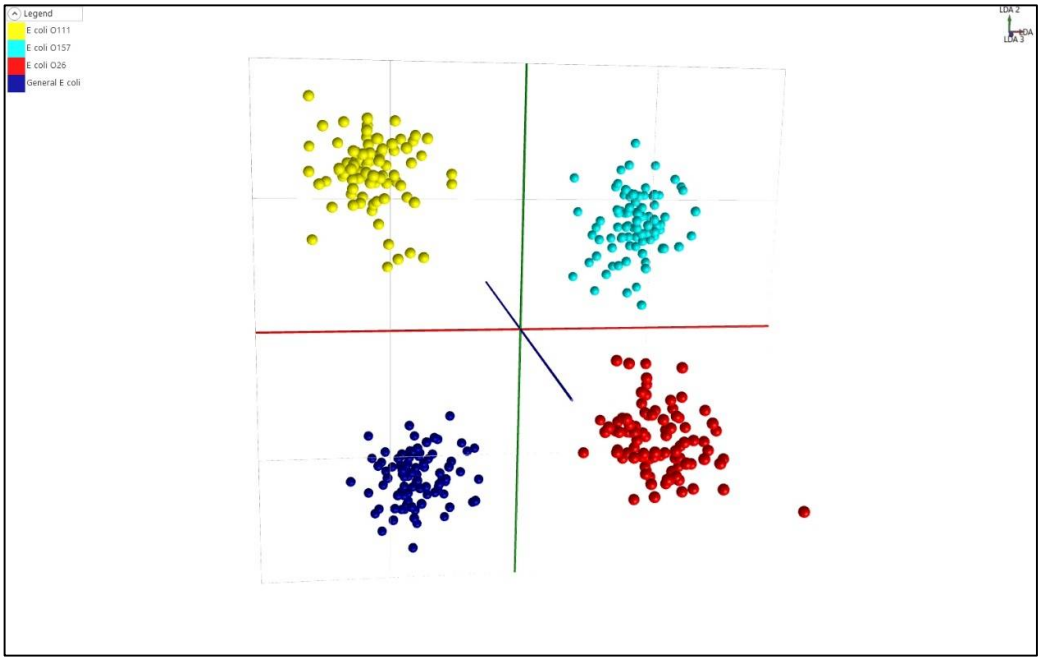

Figure 30. LDA plot of the *m/z* 100-1200 range of E. coli O111 (yellow), *E. coli* O157 (light blue), *E. coli* O26 (red) and Generic *E. coli* (purple) samples following REIMS analysis.

8.3.5 E. faecium v *E. faecalis* v *E. hirae* v *E. mundtii*

REIMS was evaluated for its ability to correctly classify 15 *E. faecium*, 15 *E. faecalis, 15 E. hirae* and 15 *E. mundtii*. Correct classification rates for all spectral ranges exceeded 97% with the *m/z* 600-900 generating the highest correct classification rate of 98.89% (Table 26). The LDA plot of the spectra from the *m/z* 600-900 range is shown in Figure 31.

Table 26. Cross-validation scores for LDA classification models for *Enterococcus* Subgroups for the spectral ranges *m/z* 100-1200, 100-500, and 600-900.

Spectral range (m/z)	***100-1200***	***100-500***	***600-900***
Model type	LDA	LDA	LDA
Number of spectra	360	360	360
Number of passes	352	352	356
Number of failures	2	2	4
Number of outliers	6	6	0
Correct classification - excluding outliers	99.44%	99.44%	98.89%
Correct classification - including outliers	97.98%	97.98%	98.89%

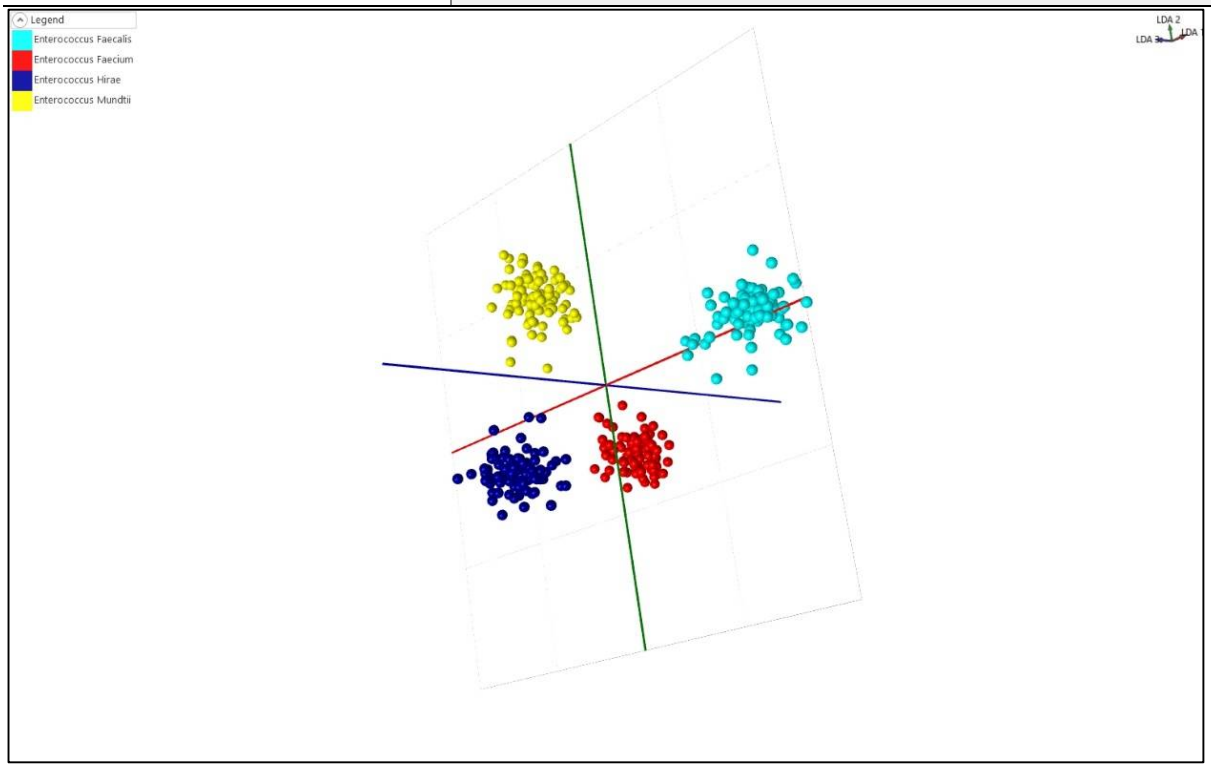

Figure 31. LDA plot of the *m/z* 600-900 range of *E. faecalis* (light blue), *E. faecium* (red), *E. hirae* (purple) and *E.mundtii* (yellow) samples following REIMS analysis.

8.3.6 RADIAN model development

A total of 89 bacterial samples comprising 29 *Salmonella*, 30 *E. coli* and 30 *Enterococcus* were subjected to Radian analysis and then subsets were used to develop the following classification models:

- Bacterial Speciation –*E. coli* v *Enterococcus* v *Salmonella*
- *Salmonella* Serovars – *S.* Anatum v *S.* Bovismorbificans v S. Typhimurium

- *E. coli* Serogroups – *E. coli* O111 v *E. coli* O157 v *E. coli* O26
- *Enterococcus* Subgroups – *E. faecium* v *E. faecalis* v *E. hirae*

8.3.7 Bacterial genus – *E. coli* v *Enterococcus* v *Salmonella*

RADIAN was evaluated for its ability to correctly classify 30 *E. coli*, 30 *Enterococcus* and 29 *Salmonella*. A LDA plot of the spectra generated across *m/z* 100-1200 range is shown in Figure 32. There is good separation of the three bacterial classes with cross validation resulting a correct classification rate of 96.44% (Table 27).

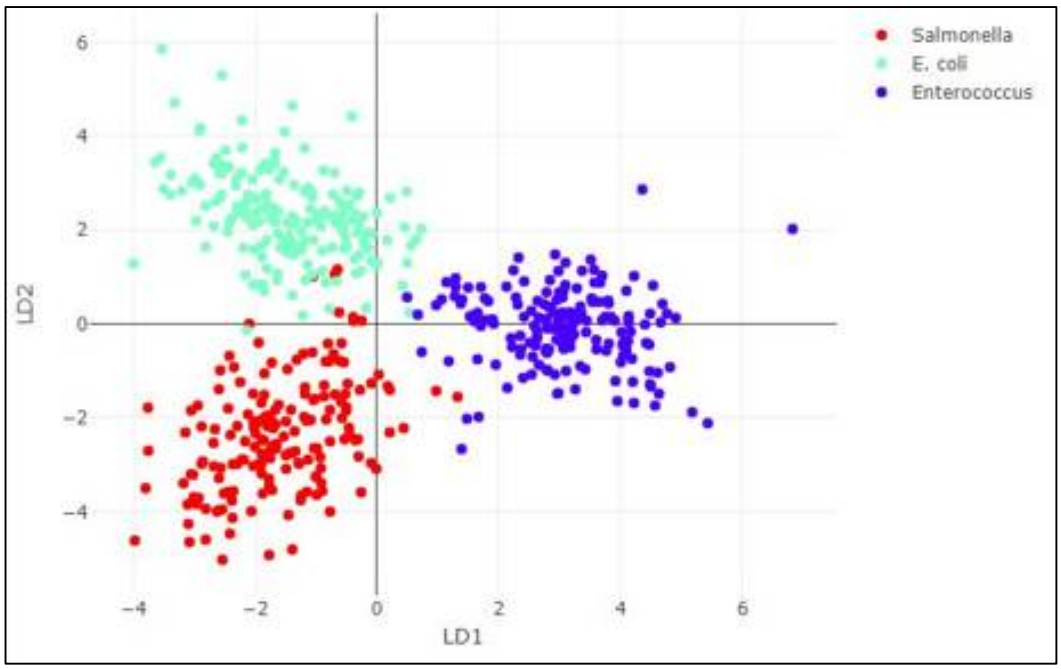

Figure 32. LDA plot of the *m/z* 100-1200 range of *E. coli* (light blue), *Enterococcus* (purple) and *Salmonella* (red) samples following RADIAN analysis.

Table 27. Cross-validation scores for LDA classification models for bacterial speciation – *E. coli* v *Salmonella* v *Enterococcus* for the spectral ranges *m/z* 100-1200.

Spectral range (m/z)	***100-1200***
Model type	LDA
Number of spectra	534
Number of passes	515
Number of failures	19
Number of outliers	0
Correct classification rate	96.44%

8.3.8 *Salmonella* serovars – *S.* Anatum v *S.* Bovismorbificans v *S.* Typhimurium

RADIAN was evaluated for its ability to correctly classify 10 *S.* Anatum,10 *S.* Bovismorbificans and nine *S.* Typhimurium. The LDA plot of the spectra across the *m/z* 100-1200 range (Fig. 33) shows good separation of the three *Salmonella* serovars. However, cross-validation performance did not reflect this observation with a correct classification rate of 81.25% achieved (Table 28). Whilst it is likely that model performance would improve with addition of more isolates, it was noted that cross-validation performance was lowest for *Salmonella* using either the REIMS or RADIAN systems. The reasons for the lower performance of the *Salmonella* models are not known.

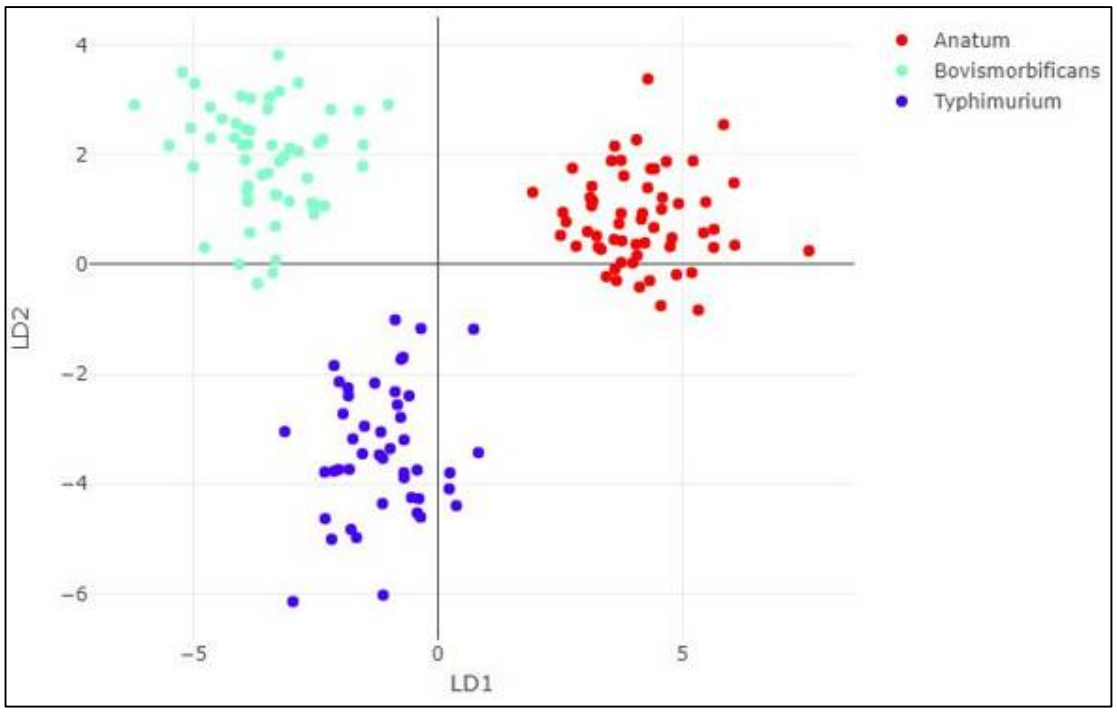

Figure 33. LDA plot of the *m/z* 100-1200 range of *S.* Anatum (red) *S.* Bovismorbificans (light blue), and *S.* Typhimurium (purple) samples following RADIAN analysis.

Table 28. Cross-validation scores for LDA classification models for *Salmonella* serovars – *S,* Anatum*, S.* Bovismorbificans and *S.* Typhimurium *f*or the spectral ranges *m/z* 100-1200.

Spectral range (m/z)	***100-1200***
Model type	LDA
Number of spectra	160
Number of passes	130
Number of failures	26
Number of outliers	4

Correct classification rate	81.25%

8.3.9 *E. coli* serogroups – O111 v O157 v O26

Radian was evaluated for its ability to correctly classify six *E. coli* O111, seven *E. coli* O157 and seven *E. coli* O26. A LDA plot of the spectra generated in the *m/z* 100-1200 range (Fig. 34) demonstrates good separation of the three *E. coli* serogroups and resulted in a correct classification rate of 85.37% (Table 29).

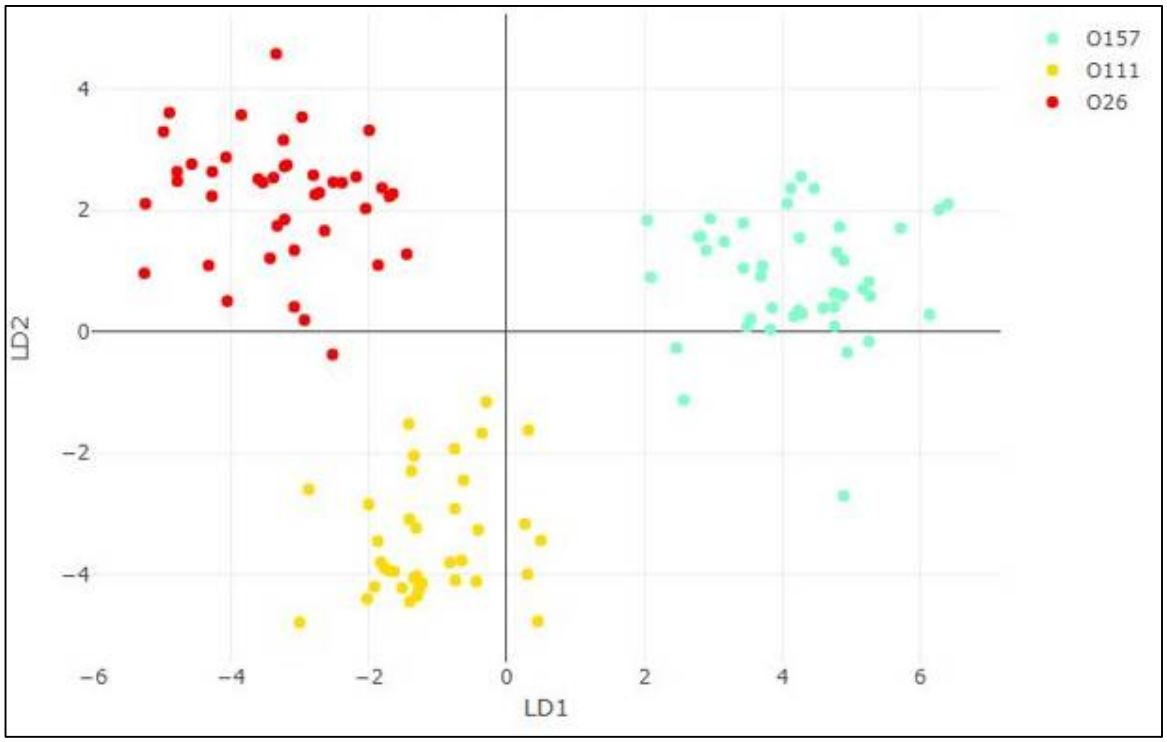

Figure 34. LDA plot of the *m/z* 100-1200 range of *E. coli* O111 (yellow), *E. coli* O157 (light blue) and *E. coli* O26 (red) samples following radian analysis.

Table 29. Cross-validation scores for PCA- LDA classification models for *E. coli* serogroups – *E. coli* O111, *E. coli* O157 and *E. coli* O26 for the spectral ranges *m/z* 100-1200.

Spectral range (m/z)	***100-1200***
Model type	LDA
Number of spectra	123
Number of passes	105
Number of failures	18
Number of outliers	0
Correct classification rate	85.37%

8.3.10 *Enterococcus* species – *E. faecium* v *E. faecalis* v *E. hirae*

Radian was evaluated for its ability to correctly classify 10 E. faecalis, 10 E. faecium and 10 E. hirae. A PCA-LDA plot of the ions identified in the *m/z* 100-1200 range (Fig. 35) demonstrates reasonable separation of the three *Enterococcus* species and resulted in a correct classification rate of 87.50% (Table 30).

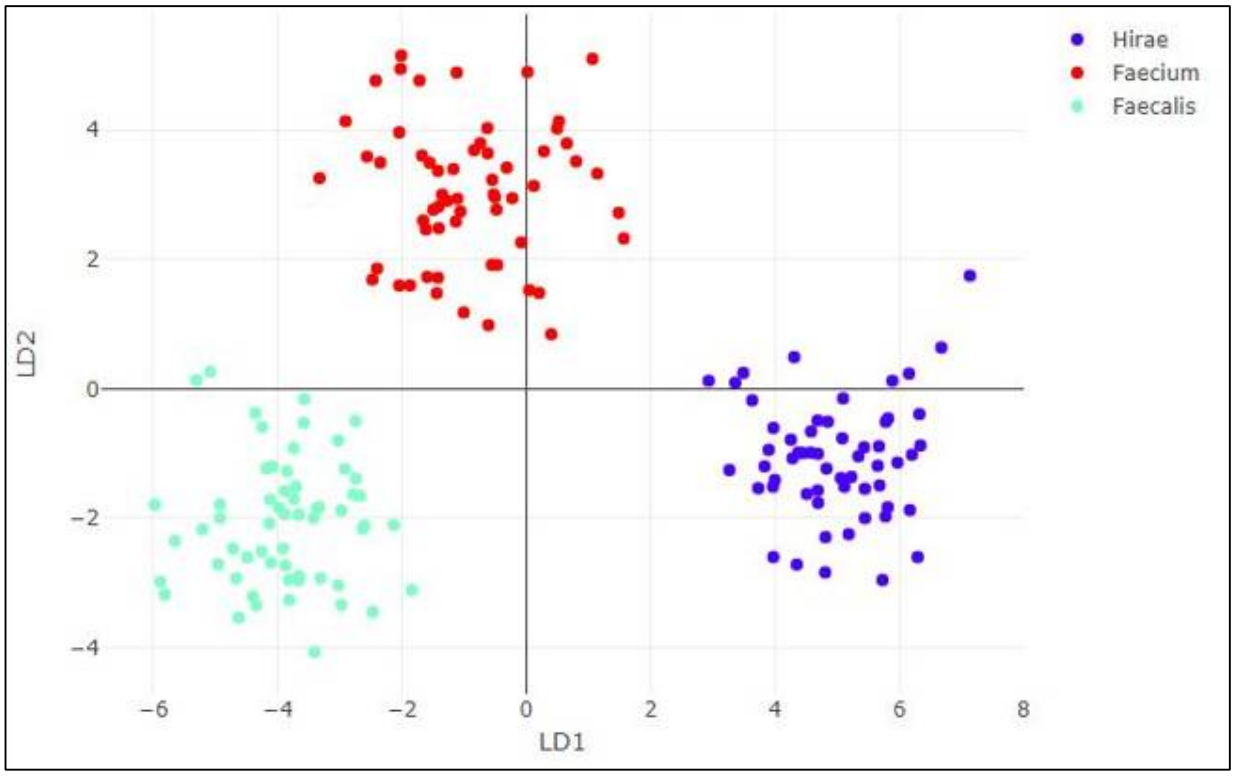

Figure 35. PCA- LDA plot of the *m/z* 100-1200 range of *E. faecalis* (light blue), *E. faecium* (red) and *E. hirae* (purple) samples following radian analysis.

Table 30. Cross-validation scores for PCA- LDA classification models for *Enterococcus* species – *E. faecalis, E. faecium* and *E. hirae* spectral ranges *m/z* 100-1200.

Spectral range (m/z)	***100-1200***
Model type	LDA
Number of spectra	176
Number of passes	154
Number of failures	19
Number of outliers	3
Correct classification rate	87.50%

8.3.11 Bacterial mixtures

Attempts were made to detect serogroups of *E. coli*, serovars of *Salmonella*, and *Enterococcus* species at reducing concentrations in a background of 10^8 CFU/mL of bacteria using the AMX recognition software. Whilst it was possible to see evidence of the target organism when equal amounts of the target and background were mixed (data not shown), there was an inability to accurately identify spectra relating to the target organism when concentrations of the target serogroup/serovar/species dropped below 10% of the overall concentration.

Despite not being able to accurately identify specific serogroups, serovars, or species of the target organisms when reduced concentrations were assessed, there was an ability to detect organisms at the genus level at reduced concentrations. For example, *Enterococcus* could be identified in backgrounds of *E. coli* or *Salmonella* at concentrations down to 1:10,000 CFU/mL. This represents an equivalent limit of detection to typical polymerase chain reaction (PCR) approaches to microbiological testing and may be applicable as a proactive food safety tool. It is also plausible to suggest that the continued development of models for serogroups, serovars or species via the addition of more samples could enhance the specificity such that the ability to provide specific insights to the type of target organisms present is achieved.

8.4 Conclusions

Previous assessments of the REIMS system for its ability to correctly classify provenance and detect red meat quality attributes have been successful and have demonstrated the capacity of REIMS to be applied for rapid food credential verification. The purpose of this study was to evaluate the REIMS system for its utility in food safety applications relevant to red meat supply chains. Australian red meat processors verify hygiene performance and meet regulatory requirements for bacterial pathogen presence via participation in the national carcase microbiological monitoring program. Microbiological testing is complex, laborious, and costly, often occurring at the end of production and processing where there are limited opportunities to deploy interventions which may offset food safety risks. In addition to assessing the opportunity for REIMS to create change in food safety systems, there was also opportunity during this study to assess a complementary ambient mass spectrometry system called RADIAN.

The REIMS and RADIAN systems were both able to develop classification models for bacterial genus, pathogenic *E. coli* serogroups, *Salmonella* serovars, and *Enterococcus* speciation. Models generated using spectra profiles from REIMS analysis resulted in correct classification rates for all models exceeding 93.33%. By comparison, models developed using spectra from the RADIAN system achieved correct classification rates exceeding 81.25%. It is important to note that the RADIAN system was loaned to CSIRO for a small portion of this study and therefore the number of samples that contributed to each model was lower than the corresponding REIMS-based model. Similarly, there was limited opportunity to refine the RADIAN workflow to optimise the outputs.

The transition from using REIMS to assess beef samples for provenance and quality attributes to food safety applications proved challenging. Sampling of beef products occurs via the use of a monopolar electrosurgical knife. In contrast, Waters recommend the use of bipolar forceps for sampling bacterial colonies or pellets. Sampling of cultures using the bipolar forceps was largely ineffective and further discussion with Waters resulted in the trialling of a Direct REIMS Inlet Assembly (DRIA) in combination with a cell line electrode. The DRIA assists in overcoming the

issue of bacterial samples producing much less vapour for analysis than beef samples typically would. Similarly, the cell line electrode fixes the distance between the ends of the sampler and enables a consistent voltage to be passed through the sample. The combination of the DRIA and cell line electrode facilitated the generation of spectra from which the models presented in this report were developed. However, sampling was conducted on a relatively high concentration of bacterial cells to achieve sufficient signal strength. Ultimately, this would limit the application of REIMS to food safety applications, particularly as food safety testing responds to a need to analyse lower concentrations of bacterial cells in greater detail.

Conversely, the RADIAN system is likely to have strong applicability for food safety applications in red meat supply chains. In contrast to the REIMS system, sampling for the RADIAN is non-destructive, requires lower numbers of organisms and can be achieved by simply swabbing a surface, product or bacterial colony and subsequently suspending it in water. A glass rod is then dipped into the water sample and then placed into the RADIAN system for analysis. The simple sampling approach combined with a relatively straight forward analysis workflow that could be navigated by personnel with limited training, makes RADIAN a suitable candidate for use in red meat processing environments. Furthermore, it is possible to identify opportunities where the RADIAN system could be used to simultaneously assess beef products in-line to confirm an attribute (e.g. Wagyu) and identify any food safety implications (e.g. the presence of STEC) using a single sample.

Ambient mass spectrometry systems continue to demonstrate promise for provenance, attribute and food safety applications in the red meat industry. In a proof of concept setting, both the REIMS and RADIAN systems were able to demonstrate their utility for classification of microorganisms of high relevance to the red meat industry. However, when practicality is considered, it is not possible to suggest that the REIMS system could create practice change within the red meat industry that would provide opportunity to proactively identify risks and to deploy interventions that ensure the safety of final products. On the other hand, the RADIAN system demonstrated substantial promise for food safety applications in red meat processing environments. Further it is possible to extend the application of the RADIAN system to the verification of provenance and additional food credentials using a single sample sampling approach. A more thorough assessment of the RADIAN system is therefore encouraged.

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10.0 Appendices

10.1 Appendix 1

Table 31. Trial design for the 12 week storage trial.

			Storage Time @ -0.5 °C					
Breed	Marble score	Animal	Week 1	Week 2	Week 4	Week 6	Week 8	Week 12
Angus	4+	1	A - 1 - T1	A - 1 - T2	A - 1 - T4	A - 1 - T6	A - 1 - T8	A - 1 - T12
Angus	4+	2	A - 2 - T1	A - 2 - T2	A - 2 - T4	A - 2 - T6	A - 2 - T8	A - 2 - T12
Angus	4+	3	A - 3 - T1	A - 3 - T2	A - 3 - T4	A - 3 - T6	A - 3 - T8	A - 3 - T12
Angus	4+	4	A - 4 - T1	A - 4 - T2	A - 4 - T4	A - 4 - T6	A - 4 - T8	A - 4 - T12
Angus	4+	5	A - 5 - T1	A - 5 - T2	A - 5 - T4	A - 5 - T6	A - 5 - T8	A - 5 - T12
Angus	4+	6	A - 6 - T1	A - 6 - T2	A - 6 - T4	A - 6 - T6	A - 6 - T8	A - 6 - T12
Angus	3+	7	A - 7 - T1	A - 7 - T2	A - 7 - T4	A - 7 - T6	A - 7 - T8	A - 7 - T12
Angus	3+	8	A - 8 - T1	A - 8 - T2	A - 8 - T4	A - 8 - T6	A - 8 - T8	A - 8 - T12
Angus	3+	9	A - 9 - T1	A - 9 - T2	A - 9 - T4	A - 9 - T6	A - 9 - T8	A - 9 - T12
Angus	3+	10	A - 10 - T1	A - 10 - T2	A - 10 - T4	A - 10 - T6	A - 10 - T8	A - 10 - T12
Angus	3+	11	A - 11 - T1	A - 11 - T2	A - 11 - T4	A - 11 - T6	A - 11 - T8	A - 11 - T12
Angus	3+	12	A - 12 - T1	A - 12 - T2	A - 12 - T4	A - 12 - T6	A - 12 - T8	A - 12 - T12
Angus	2+	13	A - 13 - T1	A - 13 - T2	A - 13 - T4	A - 13 - T6	A - 13 - T8	A - 13 - T12
Angus	2+	14	A - 14 - T1	A - 14 - T2	A - 14 - T4	A - 14 - T6	A - 14 - T8	A - 14 - T12
Angus	2+	15	A - 15 - T1	A - 15 - T2	A - 15 - T4	A - 15 - T6	A - 15 - T8	A - 15 - T12
Angus	2+	16	A - 16 - T1	A - 16 - T2	A - 16 - T4	A - 16 - T6	A - 16 - T8	A - 16 - T12
Angus	2+	17	A - 17 - T1	A - 17 - T2	A - 17 - T4	A - 17 - T6	A - 17 - T8	A - 17 - T12
Angus	2+	18	A - 18 - T1	A - 18 - T2	A - 18 - T4	A - 18 - T6	A - 18 - T8	A - 18 - T12
Wagyu	7+	19	W - 19 - T1	W - 19 - T2	W - 19 - T4	W - 19 - T6	W - 19 - T8	W - 19 - T12
Wagyu	7+	20	W - 20 - T1	W - 20 - T2	W - 20 - T4	W - 20 - T6	W - 20 - T8	W - 20 - T12
Wagyu	7+	21	W - 21 - T1	W - 21 - T2	W - 21 - T4	W - 21 - T6	W - 21 - T8	W - 21 - T12
Wagyu	7+	22	W - 22 - T1	W - 22 - T2	W - 22 - T4	W - 22 - T6	W - 22 - T8	W - 22 - T12
Wagyu	7+	23	W - 23 - T1	W - 23 - T2	W - 23 - T4	W - 23 - T6	W - 23 - T8	W - 23 - T12
Wagyu	7+	24	W - 24 - T1	W - 24 - T2	W - 24 - T4	W - 24 - T6	W - 24 - T8	W - 24 - T12
Wagyu	5+	25	W - 25 - T1	W - 25 - T2	W - 25 - T4	W - 25 - T6	W - 25 - T8	W - 25 - T12
Wagyu	5+	26	W - 26 - T1	W - 26 - T2	W - 26 - T4	W - 26 - T6	W - 26 - T8	W - 26 - T12
Wagyu	5+	27	W - 27 - T1	W - 27 - T2	W - 27 - T4	W - 27 - T6	W - 27 - T8	W - 27 - T12
Wagyu	5+	28	W - 28 - T1	W - 28 - T2	W - 28 - T4	W - 28 - T6	W - 28 - T8	W - 28 - T12
Wagyu	5+	29	W - 29 - T1	W - 29 - T2	W - 29 - T4	W - 29 - T6	W - 29 - T8	W - 29 - T12
Wagyu	5+	30	W - 30 - T1	W - 30 - T2	W - 30 - T4	W - 30 - T6	W - 30 - T8	W - 30 - T12
Wagyu	3+	31	W - 31 - T1	W - 31 - T2	W - 31 - T4	W - 31 - T6	W - 31 - T8	W - 31 - T12
Wagyu	3+	32	W - 32 - T1	W - 32 - T2	W - 32 - T4	W - 32 - T6	W - 32 - T8	W - 32 - T12
Wagyu	3+	33	W - 33 - T1	W - 33 - T2	W - 33 - T4	W - 33 - T6	W - 33 - T8	W - 33 - T12
Wagyu	3+	34	W - 34 - T1	W - 34 - T2	W - 34 - T4	W - 34 - T6	W - 34 - T8	W - 34 - T12
Wagyu	3+	35	W - 35 - T1	W - 35 - T2	W - 35 - T4	W - 35 - T6	W - 35 - T8	W - 35 - T12
Wagyu	3+	36	W - 36 - T1	W - 36 - T2	W - 36 - T4	W - 36 - T6	W - 36 - T8	W - 36 - T12

10.2 Appendix 2

Table 32. Pearson correlations between marbling score, and meat quality and fatty acid traits – Angus data

	ACROSS STORAGE WEEKS	WEEK1	WEEK2	WEEK4	WEEK6	WEEK8	WEEK12
TRAIT	Marbling Score	Marbling Score	Marbling Score	Marbling Score	Marbling Score	Marbling Score	Marbling Score
C12:0	0.424	-0.123	0.641	0.397	0.585	0.659	0.426
C14:0	0.531	0.35	0.645	0.463	0.628	0.739	0.442
C14:1	0.325	0.196	0.528	0.221	0.364	0.456	0.244
C15:0	0.456	0.297	0.653	0.294	0.558	0.671	0.34
C16:0	0.569	0.465	0.682	0.444	0.681	0.831	0.435
C16:1	0.528	0.417	0.658	0.452	0.577	0.687	0.419
C17:0	0.459	0.377	0.676	0.243	0.584	0.753	0.317
C17:1	0.448	0.387	0.659	0.218	0.537	0.604	0.353
C18:0	0.514	0.457	0.625	0.365	0.643	0.801	0.367
C18:1 CIS 9	0.552	0.508	0.686	0.369	0.659	0.804	0.421
C18:2	0.508	0.525	0.643	0.379	0.565	0.767	0.359
C20:0	0.515	0.463	0.62	0.358	0.631	0.781	0.415
C18:3	0.459	0.475	0.582	0.292	0.498	0.783	0.369
CIS 9, TRANS 11 C18:2	0.265	0.211	0.433	0.088	0.258	0.521	0.14
C20:4	-0.243	-0.351	0.007	0.018	-0.024	-0.359	-0.736
C20:5	-0.544	-0.635	-0.621	-0.302	-0.475	-0.669	-0.73
C22:6	-0.332	-0.411	-0.364	-0.296	0.03	-0.333	-0.637
SFA	0.552	0.457	0.665	0.416	0.677	0.829	0.416
MUFA	0.542	0.479	0.676	0.358	0.648	0.819	0.414
PFA	0.477	0.484	0.663	0.369	0.537	0.762	0.28
DRIP %	-0.326	0.365	0.236	-0.456	-0.451	-0.696	-0.731
PH	-0.063	-0.516	0.037	0.119	-0.292	0.189	0.169
L	-0.008	-0.058	-0.008	-0.158	0.018	0.257	-0.092
A	-0.036	0.212	0.003	0.827	-0.612	-0.616	-0.143
B	-0.04	0.222	0.067	0.842	-0.659	-0.621	-0.071
COOK LOSS %	-0.148	-0.173	-0.515	-0.288	-0.67	0.172	0.404
MOISTURE LOSS %	-0.293	-0.042	-0.458	-0.466	-0.701	-0.273	0.074
PEAK FORCE N	-0.023	0.064	-0.031	-0.214	0.542	0.123	-0.565
TBARS	-0.097	0.002	0.428	0.086	-0.885	0.009	-0.337
ESTIMATED IMF %	0.517	0.472	0.63	0.655	0.55	0.604	0.293

Table 33. Pearson correlations between marbling score, and meat quality and fatty acid traits – Wagyu data

	ACROSS STORAGE WEEKS	WEEK1	WEEK2	WEEK4	WEEK6	WEEK8	WEEK12
TRAIT	Marbling Score	Marbling Score	Marbling Score	Marbling Score	Marbling Score	Marbling Score	Marbling Score
C12:0	0.177	0.227	0.256	0.256	0.151	0.095	0.048
C14:0	0.082	0.114	0.138	0.179	0.018	-0.001	0.011
C14:1	-0.197	-0.227	-0.106	-0.059	-0.263	-0.286	-0.279
C15:0	0.057	0.11	0.119	0.155	0.008	-0.035	-0.062
C16:0	-0.008	-0.004	0.032	0.099	-0.07	-0.078	-0.082
C16:1	-0.051	-0.062	-0.01	0.094	-0.082	-0.147	-0.149
C17:0	0.053	0.107	0.086	0.12	0	0.005	-0.025
C17:1	0.027	0.033	0.039	0.132	0.03	-0.066	-0.04
C18:0	0.094	0.116	0.119	0.147	0.068	0.034	0.057
C18:1 CIS 9	-0.052	-0.062	-0.019	0.059	-0.116	-0.121	-0.123
C18:2	0.261	0.264	0.299	0.258	0.303	0.26	0.188
C20:0	0.131	0.12	0.17	0.19	0.143	0.09	0.063
C18:3	0.065	0.046	0.118	0.113	0.08	0.055	-0.02
CIS 9, TRANS 11 C18:2	-0.078	-0.133	-0.01	0.059	-0.17	-0.108	-0.176
C20:4	0.433	0.099	0.862	0.365	0.43	0.536	0.359
C20:5	0.162	-0.109	0.491	0.053	0.266	0.293	-0.093
C22:6	0.238	-	0.297	0.169	0.542	0.297	-
SFA	0.009	0.045	0.063	0.119	-0.032	-0.041	-0.177
MUFA	-0.038	-0.036	-0.005	0.071	-0.097	-0.11	-0.116
PFA	0.293	0.239	0.37	0.268	0.356	0.317	0.232
DRIP %	0.086	0.167	0.029	0.129	0.084	-0.016	0.186
PH	0.425	0.33	0.64	0.091	0.863	0.657	0.26
L	0.121	0.255	0.012	0.028	0.142	0.236	0.063
A	-0.191	-0.375	0.082	-0.304	-0.479	-0.454	0.065
B	-0.141	-0.267	0.15	-0.402	-0.441	-0.304	0.16
COOK LOSS %	-0.032	-0.36	-0.472	0	0.307	0.1	0.339
MOISTURE LOSS %	0.022	-0.323	-0.48	0.036	0.293	0.068	0.314
PEAK FORCE N	-0.33	-0.356	-0.269	-0.584	-0.351	-0.407	-0.397
TBARS	0.162	-0.804	0.386	0.913	-0.049	-0.552	0.55
ESTIMATED IMF %	-0.058	-0.064	-0.008	0.106	-0.179	-0.106	-0.155