Investigation of the Confinement Odour Problem in Exported Lamb using NMR-based Metabolomics

A thesis presented in partial fulfilment of the requirements for the degree of

Master of Science

in

Chemistry

at Massey University, Manawatū, New Zealand.

Natalia Olivecrona

2015

Dedicated to the Memory of Moira Lynette Fay Olivecrona

Abstract

Recent changes to the supply chain practices of meat exporters has increased the potential for consumers to be exposed to the phenomenon of confinement odour, the smell produced by vacuum or modified atmosphere packaged meat which has been chilled and stored for extended periods. This harmless odour, which does not indicate meat spoilage, can lead to the rejection of the product by consumers. This is a problem for NZ lamb meat producers as they form the largest group of exporters of lamb meat in the world, and their largest market is the UK and other EU countries.

The processes behind confinement odour development are poorly understood. In this thesis, NMR spectra were acquired of meat, and drip extracts of meat from two different processing plants stored under different temperatures for 11-13 weeks to simulate conditions of exported meat during overseas shipment, transport to warehouse and retail display. The spectra were analysed by multivariate data analysis to find metabolic differences between meat which produces confinement odour and meat which produces either spoilage odour or no odour. Optimisation of extraction of metabolites from meat and drip samples was also carried out.

The best sample preparation method for meat and drip included homogenisation by bead beating (meat samples only), protein precipitation using an acetonitrile, methanol and acetone solvent mixture, and removal of solvent by vacuum centrifugation.

Multivariate data analysis demonstrated the ability to discriminate drip samples with confinement odour from spoiled samples and the former showed increased lactate concentration with low levels of leucine indicating the presence of Lactic Acid bacteria. The spoiled samples had increased butyrate levels which is indicative of the presence of *Clostridium spp.* Both bacterial populations were in a late stage of growth. This is consistent with confinement odour as an early indicator of spoilage. This result indicates the potential for drip to be utilised more widely for the analysis of meat metabolites.

Additionally, samples could be discriminated by processing plant of origin using multivariate data analysis. Increased levels of pyruvate and decreased levels of glucose in samples from Plant 2 indicated their bacterial populations had progressed to a later stage of growth than the bacterial populations in samples from Plant 1.

Acknowledgements

First I would like to thank my supervisor Dr Patrick Edwards who encouraged me to pursue this project and introduced me to the field of metabolomics. His wealth of knowledge of NMR spectroscopy and valuable time was freely shared and hugely appreciated. I would also like to thank my supervisor Dr Linda Samuelsson, who provided endless encouragement, proof-reading of my thesis and expert knowledge of so many of the techniques and methods used in this research. Many thanks to Dr Mariza Reis and my supervisor Dr Marlon Reis for providing the framework and samples for this work, and their guidance throughout the entirety of my research. Thanks to the C. Alma Baker Trust for the provision of a scholarship, and AgResearch for providing a stipend without which I would not have been able to complete these studies. Particular thanks go to Assoc. Prof. Kathryn Stowell for her assistance and guidance in helping me obtain a suspension to my studies during a particularly distressing time. I would like to thank my mum Moira Olivecrona for her constant support and encouragement; she was my greatest rock and my strongest advocate. Sadly she passed away before this could be completed but she never doubted for a moment that it would be. Thanks to my dad Stenfinn and my sister Courtney who contributed with their love, support and in so many other innumerable ways. Without them this would never have happened. Finally I would like to thank Jim for sharing every happy, sad and frustrating moment and supporting me every step of the way.

Contents

Abstract ii

Acknowledgements iii

List of Figures vii

Glossary of Abbreviations x

NMR Experiments x

Statistical Analysis x

Meat Science x

Chapter One Introduction 1

1.1. Export of NZ Meat 1

1.1.1. Meat Spoilage 2

1.1.2. Acceptability Characteristics in Meat 3

1.1.3. Confinement Odour and Sensory Evaluation 3

1.1.4. Packaging and Storage for Improved Meat Quality 4

1.2. Metabolomics 5

1.2.1. Analysis Methods 7

1.2.2. Meat Metabolomics 9

1.2.3. Previous Comparison of Drip and Meat 11

1.2.4. Experimental Design Considerations 11

1.3. NMR 12

1.3.1. Underlying Principle 12

1.3.2. Experimental Variations 13

1.3.3. Benefits for Use with Metabolomics 14

1.3.4. Experimental Considerations 14

1.4. Multivariate Statistical Analysis 15

1.4.1. Data Pre-Processing for NMR and Multivariate Statistical Analysis 16

1.4.2. Principal Components Analysis 21

1.4.3. Outliers 23

1.4.4. Partial Least Squares – Discriminant Analysis 24

1.4.5. Orthogonal Partial Least Squares – Discriminant Analysis 25

1.4.6. Limitations and Common Pitfalls 26

1.4.7. Validation 26

1.4.8. Univariate Statistical Analysis 28

1.5. Aims and Objectives 28

Chapter Two Materials and Methods 29
2.1. Evaluation of sample preparation methods 29
2.1.1. Chemicals 29
2.1.2. Methods for preparation of drip samples 29
2.1.3. Methods for preparation of meat samples 30
2.2. Preparation of confinement odour samples 33
2.2.1. Preparation of drip samples 34
2.2.2. Preparation of meat samples 34
2.3. NMR Analysis 34
2.3.1. Spectral Acquisition 34
2.3.2. Spectral Processing 35
2.4. Statistical Analysis 35
2.4.1. Processing for Metabolomics 35
2.4.2. Statistical Analysis Methods 37
2.4.3. Metabolite Identification 38
Chapter Three Results and Discussion 43
3.1. Evaluation of Sample Preparation Methods 43
3.1.1. Methods for Preparation of Drip Samples 44
3.1.2. Methods for Preparation of Meat Samples 49
3.1.3. Chosen Sample Preparation Methods 52
3.2. Confinement Odour Study 54
3.2.1. Confinement Odour and Spoilage Odour Metabolite Differences 54
3.2.2. Metabolite Differences between Processing Plants 62
3.2.3. Metabolite Differences between Drip and Meat 73
Chapter Four Conclusion and Future Work 80
4.1. Conclusions 80
4.1.1. Optimal Preparation Protocol for NMR Analysis of Lamb Drip and Meat 80
4.1.2. Confinement Odour Metabolites in Lamb Drip and Meat 80
4.1.3. Metabolites Differentiating Processing Plant of Lamb Drip and Meat 81
4.1.4. Metabolites Differentiating Lamb Drip and Lamb Meat 81
4.2. Future Work 81
4.2.1. Sampling of Lamb Drip and Meat at Various Time Points for Time Series Analysis of Confinement Odour Metabolites 81

4.2.2. Mass Spectrometry Analysis of Meat and Drip for Confinement Odour Metabolites 81
4.2.3. Treatment Changes to Increase Control Samples 82
Appendix A PLS-DA Analysis and Validation 87
Analysis of Drip for Confinement Odour Metabolites 87
Analysis of Drip for Processing Plant Metabolites 87
Analysis of Meat for Confinement Odour Metabolites 89
Analysis of Meat for Processing Plant Metabolites 89
Analysis of Drip and Meat for Confinement Odour Metabolites 91
Analysis of Drip and Meat for Partitioning of Metabolites 92
Analysis of Drip and Meat for Processing Plant Metabolites 93

List of Figures

Figure 1 Interaction of the Metabolome (Griffin & Shockcor, 2004) 6
Figure 2 Splitting of Nuclei Spin States in an External Magnetic Field ("Nuclear Magnetic Resonance,") 12
Figure 3 1H NMR Spectrum of a Mixture of α-glucose and β-glucose in D2O Acquired at 400MHz 13
Figure 4 Overlay of ^{1}H NMR Spectra of Filtered Plasma and Unfiltered Plasma 15
Figure 5 Section of Overlaid Plasma ^{1}H NMR Spectra from 7.71 - 7.95 ppm 17
Figure 6 Section of Overlaid Plasma ^{1}H NMR Spectra from 7.71 - 7.95 ppm with Local Alignment 18
Figure 7 Section of Full Resolution Plasma ^{1}H NMR Spectrum Showing 0.8 - 2.75 ppm 19
Figure 8 Section of Plasma ^{1}H NMR Spectrum Showing 0.8 - 2.75 ppm Binned to 0.04 ppm 19
Figure 9 Example of Principal Components Scores Plot, Outliers are Shown Circled in Red 22
Figure 10 Example of Principal Component Analysis Loadings Plot 23
Figure 11 PCA Scores Plot of Plasma Coloured by Year of Collection 24
Figure 12 PLS-DA Scores Plot of Plasma Coloured by Year of Collection 25
Figure 13 Permutation Test for a Valid PLS-DA Model 28
Figure 14 1D Loadings Plot of an Example OPLS-DA Model with Loadings Coloured by VIP Value 39
Figure 15 1D Loadings Plot of an Example OPLS-DA Model with Loadings Coloured by VIP Value, Loadings with VIP≥1.5 Shown and a Loading with Error Greater than its Value Circled in Red 40
Figure 16 S-Plot From an Example OPLS-DA Model with Loadings Coloured by VIP Value, Loadings Combining High Correlation and High Covariance Shown Circled in Red 41
Figure 17 Overlaid ^{1}H NMR Spectra of Drip with Protein Removed by Centrifugation (Dark Red) or Ultrafiltration (Light Blue) 43
Figure 18 Overlaid ^{1}H CPMG (Dark Red) and NOESY (Light Blue) NMR Spectra of a Drip Sample with Protein Removed by Centrifugation 45
Figure 19 Overlaid Spectra of Three Experimental Replicates of Drip with Protein Removed by Centrifugation (Enlargement of Section 8.14 - 8.42 ppm) 46
Figure 20 Overlaid Spectra of three Experimental Replicates of Drip with Protein Removed by Ultrafiltration (Enlargement of Section 8.13 - 8.40 ppm) 47
Figure 21 Overlaid Spectra of Three Experimental Replicates of Meat with Protein Removed by Perchloric Acid Solvent Precipitation (Enlargement of Section 8.06 - 8.58 ppm) 51
Figure 22 PCA Scores Plot of Drip and Meat Samples Coloured According to the Procedures Outlined in the Sample Preparation Evaluation 53
Figure 23 PCA Scores Plot of Drip Coloured by Odour Status 54
Figure 24 PCA Loadings Plot of Drip Coloured by Odour Status, Bins with Largest Variation across Each Component Circled in Red 55
Figure 25 Box Plots of Significant Bins from T-Test of Drip Samples Classed by Odour Status (Red Indicates Samples with Confinement Odour, Green Indicates Samples which are Spoiled) 56
Figure 26 PCA Scores Plot of Meat Coloured by Odour Status 57

Figure 27 PCA Loadings Plot of Meat Coloured by Odour Status, Bins with Largest Variation across Each Component Circled in Red 57
Figure 28 PCA Scores Plot of Drip and Meat Samples Coloured by Odour Status 58
Figure 29 PCA Loadings Plot of Drip and Meat, Bins with Largest Variation across Each Component Circled in Red 59
Figure 30 PCA Scores Plot of Drip Coloured by Processing Plant of Origin 62
Figure 31 OPLS-DA Scores Plot of Drip Samples Classified by Processing Plant of Origin, Samples which are More Similar to Samples from the Other Plant than from Their Own Shown Circled in Red 63
Figure 32 OPLS-DA 1D Full Loadings Plot of Drip Samples Classified by Processing Plant of Origin, Loadings Coloured by VIP Value 64
Figure 33 OPLS-DA 1D Loadings Plot of Drip Samples Classified by Processing Plant of Origin, Loadings with VIP Value Greater than 1.5 Shown 65
Figure 34 S-Plot of Drip Samples Classified by Processing Plant of Origin, Coloured by VIP value, Loadings which combined High Covariance with High Correlation are Shown Circled in Red ... 65
Figure 35 PCA Scores Plot of Meat Coloured by Processing Plant of Origin 66
Figure 36 OPLS-DA Scores Plot of Meat Samples Classified by Processing Plant of Origin 67
Figure 37 OPLS-DA 1D Full Loadings Plot of Meat Samples Classified by Processing Plant of Origin, Loadings Coloured by VIP Value 67
Figure 38 OPLS-DA 1D Loadings Plot of Meat Samples Classified by Processing Plant of Origin, Loadings Coloured by VIP Value, Loadings with VIP values greater than 1.5 shown 68
Figure 39 S-Plot of Meat Samples Classified by Processing Plant of Origin, Coloured by VIP value, Loadings Combining High Covariance with High Correlation Shown Circled in Red 69
Figure 40 PCA Scores Plot of Drip and Meat Coloured by Processing Plant Origin 69
Figure 41 OPLS-DA Scores Plot of Drip and Meat Classified by Processing Plant of Origin 70
Figure 42 OPLS-DA 1D Full Loadings Plot of Drip and Meat Classified by Processing Plant of Origin, Loadings Coloured by VIP Value 71
Figure 43 OPLS-DA 1D Loadings Plot of Drip and Meat Classified by Processing Plant of Origin, Loadings Coloured by VIP Value, Loadings with VIP values greater than 1.5 shown 71
Figure 44 S-Plot of Drip and Meat Samples Classified by Processing Plant of Origin, Coloured by VIP value, Loadings Combining High Covariance with High Correlation Shown Circled in Red.. 72
Figure 45 PCA Scores Plot of Drip and Meat Coloured by Sample Type 73
Figure 46 OPLS-DA Scores Plot of Drip and Meat Classified by Sample Type 74
Figure 47 OPLS-DA 1D Full Loadings Plot of Drip and Meat Classified by Sample Type, Loadings Coloured by VIP Value 74
Figure 48 OPLS-DA 1D Loadings Plot of Drip and Meat Classified by Sample Type, Loadings Coloured by VIP Value, Loadings with VIP Value Greater than 1.5 Shown 75
Figure 49 S-Plot of Drip and Meat Samples Classified by Sample Type, Coloured by VIP value, Loadings Combining High Covariance with High Correlation Shown Circled in Red 76
Figure 50 PLS-DA Scores Plot of Drip Classified by Odour Status 87
Figure 51 PLS-DA Scores Plot of Drip Samples Classified by Processing Plant 88
Figure 52 Permutation Test Plot Showing 999 Permutations for PLS-DA model of Drip Samples Classified by Processing Plant 88
Figure 53 PLS-DA Loadings Plot of Drip Samples Classified by Processing Plant 89
Figure 54 PLS-DA Loadings Plot of Meat Samples Classified by Processing Plant of Origin 90

Figure 55 Permutation Test Plot Showing 999 Permutations for PLS-DA model of Meat Samples Classified by Processing Plant 90
Figure 56 PLS-DA Loadings Plot of Meat Samples Classified by Processing Plant 91
Figure 57 PLS-DA Scores Plot of Drip and Meat Classified by Odour Status..... 91
Figure 58 PLS-DA Scores Plot of Drip and Meat Classified by Sample Type 92
Figure 59 Permutation Test Plot Showing 999 Permutations for PLS-DA model of Drip and Meat Samples Classified by Sample Type..... 93
Figure 60 PLS-DA Loadings Plot of Drip and Meat Classified by Sample Type..... 93
Figure 61 PLS-DA Scores Plot of Drip and Meat Classified by Processing Plant of Origin..... 94
Figure 62 Permutation Test Plot Showing 999 Permutations for PLS-DA model of Drip and Meat Samples Classified by Processing Plant..... 94
Figure 63 PLS-DA Loadings Plot of Drip and Meat Classified by Processing Plant of Origin..... 95

Table 1 Method Test Treatment Conditions..... 30
Table 2 Storage Conditions of Meat Samples and Subsequent Odour Types 33
Table 3 Summary of the Result of T-Test Analysis on Drip Samples Classed by Odour Status... 55
Table 4 Summary of Metabolites Characterised from ^{1}H and ^{13}C NMR Signals for Reference .. 77

Glossary of Abbreviations

NMR Experiments

HSQC ..... Heteronuclear Single Quantum Coherence

NOESY ..... Nuclear Overhauser Effect Spectroscopy

TOCSY ..... Total Correlation Spectroscopy

CPMG ..... Carr Purcell Meiboom Gill

Statistical Analysis

PCA ..... Principal Components Analysis

PLS-DA ..... Partial Least Squares Projection to Latent Structures-Discriminant Analysis

OPLS-DA ..... Orthogonal Partial Least Squares-Discriminant Analysis

UV ..... Unit Variance (Scaling)

VIP ..... Variable Importance in Projection

Meat Science

MAP ..... Modified Atmosphere Packaging

LAB ..... Lactic Acid Bacteria

GN ..... Gram Negative

GP ..... Gram Positive

Chapter One Introduction

1.1. Export of NZ Meat

Meat is New Zealand's second highest value export after dairy products (NZ Trade and Enterprise), and New Zealand is the world's largest exporter of mutton and lamb meat (Meat and Livestock Australia). With more than 340 million kilograms of lamb meat shipped to around 110 countries every year (The Lamb Company), and ensuring the quality of meat when it reaches its export destination is incredibly important. By volume the European Union is the largest importer of New Zealand meat (Beef and Lamb NZ, 2015), and so most exported meat travels for about forty to fifty days to reach market (Richie, 2014). Taking into account the time required for the product to be processed before shipment, move through customs and be distributed the remaining shelf life is around two weeks and any delays reduce this time (The Meat Industry Association, 2011).

Recently, the time taken to deliver meat from producer to market has increased as fuel prices and environmental pressures result in exporters reducing the speed of the ships transporting the product by up to 20% (Mills, Donnison, & Brightwell, 2014), a measure termed "slow steaming" (Psaraftis & Kontovas, 2013). There is a non-linear relationship between ship speed and emissions; not only is it more environmentally friendly to reduce ship speed but also financially worthwhile (Psaraftis & Kontovas, 2013). Therefore the life of chilled meats is being pushed beyond the recognized, and achievable, time of sixty to seventy days (Mills et al., 2014). Meat is usually stored at temperatures of -1.5-0°C (Johnson, 1991) during export, with the minimum value set to stop the meat from freezing.

A positive side effect of longer storage times for exported red meat is that the prolonged aging period improves consumer reported scores for attributes such as tenderness and flavour (Graham et al., 2010), both of which increase with aging. This has been linked to the proteolysis of myofibrillar proteins in the meat (Graham et al., 2010). Higher levels of amino acids, nucleotides and sugars are found in aged meat and these help to impart flavour in the final cooked product.

Another adjustment to the procedures surrounding the export of NZ meat relates to the changing practices of retailers. Previously it was common for retailers to repackage transported primal meat cuts into retail-ready portions at in-house butcheries, however many now choose to do away with the additional space and labour this requires in favour of the

more convenient and economical option of purchasing retail-ready cuts directly from the supplier (Jeremiah & Gibson, 2001).

1.1.1. Meat Spoilage

Meat spoilage is linked to the growth of certain classes of bacteria on the surface of meat. These bacteria can contaminate the meat at any stage from the farm to the final packaging. Tearing of packaging can also allow bacterial contamination. Inhibiting the growth of these bacteria is the goal of storage temperature and packaging systems for exported meat. Removal of oxygen has one of the biggest effects on the rate of bacterial growth (Gill, 1989); therefore packaging is designed to achieve this. Meat will also show discolouration due to oxidation of muscle pigment in the presence of oxygen (Gill, 1989), and this is another characteristic that results in rejection of product by the consumer.

1.1.1.1. Meat Spoilage Characteristics

Certain groups of bacteria are associated with the production of off odours and spoilage in meat upon reaching sufficient concentration. Precursors for the metabolites implicated in spoilage are glucose, lactic acid, water soluble proteins and amino acids. As the quantity of these precursors increases the rate and extent of spoilage also increases (Ercolini et al., 2011).

1.1.1.2. Spoilage Bacteria

Meat which has been processed and stored in adherence with industry standard best practice (Devine, 2014) will nonetheless contain a wide range of bacterial species. Which of these species is able to predominate is dependent on the meat environment; this is in turn determined by factors such as temperature, atmosphere and pH (Davies, Board, & Board, 1998). Strong off-odours are largely caused by gram negative (GN) bacteria: these bacteria are fast growing and proliferate in aerobic environments. Gram positive (GP) bacteria, such as many strains of lactic acid bacteria (LAB) and *Micrococcaceae*, are slower growing than GN bacteria but are able to grow in both aerobic and anaerobic conditions, although they generally form only a small proportion of the overall meat bacterial ecosystem in the presence of oxygen. LAB species are also tolerant of low temperatures (Davies et al., 1998). Therefore refrigeration (around -1.5-1 degree C) and vacuum or modified atmosphere packaging (MAP) of meat will cause LAB to thrive relative to GN bacteria. However, LAB will still grow slowly under these conditions.

LAB utilise carbohydrate fermentation for energy production to produce a range of organic acids which produce sour or acid smells (Davies et al., 1998). The acid production lowers the pH of the meat surface and, in conjunction with other ‘antimicrobial’ metabolites produced by

LAB; this has the effect of creating an inhospitable environment for pathogenic, putrefactive and toxinogenic bacteria(Davies et al., 1998). LAB also competes for resources with these undesirable bacteria.

Both types of bacteria produce malodorous substances, for example LAB produce small amounts of dimethlysulphide and methanethiol in addition to organic acids which contribute to the distinctive smell that accompanies these colonies(Davies et al., 1998). As part of their metabolism pathogenic GN bacteria produce sulphurous compounds which have an offensive smell; therefore the odours of meats with greater proportions of LAB present will be less offensive than meat where GN bacteria proliferate(Davies et al., 1998). This means that meat on which LAB is the dominant bacterial species will have a longer shelf life than other meat, therefore meat environments are generally manipulated to provide conditions in which GP bacteria will grow in preference to GN bacteria(Davies et al., 1998).

However, once bacterial counts reach around 10^7 to 10^8 cells/cm^2 on the meat surface glucose is generally exhausted meaning that bacteria turn to amino acids for metabolism, breaking these down in to foul smelling sulphides, esters and amines(Davies et al., 1998).

1.1.2. Acceptability Characteristics in Meat

For the majority of consumers the acceptability of meat is mostly based on visual and olfactory cues, since information on its age or quality is normally not provided. The appearance of 'freshness' is very important and can be suggested by colour (deep red for beef and lamb) or state (frozen is suggestive of long transport times and old meat). Meat is expected to have little smell, as a strong odour is most often associated with spoilage.

From a manufacturing perspective freezing meat for transport is preferable to chilling due to the increased shelf life of frozen meat. As a consequence, frozen meat can be shipped slower which reduces costs. For chilled meat some storage time is preferable as it increases the flavour of the meat and results in a more tender product (Graham et al., 2010). Chilling also increases the water holding content of the meat compared with freezing/thawing, meaning that the same quantity of meat will weigh more and can therefore be sold for a higher price.

1.1.3. Confinement Odour and Sensory Evaluation

Confinement odour (CO) is a phenomenon that has always been an issue with vacuum and modified atmosphere packaged meat. Meat that has been stored in these ways will sometimes produce a possibly unpleasant smell immediately upon the packaged being opened; however this doesn’t indicate that the meat is unfit for consumption as other odours might. As it is a

well-known phenomenon in the meat industry its detection does not result in meat being rejected by a retailer. However, changes to supply chain practices have resulted in the consumer being exposed to this phenomenon in place of the retailer. As consumer have not been educated about CO they are most likely to equate the smell to spoilage.

Odour assessment of meat is often carried out by selected panels using a hedonic scale, in which the odour is categorised over a point system ranging from acceptable to unacceptable (Gill & Penney, 1986). In the assessment of CO, it is necessary to evaluate the meat immediately upon opening its packaging and again after a short wait period. Meat exhibiting CO can then be differentiated from spoiled meat as it will produce unacceptable odours only upon first opening the package, which will dissipate over the wait period and not be detectable at the second assessment. It may also be possible to distinguish CO from spoilage odour by the notes each produces. CO has been described as cheesy, dairy or milky with additional sour or acid notes (Johnson, 1991). In comparison spoilage odours are generally referred to as putrid, sweet or sulfurish (Gill & Penney, 1986; Seman, Drew, Clarken, & Littlejohn, 1988).

Hydrogen sulfide is the cause of sulfur notes associated with spoilage odours; it can be produced by particular strains of *Lactobacillus sakei* (a species of LAB) (Mills et al., 2014). Sulfur notes are also linked to the depletion of glucose levels in meat(McMullen & Stiles, 1994).

It has been suggested that LAB proliferation in meat is linked to confinement odour and that the specific odour produced is dependent on the particular type of LAB present (Mills et al., 2014).

1.1.4. Packaging and Storage for Improved Meat Quality

Reduction of bacterial growth in meat packaging is often achieved in one of two ways – vacuum packaging and MAP. Vacuum packaging involves the complete removal of air from the product and effective sealing of the package. MAP involves the complete removal of air from the product followed by replacement with an alternative gas, or mixture of gases. A common MAP technique uses the addition of carbon dioxide (Gill, 1989), a gas which inhibits microbial growth (Bill B.A.; Small, Sikes, & Doral, 2008) through slowed respiration and increased lag phase/generational times. For both types of packaging it is important that the packaging film is strong, to reduce the chance of breakage, and has a very low permeability, to reduce the transmission of oxygen back into the meat during storage (Gill, 1989) as well as reducing the transmission of carbon dioxide out of the film. Trace amounts of gases remain in vacuum packaged products; however tissue and microbial respiration will reduce any oxygen to less

than 1% and increase carbon dioxide to around 20% (Davies et al., 1998). The rest of the modified atmosphere consists of nitrogen gas.

Storing meat at temperatures low enough to freeze (<-1.5°C) significantly extends the acceptable storage time when compared to meat that is only chilled (-1.5°C-4°C) (Small et al., 2008). However, chilled meat displays a number of beneficial characteristics that are less evident in meat which has been frozen. For example the water holding capacity (WHC) is higher in chilled meat, which results in less water loss during storage and increases the tenderness of the final product (Trout, 1988). Tender meat is more desirable to the consumer. A high WHC also means that, for the same pre-storage weight, chilled meat can be sold for a higher price than frozen and thawed meat as the water that stays in the muscle tissue is included in the weight of the final product sold to the consumer. This makes chilled meat a more desirable product for the producer. Spoilage bacteria are still able to grow at temperatures slightly below -1.5°C; therefore chilling meat can only extend the shelf life of meat (Davies et al., 1998).

1.2. Metabolomics

Metabolomics is concerned with the investigation of all the low molecular weight compounds present within a cell or organism as a result of metabolism or ingestion (Dixon et al., 2006), and more particularly with the changes in metabolite concentrations that occur due to a perturbation to the genetic or environmental conditions of the organism. These compounds are called metabolites and are the starting materials, intermediates and products of cellular metabolism. They include a number of classes of compounds such as amino acids, sugars, lipids and organic acids. Metabolites are affected not just by gene expression but also by the interaction of the environment with the genome, transcriptome and proteome. Figure 1 also shows that while signalling is generally thought of as occurring from genome to transcriptome to proteome it is possible for it to go in the opposite direction and from any of these to any other. The entire collection of an organism's metabolites is called its metabolome.

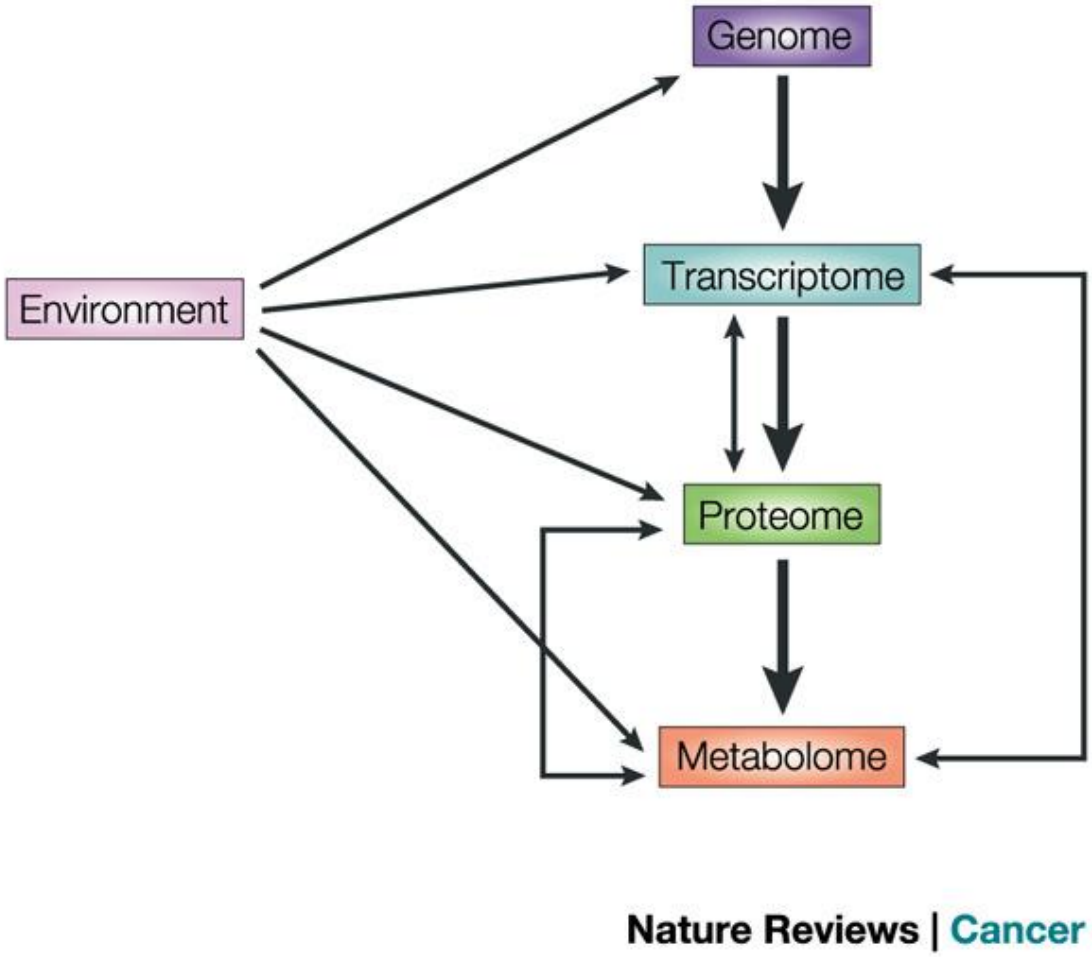

Figure 1 Interaction of the Metabolome (Griffin & Shockcor, 2004)

The goal of metabolomics can generally be considered to be the investigation of the metabolome to determine the metabolic response to various forms of stimulus (for example disease, diet intervention or drug exposure) (Lindon & Nicholson, 2008). Traditional statistical approaches follow the changes to a single metabolite concentration when the system is perturbed, for example monitoring the level of lactate in human sweat before and after an exercise intervention. The metabolomics approach is to follow the changes that occur to the entire metabolome when the system is perturbed. This means that there is no need to narrow down the field of possible effects on the system in order to perform an analysis, therefore unexpected changes can be discovered. All of the metabolite concentration information which a particular analysis technique can provide is able to be analysed together, which means that a holistic view of the metabolic response is obtained.

In animal systems the metabolome is made more complex due to the presence of symbiotic microorganisms or parasites which produce their own metabolites and interact with the host's metabolism (van der Greef & Smilde, 2005).

There are two main types of metabolomic analysis. The first is metabolic profiling, in which the biological sample is analysed and as many of the metabolite signals as possible identified. In targeted metabolic profiling these metabolites are quantified using internal standards at known concentrations (Savorani, Rasmussen, Mikkelsen, & Engelsen, 2013). The second of these techniques is metabolic fingerprinting, in which the analysis of the metabolome is coupled with multivariate analysis techniques in order to compare the metabolic patterns of different classes of samples. The metabolites are not initially identified; instead the signals they produce due to the particular analytical technique used are all used to provide a 'fingerprint' of the samples' metabolomes. The mean metabolome is computed for each class and these means are compared to produce a list of metabolites which describe the perturbation produced by the stimulus (Broadhurst & Kell, 2006). The metabolites are used to discover the biological mechanisms activated by the stimulus (Savorani et al., 2013).

1.2.1. Analysis Methods

Two analysis platforms are used for the majority of metabolomics studies – Nuclear Magnetic Resonance (NMR) spectroscopy and Mass Spectrometry (MS). Both platforms offer a large number of protocols which have their own strengths and weaknesses. NMR can be applied both to solid and liquid state samples, many different elements can be probed to produce numerous one-dimensional and two-dimensional experiments. MS is coupled to many separation and infusion methods. These combinations include gas chromatography MS (GC-MS), liquid chromatography MS (LC-MS), two dimensional GC time-of-flight MS (2D-GC-TOF-MS), high performance LC-MS (HPLC-MS), direct infusion electrospray ionization TOF-MS (DI-ESITOF-MS), capillary LC-ESI-TOF-MS, ESI-MS, capillary electrophoresis ESI-MS and Fourier transform ion cyclotron resonance MS (FTI-CR-MS) (Rochfort, 2005Rochfort, 2005).

Less commonly utilised platforms are Fourier transform infrared spectroscopy (FTIR), high performance liquid chromatography-ultraviolet (HPLC-UV) and fluorescence microscopy (Rochfort, 2005Rochfort, 2005).

NMR and MS differ in a number of fundamental ways (Lindon & Nicholson, 2008Lindon & Nicholson, 2008) but are both useful for metabolomics applications as they are considered complementary techniques {Rochfort, 2005metabolome, and therefore many platforms are necessary for application to metabolomic studies.). The strengths of one platform are related

to the weaknesses of the other, and vice-versa. There is currently no technique which is able to measure the entire metabolome, and therefore many platforms are necessary for application to metabolomic studies.

Because of the techniques' specific limitations and strengths it is necessary for the analysis method to be chosen based on the established needs of the study. A one size fits all recommendation should not be made, with some papers (Ercolini et al., 2011; Rochfort, 2005) reporting the use of both analysis methods on the same sample sets to take advantage of the benefits of both platforms.(Ercolini et al., 2011; Rochfort, 2005) reporting the use of both analysis methods on the same sample sets to take advantage of the benefits of both platforms.

NMR is generally only sensitive enough to pick up metabolites at micro-molar quantities, although improvements to the detection limit down to nano-molar can be made if the spectrometer is fitted with a cryoprobe. The techniques generally require a sample size of a few hundred microlitres or milligrams (with the use of a microprobe only tens of microlitres are required) which for certain studies can be very difficult or time consuming to obtain (Young, Berdagué, Viallon, Rousset-Akrim, & Theriez, 1997Young, Berdagué, Viallon, Rousset-Akrim, & Theriez, 1997).

However, NMR does have a number of characteristics which are advantageous when applied to metabolomics. It is a non-destructive technique (Rochfort, 2005Rochfort, 2005)(Worley, 2013). This can be important for metabolomics experiments where previous biological knowledge of the samples can lead to false (but seemingly reliable) interpretations of the data (Worley, 2013). The technique provides high analytical reproducibility, which removes the need for machine replicates (Rochfort, 2005). This robustness also means that comparison of sample data taken in different labs using different machines is possible (Rochfort, 2005). NMR also allows for fairly easy identification of metabolites through the use of online databases containing spectra of pure metabolites, in addition to 2D experiments as described elsewhere. which means the same sample can be reanalysed at a later date to investigate stability. Minimal sample preparation is necessary for NMR analysis, and many different classes of compounds can be analysed together without the need for separation techniques. It allows many biofluids, cell lysates and tissues (whether intact or not) to be analysed without bias for particular classes of compounds. This can be important for metabolomics experiments where previous biological knowledge of the samples can lead to false (but seemingly reliable) interpretations of the data. The technique provides high analytical reproducibility, which removes the need for machine replicates.. This robustness also means that comparison of

sample data taken in different labs using different machines is possible. NMR also allows for fairly easy identification of metabolites through the use of online databases containing spectra of pure metabolites, in addition to 2D experiments as described elsewhere.

It should be noted that analysis can be performed using many types of animal tissues and fluids including muscle (Graham et al., 2010; Mannina et al., 2008; Savorani et al., 2010), organs, adipose tissue (Atherton et al., 2006), hemolymph, glands (Viant, Rosenblum, & Tjeerdema, 2003), cerebrospinal fluid (CSF), blood, urine (Osorio, Moloney, Brennan, & Monahan, 2012) and freeze exudate (Straadt, Aaslyng, & Bertram, 2011).(Graham et al., 2010; Mannina et al., 2008; Savorani et al., 2010), organs, adipose tissue , hemolymph, glands , cerebrospinal fluid (CSF), blood, urine and freeze exudate.

1.2.2. Meat Metabolomics

Metabolomic meat analysis has been carried out in a diverse range of animal species for an equally diverse range of applications.

Beef and pork have been frequently investigated, providing the ability to authenticate breed (Straadt et al., 2011), production system (Osorio et al., 2012) and storage length (Graham et al., 2010); this being a common source of research for food and nutrition science on products of both animal and plant origin. Food authenticity is a hugely important issue for many countries globally as they seek to maintain premiums on food and drink produced in certain geographical locations, using specific techniques, or made with particular constituents.

Discrimination of meat based on the breed of the animal which it came from is not only useful for food authenticity analysis but also investigating the effect of breed on meat quality characteristics. This was the objective of a study by (Straadt et al., 2011) in which the NMR and metabolomics were used to probe biophysical and biochemical factors of pork quality. NMR T_2 relaxation was used to discover the water holding content (WHC) of five pig cross breeds, in order to determine which breed produced the most tender meat, and this result was combined with scores from sensory analysis. ^{1}H NMR spectroscopy was also used to discover the metabolic profile differences between the breeds; it was found that a number of amino acids, choline-containing compounds, lactic acid, inosine, inosine-monophosphate (IMP) and glycerol differed significantly between the breeds.

Metabolomics has been utilised for investigating the possibility of discriminating meat samples based on production system and type of feed for a number of different meat types. Both of

these factors can influence meat quality and as such consumers are willing to pay different prices based on the treatment of the animal before slaughter. For example, meat from animals which have been raised outdoors and fed on pasture commands a high price as consumers become more concerned about animal welfare and are made aware of its nutritional superiority over more intensively produced meat. NMR metabolomics has been used to discriminate between beef from pasture-fed cows which had been reared outdoors and barley concentrate fed cows which had been confined indoors (Osorio et al., 2012). The ability to discriminate meat based on the type of feed used in its production not only prevents producers from being able to mislead consumers but can also detect illegal practices. One such practice is adding chicken bone meal to chicken feed for a cheap source of nutrients; it is illegal in many cases as an animal cannot have feed adulterated with processed animal protein from its own species. Direct Analysis in Real Time Ionization-Mass Spectrometry (DART-MS) used in conjunction with chemometric methods is able to differentiate between the meat of chickens which have been fed with chicken bone meal and the meat of those which have been fed the same diet, under the same conditions, without the addition of bone meal (Cajka, Danhelova, Zachariasova, Riddellova, & Hajslova, 2013). The analysis showed that while the polar meat metabolites differed corresponding to the feed type the non-polar meat metabolites were more influenced by seasonal differences.

A number of food products have a Protected Designation of Origin (PDO) to indicate they have been produced from a certain geographical location with a reputation for quality. Proton transfer reaction time-of-flight mass spectrometry (PTR-ToF-MS) has been used in conjunction with multivariate analysis techniques in order to discriminate the headspace gas from dry-cured ham from PDOs from dry-cured hams from other regions (del Pulgar et al., 2011). In order for producers in these regions to profit from their PDOs it is important that imposters are easily discriminated and the illegal practice punished.

A number of studies have focussed on the discrimination of meat based on post-slaughter treatment. For example, NMR metabolomics has been applied to ground beef to determine whether the meat has been irradiated (Zanardi et al., 2015), a treatment which extends the shelf-life of the meat but is not legal in a number of countries. Principal Components Analysis (PCA) and Classification Trees were applied to the data and revealed that glycerol, lactic acid esters and tyramine (or a *p*-substituted phenolic compound) were discriminating metabolites between irradiated and non-irradiated beef. Another example shows that the chilling process applied to lamb meat immediately after slaughter can affect the tenderness of the meat by an

altered energy metabolism (Warner et al., 2015). This change can be assessed by a number of NMR and MS-based methods which reveal the differences between the meat metabolites based on the chilling process.

It has been shown that the age of duck meat can be ascertained from inspecting the metabolite profiles of equivalent reference samples (Liu, Pan, Ye, & Cao, 2013).

It is possible to monitor the microbial populations in meat over its storage time. This has been performed by Ercolini *et al.* who compared this information with metabolomic analysis in order to assess the effects of various forms of packaging conditions on beef (Ercolini et al., 2011). They were able to follow the changes in the microbial population as the storage progressed and showed that the storage conditions had a great effect on which species predominated and the metabolites which were produced.

1.2.3. Previous Comparison of Drip and Meat

A previous study sought to determine the relative merits of analysing meat or drip ^{1}H NMR spectra to differentiate the metabolite profiles of various pork producing pig breeds. Based on multivariate statistical analysis it appears that analysis of the drip was able to yield a better grouping of samples based on breed. The authors suggest that this may be the result of many metabolites important to the discrimination being lost from the meat in the form of drip (Straadt et al., 2011).

1.2.4. Experimental Design Considerations

For a robust analytical method (such as NMR spectroscopy) generally only one analytical replicate per sample is necessary (Savorani et al., 2013).

It is necessary to minimise variation introduced to the samples during preparation and analysis. Any variation unrelated to wanted classification can confound the efforts of the statistical analysis to discriminate the samples into groups. Examples of unwanted sample variation include differences in collection, storage, temperature, pH, post-collection metabolism and the presence of precipitate (Savorani et al., 2013). Sample preparation and analysis is an important area to consider in minimising variation. For instance, if different sample classes are prepared separately it would be impossible to discriminate whether any variation found was due to inherent differences between the classes or if unwanted variation had been introduced during preparation. Problems of this kind can be mitigated by standardising all aspects of preparation and analysis, for example the order/time of collection, preparation, transport and spectral acquisition (Liland, 2011). In addition, samples are

normally randomised during preparation and analysis to avoid run order and/or batch effects. This allows the analysis to be robust, reproducible and easily interpretable (Trygg, Holmes, & Lundstedt, 2006).

1.3. NMR

1.3.1. Underlying Principle

Certain isotopes, specifically those with an odd number of protons and/or neutrons, have a non-zero spin (an intrinsic quantum property of nucleons) which allows for their spin systems to be perturbed by an external magnetic field, these are said to be magnetically susceptible. Examples of such isotopes include ^{1}H, ^{2}H, ^{13}C, ^{19}F and ^{31}P. This perturbation comes in the form of splitting of the spin energy levels, proportional to the size of the applied field, within the nucleus of the atoms (Figure 2). This splitting behaviour creates a small magnetic field. The nuclei can absorb and re-emit applied radio frequency pulses which match the frequency of the energy transition between the two spin states. The frequency of the energy transition depends on the size of the effective magnetic field at the nucleus; electron density shields the nucleus from the external magnetic field thereby lowering the size of the effective field. If the effective magnetic field at the nucleus is lowered then the spin system will be split less, meaning that the matching radio frequency will be less. The difference between the amount of splitting that is expected from the applied magnetic field, and the amount of splitting that is observed through the emitted radio frequency is termed the chemical shift. The chemical shift is highly dependent on the chemical environment of the nucleus and is therefore useful in molecular structure elucidation (Savorani et al., 2013). Nuclei in many common compounds have known chemical shift values and this information can be found in many online databases. A large number of metabolites are catalogued this way, and therefore analysis of a sample by NMR can show the metabolite components.

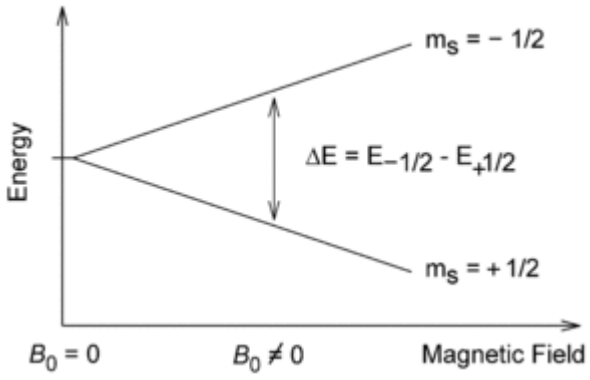

Figure 2 Splitting of Nuclei Spin States in an External Magnetic Field ("Nuclear Magnetic Resonance,")

Additional structural information is contained in the NMR spectrum, for example the multiplicity of a peak in a 1D spectrum indicates the number of nuclei neighbouring the nucleus of interest, while the integrals of all the individual peaks for a given metabolite indicate the proportions of nuclei corresponding to those signals. In Figure 3 the spectrum corresponding to a mixture of α-glucose and β-glucose shows the various peaks which are produced by the molecules and their different multiplicities can clearly be observed. The various anomeric signals have been annotated.

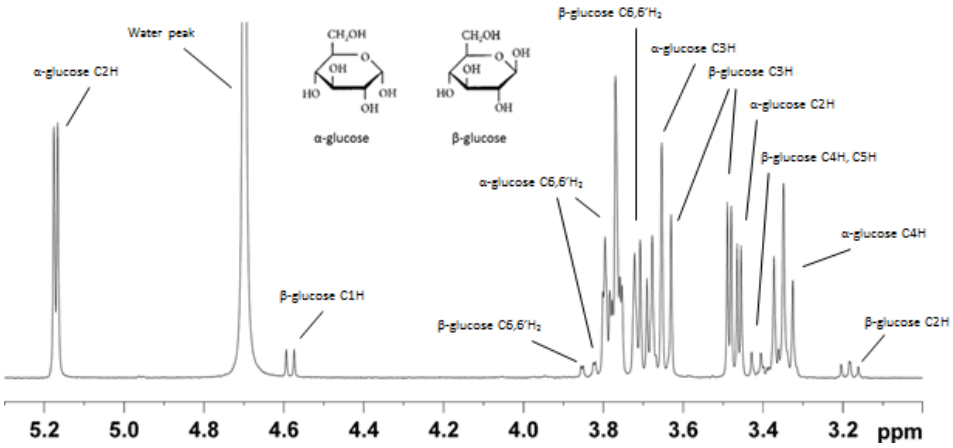

Figure 3 1H NMR Spectrum of a Mixture of α-glucose and β-glucose in D2O Acquired at 400MHz

Many types of biological samples can be analysed by NMR, for example cell lysates, whole tissue, tissue extracts and biofluids such as blood, urine and cerebrospinal fluid. The most common technique for metabolomics is 1D ^{1}H NMR spectroscopy which investigates liquid samples. However, other elements (such as ^{13}C and ^{31}P) can also be targeted, and these can be combined with ^{1}H in order to enable 2D exploration (Rochfort, 2005). These elements are generally less abundant than ^{1}H and their ability to produce quantitative information is limited. 2D ^{1}H-^{13}C experiments can be employed to improve metabolite identification as the two dimensions reduce the risk of peaks from different metabolites overlapping (Rochfort, 2005). Intact tissues and cells can also be used if high resolution magic angle spinning (HRMAS) NMR is available (Straadt et al., 2011).

1.3.2. Experimental Variations

High resolution magic angle spinning (HR-MAS) NMR spectroscopy is a technique which allows for the analysis of solid samples such as intact muscle tissue (Lindon & Nicholson, 2008). The sample must be rapidly spun (around four to six kHz) at an angle of 54.7 degrees in relation to the applied magnetic field, to mitigate the line broadening effect otherwise seen in solid samples. This effect is not seen in liquids as, unlike in solids, molecules are able to tumble freely in solution which averages out anisotropic NMR parameters.

Recent experiments using HR-MAS NMR spectroscopy (Savorani et al., 2013) have shown that solid tissue samples can be directly analysed with only the addition of D_2O to the sample. This incredibly simple preparation is ideal for metabolomics as the sample is analysed in as natural a state as possible. Many major signals can be identified; these include lactic acid, creatine, fatty acids, amino acids, organic acids and nucleosides. The information obtained from this analysis has been used, in conjunction with stable ratio isotope analysis and multivariate analysis, to discriminate the geographic origin of meat with a 96% predictive ability (Sacco, Brescia, Buccolieri, & Caputi Jambrenghi, 2005). The robustness and reliability of this method suggest that HR-MAS NMR is a very useful technique for analysis of meat metabolite characteristics. However, logistically it is difficult to analyse the large number of samples that is required for metabolomic analysis without a sample changer.

1.3.3. Benefits for Use with Metabolomics

Simple sample preparation is obviously very important for metabolomics studies involving multivariate analysis as these require high numbers of samples for statistical robustness. In order to reduce preparation induced variability it is important to complete preparation quickly and efficiently. There is also very little variation introduced by NMR analysis due to its reproducibility and uniformity (Dixon et al., 2006).

1.3.4. Experimental Considerations

Proteins are generally not included in metabolomic analysis and there are a number of reasons why physical protein removal is done before NMR analysis. For instance many low molecular weight metabolites will remain bound to the proteins present and will therefore not be detected in the spectra; this hinders the ability to quantify the concentrations of these species. High molecular weight molecules can also distort the spectral baseline, protein-bound metabolites can exchange with free metabolites over the period of acquisition resulting in broadened peaks, and a high concentration of protein will lower transverse relaxation (T_2) times, all phenomena leading to a reduction in quantitative accuracy. Protein can be removed from a biological sample in a number of ways each with varying levels of efficacy and appropriateness depending on the sample type, for example a chemical method involves precipitating proteins out of solution using a solvent, while a physical method for removal involves filtration of the sample.

In Figure 4 a spectrum of drip which includes protein (blue) overlays a spectrum of the same drip sample where the protein has been physically removed by ultrafiltration (red). The sharp low molecular weight metabolite peaks can be seen to be partially or completely obscured by

the broad resonances of the protein. Not only is quantification of the metabolites impossible from the protein-containing spectrum, but some metabolites are so obscured as to not be identifiable. For analysis which would seek to monitor the concentration differences of these metabolites based on NMR spectroscopy the protein-containing samples would be useless.

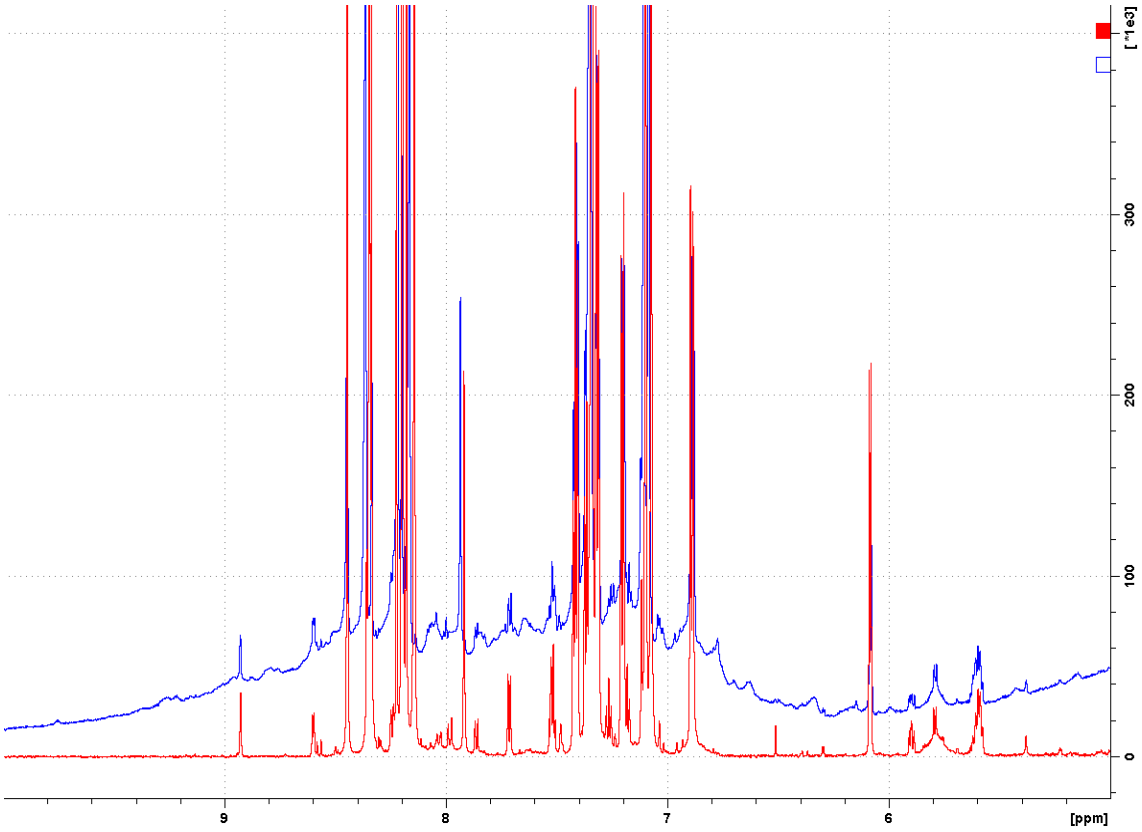

Figure 4 Overlay of ^{1}H NMR Spectra of Filtered Plasma and Unfiltered Plasma

In addition to the above methods the Carr Purcell Meiboom Gill (CPMG) pulse sequence is often used for analysis of samples containing protein where it is undesirable to remove the protein prior to spectral analysis. For metabolomics this approach is often not appropriate due to its varied effects on both quantitative and qualitative investigation (Savorani et al., 2013).

1.4. Multivariate Statistical Analysis

Multivariate statistical analysis describes the analysis of more than one statistical variable at a time (Liland, 2011) to obtain information about the differences between groups (classes) of samples, which means that it is particularly useful for applying to metabolomics research. Metabolomics experiments produce a very large number of variables (in the case of NMR, bin integrals or metabolite concentrations) compared to the small number of samples. Each of these variables can be considered as representing a dimension in the metabolic space, and therefore an entire spectrum can be thought of as a point in a multidimensional metabolic

hyperspace (Lindon & Nicholson, 2008). A lot of these variables do not relate to the class differences in the experiment and can interfere with the analysis. This produces the need for methods of dimension reduction in order to expose the information most relevant to the experiment.

Dimensionality reduction can be achieved by combining the variables from input data space (*i.e.* the chemical shift bins) with a linear transformation to lower dimensional output space (*i.e.* scores/principal components) (Worley, 2013).

There are two broad categories of model-based methods. Unsupervised methods (such as principal components analysis, hierarchical cluster analysis and nearest-neighbour clustering) require no previous knowledge of the sample characteristics and therefore don't incorporate a hypothesis bias. Supervised methods (for example partial least squares and orthogonal partial least squares analysis) require prior knowledge of expected sample group variation (*i.e.* class membership) (Worley, 2013).

A commonly used statistical analysis program for metabolomics is SIMCA 13 (Umetrics; Umeå, Sweden).

1.4.1. Data Pre-Processing for NMR and Multivariate Statistical Analysis

The following techniques are used to transform the raw data before statistical analysis in order to create fewer data points, reduce the impact of experimental variation and make the spectra more comparable. This vastly improves the ease of model interpretation.

1.4.1.1. Exclusions

It may not be suitable to use the entire NMR spectrum for analysis. There are certain regions which may have to be excluded prior to the steps described below. One region with is often excluded from all spectra is the region around the water peak. This peak varies in intensity primarily due to acquisition parameters, not the sample properties. Unless accounted for through spectroscopic techniques, proteins will appear as large broad peaks. Often these molecules are not specifically investigated, as is the case in this work, and so are excluded so as not to have a confounding effect on the normalisation (described in 1.4.1.4). Because protein signals are especially broad it can be difficult to exclude them from spectra without also excluding areas which contain other metabolites, therefore it is best in this situation to physically remove proteins from samples before spectroscopy is performed. Additional exclusions may be made considering the properties of the samples and the needs of the study.

1.4.1.2. Alignment

Peak positions can vary, by small or large amounts depending on the metabolite, due to a number of factors that can affect chemical environments. These factors include pH and temperature (the effect of pH differences can be mitigated somewhat with the addition of buffer to the samples before NMR analysis). Because of this, comparing unprocessed spectra against one another can result in position differences obscuring the intensity differences that are the object of metabolomic analysis. These position shifts must therefore be either compensated for (see 1.4.1.3 below) or reduced. Position shift reduction can be achieved by certain programs which incorporate peak alignment features (*e.g.* MestReNova Suite NMR Plugin v. 10.0.1). Alignment is often applied one of two ways; globally, whereby the entire spectrum is shifted by a set amount in order to optimise overall peak overlap, or locally, by which individual peaks are selected and shifted relative to the rest of the spectrum. Figure 6 shows the effect of applying local alignment on a peak in overlaid NMR spectra; Figure 5 shows the same peak pre-alignment.

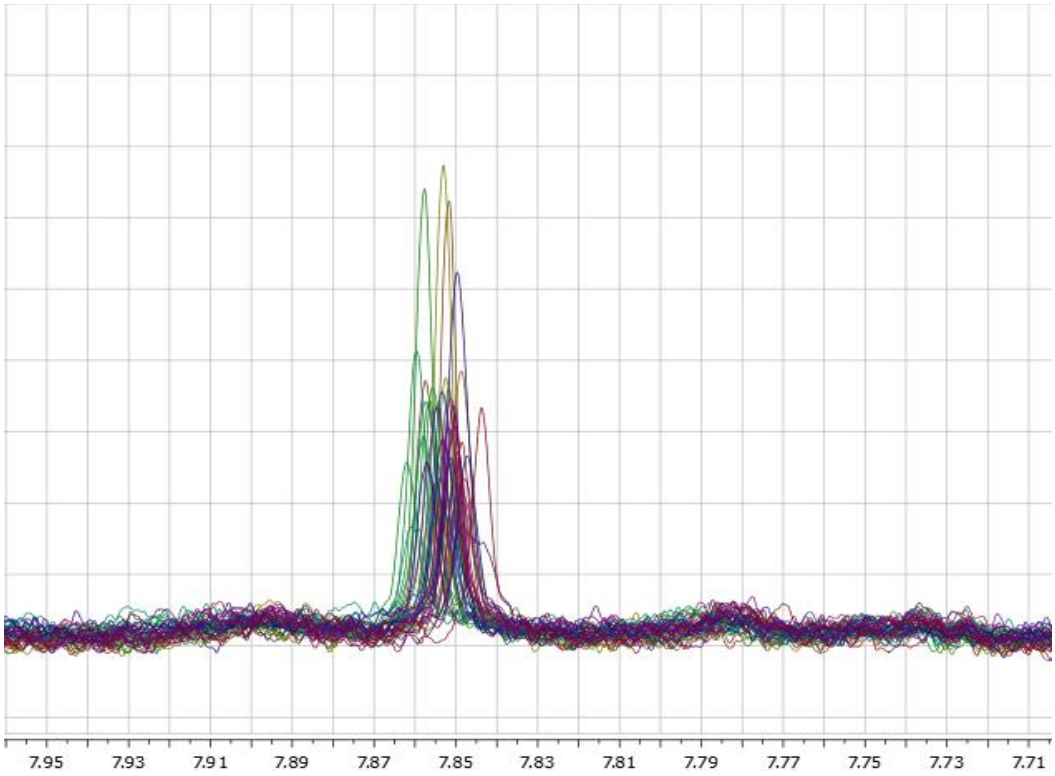

Figure 5 Section of Overlaid Plasma ^{1}H NMR Spectra from 7.71 - 7.95 ppm

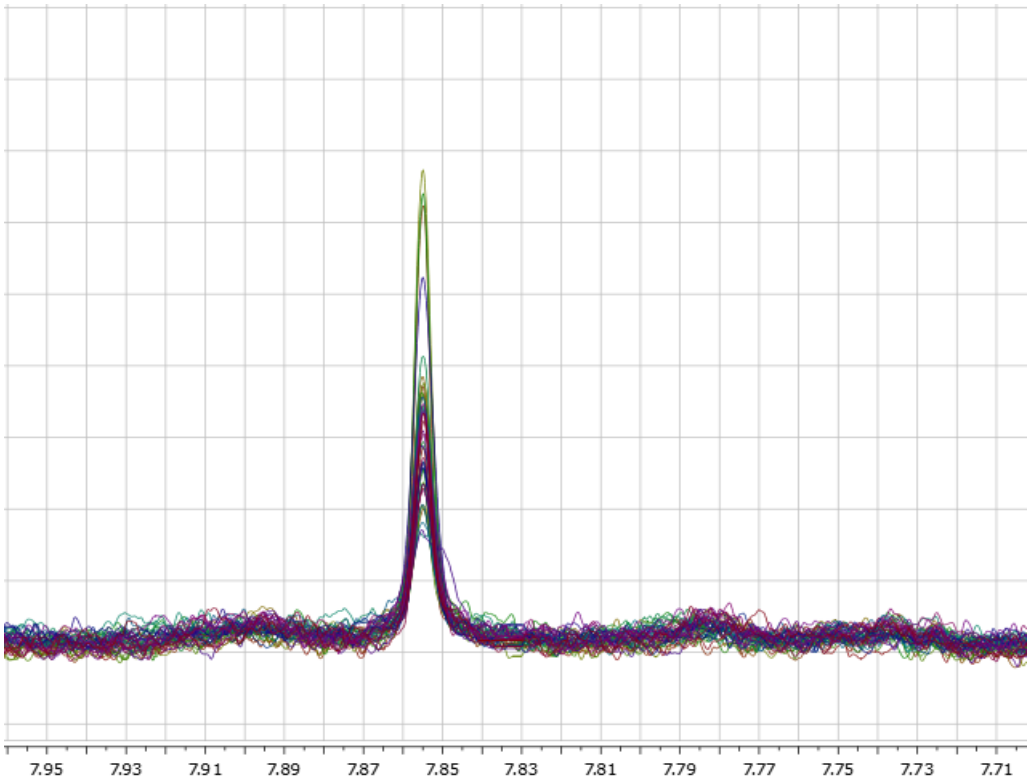

Figure 6 Section of Overlaid Plasma ^{1}H NMR Spectra from 7.71 - 7.95 ppm with Local Alignment

1.4.1.3. Binning

The procedure of binning, alternatively called bucketing, is used to reduce the dimensionality of spectra while also compensating for pH-induced chemical shift differences. Spectra are divided by chemical shift into sections, or "bins", generally of equal width (although some sophisticated software programs can provide variable bin widths), the sum of the intensities of all the individual plot points within that section are then used to give a single variable for each bin. In this way a spectrum containing many thousands of variables (spectral points) may now be represented using only a few hundred (bins). Figure 8 shows the effect binning to 0.04 ppm has on the spectra in Figure 7. It can be seen that while the fine detail of the peak multiplicity has been lost the number of variables in the same section of a spectrum has decreased dramatically.

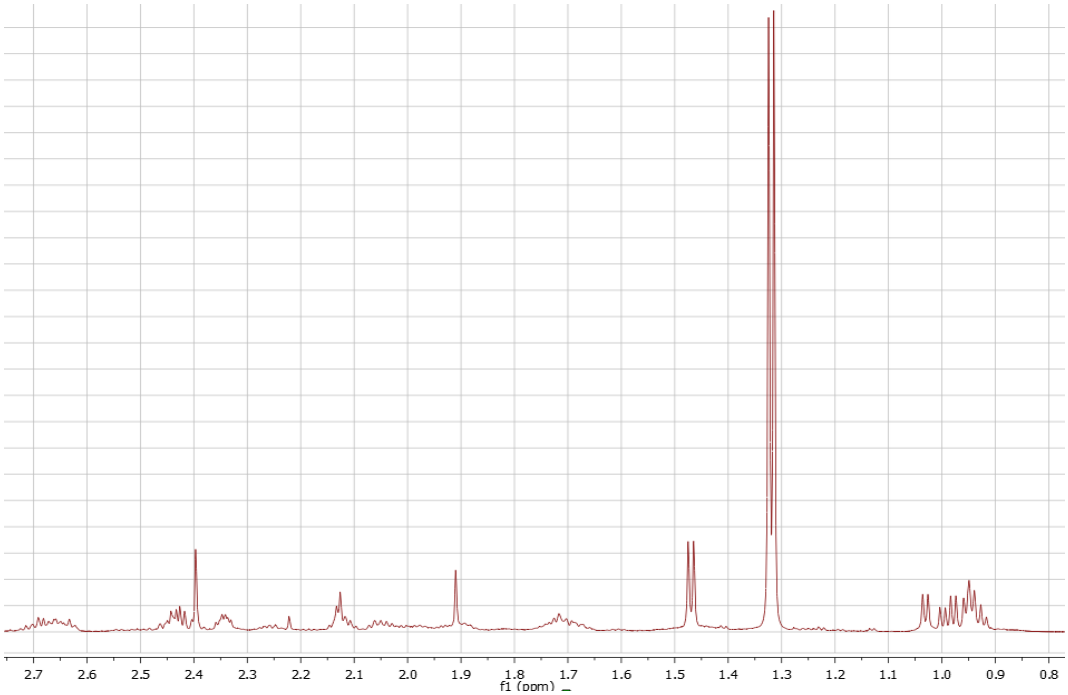

Figure 7 Section of Full Resolution Plasma ^{1}H NMR Spectrum Showing 0.8 - 2.75 ppm

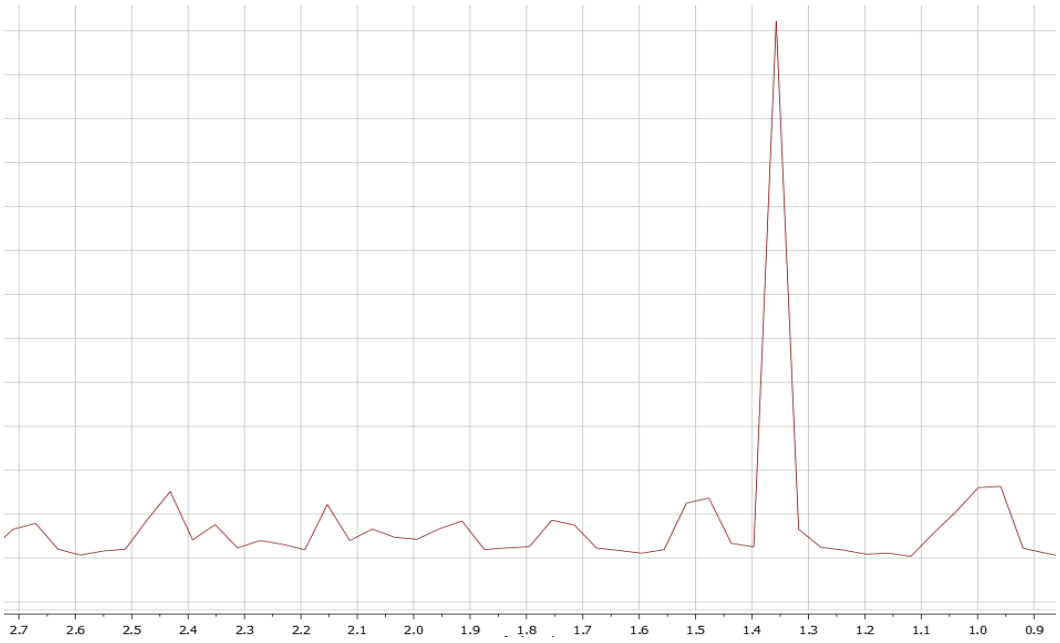

Figure 8 Section of Plasma ^{1}H NMR Spectrum Showing 0.8 - 2.75 ppm Binned to 0.04 ppm

A bin width of 0.04 ppm is predominantly used for metabolomics studies. This value is a good compromise between too narrow bins, where the bin width is insufficient to contain misaligned peaks, and too wide bins, where many peaks may be contained within the same bin and therefore individual peak changes are masked. However, it is important to choose the optimal bin width with consideration to the particular needs of the study. If peaks are very well

aligned and not very wide then narrower bins would be appropriate, while spectra with broad and/or badly aligned peaks would require wider bins.

1.4.1.4. Normalization

Samples for metabolomics studies can, and often do, have differences in their total concentration. More concentrated samples will show higher peak intensities, possibly for all metabolites, than less concentrated samples. Relative changes in metabolites will then be obscured. It is therefore necessary to normalise the spectra in order to apply direct comparison. Normalisation is a process whereby every point in a spectrum is either multiplied or divided by a constant. The most common normalisation methods for metabolomics involve either dividing the intensity of each point by the total sum of intensities over the whole spectrum, by the intensity of the largest peak, or by the area from an internal standard. The third requires a reference chemical of known concentration, while the first assumes that the total concentration of metabolites is approximately constant. Normalisation to total spectral intensity is suitable for most data however very large peaks with highly variable intensities can confound this approach.

1.4.1.5. Scaling

The goal of multivariate analysis for metabolomics is, in part, to find metabolites with systematic quantity differences between two, or more, groups. These differences can be very large, and obvious to the eye, or very small, and difficult to find without the assistance of a statistical program. Even with a sophisticated analysis package it is possible for small, but significant, variation to go unnoticed amidst the larger variation of high abundance metabolites. Often this large variation is unrelated to the biological difference of interest. To mitigate this, a technique known as scaling is employed; this is a process whereby each variable (bin) is divided by a scaling factor which is different for each bin. Initially it is common to subtract the mean of the value of the bin across all samples from the corresponding bin in each spectrum which produces mean-centred data. A variety of scaling factors can then be applied; which factor is chosen depends on the data and the objectives of the study. The two most common scaling methods are described below.

Unit variance (UV) scaling, also known as autoscaling, uses the standard deviation of each bin as the scaling factor; this allows metabolites with small abundance changes to be detected with equal weight as those which vary by a large amount. The principal disadvantage of this method is that it amplifies the influence of noise.

Pareto scaling is similar to UV scaling, but it uses the square root of the standard deviation of each bin as the scaling factor. This leaves the data closer to its original form than UV; it still increases the influence of low abundance metabolites without inflating noise.

It is important to note that the type of data and multivariate analysis method used must be taken into consideration when selecting a scaling method. At times it can be necessary to employ no scaling method.

1.4.2. Principal Components Analysis

PCA is generally the first multivariate analysis method to be performed as it provides an unbiased overview of groupings, trends and outliers within the given data (Trygg et al., 2006).

The process seeks to find the most important dimensions of variability in the data and present this information in plots which allow for simple interpretation. This is achieved by creating a multidimensional space where each variable represents a dimension and each spectrum is represented by a point in this space. The origin of the coordinate system is moved to the average data point and a new variable is created by finding the direction in the data space which describes the greatest variance. This variable is called the first principal component (PC). This step is then repeated, with the condition that each PC is orthogonal to each other PC, until the desired number of components are produced (or the desired level of variability is explained). The requirement for orthogonality means that the components are uncorrelated. Each component may be described as explaining a certain percentage of the variability. For the most part the correlation between the variables is strong enough that only a few components are needed to be calculated to account for the vast majority of the variance.

Two plots are produced to represent the output of PCA.

The scores plot (an example of which is shown in Figure 9) shows the projection of the data onto the new coordinate system, the axes represent the PCs and each spectrum is represented on the plot as a single point. This shows the relationships between the samples (sometimes called objects), if the explained variance is sufficiently high then samples which are close together in this plot share similar properties, and those which are far apart have very different properties.

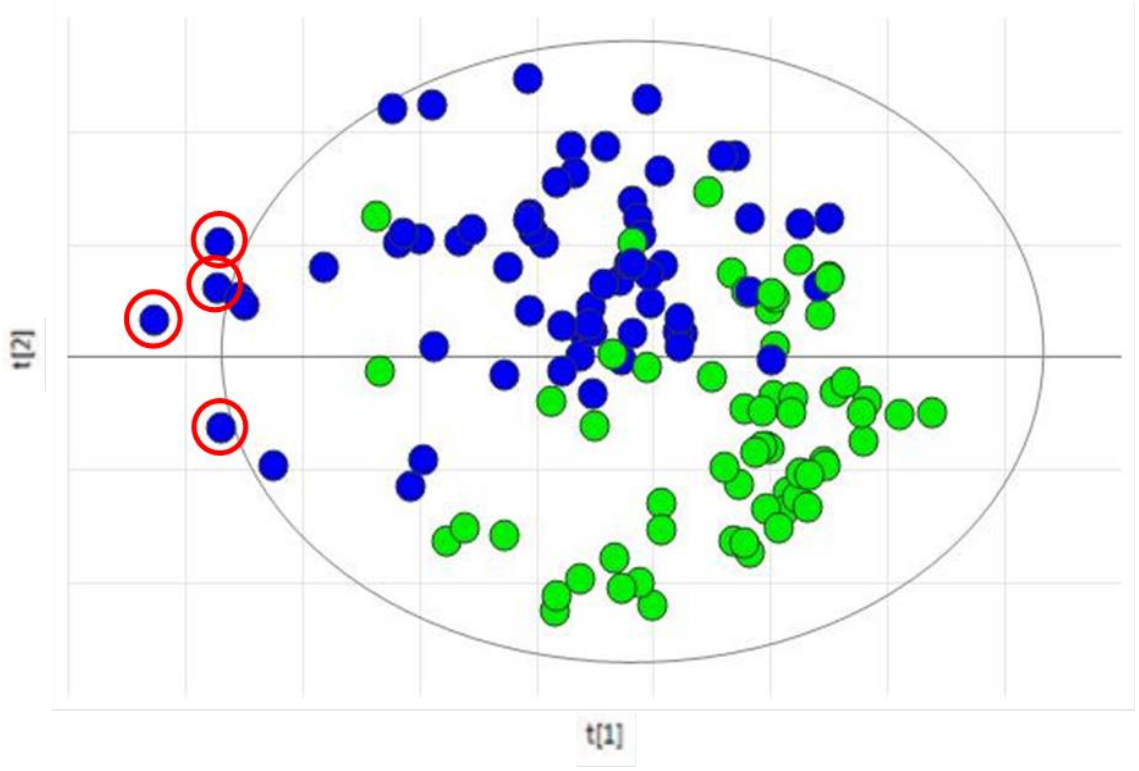

Figure 9 Example of Principal Components Scores Plot, Outliers are Shown Circled in Red

Like the scores plot, the loadings plot (an example of which is shown in Figure 10) has axes representing the PCs however here each point represents a single variable. It therefore shows the relationships between the original variables and the PCs. Each point (variable) has a loading value which shows how much the original variable contributed to the component. A high loading value indicates that the PC is strongly related to the variable. If the model explains a sufficiently large amount of the variance then variables which are close together in the loadings plot have a high correlation, and variables which fall on opposite sides of the plot have a negative correlation.

The scores and loadings plots are used in conjunction, samples which are positioned on a particular side of the scores plot have high values of variables which are located on the same side of the loadings plot. The combination of the scores, loadings and residuals values gives all the variation in the original data set.

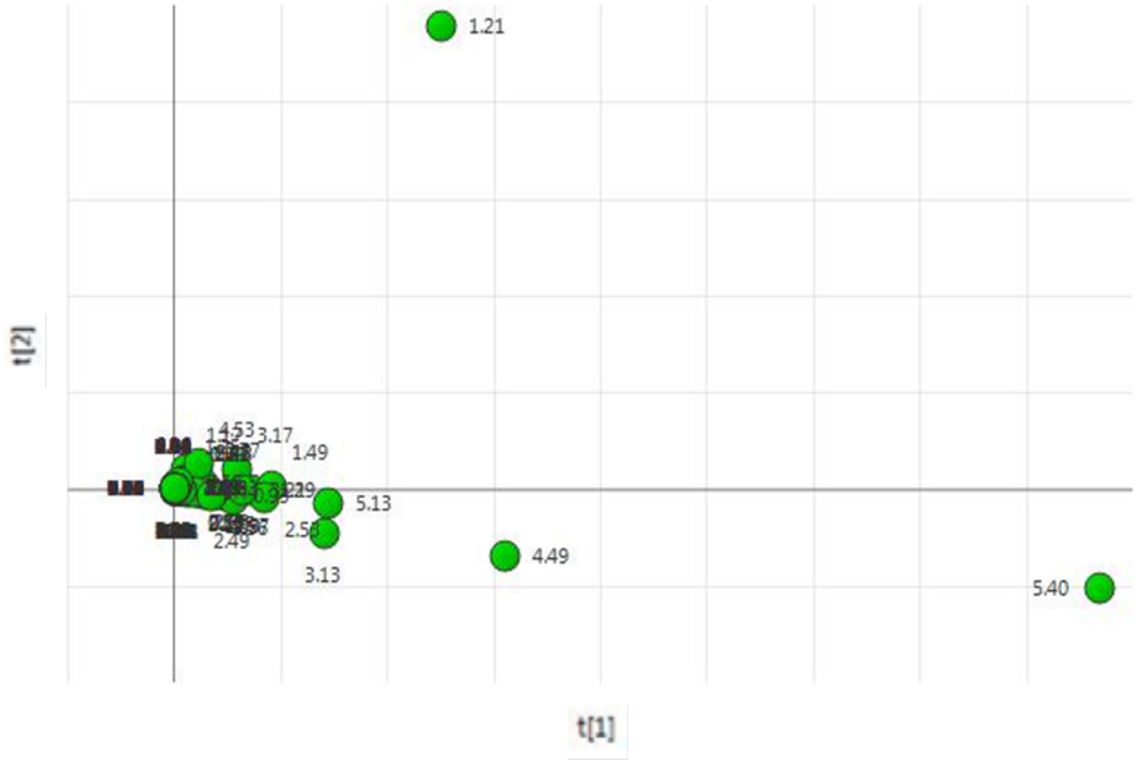

Figure 10 Example of Principal Component Analysis Loadings Plot

While PCA is a very useful analysis tool, it is not frequently used in isolation from the other methods described below. There are a few disadvantages associated with this method, for example the loadings plots are often not easy to interpret and local patterns in the data can be obscured by global patterns (van der Greef & Smilde, 2005).

1.4.3. Outliers

Samples which are positioned far from the other samples in the PCA scores plot are likely candidates to be outliers (Næs, Brockhoff, & Tomic, 2011), especially those which fall outside of the Hotelling's T^2 region (an ellipse on the scores plot which shows the 95% confidence interval of the modelled variation) (Trygg et al., 2006). Figure 9 shows an example of a scores plot with potential outliers (circled in red). However, it is not enough to say that a sample is an outlier based only on this condition. It is necessary to investigate the original spectrum to find reasons which would qualify the sample to be excluded. These could include contaminant compounds, solvent residues, noise overwhelming the metabolite signals, poor water suppression and poor spectral quality. To narrow down the portions of the spectrum which must be examined to find the way in which the candidate outlier differs from the rest of the samples the loadings plot can be used.

1.4.4. Partial Least Squares – Discriminant Analysis

Partial Least Squares or Projections to Latent Structures (Worley, 2013) (PLS) is an extension of PCA in which the principal components are calculated by maximising the covariance of the linear functions of X (*e.g.* the values of the bins) and the response Y (*i.e.* the classes). As with PCA the subsequent latent variables (similar to principal components from PCA) are found from the residuals of the previously calculated component with the condition that each component is independent of all other components (Næs et al., 2011).

PLS-DA is the combination of PLS with discriminant analysis, a technique which results in the discriminant surface being placed in the best position to allow for the greatest separation of the classes (Lindon & Nicholson, 2008). This is achieved by first making the Y variables (classes) into dummy variables (for the most common case of two classes the labels will be set as vectors of length zero and one respectively (Næs et al., 2011)) and then the latent variables are calculated from the relation between the vectors of X and the dummy variables (maximising the between-group covariance matrix (Liland, 2011)).

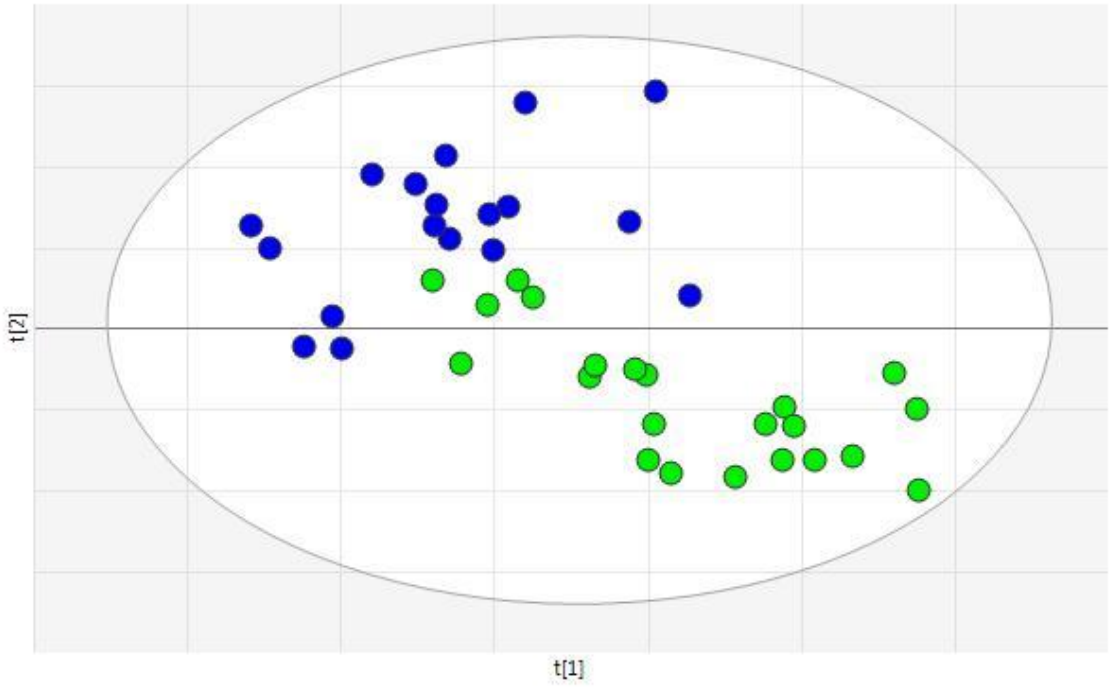

Figure 11 PCA Scores Plot of Plasma Coloured by Year of Collection

Figure 11 is the scores plot of a PCA model of sheep plasma that was collected in two separate years, this same data was used to construct the PLS-DA model whose scores plot is shown in Figure 12. It is clear to see that the PLS-DA model has a scores plot in which the samples are separated by their year of collection, while the PCA model shows some mixing of the samples

and therefore an incomplete separation. Therefore the PLS-DA model is more likely to be able to give accurate information on the metabolites which cause this separation.

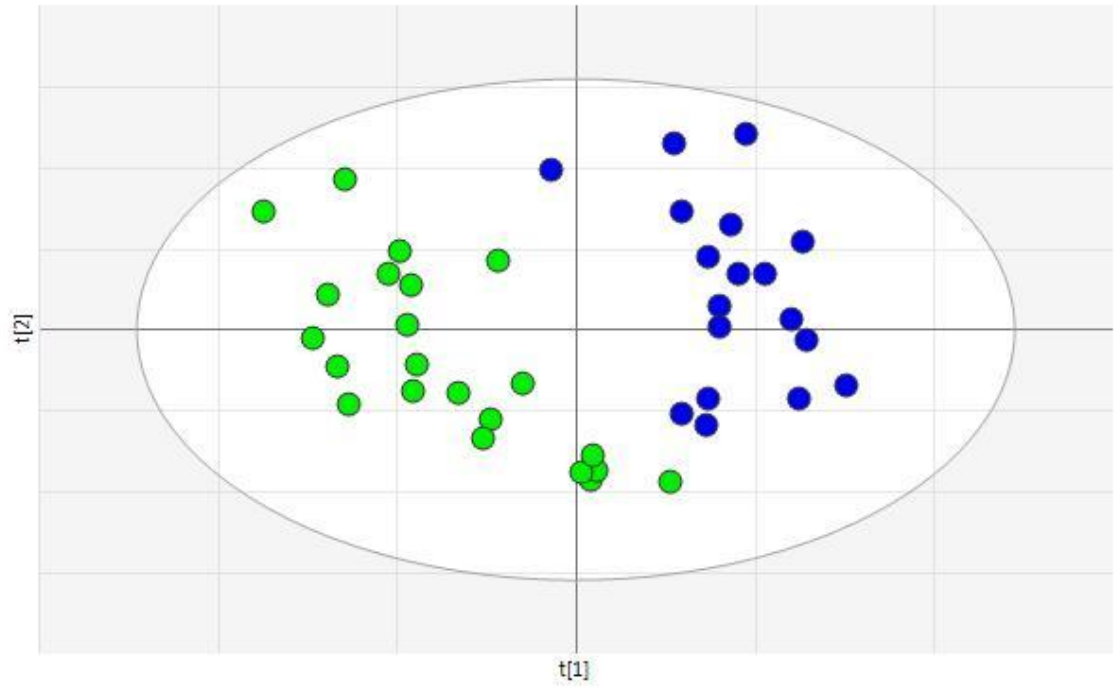

Figure 12 PLS-DA Scores Plot of Plasma Coloured by Year of Collection

1.4.5. Orthogonal Partial Least Squares – Discriminant Analysis

While PLS-DA gives better definition of the classes than PCA, allowing for improved classification of samples, it can be difficult to interpret the scores and loadings plots. This is because the latent variables combine variation correlated with the Y-response (class) with variation which is uncorrelated to Y (Trygg et al., 2006). A technique called OPLS-DA combines Orthogonal Signal Correction (OSC) with PLS-DA (Worley, 2013). This results in all Y-correlated variation being contained in the first component (called the predictive component) while systematic variation uncorrelated to Y is confined to the higher orthogonal principal components (Trygg et al., 2006). This process can be shown to amount to a rotation of the PLS-DA solution (Liland, 2011), which makes it clear that while OPLS-DA increases interpretability it does not improve classification (Savorani et al., 2013). If there is no Y-uncorrelated variation in the data-set then OPLS-DA will produce models identical to PLS-DA (Worley, 2013).

1.4.6. Limitations and Common Pitfalls

Although multivariate statistical methods can be very useful in investigating metabolomic datasets there are a number of issues that must be kept in mind in order to prevent spurious deductions.

As previously mentioned, PLS-DA and OPLS-DA are very powerful analysis tools that are prone to over-fitting models to data. Validation is an absolutely necessary component of any analysis involving these methods. These validation steps are most robust when used on two-class problems (for example diseased and control populations).

Because variation correlated directly with the class differences is the subject of metabolomic analysis it is very important to minimise all other sources of variation and be aware of the effects of those which are still present.

Once metabolites have been identified from these analysis methods it is important to consider them only as candidates for biomarkers until they have been validated against correlations from other sources. Statistical analysis is unable to distinguish between causal effects and indirect correlations.

1.4.7. Validation

A well-known downside to supervised analysis techniques (*i.e.* PLS-DA and OPLS-DA) is their potential to over-fit data to produce unreliable models. How well a model performs is generally based on two parameters – the goodness of fit statistic R^2 (an indication of how much of the variance in the data is explained by the model) and the prediction statistic Q^2 (how well the model will be able to correctly predict the class assignment of new samples). It is possible however, that models with high R^2 and Q^2 values can be formed from samples with randomly assigned group labels. There is no absolute value above which a Q^2 value can be deemed to indicate a statistically valid model (Westerhuis et al., 2008). A sign of possible over-fitting is when a model's R^2 and Q^2 values differ by a large amount (*i.e.* more than 0.2) (Worley, 2013) However, this in itself is not conclusive. It is therefore necessary to validate the reliability of models generated from supervised analysis. Described below is a common validation method.

1.4.7.1. Cross Validation

If all possible latent variables are included in PLS-DA model building, there is a high chance that they will be over-fit. In order to compensate for this SIMCA 13 chooses an appropriate number of significant components for each model through cross validation. There are a number of

cross validation methods with SIMCA 13 utilising 7-fold cross validation. This method consists of initially splitting the dataset into 7 equal subsets, removing one of the subsets and building a model with a set number of components using the other 6 subsets (calibration set). The Y values for all members of the excluded subset are predicted using this model. This procedure is repeated for each subset. The differences between the observed and predicted Y values of the data are used to calculate the Q^2 value for this particular number of components. The number of significant components is chosen by repeating this for an increasing number of components until the Q^2 increase is below a certain threshold set by a particular set of rules.

1.4.7.2. Permutation Testing

Permutation testing can only be applied to PLS-DA models; however the inference of validation can be extended to OPLS-DA models with entirely identical parameters/data. It is for this reason that PLS-DA models were built for each of the OPLS-DA models analysed in this study.

A permutation test consists of a specified number of iterations of creating models where the class labels have been randomly assigned. Often two hundred permutations is considered sufficient, however the more permutations used, the clearer the result can be (Westerhuis et al., 2008). The R^2 and Q^2 values for these models are then plotted against those values calculated from the original model. The class assignments in the permuted models are assumed to have no biological significance, and therefore no inherent differentiation. The y-axis of the graph represents the values of the parameters R^2 and Q^2 while the x-axis represents the degree of similarity of the permuted models to the original model. Regression lines are drawn from the original values as a line of best fit through the permuted values. An example of a permutation plot for a valid model is shown in Figure 13.

The information presented in this plot must be interpreted to discover whether the model can be considered statistically valid. As mentioned above, model over-fit is indicated if the original model's R^2 and Q^2 values differ by more than 0.2. This is because adding latent variables to the model will optimise its parameters and R^2 and Q^2 will increase toward 1, however at a certain point the two values will diverge with Q^2 trending toward 0 as the model becomes over-fitted (Broadhurst & Kell, 2006). Therefore it can be assumed that where the values begin to diverge indicates the optimal parameters for prediction ability. Secondly, all permuted Q^2 values must be lower than the original model's Q^2 value. However for R^2 it is only necessary that the majority of the permuted values are lower than the original value. Additional requirements have been proposed – the y intercept of the Q^2 regression line must be lower than -0.2 and the

R^2 intercept must be lower than 0.3 (Worley, 2013). The example in Figure 13 shows that this model is valid.

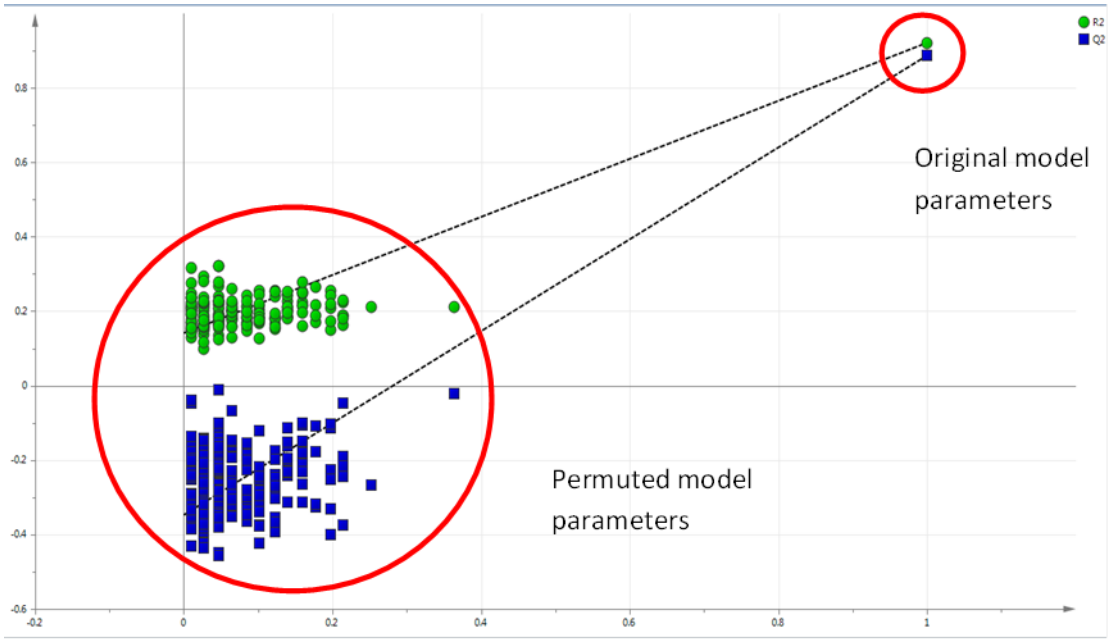

Figure 13 Permutation Test for a Valid PLS-DA Model

1.4.8. Univariate Statistical Analysis

A common analytical technique for determining the variance between two classes of samples is the *t*-Test. This test individually compares the means of the variables of the two classes and establishes the significance of the differences between them. Type I errors (rejection of a true null hypothesis) are a hazard of metabolomic studies where the number of variables (*e.g.* spectral bins) is far greater than the number of samples. The term False Discovery Rate (FDR) is used to describe the number of Type I errors. To counter the FDR it is necessary to apply a suitable correction to the *p*-value (for example a Bonferroni correction which decreases the *p*-value relative to the number of variables) (Broadhurst & Kell, 2006).

1.5. Aims and Objectives

The primary goal of this research is to use multivariate data analysis to find metabolic differences between meat which produces confinement odour and meat which produces either spoilage odour or no odour. An additional goal is to optimise the method of extraction of metabolites from meat and drip.

Chapter Two Materials and Methods

2.1. Evaluation of sample preparation methods

A trial of the available methods for preparation of meat and drip (or drip-like substances) for NMR analysis from the literature was conducted in order to establish the quickest, safest and most convenient polar metabolite extraction protocol. Procedures for homogenising the meat and removing protein and solvent from both sample types were tested.

Meat and drip (from a leg of lamb) were obtained from one of the commercial meat processing plants providing samples for the confinement odour trial. The leg was vacuum-packaged and stored at -1.5°C for 16 weeks before the packaging was opened and the meat and drip sampled. The samples were then stored at -80°C until analysis.

2.1.1. Chemicals

Ultrapure water was obtained from a Milli-Q® system (Millipore, Bedford, MA). Acetonitrile and methanol were purchased from Thermo Fisher Scientific (Auckland, New Zealand) and were of Optimal LC-MS grade. Acetone and chloroform were purchased from BDH Laboratory Supplies (Poole, England). 3-(Trimethylsilyl)-1-propanesulfonic acid sodium salt (DSS) and imidazole were purchased from Sigma. Deuterium oxide (D_2O, 99.5 atom%) was purchased from Cambridge Isotope Laboratories (Tewksbury, MA). Perchloric acid (70%) was purchased from Univar (Auburn, Australia).

2.1.2. Methods for preparation of drip samples

As there is very little in the literature concerning preparation of drip for NMR analysis (Straadt et al., 2011), the methods for the drip methods test were adapted from methods for protein removal from plasma.

2.1.2.1. Centrifugation

400 µL of drip was centrifuged at 20,000 x g for 15 minutes at 4°C, 300 µL of supernatant was then added to 240 µL of phosphate buffer (187.5 mM, pH 7.4) and 60 µL of DSS (5 mM) in D_2O (with 100 µM imidazole).

2.1.2.2. Ultrafiltration

Traces of glycerol were removed from 3,000 MWt cut-off centrifugal filters (Nanosep 3K Omega Microcentrifuge filters; Pall, USA) by soaking in MilliQ water overnight (Chenomx, 2001). The next morning the filters were replaced in their tubes and were centrifuged at 20,000 x *g* for 5 min to remove any excess water. 400 µL of drip was immediately added to the

filters and centrifuged at 20,000 x *g* for 18 hours at 4°C. 300 µL of supernatant was then added to 240 µL of phosphate buffer (187.5 mM, pH 7.4) and 60 µL of DSS (5 mM) in D_2O (with 100 µM imidazole).

2.1.2.3. Solvent

Four solvents or solvent mixtures were used to precipitate protein from the drip samples. The first solvent was acetonitrile, the second methanol, the third a mixture of acetonitrile, methanol and acetone, and the fourth a mixture of methanol and chloroform.

400 µL of drip was added to one of the four ice cold solvents or solvent mixtures in either 1:2 or 1:3 ratios. The drip-solvent mixtures were vortexed for 10 seconds before being left to partition on ice for 10 minutes. The mixtures were then centrifuged at 14,000 rcf for 10 minutes at 4°C. The supernatant was collected and either freeze-dried or speed-vacuumed overnight as described below in 3.1.2.3. The remaining pellet was resuspended in 240 µL of phosphate buffer (187.5 mM, pH 7.4), 60 µL of DSS (5 mM) in D_2O (with 100 µM imidazole) and 300 µL of MilliQ water.

2.1.3. Methods for preparation of meat samples

The protein removal methods adapted for the meat preparation test were not only taken from established procedures on meat but also other forms of tissue (liver, heart and adipose tissue(Atherton et al., 2006)).

Table 1 summarises the different methods applied to the meat samples. A total of sixteen methods were applied with combinations of each of the described components.

Table 1 Method Test Treatment Conditions

	Homogenisation		Solvent Combination				Drying	
Method	Liquid Nitrogen	Bead Beater	Methanol/Chloroform/Water	Methanol/Water	Perchloric Acid	Acetonitrile/Methanol/Acetone	Vacuum Centrifugation	Freeze Drying
1								
2								
3								
4								
5								
6								
7								
8								
9								
10								
11								
12								
13								
14								
15								
16								

2.1.3.1. Homogenisation

Homogenisation of meat samples was achieved through either liquid nitrogen grinding or bead beating. Liquid nitrogen grinding was conducted at AgResearch Ruakura Research Centre Hamilton.

Liquid nitrogen homogenisation

Samples were first frozen and then ground under liquid nitrogen using a SPEX Sample Prep Freezer/Mill 6970 (SPEX; Stanmore, United Kingdom). Once ground the samples were stored at -80°C until weighing. 200 mg of the ground tissue was weighed directly into 2 mL microtubes with screw caps and frozen in liquid nitrogen. The samples were kept frozen at -80°C until solvent extraction at which point they were thawed on ice.

Bead beater homogenisation

For the second homogenisation method 200 mg of tissue was weighed directly into 2 mL microtubes with screw caps (Sarstedt; Nümbrecht, Germany) containing three high density zirconium oxide beads of 1.4-1.7 mm in diameter (Glen Mills Inc.; Clifton NJ, USA) and frozen in liquid nitrogen. The sample was kept frozen at -80°C until solvent extraction. The tissue was thawed on ice prior to extraction and then a solvent mixture was added to the tissue which was homogenised in a Mini-Beadbeater-96 (Biospec Products, Inc.; Bartlesville OK, USA) at 2100 oscillations per minute for four minutes.

2.1.3.2. Solvent Extraction

Methanol/Chloroform/Water

The method of Mannina *et al.* (2008) was followed with slight alterations. 400 µL of ice cold methanol and 85 µL of ice cold MilliQ water were added to 200 mg of either liquid nitrogen ground tissue or unground tissue. The unground tissue was then homogenised in a bead beater as described above. 400 µL ice cold chloroform and an additional 200 µL of ice cold MilliQ water were then added to the homogenised tissue, which was vortexed for 20 seconds. The samples were left on ice for 10 minutes to partition then centrifuged at 16,000 x *g* for 10 minutes at 4°C. The supernatant was transferred to an eppendorf tube and kept on ice until solvent evaporation.

Methanol/Water

The protein precipitation method of Straadt *et al.* (2011) was used for the methanol/water extraction. 1.2 ml of aqueous methanol (v/v, 2:1 methanol to water) was added to 200 mg of liquid nitrogen ground tissue or unground tissue. The unground tissue was homogenised in a

bead beater as described above. The samples were left on ice to partition for 10 minutes and then centrifuged at 16,000 x *g* for 10 minutes at 4°C. The supernatant was transferred to an Eppendorf tube and kept on ice until solvent evaporation.

Perchloric Acid

400 µL of perchloric acid was added to 200 mg of liquid nitrogen ground tissue or unground tissue. The unground tissue was homogenised in a bead beater as described above. The samples were centrifuged at 16,000 x *g* for 10 minutes at 4°C, and then the supernatant was removed to an Eppendorf tube and neutralized to pH 7.8 with KOH (6 M). The samples were centrifuged at 16,000 x g for a further 10 minutes at 4°C, and then the supernatant was transferred to an eppendorf tube and kept on ice until solvent evaporation.

Acetonitrile/Methanol/Acetone

200 µL each of ice cold acetonitrile, methanol and acetone were added to 200 mg of tissue. Unground tissue mixtures were homogenised in the bead beater. All mixtures were then vortexed for 10 seconds and left to partition on ice for 10 minutes. The mixtures were then centrifuged at 14,000 rcf for 10 minutes at 4°C. The supernatant was transferred to an Eppendorf tube and kept on ice until solvent evaporation.

2.1.3.3. Solvent Evaporation

Two solvent evaporation methods were trialled.

Vacuum centrifugation

Samples were centrifuged in a Speed Vac Plus (SC110A) Concentrator (GMI; Minnesota, USA) speed vacuum with a Savant^{TM} Refrigerated Vapor Trap (RVT400) Vapornet (VN100) and Digital Vacuum Gauge (DVG50) (Thermo Scientific) with a vacuum supplied by a VacuuBrand RZ-6 vacuum pump (VacuuBrand; Wertheim, Germany); tube lids were left open and samples were dried for a minimum of 16 hours. The remaining residue was resuspended in 240 µL of phosphate buffer (187.5 mM, pH 7.4), 60 µL of DSS (5 mM) in D_2O (with 100 µM imidazole) and 300 µL of MilliQ water.

Freeze drying

Samples were first frozen in liquid nitrogen for ten minutes and then freeze dried with their tube lids open in a Dura-Dry MP (600-4413) (FTS Systems; Warminster PA, USA) freeze drier for a minimum of 16 hours. The remaining residue was resuspended in 240 µL of phosphate buffer (187.5 mM, pH 7.4), 60 µL of DSS (5 mM) in D_2O (with 100 µM imidazole) and 300 µL of MilliQ water.

2.2. Preparation of confinement odour samples

Ninety meat samples were obtained from two commercial meat processing plants and were stored at various chilled temperatures to simulate the conditions of exported meat during shipment overseas, transport to warehouse, and retail display. The various conditions are shown in Table 2. The number of samples and conditions were chosen to produce meat with a variety of odour types. At the end of the storage time meat and drip samples were collected just after opening the packs and immediately frozen in liquid nitrogen. Some samples did not produce enough drip for analysis. Immediately upon opening the packages the meat was assessed for odour and again ten minutes later, those with persistent odour were classified as having spoilage-related odour, those with an odour upon opening which dissipated before the second assessment were classified as having confinement odour and those which had no odour at either assessment were classified as having no odour. Samples with no odour made up 10% of the samples, while those with confinement odour or spoilage odour made up 22% and 68% respectively.

Table 2 Storage Conditions of Meat Samples and Subsequent Odour Types

	Treatment	No of samples from Plant 1	No of samples from Plant 2	No of Odour Type		
				None	CO	Spoil.
Control 1	11 weeks -1.5°C	5	5	3	1	6
Control 2	12 weeks -1.5°C	5	5	1	4	5
Control 3	13 weeks -1.5°C	5	5	1	0	9
Treatment 1	7 weeks -1.5°C 4 weeks +2.0°C	5	5	2	3	5
Treatment 2	7 weeks -1.5°C 2 weeks +2.0°C 2 weeks +4.0°C	5	5	0	5	5
Treatment 3	7 weeks -1.5°C 5 weeks +2.0°C	5	5	0	5	5
Treatment 4	7 weeks -1.5°C 2 weeks +2.0°C 3 weeks +4.0°C	5	5	2	2	6
Treatment 5	7 weeks -1.5°C 6 weeks +2.0°C	5	5	0	0	10

Treatment 6	7 weeks -1.5°C 2 weeks +2.0°C 4 weeks +4.0°C	5	5	0	0	10

Before preparation all drip and meat samples were separately randomised into six groups using a random number generator (random.org). The order of preparing the groups was also randomized; this was to prevent any possible systematic variation being introduced to the samples which could confound the statistical analysis.

2.2.1. Preparation of drip samples

400 µL of drip was added to 800 µL of a mixture of acetonitrile, methanol and acetone in equal parts. The drip-solvent mixtures were vortexed for 10 seconds before being left to partition on ice for 10 minutes. The mixtures were then centrifuged at 14,000 rcf for 10 minutes at 4°C. The supernatant was collected and kept on ice until solvent evaporation. Samples were vacuum centrifuged, with their tube lids open, for a minimum of 16 hours. The remaining powder was resuspended in 240 µL of phosphate buffer (187.5 mM, pH 7.4), 60 µL of DSS (5 mM) in D_2O (with 100 µM imidazole) and 300 µL of MilliQ water.

2.2.2. Preparation of meat samples

For the sample preparation itself 200 mg of tissue was weighed directly into 2 mL microtubes with screw caps (Sarstedt) containing three beads and frozen in liquid nitrogen. The samples were kept frozen at -80°C overnight. The following day the samples were thawed on ice prior to extraction and 200 µL each ice cold acetonitrile, methanol and acetone were added to each. The tissue was homogenised in the bead beater for 4 minutes at 2100 oscillations per minute. All mixtures were then vortexed for 10 seconds and left to partition on ice for 10 minutes. The homogenates were then centrifuged at 14,000 rcf for 10 minutes at 4°C. The supernatant was removed into an Eppendorf tube and kept on ice until solvent evaporation. Samples were vacuum centrifuged, with their tube lids open, for a minimum of 16 hours. The remaining powder was resuspended in 240 µL of phosphate buffer (187.5 mM, pH 7.4), 60 µL of DSS (5 mM) in D_2O (with 100 µM imidazole) and 300 µL of MilliQ water.

2.3. NMR Analysis

2.3.1. Spectral Acquisition

All spectra were recorded using a Bruker Avance 700 MHz NMR spectrometer (Bruker-Biospin, GmbH, Rheinstetten, Germany) operating at 700.13 MHz equipped with a three channel

inverse detection cryo-probe. The temperature for all measurements was 298 K, which was calibrated using the separation of the residual ^{1}H signals from a standard sample of d4-methanol.

Water suppression was achieved during the recycle delay in all drip and meat experiments, *via* pre-saturation at the water offset frequency (4.71 ppm), using a field strength of 50 Hz. All methods used a recycle delay of 1.50 s at the end of each scan.

1D Nuclear Overhauser Effect Spectroscopy (NOESY) spectra were recorded for all samples. The spectra were recorded using the standard Bruker 'noesygppr1d' pulse sequence, with a spectral width (SW) of 8.33 kHz (11.90 ppm) and 58 k points, with an acquisition time of 3.50 s and averaged for 128 scans with 4 dummy scans. A mixing time of 100 ms was used for all samples.

2D Heteronuclear Single Quantum Coherence (HSQC) spectra were recorded using the standard Bruker 'hsqcetgpsisp2.2' pulse program with a SW of 8.39 kHz (11.98 ppm) in the F2 domain and 2048 points using an acquisition time of 0.12 s, and SW of 2.92 kHz (166.05 ppm) and 512 points in the F1 domain, using an acquisition time of 0.01 s. 44 scans were used for drip preceded with 32 dummy scans and 160 scans for meat preceded with 128 dummy scans.

2D Total Correlation Spectroscopy (TOCSY) spectra were recorded using the standard Bruker 'mlevesgpph' pulse program with a SW of 8.39 kHz (11.98 ppm) in the F2 domain and 4096 points using an acquisition time of 0.24 s, and SW of 8.40 kHz (12.00 ppm) in the F1 domain and 400 points using an acquisition time of 0.02 s. A mixing time of 60 ms was used. 48 scans were used for drip and 128 scans for meat, both were preceded by 256 dummy scans.

2.3.2. Spectral Processing

All 1D NOESY spectra were apodised using an exponential function with line broadening of 1 Hz, and zero filled to 128 k points. All HSQC and TOCSY spectra were apodised using a cosine bell in both dimensions, Fourier transformed, and zero filled to 16k points in the F2 dimension and 256 points in the F1 dimension.

Prior to statistical analysis, all spectra were referenced, phased and baseline corrected using Topspin (version 2.5; Bruker-Biospin, GmbH, Rheinstetten, Germany).

All spectra were referenced to the methyl peak from DSS (0.00 ppm).

2.4. Statistical Analysis

2.4.1. Processing for Metabolomics

The following pre-processing steps were applied to all NMR spectral data in batch mode. The order in which they are presented is the order in which they were performed. Alignment and

region exclusions must be carried out before binning and normalization. Scaling is applied for each model individually so is necessarily performed last.

2.4.1.1. Region exclusions

For every spectrum the area around the water peak (approximately 4.65-5.15 ppm) and the methyl peak from the reference DSS (-0.2-0.2 ppm) were excluded. The size and shape of the water peak has no correlation with the water concentration of the sample and if included will confound the normalization step.

Other region exclusions were applied for each sample type (meat or drip) individually for the initial analysis, and to compare the meat and drip directly the exclusions for each were combined.

The additional exclusions that were made for the drip spectra were 3.33-3.38 ppm (scyllo-inositol), 3.70-3.80 (pH shifted peaks), 5.65-5.88 ppm (urea, too broad for analysis), 6.92-7.15 ppm (pH shifted carnosine peaks), 7.21-7.37 ppm (pH shifted unidentified peaks), 7.43-7.50 ppm (pH shifted unidentified peaks) and 7.62-8.21 ppm (pH shifted carnosine peaks).

The peaks corresponding to the metabolite carnosine are very pH sensitive (15), so much so that buffering the samples was not enough to prevent the shifts mentioned above.

The scyllo-inositol peak at 3.35 ppm was excluded from the drip spectra as it was varying randomly and as one of the largest peaks in the spectrum was adversely affecting the normalisation process.

The additional exclusions that were made for the meat spectra were 7.19-7.30 ppm (pH shifted peaks), 7.60-7.65 ppm (pH shifted peaks) and 7.74-8.21 ppm (pH shifted peaks).

In some cases it is possible to align peaks whose chemical shift varies across a batch of samples, but this is only possible when the sample-to-sample chemical shift variations are small. In the current study, spectral alignment was not possible since the carnosine and unidentified peaks were shifted over such a large chemical shift range and therefore overlapped with unrelated neighbouring peaks.

2.4.1.2. Binning

All spectra were divided into bins of 0.04 ppm width from 10 ppm to -0.5 ppm. Within the bins the data points were summed to provide the new value for the bin.

2.4.1.3. Normalization

All spectra were normalized by dividing the intensity of each point on each spectrum by the total sum of intensities over that whole spectrum.

2.4.1.4. Scaling

All data was mean-centred prior to model building. For PCA, PLS-DA and OPLS-DA models the data was Pareto scaled: every variable was divided by the square root of the standard deviation for that particular variable to help prevent large peaks from obscuring systematic variation in the lower concentration metabolites.

2.4.2. Statistical Analysis Methods

Metabolomics experiments produce a very large number of variables (in the case of NMR, spectral data points) in comparison to their generally low number of samples. These variables each represent a dimension and a large number of them are often superfluous to model building. This produces the need for methods of dimension reduction in order to expose the information most relevant to the experiment. One of these methods is binning (2.4.1.2) but this must be used in concert with the techniques described below in which the variables are combined in order to produce fewer points in lower dimensional space. This improves model interpretation.

All statistical analysis was performed in SIMCA 13 (Umetrics; Umeå, Sweden).

2.4.2.1. Principal Components Analysis

All PCA models were built from Pareto scaled data for exploratory analysis. All samples of interest and the entire recorded NMR spectrum from -0.5-10.00 ppm were included in order to discover outliers and trends within the data.

2.4.2.2. Partial Least Squares – Discriminant Analysis

Data for PLS-DA was Pareto scaled. PLS-DA models were built for two classes at a time (*e.g.* meat and drip, or confinement odour and non-confinement odour). Observations which were found to be outliers based on PCA and spectrum inspection were excluded from PLS-DA analysis.

PLS-DA models were assessed for possible over-fit by permutation testing (2.4.2.6).If the model met the stipulated criteria for this test an OPLS-DA model was built.

2.4.2.3. Orthogonal Partial Least Squares – Discriminant Analysis

OPLS-DA models were built following PLS-DA model validation in order to improve interpretability of the results. These models were built using all the same parameters as the PLS-DA model on which it was based.

2.4.2.4. Univariate Analysis

Data was entered into MetaboAnalyst (Xia, Sinelnikov, Han, & Wishart, 2015) and was processed prior to analysis by normalization to constant sum, log normalization and Pareto scaling. The data was then analysed by t-test and significant bins were chosen based on the criterion of a false discovery rate correction (FDR) value of less than 0.05.

2.4.2.5. PLS-DA Model Validation

A well-known downside to supervised analysis techniques (*i.e.* PLS-DA and OPLS-DA) is their potential to over-fit data to produce unreliable models. It is possible that models with high R^2 and Q^2 values are formed from samples with randomly assigned class labels. There is no absolute value above which a Q^2 value can be deemed to indicate a statistically valid model. A sign of possible over-fitting is when a model's R^2 and Q^2 values differ by a large amount (*i.e.* more than 0.2) (Ritota, Casciani, Failla, & Valentini, 2012) however this in itself is not conclusive. It is therefore necessary to validate the reliability of models generated from supervised analysis. The two validation methods used are described below.

2.4.2.6. Permutation Testing

All PLS-DA models were validated using a permutation test. 999 models, with randomly assigned class labels, were built from the dataset. These 999 models were then compared with the original model with correct class labels. The validity of the original model was judged based on three criteria; the R^2 and Q^2 values had to be within 0.2 units of each other, the Q^2 value had to be larger than all permuted Q^2, and the R^2 value had to be larger than at least half of the permuted R^2.

2.4.3. Metabolite Identification

2.4.3.1. Significant Bins

To identify bins which had a significant influence on the OPLS-DA model's classification ability, and therefore the potential of the associated metabolite as a biomarker for the differentiation of the samples, the following procedure was used:

VIP values

Loading variables have a Variable Importance on Projection (VIP) parameter, which is used to indicate the influence of the variable on the classification of the classes based on the model. If the variable has a VIP value greater than or equal to 1, then it has a greater than average influence on the model classification (Weljie, Bondareva, Zang, & Jirik, 2011). In this study bins with VIP≥1.5 were selected as they were having the greatest influence on the model, and these bins were investigated further (Osorio et al., 2012). Figure 15 shows the selection of only those loadings with VIP≥1.5 from Figure 14.

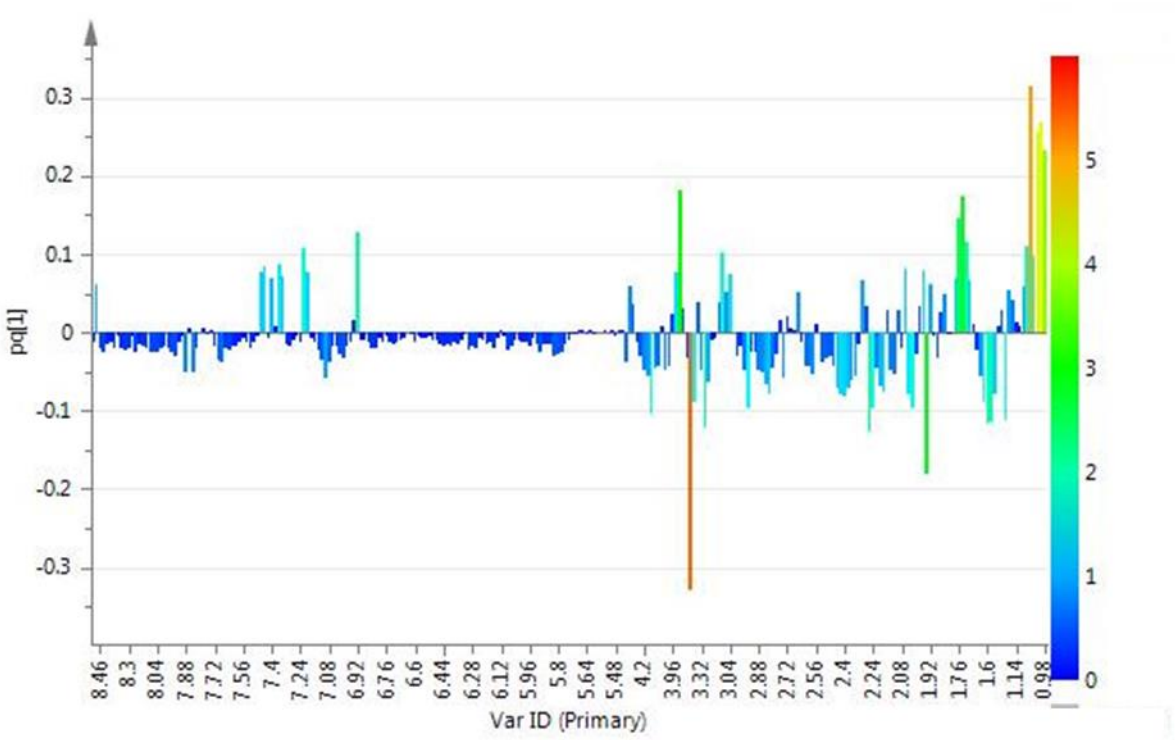

Figure 14 1D Loadings Plot of an Example OPLS-DA Model with Loadings Coloured by VIP Value

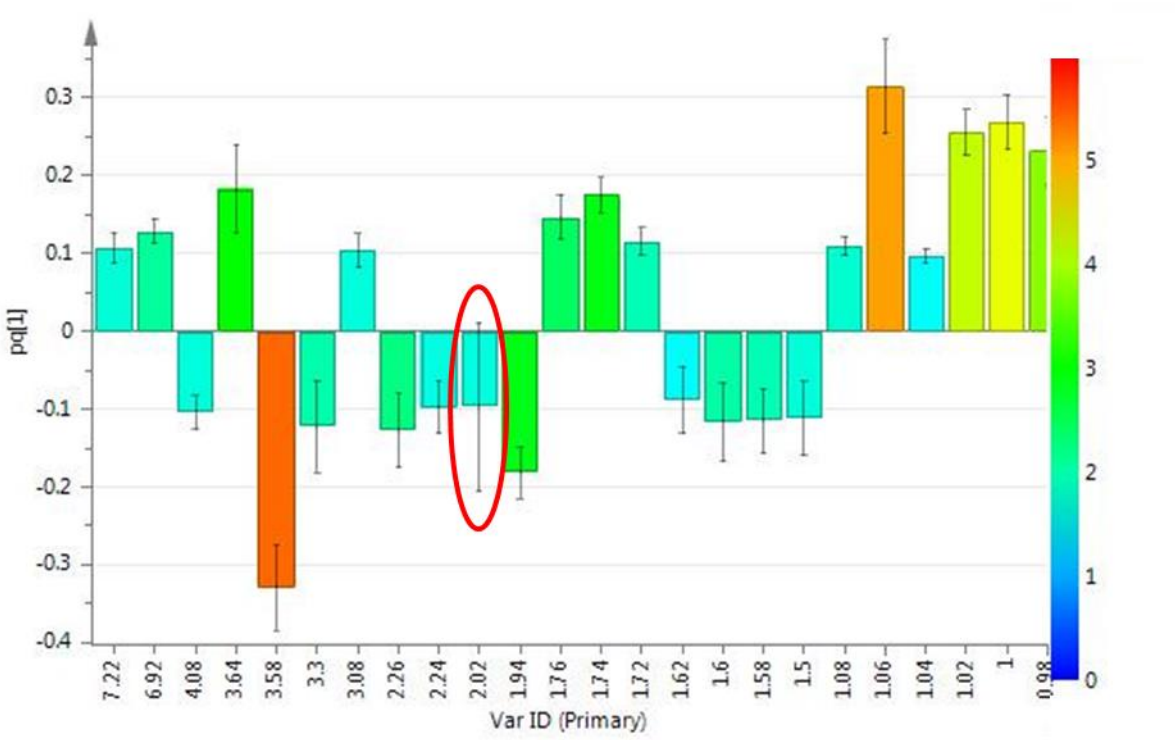

Figure 15 1D Loadings Plot of an Example OPLS-DA Model with Loadings Coloured by VIP Value, Loadings with VIP≥1.5 Shown and a Loading with Error Greater than its Value Circled in Red

Loadings Plot

For OPLS-DA models all the predictive variance is combined into the first principal component, therefore a 1D loadings plot representing only the predictive component shows how much each bin contributes to the predictive ability. The further away the loading is from the axis, the more the bin it represents contributes to class separation. This type of plot also shows the error associated with the loading value. These error measures are calculated by jack-knifing (a method used to find the precision of an estimate) the multiple models calculated from the cross validation of the model (Efron & Gong, 1983). Variables with a VIP value greater than 1.5, but with an associated error that was larger than the size of the loading value, were excluded as a significant bin (circled in red in Figure 15).

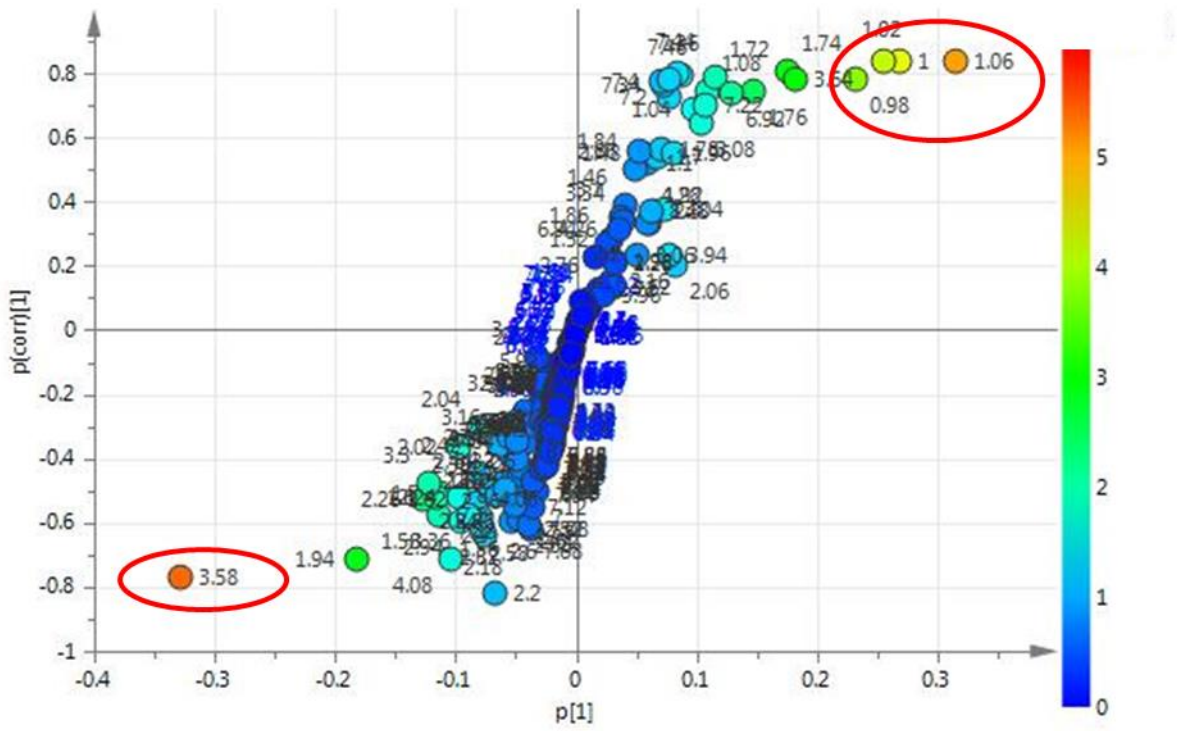

Figure 16 S-Plot From an Example OPLS-DA Model with Loadings Coloured by VIP Value, Loadings Combining High Correlation and High Covariance Shown Circled in Red

S-Plot

The S-plot is another way of representing the loadings of an OPLS-DA model. The x axis represents the covariance of the data, which is the influence of the bin on the predictive component of the model. The y axis represents the correlation, which is a measure of the loading's confidence interval and its reliability for class separation. If the variables have been Pareto scaled the loadings will form an 'S' shape. Those bins which fall on the wings of the S (circled in red in Figure 16) combine high reliability with a strong influence on the class separation; a cut-off of 0.2 for both values is used to determine the position of the wings. Therefore loadings which do not combine these two characteristics will be removed as indicators of potential biomarkers.

2.4.3.2. Metabolite Identification

The list of bins that are significantly different between the two classes was examined further. The areas of the spectrum represented by these bins were examined in order to determine which metabolite peaks they contain. For each significant bin the chemical shift and multiplicity of the peak(s) within that area were noted, for some of the bins multiple peaks were present and all of these were noted. Metabolites which were identified from bins with many peaks in them were noted and only considered to be candidates for biomarkers if they also had peaks which were isolated in other bins. Those that didn't fulfil this criterion were not

considered further because it could not be established that it was their concentration which was significantly different between the groups. It can only be shown that the concentrations of all the metabolites represented by the peaks within the bin added together to have an influence on model building.

Metabolites were tentatively annotated through spectrum inspection by comparing the chemical shift value and peak multiplicity information against a list of metabolites previously identified from human blood plasma and beef tissue. However, this information does not completely confirm a peak's identity as a number of metabolites can have peaks with the same multiplicity in approximately the same chemical shift position, and the chemical shift values of peaks can differ by small amounts when pure compared to in a mixed solution.

Peak identities, and thereby metabolite identities, were confirmed by comparing the HSQC spectrum of the pooled meat or drip samples with the HSQC spectrum of the pure metabolite of interest from one of two online databases (Ulrich et al., 2008; Wishart et al., 2013). The combination of the proton and carbon chemical shift values for each peak gives more certainty to the identification than the proton chemical shift values alone.

Chapter Three Results and Discussion

3.1. Evaluation of Sample Preparation Methods

The tested sample preparation methods were judged on five criteria. First, the ease with which the samples could be prepared using the method. This was an important consideration due to the large number of samples that would need to be prepared in a short time frame. The second, the risk of metabolism occurring. Between the time the samples are defrosted and the acquisition of their spectra any additional metabolism of their constituents will result in an inaccurate picture of the metabolism effects of storage. The third criterion concerns the extent of protein removal. The importance of protein removal is twofold – broad peaks in NOESY spectra (Figure 17) belonging to proteins obscure the presence of the lower molecular weight metabolites that are the focus of this study, and proteins will generate unwanted metabolism within the sample.

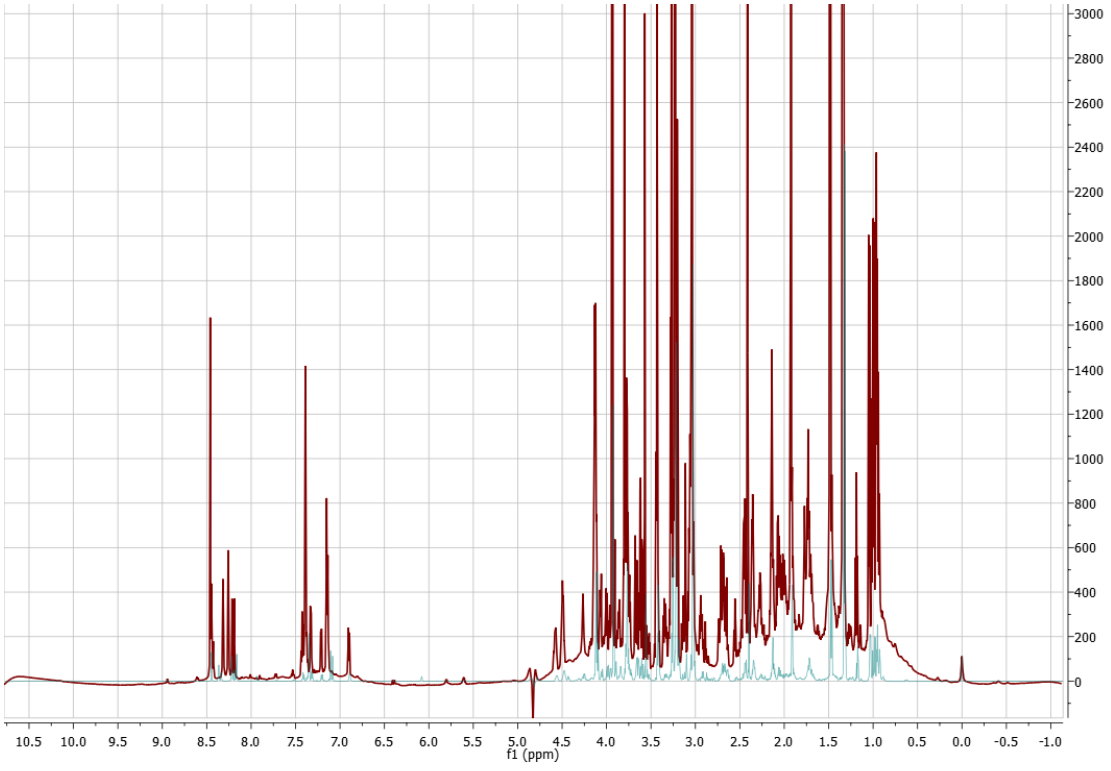

Figure 17 Overlaid ^{1}H NMR Spectra of Drip with Protein Removed by Centrifugation (Dark Red) or Ultrafiltration (Light Blue)

The next criterion is reproducibility; the method must provide consistent results across technical replicates as this suggests that unwanted variation is not introduced at this stage. This was judged both by visual inspection of the spectra and by performing PCA analysis on all samples produced by these methods in order to assess the variation within each method

(Figure 22). The fifth is spectral quality. The spectra were judged on their signal to noise ratio, degree of water suppression, angle of baseline and the peak resolution achieved. The last criteria by which the samples will be judged is stability. Spectra of the samples were taken, under identical conditions and settings, one month after the initial acquisition date. The spectra were then overlaid and any changes were noted.

3.1.1. Methods for Preparation of Drip Samples

3.1.1.1. Centrifugation

The centrifugation method scored very highly in terms of ease; this method had the lowest amount of sample handling and was by far the fastest from unprocessed drip to NMR ready samples. This method was the best at limiting the overall time it would take to process all the samples so reduced the variability caused by processing time. This aspect of the method also means that the risk of metabolism is very low; there is little time for further metabolism to occur when the protein is removed from the sample so quickly. However, upon inspection of the resulting 1D NOESY spectra (Figure 17), it is clear that significant amounts of protein remain in the sample after centrifugation in comparison with a sample where protein was removed by ultrafiltration. This is demonstrated by the large, broad signals upon which the sharp, low molecular weight metabolite signals are shown.

A CPMG spectrum (Figure 18) shows that the protein can be sufficiently edited out by the pulse programme to allow for unhindered analysis; however the presence of protein in the sample likely leads to unwanted further metabolism.

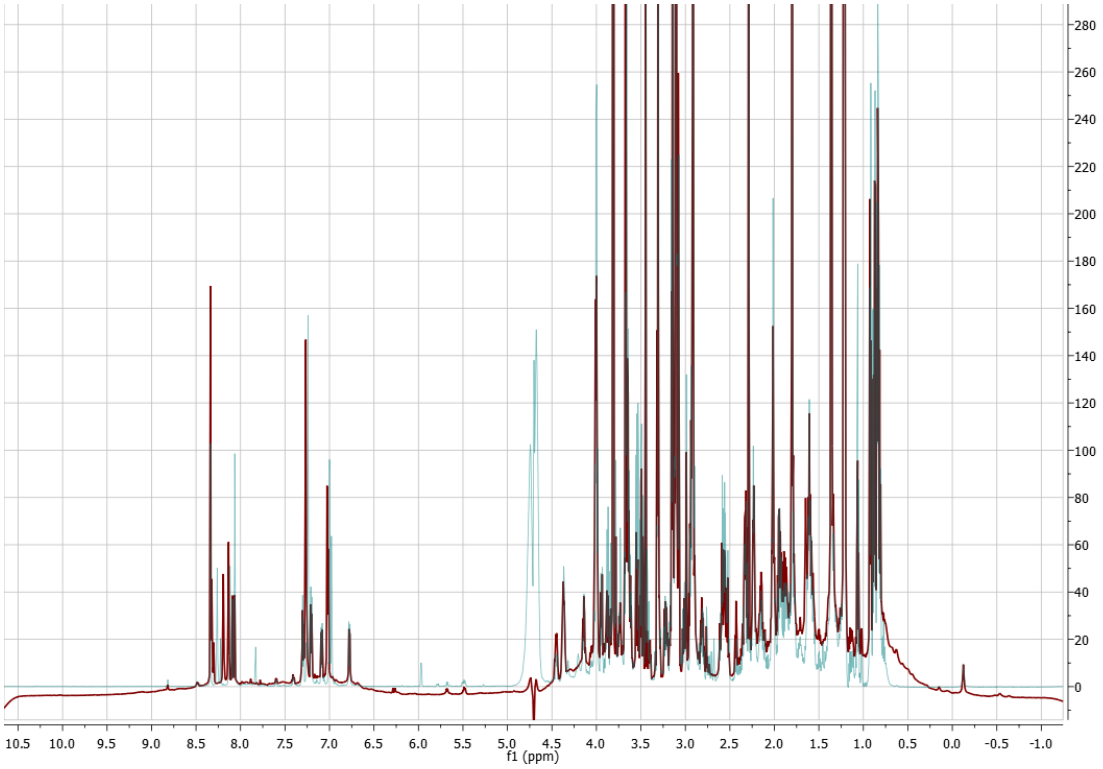

Figure 18 Overlaid ^{1}H CPMG (Dark Red) and NOESY (Light Blue) NMR Spectra of a Drip Sample with Protein Removed by Centrifugation

Technical replicates of processed samples show a high level of variability (Figure 19); many peaks are shifted over the higher ppm range and many peaks which are shifted, or have a high intensity, show variable intensities between replicates. The samples only show a slight spread in the PCA scores plot of all methods (coloured bright red in Figure 22) but they all vary significantly compared to samples from all other methods. This is a very significant issue as it suggests that the spectra cannot convey sufficiently accurate information regarding the quantities of metabolites in the original sample. The dark blue spectrum in Figure 19 has broader peaks than the other two spectra; this suggests that some of metabolites may have still been bound to protein remaining in the sample.

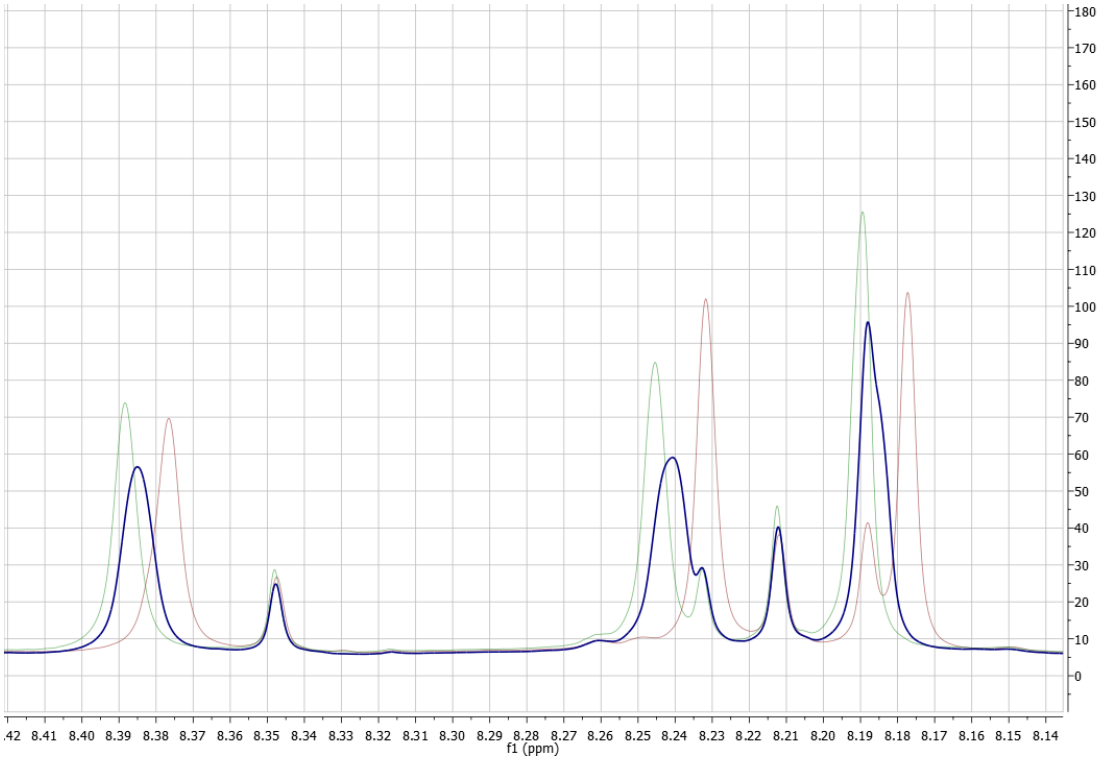

Figure 19 Overlaid Spectra of Three Experimental Replicates of Drip with Protein Removed by Centrifugation (Enlargement of Section 8.14 - 8.42 ppm)

Water suppression was generally good in the spectra however the spectra would only be useful for metabolomics if acquired using a CPMG pulse sequence. The spectral quality was therefore rated to be neither excellent nor poor.

3.1.1.2. Ultrafiltration

This method scored just after the centrifugation method in terms of ease. Only two additional steps differentiate it from the centrifugation method; however these steps are simple and require little hands-on time. Because of their buoyancy it was difficult to immerse all the filters in their soaking step, but once this was accomplished they were simply left overnight. Although the filtration was carried out at 4°C, the time (approx. 18 hr) it takes for the proteins to be filtered from the drip means that there is an increased risk of additional metabolism occurring during the filtration. The resulting NOESY spectra showed no discernible protein signals following filtration (Figure 17); so, unlike with the centrifugation method, it is expected that the samples will show a good level of stability. Of the three technical replicates two showed excellent comparability while one showed a few peak shifts and intensity differences when compared to the other two (Figure 20). Inspection of the PCA scores plot with samples from all methods (coloured yellow in Figure 22) shows that this technique produced very similar profiles across technical replicates. This was a vast improvement over the

centrifugation method but was not the most reproducible of all the techniques. Due to the absence of protein signals in the NOESY spectrum and the good water suppression the spectral quality obtainable from these samples was classified as very high.

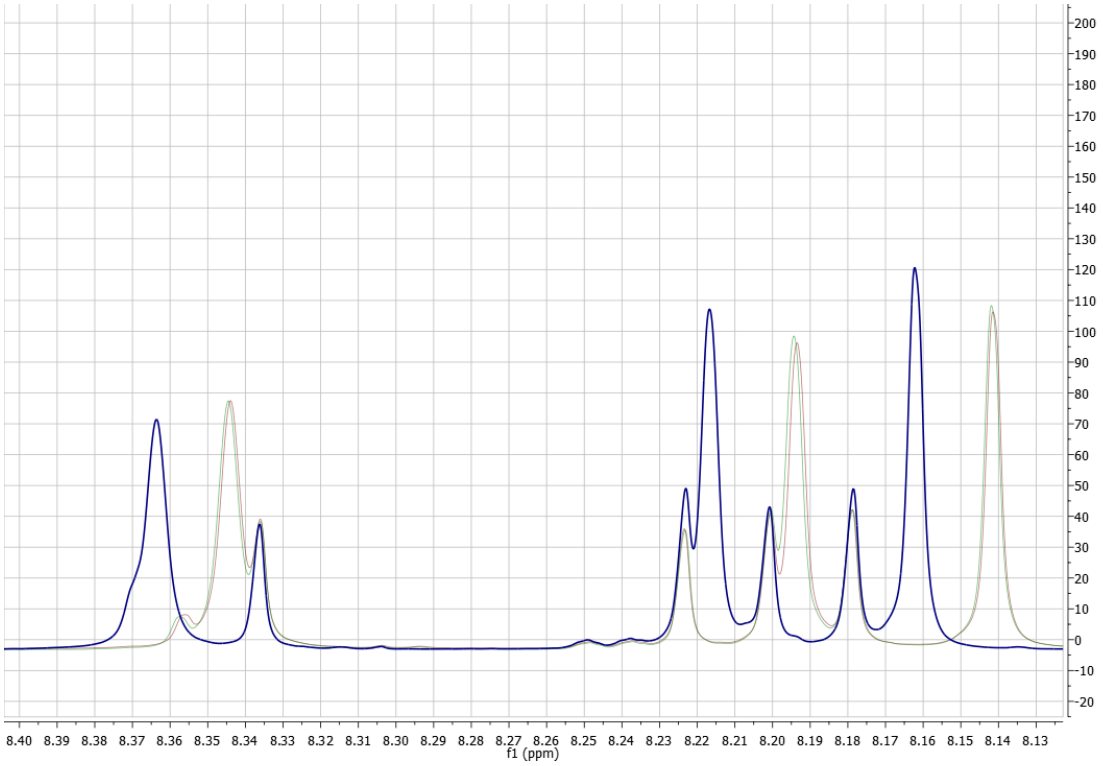

Figure 20 Overlaid Spectra of three Experimental Replicates of Drip with Protein Removed by Ultrafiltration (Enlargement of Section 8.13 - 8.40 ppm)

3.1.1.3. Solvent Protein Precipitation

Solvents were added in a ratio of either 2:1 or 3:1 (v/v) with the sample. Very little difference in protein removal efficiency was found between the ratios.

Acetonitrile

This method involved more hands-on time than either of the previous two. The large number of samples involved in the study would require considerably more time to be processed using this method. The risk of further metabolism was very low; the protein precipitated out immediately upon contact with the solvent. Protein removal was confirmed in the resulting spectra which showed very little protein signal. The technical replicates showed differences in peak height and position across the spectrum; this indicated poor reproducibility using this method. The PCA scores plot with samples from all methods showed three of these replicates (coloured green in Figure 22) clustered well, while the rest were slightly spread out. The spectral quality was also considered low as the water suppression was poor and the phasing and baseline correction was difficult.

Methanol

The time involvement of this method was on par with that of the acetonitrile method, as the only difference between the two was the type of solvent. This similarity also applied to the risk of further metabolism in the sample. The NOESY spectra confirmed that there was a very limited amount of protein present in the processed samples. However the reproducibility was judged to be very poor as there were extensive chemical shift and peak intensity differences between the replicates. The PCA scores plot of samples from all methods showed agreement with the visual inspection as the replicates (coloured turquoise in Figure 22) were very spread out compared to the other methods. The spectral quality was greater than the previous method as good water suppression was achieved and the spectra required little processing.

Acetonitrile/Methanol/Acetone

This method required one more step than the other solvent protein precipitation methods, namely mixing the solvents in the appropriate ratio before adding them to the sample. For a large number of samples this step involves a negligible addition to the total preparation time. The risk of further metabolism was comparable to the two other solvent protein precipitation methods; the NOESY spectra showed only a very low level of protein remains in the samples after processing. The spectra also showed good reproducibility for two of the technical replicates; however one of the replicates showed lower intensities of some peaks and pH shifts in others. The PCA scores plot of samples from all the methods showed that the technical replicates for this method (coloured in blue in Figure 22) were mostly well clustered with a few varying more. The spectra also revealed good water suppression in the samples and required very little processing.

Applying Meat Solvent Method – Methanol/Chloroform/Water

In order to be able to directly compare the results of the analysis of both sample types, meat and drip, it was decided that the best of the methods applied to each type of sample would be applied to the other. Therefore the methanol/chloroform/water protein precipitation method was applied to the drip. This method had the same number of steps as the acetonitrile/methanol/acetone method and so was comparable in terms of ease and time for preparation. The risk of metabolism was the same as all other solvent protein precipitation methods. The NOESY spectra showed only very little protein remained in the sample, and the reproducibility of the technical replicates was high. Inspection of the PCA scores plot of the samples from all methods agreed with the visual inspection of the spectra as the replicates (coloured in orange in Figure 22) were seen to group well. There was some shifting of pH sensitive peaks between replicates at about the same level as the

acetonitrile/methanol/acetone treated samples. Some spectral processing was required to adjust the baseline and phasing of the spectra.

3.1.2. Methods for Preparation of Meat Samples

3.1.2.1. Homogenisation

Liquid Nitrogen

The homogenisation itself was performed by AgResearch's Ruakura site from which the samples originated. The grinding was done at low temperature which prevented metabolism in the sample. Although the procedure was straightforward only two samples could be prepared at a time. The tissue needed only to be thawed and weighed before metabolite extraction was carried out, however it proved to be difficult to weigh out such small amounts of sample as the meat thawed and clumped incredibly quickly during weighing. Thawing occurred too quickly even using a liquid nitrogen-cooled spatula. This may then have led to further metabolism due to the increased time the meat was at or near room temperature.

Inspection of the spectra showed that the spectral quality and reproducibility were slightly lower than the method using the bead beater.

Bead Beater

Weighing out solid meat was much easier than pre-ground meat, and did not lead to as much thawing occurring. Some optimisation of bead size and beating time was necessary to obtain a fully homogenised sample without the sample temperature rising above room temperature or the tubes shattering. Homogenisation using three 3 mm beads and beating for 4 minutes was found to be optimal. Bead beating had the advantage that many samples could be prepared at once (approx. 30).

Inspection of the spectra showed that this method provides a high spectral quality and good reproducibility.

3.1.2.2. Solvent Protein Precipitation

Methanol/Chloroform/Water

This was the only method which required two solvent addition steps, for a large number of samples this could increase the hands on work significantly. The second addition also entailed that the samples were vortexed, which was time consuming. The risk of further metabolism using this method was very low; the protein precipitated out immediately upon contact with the solvent mixture. Spectral inspection showed that there was very good agreement between

the technical replicates, however there was still a small amount of shifting of pH sensitive peaks. Inspection of the PCA scores plot with samples from all methods agreed with the visual inspection of the spectra as the replicates (coloured in orange in Figure 22) were well clustered. The water suppression and protein removal were both good and spectral processing was easy.

Methanol/Water

This method scored the highest in terms of ease. The single solvent addition step minimised the hands on time needed and did not require the samples to be vortexed. The risk of further metabolism was very low; the protein precipitated out immediately upon contact with the solvent. Inspection of the spectra showed that the technical replicates were not quite as similar as those from the methanol/chloroform/water method. Inspection of the PCA scores plot with samples from all methods suggested that the technical replicates (coloured purple in Figure 22) may have been more similar to each other than the replicates of the methanol/chloroform/water method. The water suppression and protein removal were both good and the spectral processing was easy.

Perchloric Acid

While this method only required one solvent addition step it scored the lowest of the three methods in terms of ease and spectral quality. Perchloric acid was the most hazardous solvent used for protein precipitation. It is highly corrosive and may evolve chlorine or hydrogen chloride gas. All steps involved in the preparation, excepting the pH adjustment, were carried out in a fume hood. The pH adjustment step was the most difficult as the volume of KOH needed was different for each sample and had to be accurate down to the drop. This step would be prohibitively time consuming for a large number of samples. Some of the samples prepared with this method had a salt concentration that was too high to acquire NMR spectra. Those samples which could be analysed by NMR had spectra which were difficult to accurately phase and baseline correct. However, the water suppression and protein removal were both good. In the high ppm range there were a lot of peaks which were shifted due to pH differences between the samples, and many of those peaks had widely varying heights (Figure 21). Inspection of the PCA scores plot with samples from all methods showed that the technical replicates (coloured dark red in Figure 22) varied a lot.

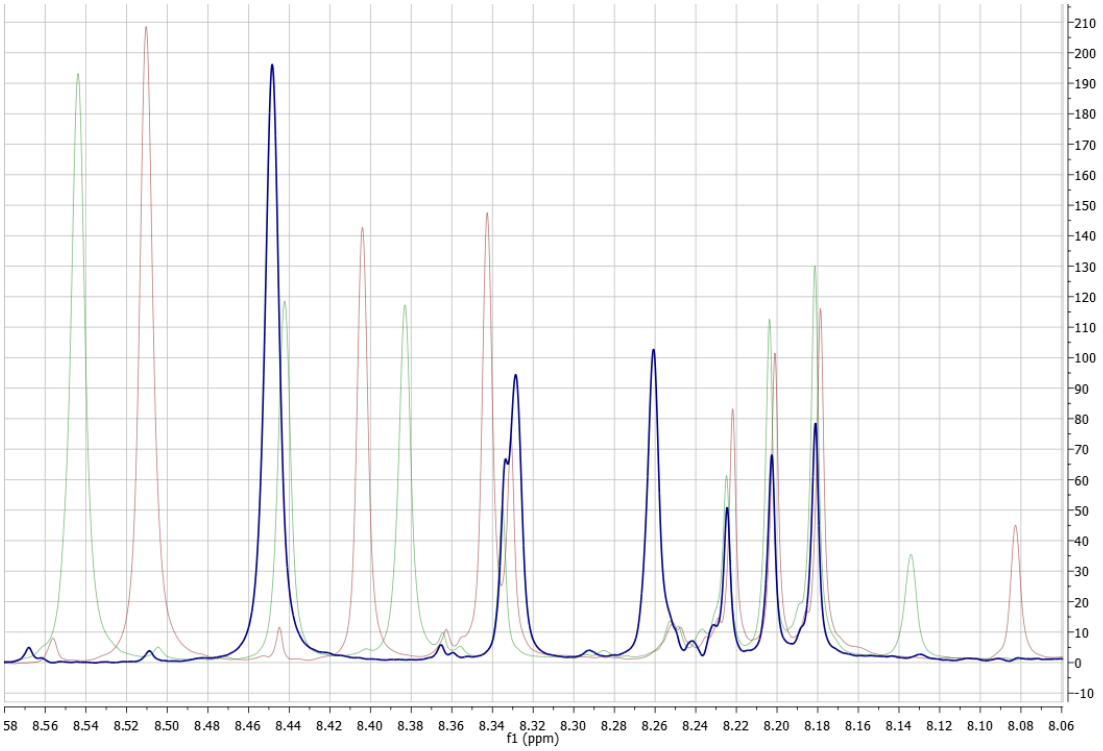

Figure 21 Overlaid Spectra of Three Experimental Replicates of Meat with Protein Removed by Perchloric Acid Solvent Precipitation (Enlargement of Section 8.06 - 8.58 ppm)

Applying Drip Solvent Method – Acetonitrile/Methanol/Acetone

The acetonitrile/methanol/acetone method used previously on drip was applied to meat samples. This method was comparable with the methanol/water method in terms of ease, and like the other protein precipitation methods applied had a very low risk of further metabolism. The technical replicates showed very good agreement, and the spectral quality was comparable to the methanol/chloroform/water method. The water suppression and protein removal was good, and the spectral processing was easy.

3.1.2.3. Solvent Evaporation

The two evaporation methods were not judged by the criterion of metabolism risk as both required equal amounts of time to evaporate the solvent. In addition, protein removal was not assessed as this was not the aim of solvent evaporation. Both methods required approximately 18 hrs.

Vacuum Centrifugation

This evaporation method was by far the easiest and required the least hands on lab work of the two. After solvent extraction the lids of the sample tubes were simply opened and the tubes were placed in the speed vacuum. This method allowed for a maximum of 72 samples to be run at a time.

Inspection of the spectra showed that this method produced highly similar technical replicates and was therefore judged to have a good level of reproducibility. The spectral quality was also judged to be very high with good water suppression and easy spectral processing.

Freeze Drying

This method required more time and effort than the first; it was found that the sample tubes needed to be stored upright in the vacuum vessel in order to prevent loss of sample. This required a sample rack which was difficult to fit into the cylindrical vessel. For the full scale trial this would necessitate splitting the samples into batches as a vessel large enough to hold ninety samples was not available.

Examination of the spectra showed that the reproducibility and spectral quality of this method was slightly lower than the vacuum centrifugation method.

3.1.3. Chosen Sample Preparation Methods

Meat Homogenisation

For meat homogenisation the bead beater method was chosen as it was clearly quicker and easier to weigh out whole meat compared to meat pre-ground with liquid nitrogen. The spectral quality was also slightly better than the alternative homogenisation method.

Protein Precipitation

The acetonitrile/methanol/acetone protein precipitation method was chosen for the full confinement odour trial. This method produced very similar results to the methanol/chloroform/water method but was chosen as it was slightly safer and more consistent across both drip and meat samples.

Solvent Evaporation

The vacuum centrifugation method was chosen for solvent evaporation as loading and unloading the samples was by far simpler and faster than for freeze drying; considering the number of samples which constituted the full confinement odour trial this was a very important factor. Across all preparation methods spectra from samples which were vacuum centrifuged, whether meat or drip, showed less shifting of pH sensitive peaks and a higher reproducibility between technical replicates.

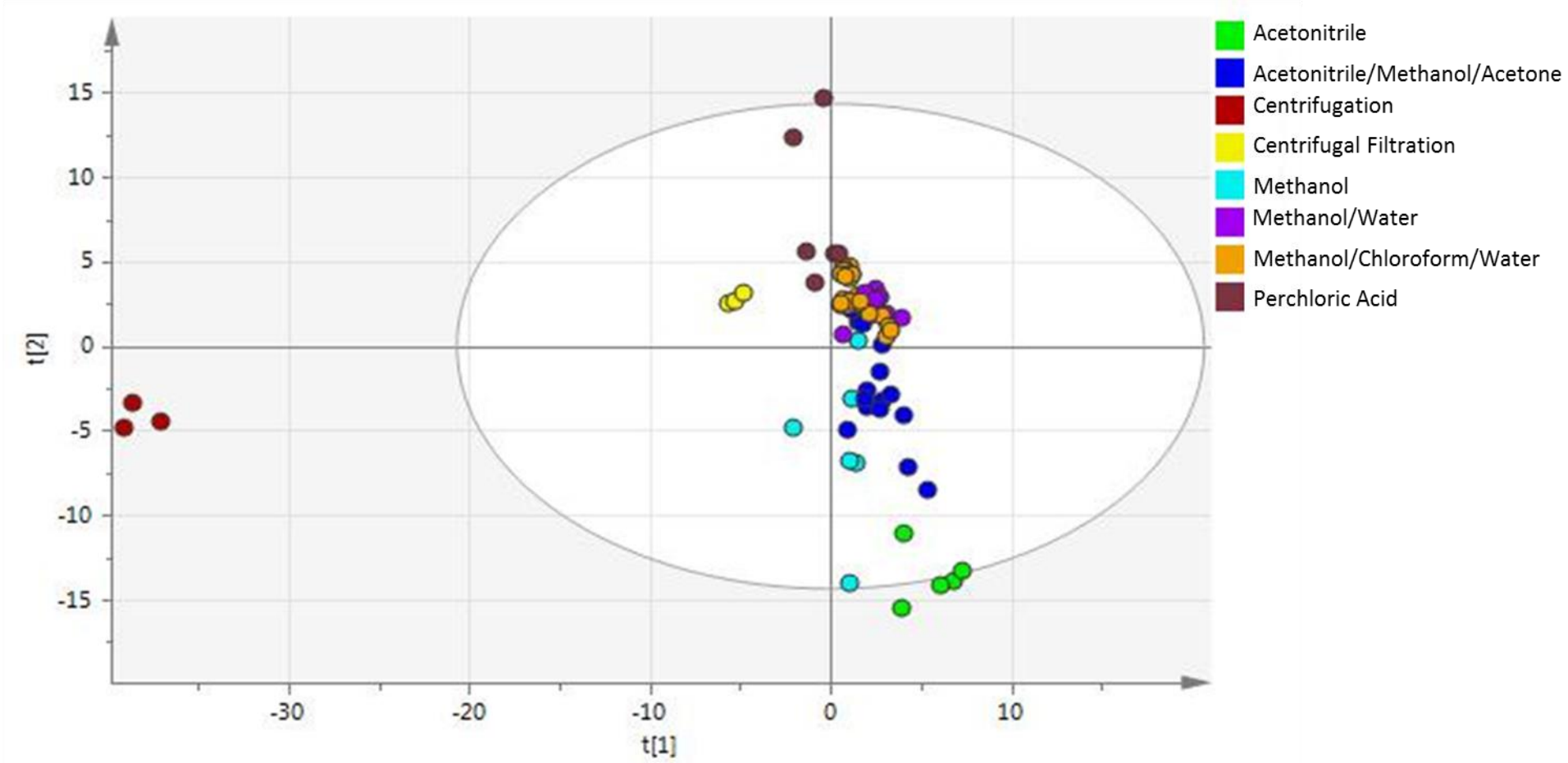

Figure 22 PCA Scores Plot of Drip and Meat Samples Coloured According to the Procedures Outlined in the Sample Preparation Evaluation

3.2. Confinement Odour Study

3.2.1. Confinement Odour and Spoilage Odour Metabolite Differences

Originally confinement odour was thought to be connected to specific groups of bacteria but recently it has been suggested that it may instead be linked to spoilage processes. For this reason the samples exhibiting confinement odour were compared to samples which exhibited spoilage odours. The samples which exhibited no odour were not included in the analysis as they constituted fewer than 10% of the total number of samples.

3.2.1.1. Analysis of Drip for Confinement Odour Metabolites

PCA

PCA was performed on the spectra of drip samples which produced confinement or spoilage odours, an initial analysis showed that three samples had to be removed as outliers after inspection of the scores plot, contribution plot and spectra. The final model (Figure 23) incorporated fifty-seven samples and had twelve calculated components that explained 92.5% of the variance. Inspection of the scores plot showed that there was no separation of the two classes along any of the components. The first two components described 31.5% and 13.2% respectively. The model had a Q^2 of 0.522.

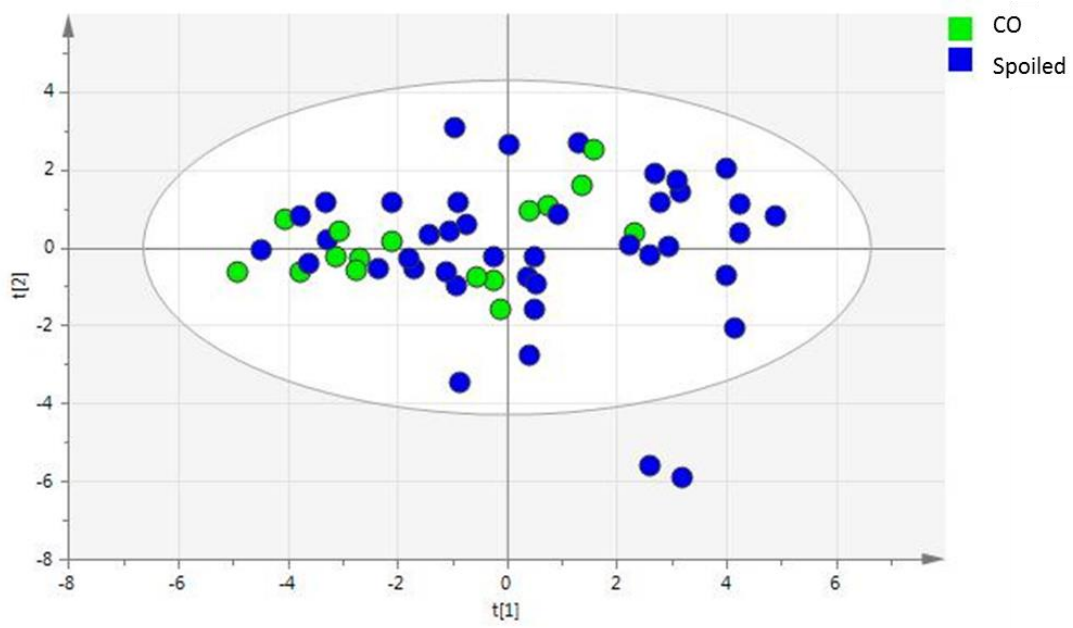

Figure 23 PCA Scores Plot of Drip Coloured by Odour Status

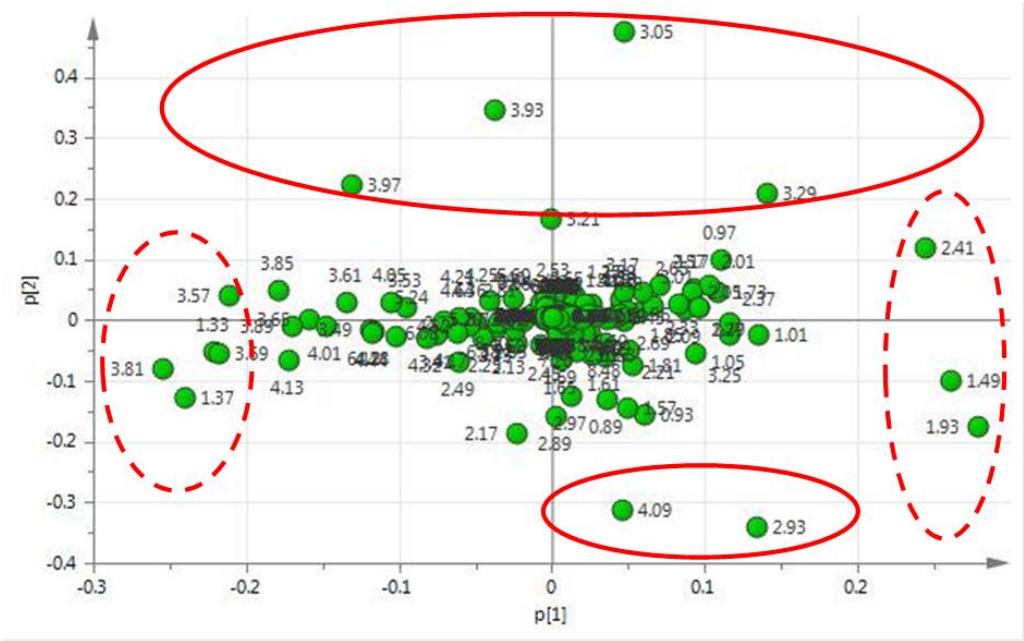

Figure 24 PCA Loadings Plot of Drip Coloured by Odour Status, Bins with Largest Variation across Each Component Circled in Red

The loadings plot for this model indicated that the bins with the largest variation across the first component (as circled in dashed red in the right hand side of Figure 24) correspond to the peaks for lactate, alanine, acetate, pyruvate, glycine, methionine and an unidentified multiplet at 3.65. The bins with the largest variation across the second component (as circled in solid red at the top and bottom of Figure 24) correspond to the peaks for creatine, inosine, choline and an unidentified singlet at 2.91.

OPLS-DA

It was not possible to produce a valid PLS-DA model for this comparison and therefor no OPLS-DA model was built.

Univariate Analysis

T-test analysis of the drip data (summarised in Table 3) showed that five bins could be said to be significantly different between the two classes after false discovery rate correction (FDR). These bins corresponded to peaks for butyrate, lactate, and an overlap of leucine and lysine.

Table 3 Summary of the Result of T-Test Analysis on Drip Samples Classed by Odour Status

Bin	False Discovery Rate Correction	Metabolite
0.89	0.023	Butyrate
1.37	0.023	Lactate

1.65	0.006	Leucine/Lysine
1.69	0.006	Leucine/Lysine
2.21	0.023	Butyrate

Inspection of the box plots associated with each bin shows that the butyrate and leucine/lysine peak concentrations are higher in the spoiled samples while the lactate concentration is higher in the confinement odour samples (Figure 25).

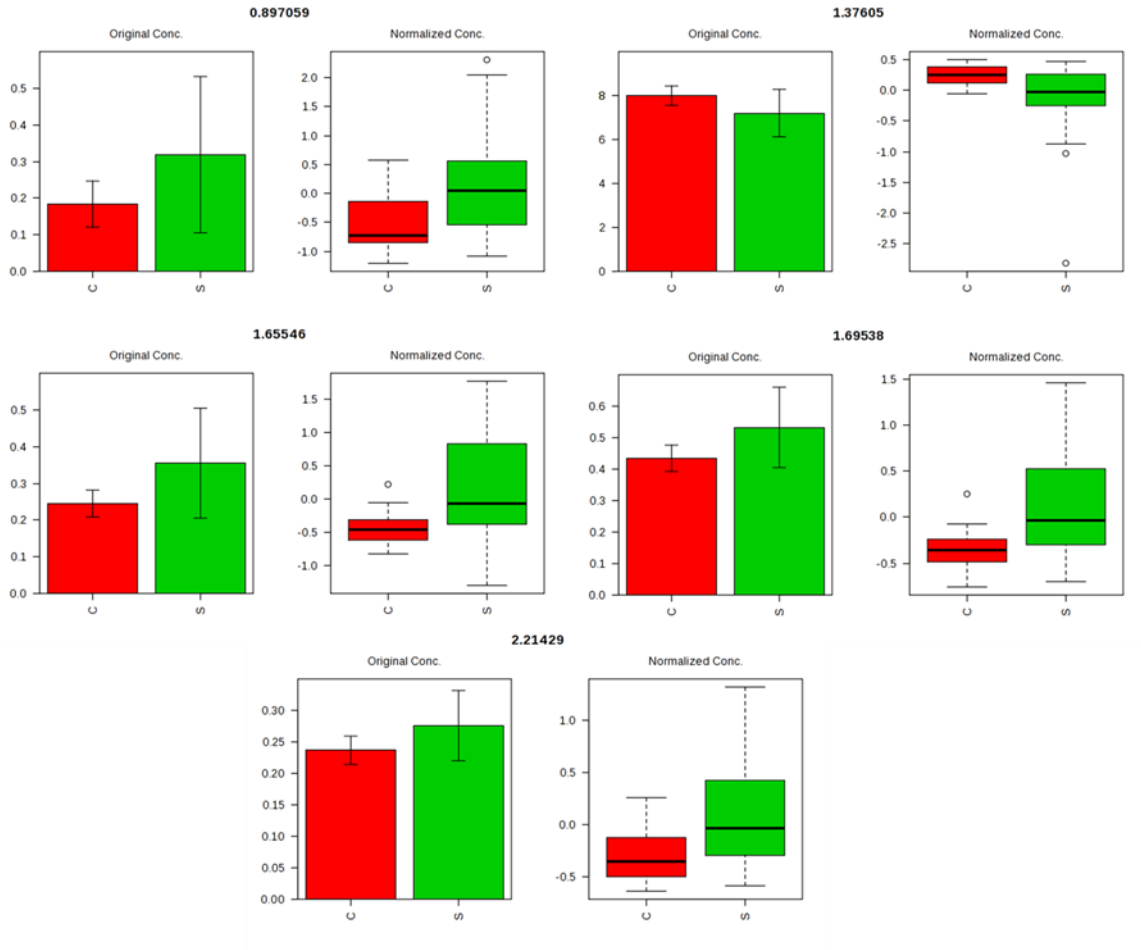

Figure 25 Box Plots of Significant Bins from T-Test of Drip Samples Classed by Odour Status (Red Indicates Samples with Confinement Odour, Green Indicates Samples which are spoiled)

3.2.1.2. Analysis of Meat for Confinement Odour Metabolites

PCA

Initial PCA analysis resulted in three samples being excluded due to possible contaminant peaks being present in their spectra. A subsequent seven component model (Figure 26) was produced which accounted for 73.2% of the variation, inspection of the model showed that none of the components produced a separation of the two classes. The first two components described 29% and 12.7% of the variation respectively. The Q^2 value for this model was 0.336.

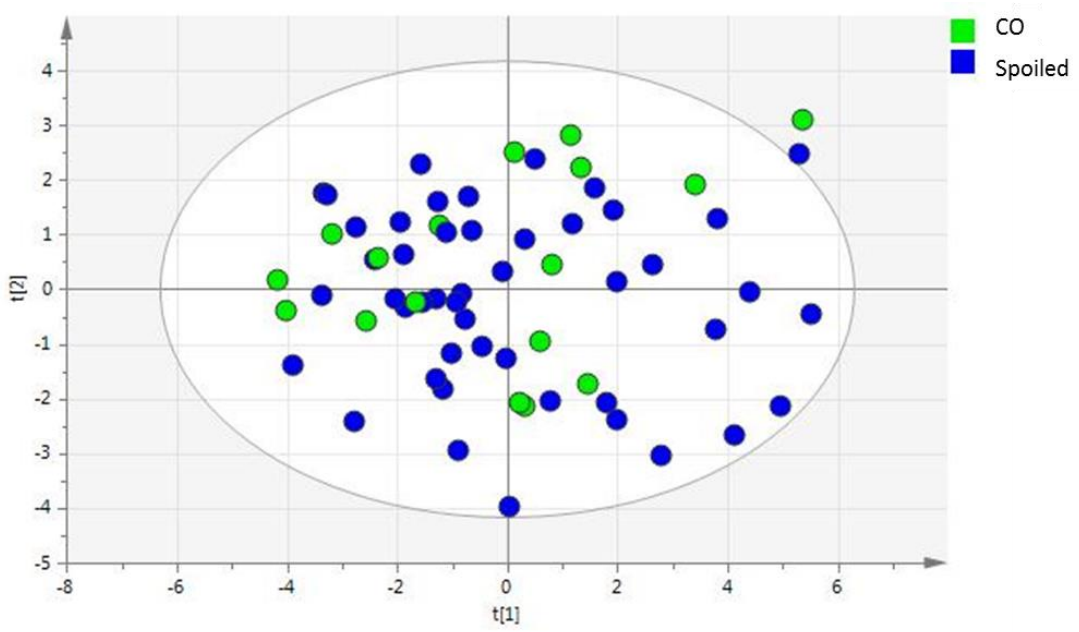

Figure 26 PCA Scores Plot of Meat Coloured by Odour Status

The loadings plot for this model indicated that the bins with the largest variation across the first component (as circled in dashed red in the right hand side of Figure 27) correspond to the peaks for alanine, acetate, pyruvate, glutamine and α-glucose. The bins with the largest variation across the second component (as circled in solid red at the top and bottom of Figure 27) correspond to the peaks for lactate.

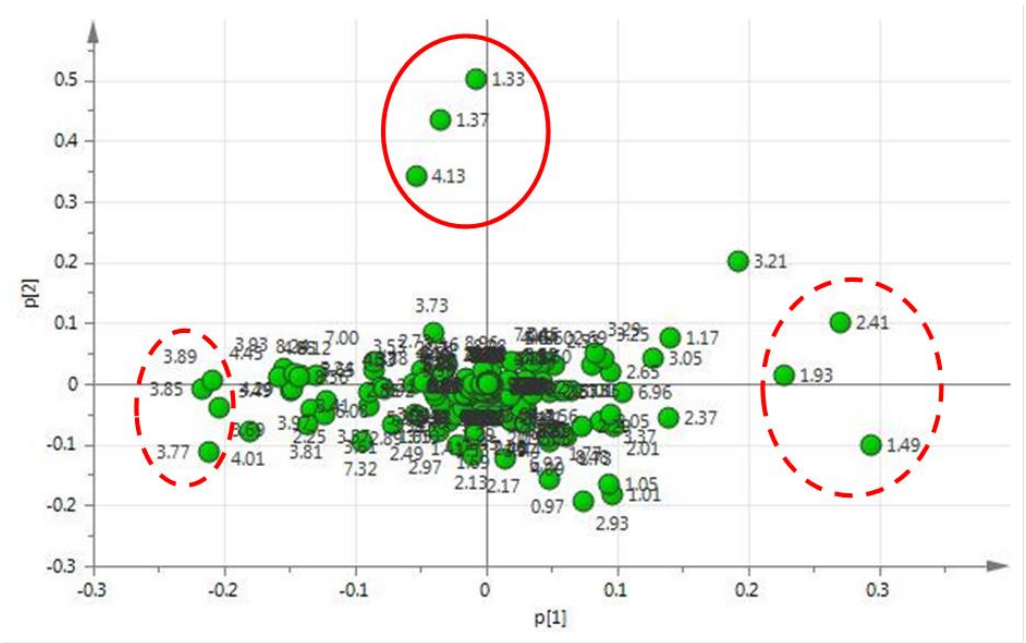

Figure 27 PCA Loadings Plot of Meat Coloured by Odour Status, Bins with Largest Variation across Each Component Circled in Red

OPLS-DA

It was not possible to produce a valid PLS-DA model for this comparison and therefor no OPLS-DA model was built.

Univariate Analysis

T-test analysis produced no bins which differed significantly between the two classes after FDR.

3.2.1.3. Combined Analysis of Drip and Meat for Confinement Odour Metabolites

PCA

A PCA model containing both meat and drip samples which showed confinement or spoilage odour was produced (Figure 28); the sixteen components described 94.6% of the variation in the data. All components were inspected; however none showed any separation of the samples based on odour type. The first two components described 37.1% and 17.1% of the variation respectively. The model's Q^2 value was 0.675.

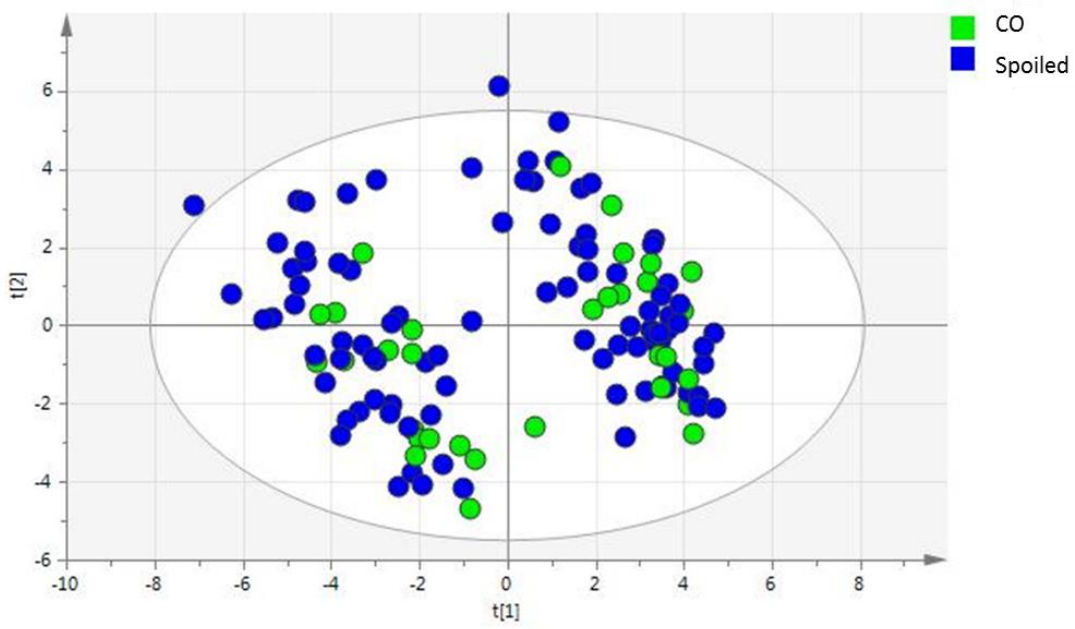

Figure 28 PCA Scores Plot of Drip and Meat Samples Coloured by Odour Status

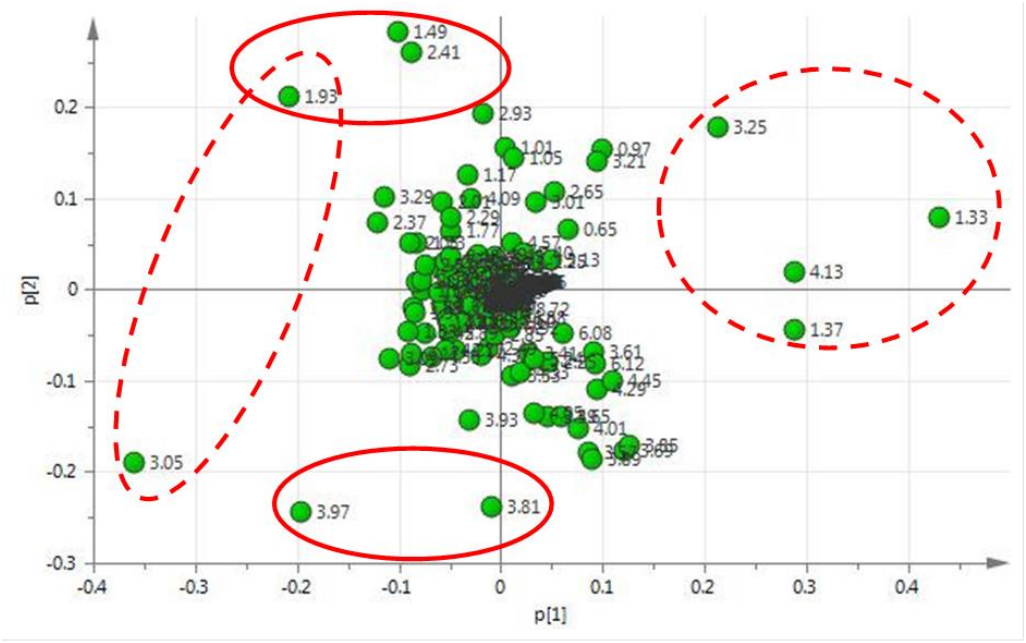

Figure 29 PCA Loadings Plot of Drip and Meat, Bins with Largest Variation across Each Component Circled in Red

The loadings plot for this model indicated that the bins with the largest variation across the first component (as circled in dashed red in the right hand side of Figure 29) correspond to the peaks for lactate, acetate, creatine and choline. The bins with the largest variation across the second component (as circled in solid red at the top and bottom of Figure 29) correspond to alanine, acetate, pyruvate, methionine and inosine.

OPLS-DA

It was not possible to produce a valid PLS-DA model for this comparison and therefore no OPLS-DA model was built.

3.2.1.4. Discussion

The metabolomic analysis of these samples was not proposed until after the study had been designed and was underway. The intention of the study design was to produce variations in the bacterial growth that could lead to the presence of confinement odour, because of this there were nine different treatment groups which comprised three storage times and temperatures (Table 2). These conditions produced many samples exhibiting confinement odour, a large number of spoiled samples and fewer than ten percent of the samples displayed no odour at all. This meant that there were insufficient samples without odour to satisfy the group size requirement of multivariate statistical analysis. While the comparison of samples with no odour and samples with confinement odour may have yielded interesting results the comparison of interest was the confinement odour and spoilage odour samples, therefore

these were the only two classes to be compared. It has been proposed that confinement odour is a side effect of a 'protective' effect in the meat which delays the onset of spoilage (Davies et al., 1998). It is therefore useful to be able to compare the samples with confinement odour to those with spoilage odour in an attempt to find differences between their metabolomic profiles. Although this research was only able to show a few metabolites in drip which have characteristic concentrations in confinement odour metabolite fingerprints the possibility of utilising other analytical techniques (such as MS which can give information on volatiles and very low concentration metabolites), or statistical techniques (PLS time-series which can track the changes in the metabolite concentrations over time) justifies further work.

It was hypothesised that multivariate data analysis could be used to discriminate between the metabolomic profiles of spoiled and confinement odour producing meat. However, this was not observed. A limitation of the statistical modelling which was used is that it can be difficult to discern between class variation when the within class variation is much larger. There were a number of sources of variation within the sample classes induced by the study design. These included the different treatment groups, the different processing plants (which produced different bacterial contamination) and potentially any differences between the rearing of the lambs. These are all sources of variation which can have an effect on the metabolome of the samples and it is possible that these are masking the effects of the meat's odour status.

Where multivariate analysis failed, univariate analysis was more effective. While analysis of the meat metabolite profiles showed no significant differences, the drip could be differentiated based on the concentrations of four metabolites – lactate, butyrate and a combination of leucine and lysine. The results indicate that lactate levels are high in samples showing confinement odour, while the leucine/lysine concentration is low. This possibly suggests a predominance of LAB in the bacterial population on the meat; these produce large quantities of lactic acid from the fermentation of glucose and switch to the catabolism of branched-chain amino acids once glucose has been exhausted (Davies et al., 1998). The spoiled samples were seen to contain higher concentrations of butyrate, a metabolite which is produced from pyruvate following glycolysis and which gives vomit its distinctive odour. This suggests that a butyric acid producing bacterial species such as *Clostridium spp.* is present in significant numbers and contaminated the meat during processing (Ray & Bhunia, 2007). These bacteria have previously been reported to be found in lamb and beef and are more likely to be found in chilled, vacuum packed meat which has become spoiled (Broda, De Lacy, Cook, & Bell, 1997; Broda, Delacy, Bell, Braggins, & Cook, 1996). Meat processing plants are operated under

stringent hygiene protocols, in order that contamination of the product is minimised, however it is still highly likely that there will be a number of bacterial species on the meat once it is packaged. The sources of these include the skins and digestive system of the animals, the workers' skin or clothing, and the machinery surfaces (Davies et al., 1998).

These results are consistent with LAB suppressing the growth of other bacterial species which cause more rapid spoilage. It has been shown that under certain conditions, such as chilled storage of MA or vacuum packaged meat, LAB will overcome other bacterial species and further hinder their growth through the production of lactic and other organic acids which lower the pH of the environment (Davies et al., 1998). LAB also produce antimicrobial peptides which also impact the growth of competing bacterial species(Davies et al., 1998). This effect is referred to protein sparing as LAB preferentially metabolise glucose through fermentation to lactic and acetic acids, while spoilage bacteria preferentially metabolise proteinaceous and non-protein nitrogenous (NPN) compounds (Ray & Bhunia, 2007).

Leucine is a branched-chain amino acid (BCAA), the metabolism of this type of metabolite occurs following glucose depletion. The end products of this metabolism are various short chain fatty acids with unpleasant odours. The increased leucine/lysine peak concentration in spoiled samples, with increased lactate in the confinement odour samples, may suggest that the bacteria associated with confinement odour are at an early stage of growth and have not yet had to switch metabolic pathways due to glucose depletion. This supports the hypothesis that confinement odour may be an early indicator of spoilage.

The fact that it was not possible to discover significant differences between the confinement and spoilage odour meat samples could potentially be a consequence of the heterogeneity of the meat compared to the drip. The distribution of fat and protein throughout a cut of meat is not consistent and it has been shown that bacteria do not penetrate into meat particularly well therefore creating a gradient of metabolite concentrations from the surface to the interior of the meat (Barnes, 1983). Drip on the other hand is the result of water loss throughout the sample.

3.2.2. Metabolite Differences between Processing Plants

3.2.2.1. Analysis of Drip for Processing Plant Metabolites

PCA

The PCA model produced in 3.2.1.1 was coloured according to the processing plant from which the samples originated, this is shown in Figure 30. It appears that there is some separation of the samples based on processing plant along the first and second components.

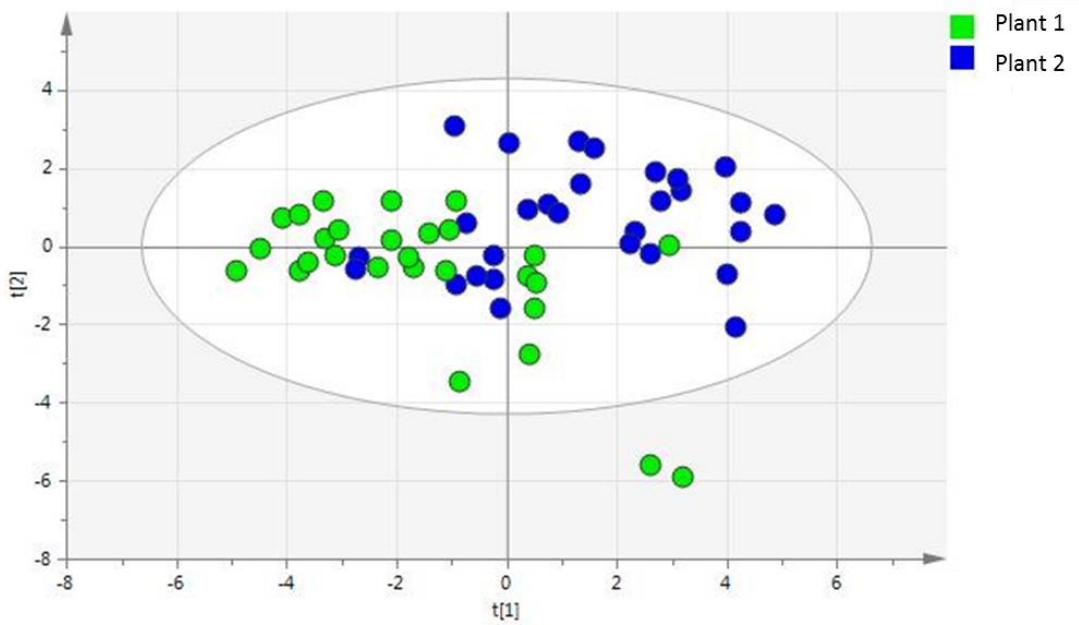

Figure 30 PCA Scores Plot of Drip Coloured by Processing Plant of Origin

OPLS-DA

An OPLS-DA model was built on the same parameters as the validated PLS-DA model produced for the comparison of the processing plant of origin for the drip samples. It was composed of one predictive component and two orthogonal components and had an R^2 value of 0.807 and a Q^2 value of 0.652. The two classes of samples are separated quite well across the predictive component, however there are three samples (circled in red in Figure 31 below) which fall on the opposite side to the rest of their class.

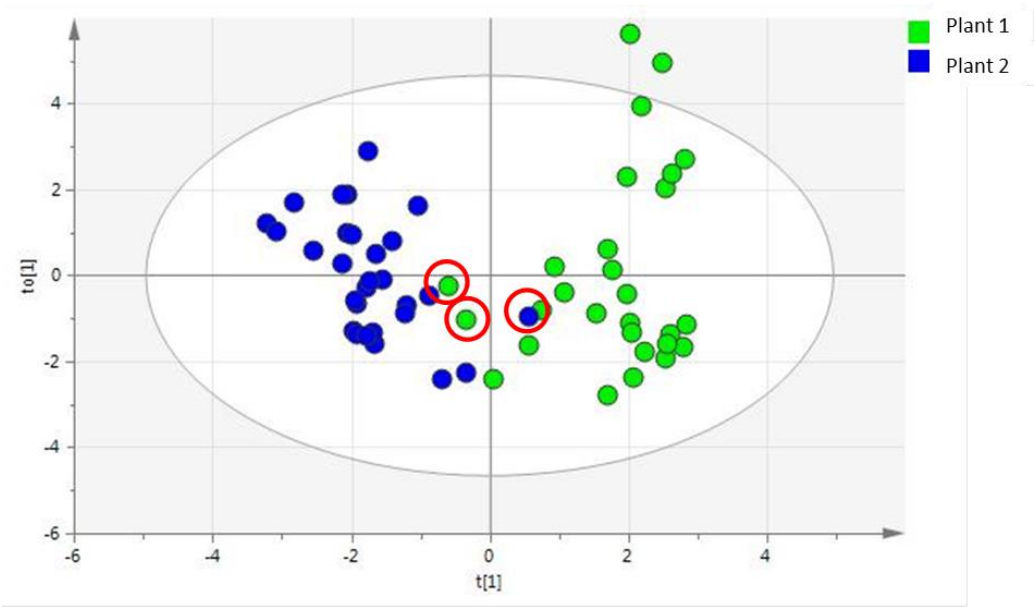

Figure 31 OPLS-DA Scores Plot of Drip Samples Classified by Processing Plant of Origin, Samples which are More Similar to Samples from the Other Plant than from Their Own Shown Circled in Red

The loadings were represented in a 1D plot for two reasons; the first is that this is visually very similar to the original NMR spectrum which helps in the interpretation of the results. The second is that for a two class problem the OPLS-DA model combines all the variance related to the class separation into a single component (*i.e.* the predictive component) which means that only the first loading dimension contains information on the differences between the classes.

The full 1D loadings plot for this model is represented in Figure 32, the loadings bars have been coloured by their VIP values and are shown in order along the x axis corresponding to their bin positions on the NMR spectrum. Those loadings which have positive values indicate the bins which have higher values in samples from plant 1, while those which have negative values are higher in samples from plant 2.

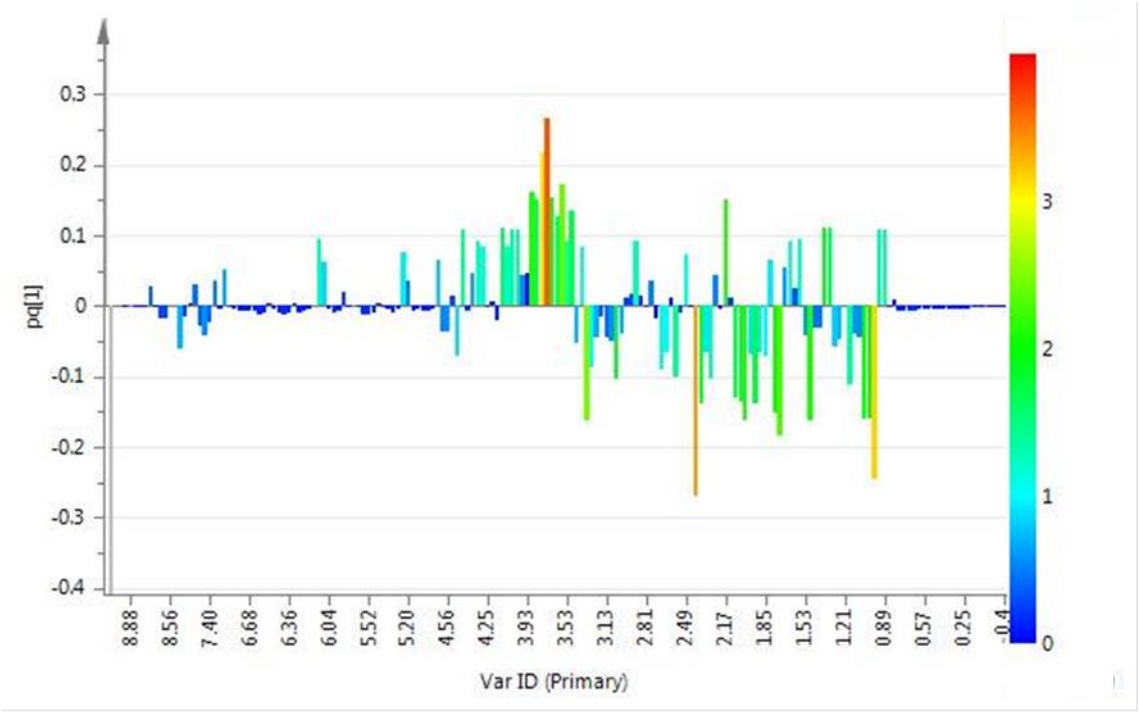

Figure 32 OPLS-DA 1D Full Loadings Plot of Drip Samples Classified by Processing Plant of Origin, Loadings Coloured by VIP Value

The 1D loadings plot was modified to display only those loadings which had a VIP value greater than 1.5; these represented the bins which were to be considered as possible indicators of biomarkers. The plot also shows the direction of the variance with respect to the classes by representing each class as a grey bar, seen at the far left of the plot.

Inspection of this plot showed that the following metabolites were at elevated concentrations in plant 1 when compared to plant 2: α-glucose, β-glucose, glutamine, glycine, lactate, threonine and glycerophosphocholine. Acetate, alanine, glutamate, isoleucine, pyruvate and valine were all shown to have lower values in plant 1 compared to plant 2. A bin containing peaks for both leucine and lysine had lower values in plant 1 when compared to plant 2, the bins which contained the other peaks for these two metabolites were not shown to significantly contribute to this model and therefore it cannot be determined from this which of the two is causing this difference between the classes.

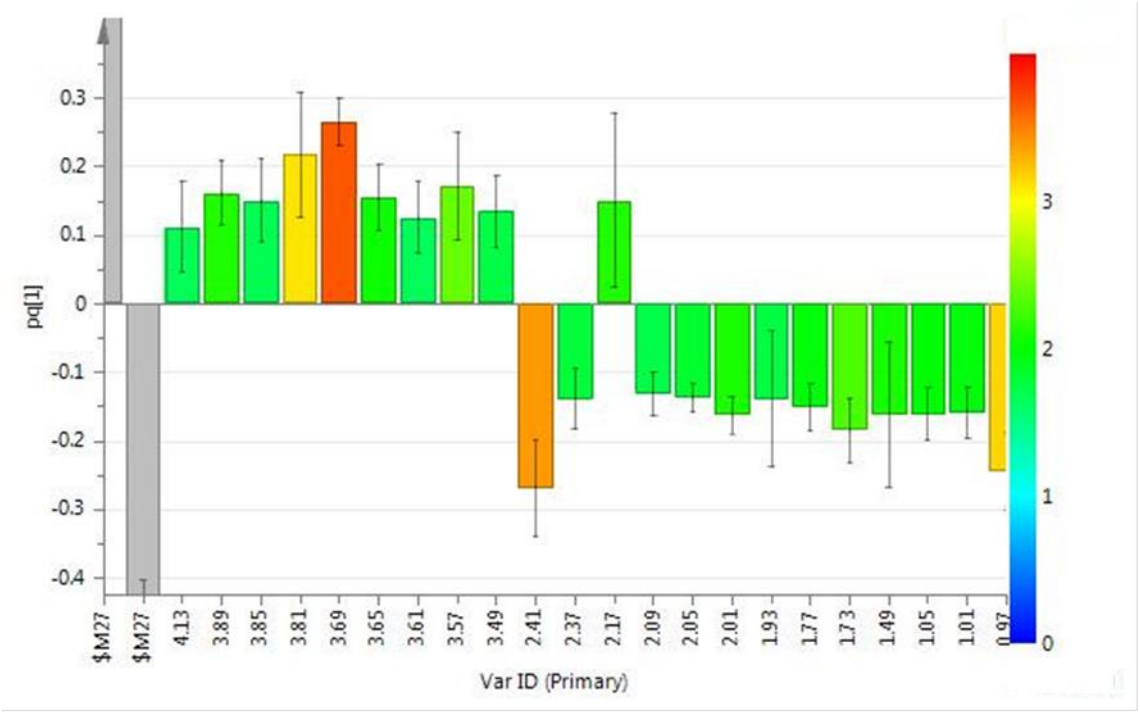

Figure 33 OPLS-DA 1D Loadings Plot of Drip Samples Classified by Processing Plant of Origin, Loadings with VIP Value Greater than 1.5 Shown

.

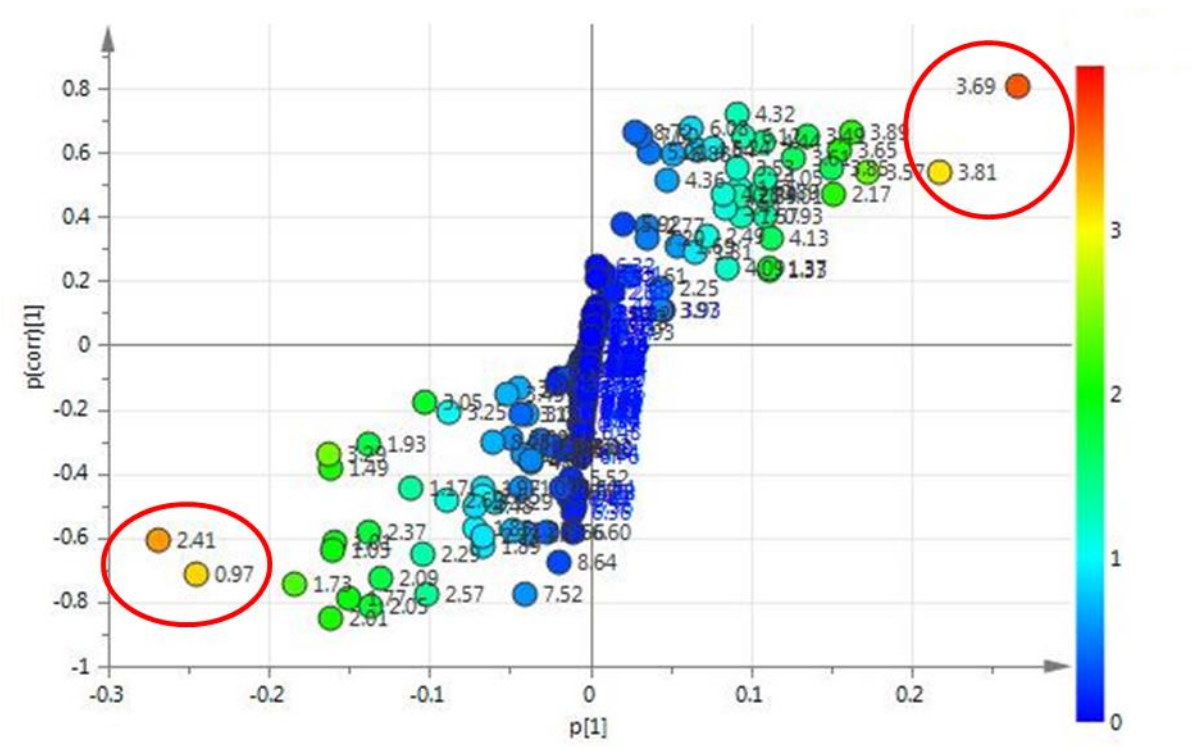

Figure 34 S-Plot of Drip Samples Classified by Processing Plant of Origin, Coloured by VIP value, Loadings which combined High Covariance with High Correlation are Shown Circled in Red

Those bins which combine both a high correlation and high covariance are circled in red in Figure 34 and relate to the peaks for isoleucine, pyruvate, glucose and the unidentified multiplet at 3.69.

3.2.2.2. Analysis of Meat for Processing Plant Metabolites

PCA

The PCA model produced in 3.2.1.2 was coloured according to processing plant of origin (Figure 35).

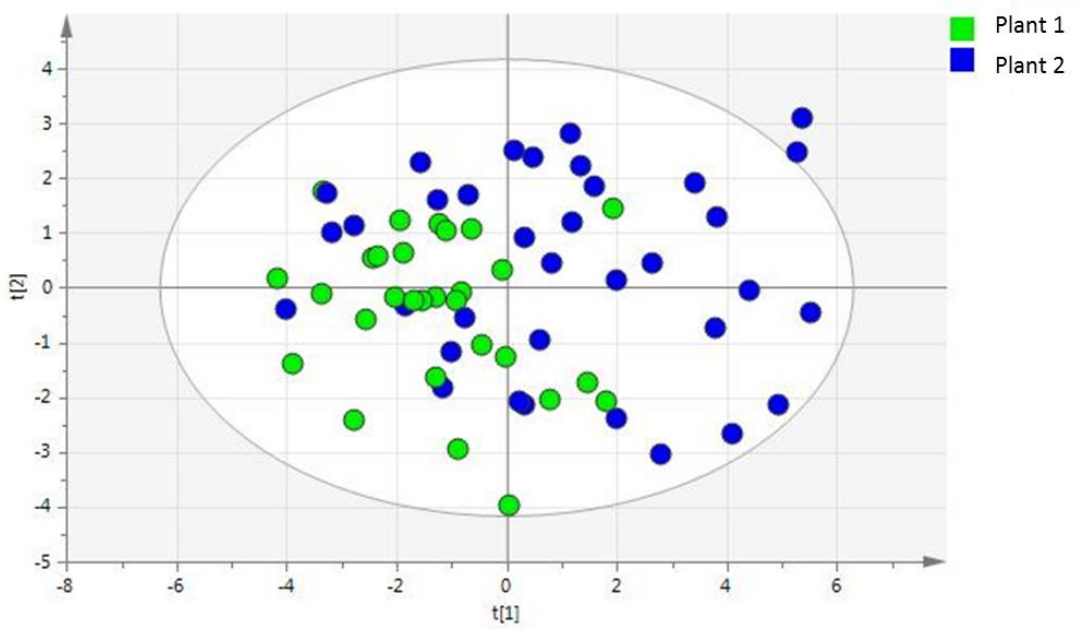

Figure 35 PCA Scores Plot of Meat Coloured by Processing Plant of Origin

OPLS-DA

An OPLS-DA model was built on the same parameters as the validated PLS-DA model produced for the comparison of the processing plant of origin of the meat samples. It was composed of one predictive component and one orthogonal component and had an R^2 value of 0.478 and a Q^2 value of 0.275. There is a certain degree of separation across the predictive component of the samples with different plant origins, but the slight mixing of the two classes is reflected in the low Q^2 value.

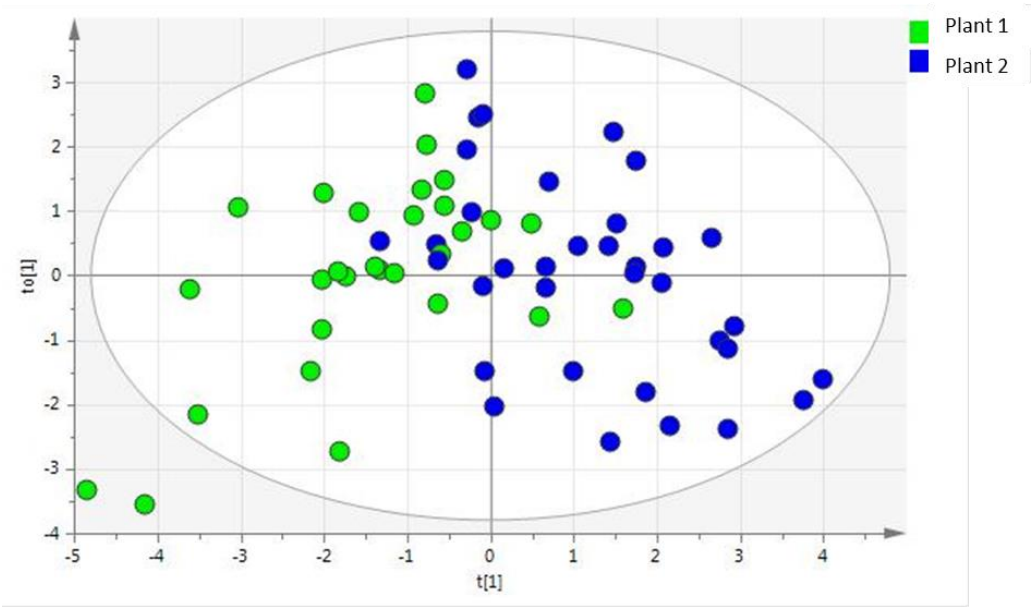

Figure 36 OPLS-DA Scores Plot of Meat Samples Classified by Processing Plant of Origin

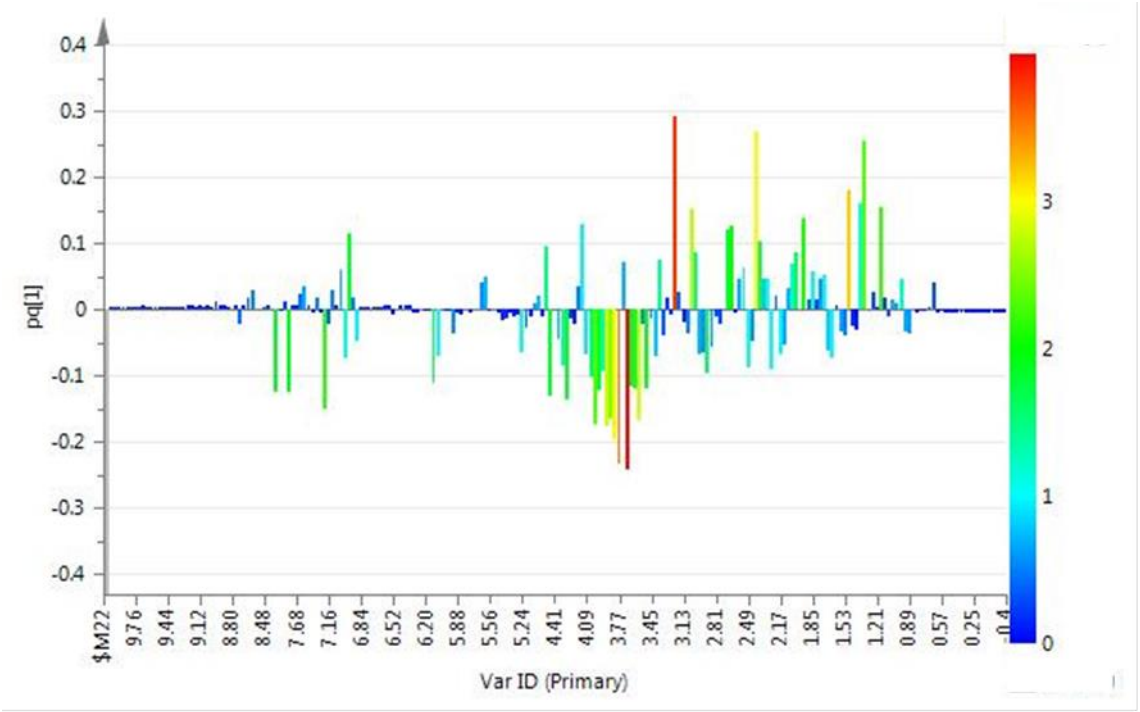

Figure 37 OPLS-DA 1D Full Loadings Plot of Meat Samples Classified by Processing Plant of Origin, Loadings Coloured by VIP Value

The full 1D loadings plot for this model is represented in Figure 37, the loadings bars have been coloured by their VIP values and are shown in order along the x axis corresponding to their bin positions on the NMR spectrum. Those loadings which have positive values indicate the bins

which have higher values in samples from plant 2, while those which have negative values are higher in samples from plant 1.

Inspection of the 1D loadings plot containing only those bins which have a VIP value greater than 1.5 shows that the following metabolites have higher concentrations in plant 1 in comparison to plant 2: α-glucose, β-glucose, creatinine, glutamine, hypoxanthine, inosine, phenylalanine, trimethylamine and glycerophosphocholine. A bin containing both valine and glycine peaks at 3.57 ppm also had larger values in samples from plant 1 compared to those from plant 2. As glycine has no other peaks and valine has others which did not significantly contribute to the model it is likely that it is the differences in glycine concentration which are producing this effect. Alanine, carnosine, choline, creatine, glutamate, isobutyrate, isoleucine, lactate and pyruvate all had lower concentrations in plant 1 samples compared to plant 2 samples.

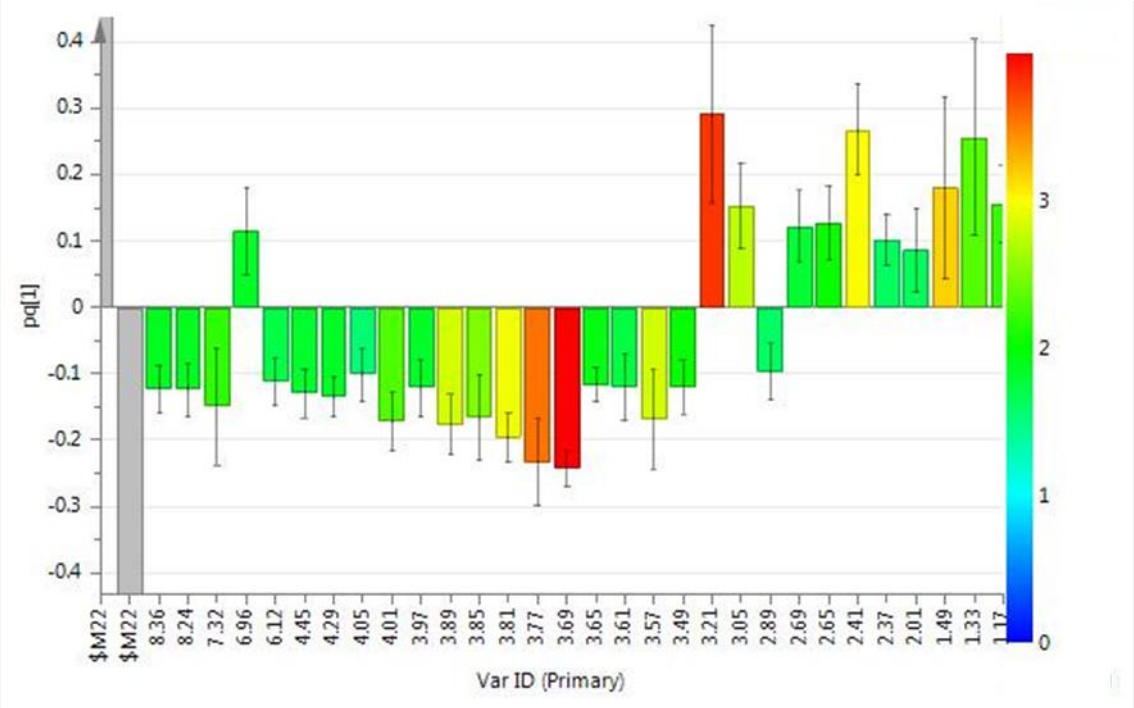

Figure 38 OPLS-DA 1D Loadings Plot of Meat Samples Classified by Processing Plant of Origin, Loadings Coloured by VIP Value, Loadings with VIP values greater than 1.5 shown

The S-plot shows that glutamine, choline, pyruvate, lactate and glycerophosphocholine combine both high covariance and high correlation.

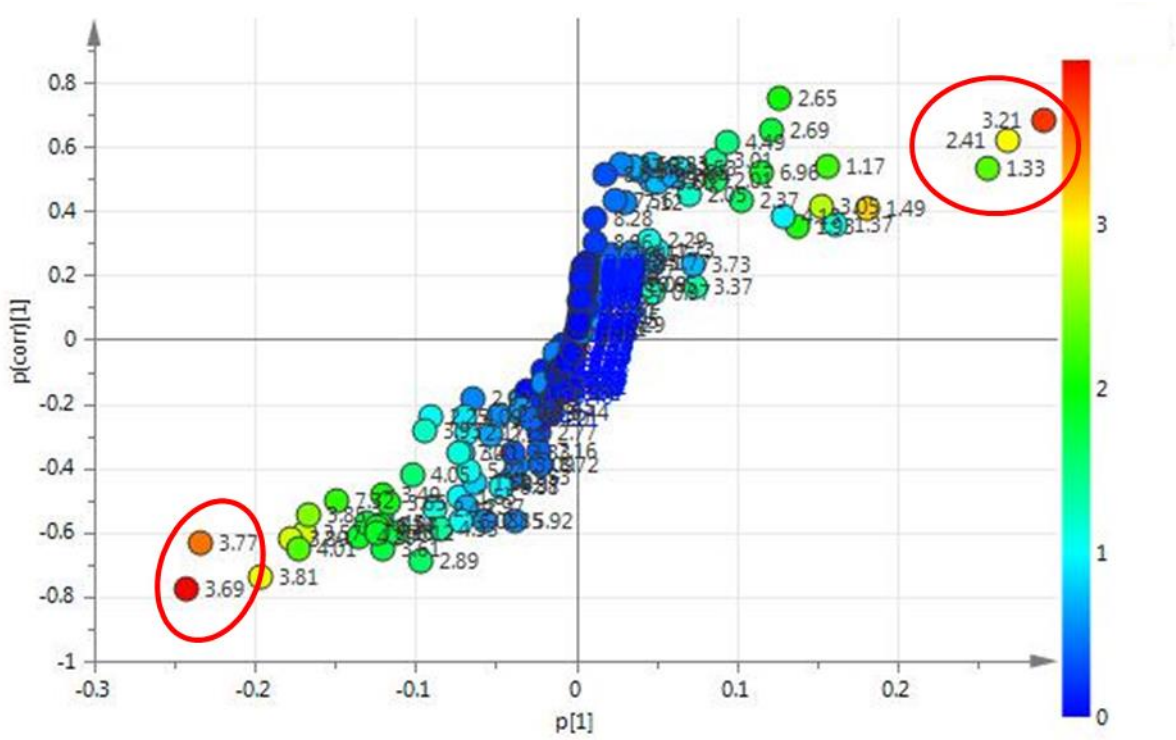

Figure 39 S-Plot of Meat Samples Classified by Processing Plant of Origin, Coloured by VIP value, Loadings Combining High Covariance with High Correlation Shown Circled in Red

3.2.2.3. Combined Analysis of Drip and Meat for Processing Plant Metabolites

PCA

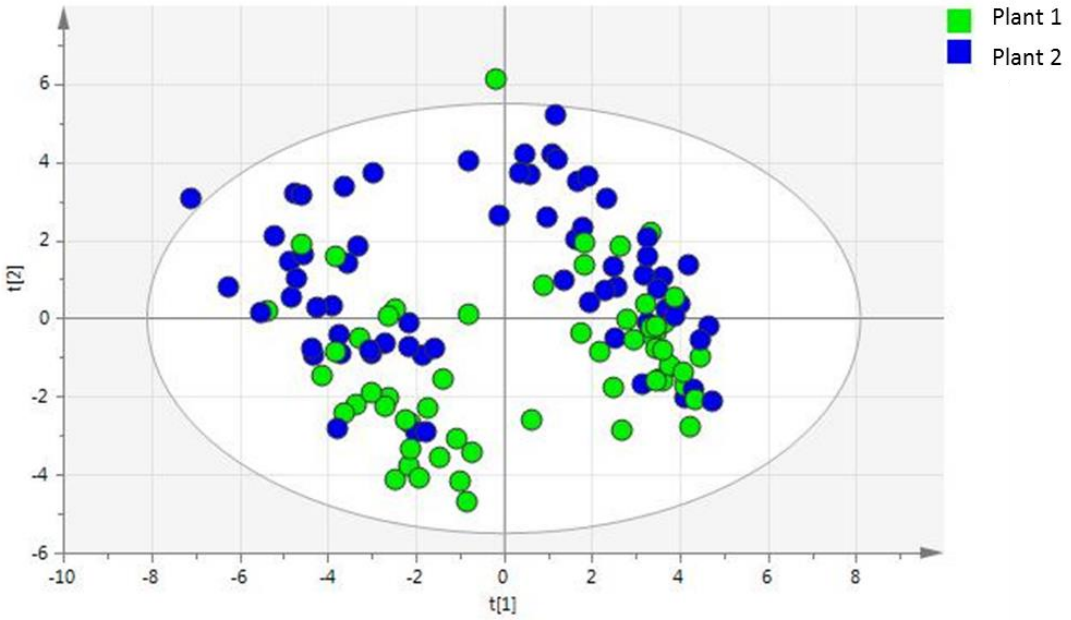

Figure 40 PCA Scores Plot of Drip and Meat Coloured by Processing Plant Origin

The PCA model created for the analysis in section 3.2.1.3 was inspected for separation of the samples based on their processing plant of origin. There was a slight separation of the samples across the second component.

OPLS-DA

An OPLS-DA model was built on the same parameters as the validated PLS-DA model produced for the comparison of the processing plant of origin for the drip and meat samples. It was composed of one predictive component and two orthogonal components and had an R^2 value of 0.580 and a Q^2 value of 0.509. The separation of the classes across the predictive component is good; however there are a number of samples which fall on the opposite side to the rest in their class.

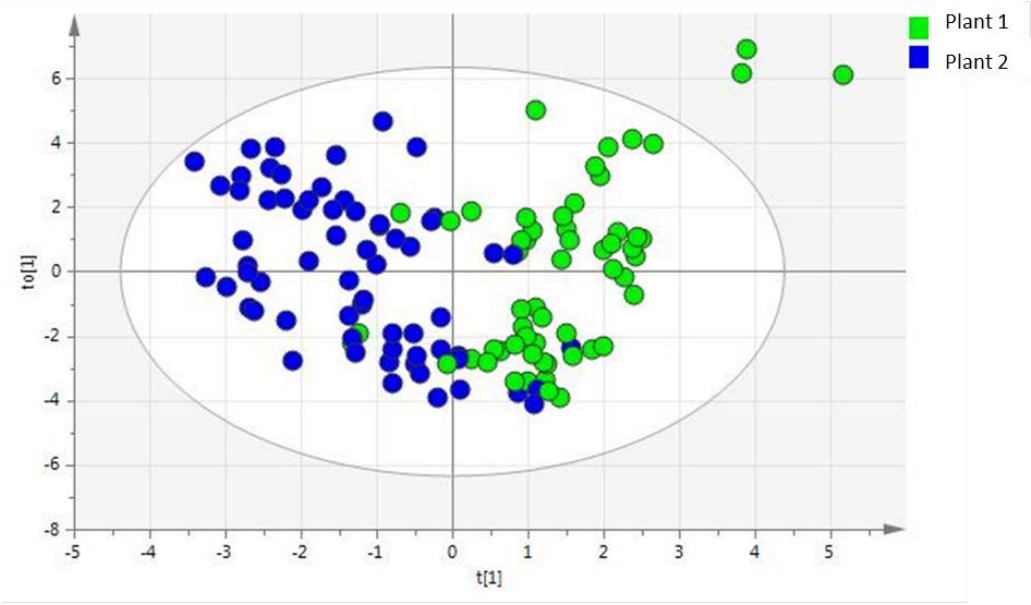

Figure 41 OPLS-DA Scores Plot of Drip and Meat Classified by Processing Plant of Origin

The full 1D loadings plot for this model is represented in Figure 42, the loadings bars have been coloured by their VIP values and are shown in order along the x axis corresponding to their bin positions on the NMR spectrum. Those loadings which have positive values indicate the bins which have higher values in samples from plant 1, while those which have negative values are higher in samples from plant 2.

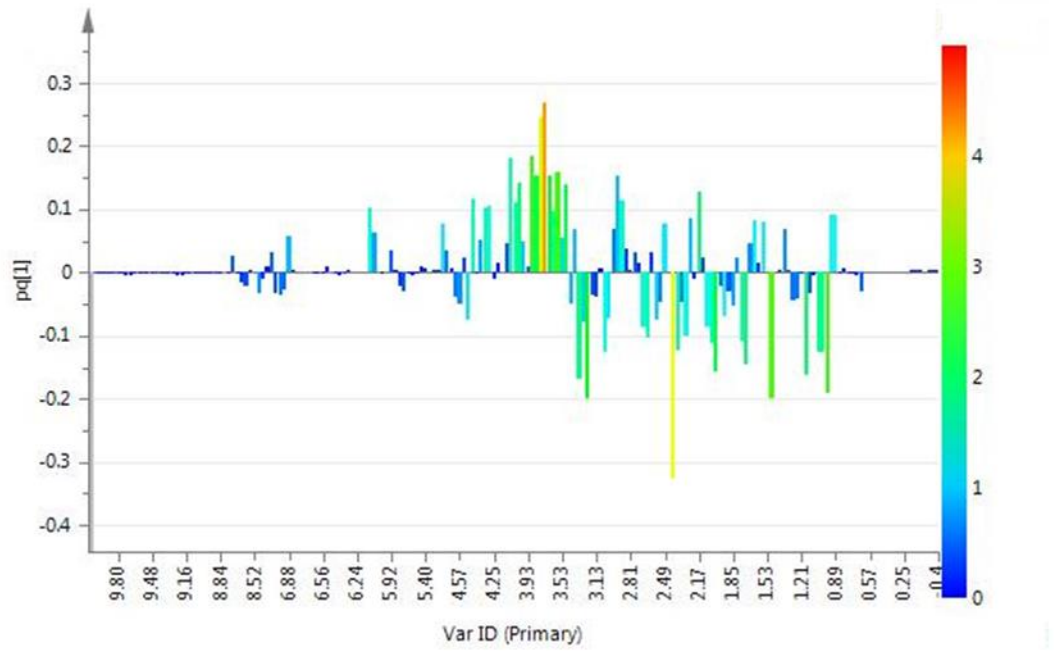

Figure 42 OPLS-DA 1D Full Loadings Plot of Drip and Meat Classified by Processing Plant of Origin, Loadings Coloured by VIP Value

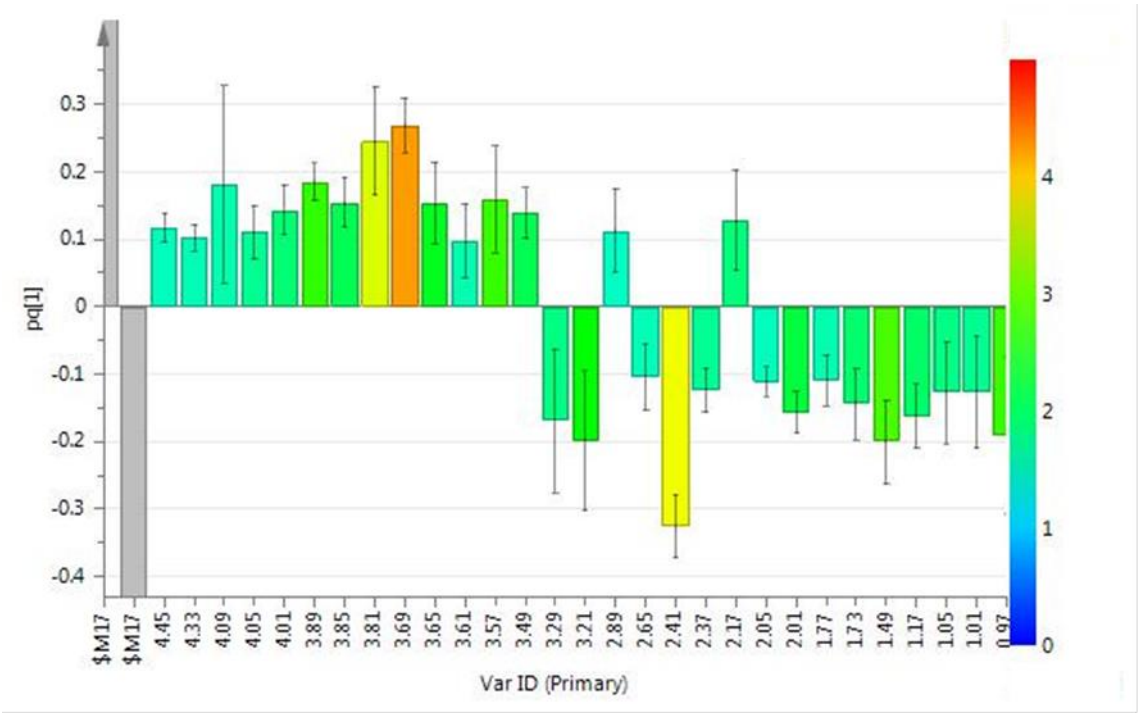

Figure 43 OPLS-DA 1D Loadings Plot of Drip and Meat Classified by Processing Plant of Origin, Loadings Coloured by VIP Value, Loadings with VIP values greater than 1.5 shown

Those metabolites which were higher in plant 1 samples than those from plant 2 were α-glucose, β-glucose, butyrate, creatinine, choline, glutamine, glycine, inosine, threonine, trimethylamine, tyrosine and glycerophosphocholine, while those which were lower were

alanine, carnosine, glutamate, isobutyrate, isoleucine, pyruvate, taurine, valine and the overlapped peaks for leucine and lysine.

Inspection of the S-plot shows that pyruvate, glutamine and glycerophosphocholine combine high covariance and high correlation.

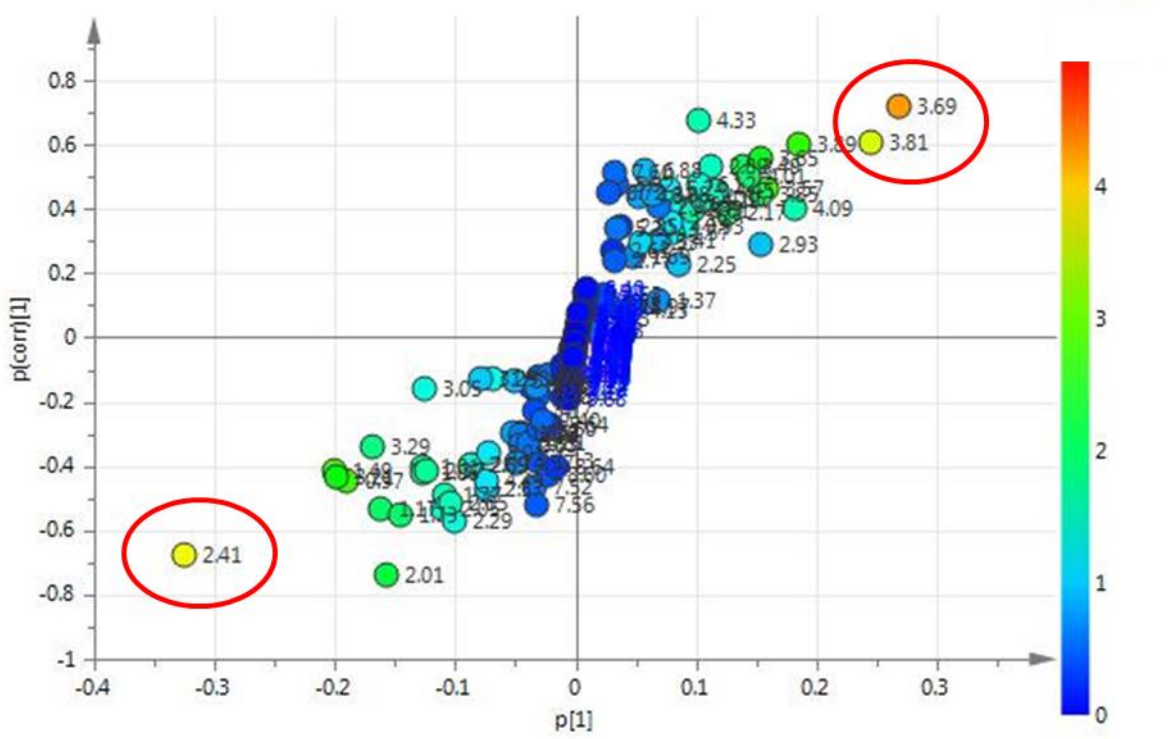

Figure 44 S-Plot of Drip and Meat Samples Classified by Processing Plant of Origin, Coloured by VIP value, Loadings Combining High Covariance with High Correlation Shown Circled in Red

3.2.2.4. Discussion

The respective analyses of drip and meat samples produce similar metabolite profile differences. They indicate that there are lower levels of glucose and higher levels of pyruvate in plant 2 samples; this suggests that the bacterial population in plant 2 has exhausted the glucose supply through glycolysis and has then turned to protein degradation and amino acid metabolism. For example, the high isobutyrate levels in samples from plant 2 are indicative of the metabolism of valine by LAB (Davies et al., 1998). The difference between the amino acid concentrations between the plants could suggest that the bacteria from plant 1 are still metabolising glucose, while the bacteria from plant 2 have moved on to a further stage of their growth.

Generally bacterial growth on meat is fuelled by the metabolism of glucose, however some bacteria preferentially metabolise proteinaceous and NPN compounds. But once bacterial density reaches a certain level the diffusion of glucose to the surface of the meat is insufficient for the needs of the bacteria and they begin to catabolise proteins for the metabolism of amino acids. This leads to the production of foul smelling esters, sulfides and amines (Davies et al., 1998) which indicates the onset of spoilage.

3.2.3. Metabolite Differences between Drip and Meat

3.2.3.1. Analysis of Drip and Meat for Partitioning of Metabolites

PCA

The PCA model created for the analysis in section 3.2.1.3 was inspected for separation of the sample types and showed clear separation across the first component.

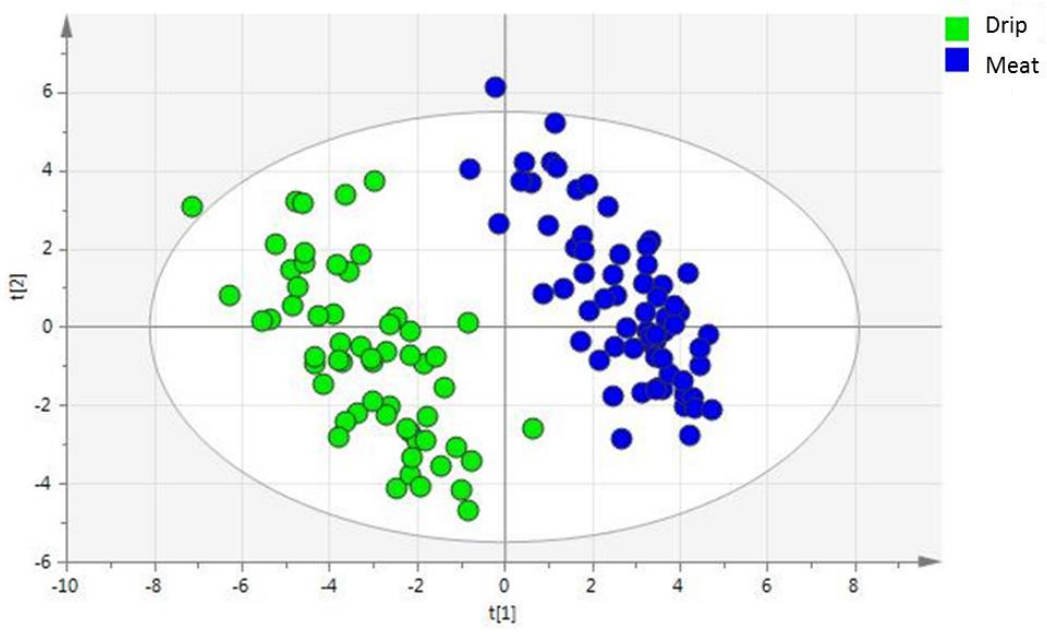

Figure 45 PCA Scores Plot of Drip and Meat Coloured by Sample Type

OPLS-DA

An OPLS-DA model was built on the same parameters as the validated PLS-DA model produced for the comparison of the drip and meat samples. It was composed of one predictive component and one orthogonal component and had an R^2 value of 0.935 and a Q^2 value of 0.924. The two classes were perfectly separated across the predictive component.

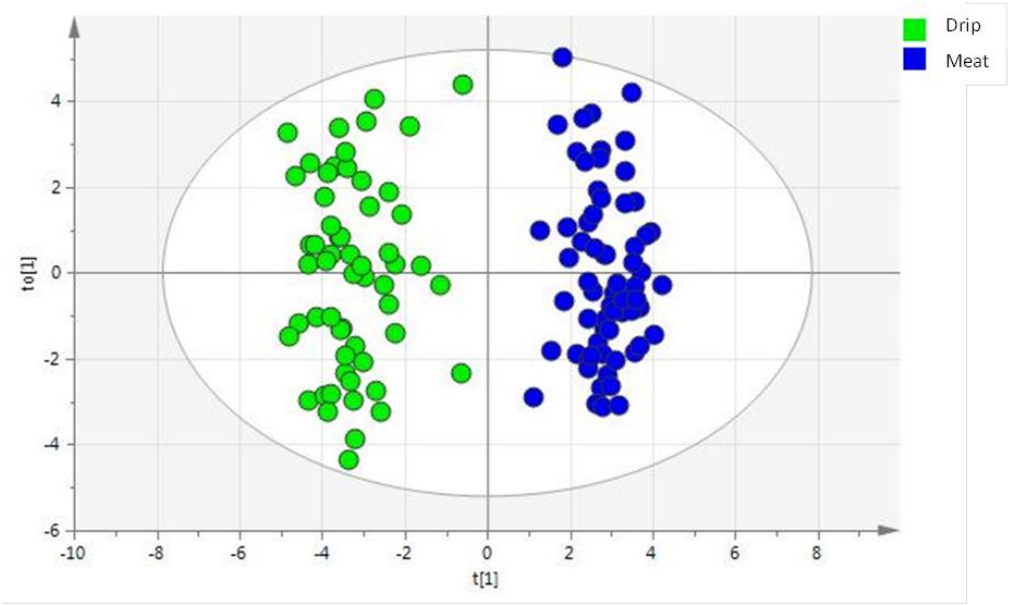

Figure 46 OPLS-DA Scores Plot of Drip and Meat Classified by Sample Type

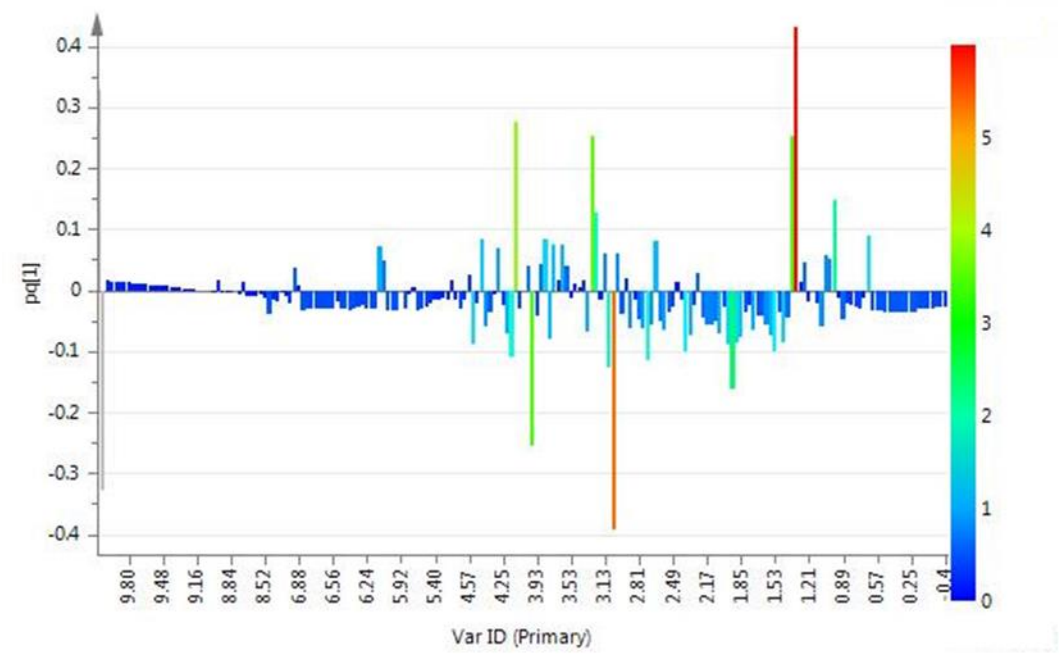

Figure 47 OPLS-DA 1D Full Loadings Plot of Drip and Meat Classified by Sample Type, Loadings Coloured by VIP Value

The full 1D loadings plot for this model is represented in Figure 47, the loadings bars have been coloured by their VIP values and are shown in order along the x axis corresponding to their bin positions on the NMR spectrum. Those loadings which have positive values indicate the bins which have higher values in meat samples, while those which have negative values are higher in drip samples.

Nine metabolites were identified from the loadings plot in Figure 48 as the greatest discriminators between the meat and drip samples. Those that were higher in the meat were choline, isoleucine, lactate and taurine, while 3-hydroxybutyrate, acetate, aspartate, creatine and inosine were all lower than in drip.

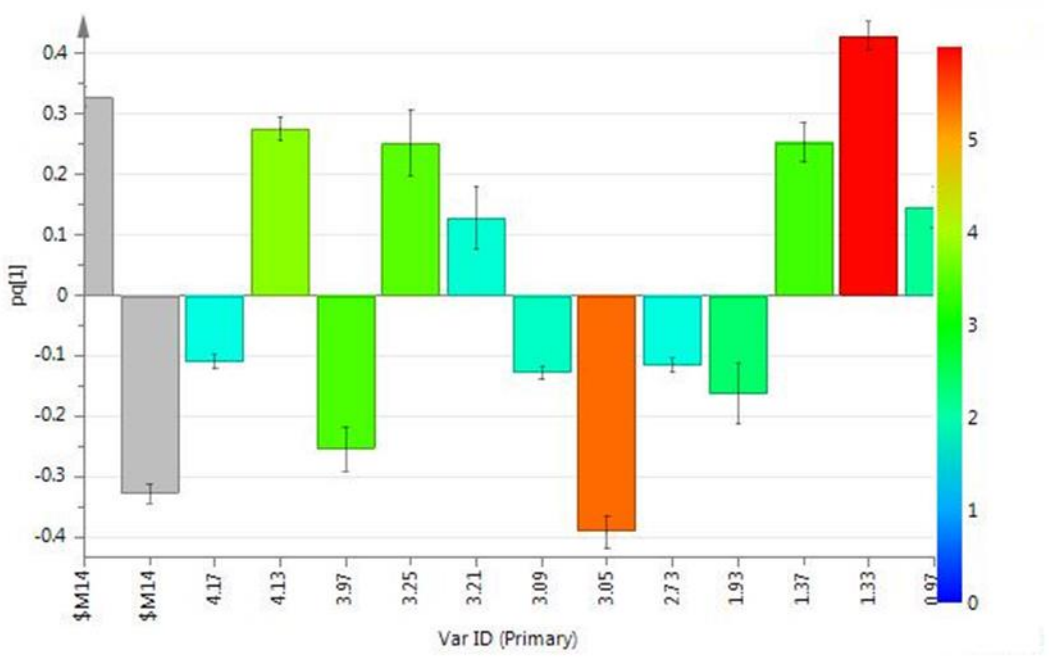

Figure 48 OPLS-DA 1D Loadings Plot of Drip and Meat Classified by Sample Type, Loadings Coloured by VIP Value, Loadings with VIP Value Greater than 1.5 Shown

The S-plot shows that lactate, choline and creatine combined high covariance with high correlation.

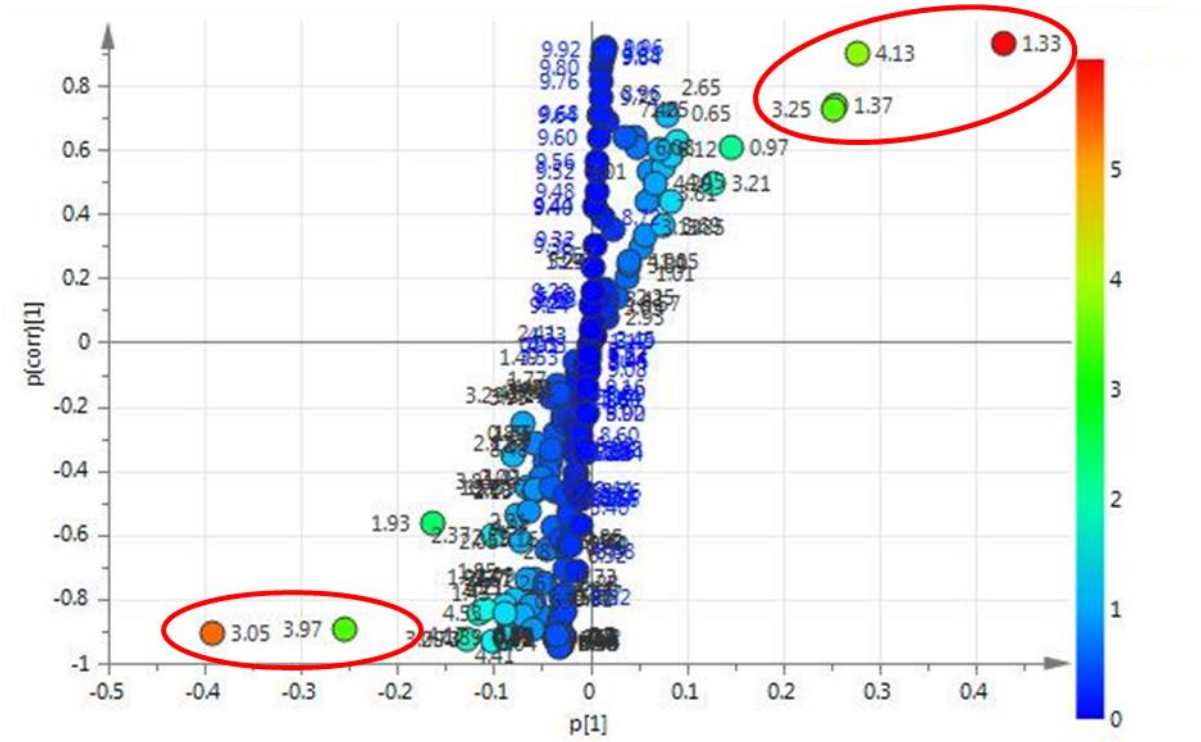

Figure 49 S-Plot of Drip and Meat Samples Classified by Sample Type, Coloured by VIP value, Loadings Combining High Covariance with High Correlation Shown Circled in Red

3.2.3.2. Discussion

PCA modelling from the analysis of all samples indicates that the greatest source of variation in the data comes from the differences between the drip and meat samples.

Meat has been assessed for a range of characteristics by metabolomic analysis, but drip appears to be a potentially useful yet so far overlooked medium. In many cases drip may be easier to sample than meat; especially considering it is an unwanted by-product in packaged meat. This ease extends to the preparation of the sample for analysis, the smaller number of steps necessary means that there are fewer avenues for introducing unwanted variation. While similar conclusions can be made about the metabolite differences associated with the processing plants using drip or meat samples, only the drip samples showed differences between the samples with spoilage or confinement odours. As mentioned above, this may be due to the greater homogeneity of the drip when compared to meat.

Table 4 Summary of Metabolites Characterised from ^{1}H and ^{13}C NMR Signals for Reference

[a] These peaks were obscured in the drip and meat spectra but were inferred based on reference values

Metabolite	Position	^{1}H (ppm)	m (multiplicity)	^{13}C (ppm)
3-hydroxybutyrate	β-CH_3	1.18	d	24.6
	γ-CH_2	2.29	m	49.2
	γ'-CH_2	2.38	m	49.2
	α-CH[a]	4.16	m	68.5
Acetate	CH_3	1.91	s	25.8
Alanine	β-CH_3	1.47	d	18.8
	α-CH	3.77	q	53.2
Aspartate	β-CH_2	2.66	m	39.4
	β'-CH_2	2.80	m	39.3
	α-CH	3.84	m	59.3
Butyrate	γ-CH_3	0.87	t	15.9
	β-CH_2	1.54	tq	22.1
	α-CH_2	2.14	t	42.3
Carnosine	C9H	2.67	m	34.8
	C6H	3.03	m	30.8
	C6H	3.17	m	30.6
	C10H	3.25	t	38.3
	C7H	4.47	m	57.5
	C4H	7.08	s	119.8
	C2H	8.09	s	137.4
Choline	NCH_3	3.22	s	56.7
	β-CH_2	3.53	dd	73.8
	α-CH_2	4.06	ddd	57.6
Creatine	NCH_3	3.03	s	39.6
	NCH_2	3.92	s	56.7
Creatinine	NCH_3	3.03	s	32.8
	NCH_2	4.04	s	59.1
Glutamate	β,β'CH	2.06	m	29.8
	γ-CH_2	2.35	m	36.1
	α-CH_2	3.73	m	56.3
Glutamine	β,β'-CH_2	2.12	m	29.0

Metabolite	Position	^{1}H (ppm)	m (multiplicity)	^{13}C (ppm)
	γ-CH_2	2.44	m	33.7
	α-CH_2	3.77	t	57.0
Glycine	α-CH_2	3.55	s	44.5
Glycerophosphocholine	N-CH_3	3.20	s	56.6
	α-CH_2	3.63	m	64.6
	ε-CH_2	3.67	m	68.6
	β-CH	3.90	m	73.3
	γ- CH_2	3.91	m	69.2
	δ-CH_2	4.30	m	62.1
Hypoxanthine	CH-8	8.19	s	144.8
	CH-2	8.22	s	139.4
Inosine	C5H’	3.84	dd	63.1
	C5H’’	3.96	dd	63.1
	C2H	4.27	dd	88.5
	C4H	4.43	dd	73.2
	C3H^{a}	4.80	t	76.8
	α-CH	6.09	d	90.9
	CH	8.18	s	148.2
	CH	8.33	s	142.9
Isobutyrate	β,γ-CH_3	1.13	d	19.3
	α-CH	2.34	m	36.3
Isoleucine	γ-CH_3	0.95	t	13.8
	β-CH_3	1.00	d	17.4
	γ-CH_2	1.25	m	27.1
	γ’-CH_2	1.45	m	27.1
	β-CH_2	1.98	m	38.5
	α-CH	3.66	d	62.4
Lactate	β-CH_3	1.32	d	22.8
	α-CH	4.11	q	71.2
Leucine	δ’-CH_3	0.94	m	23.6
	δ-CH_3	0.96	m	24.7
	γ-CH	1.70	m	24.7
	β-CH_2	1.71	m	29.1

Metabolite	Position	^{1}H (ppm)	m (multiplicity)	^{13}C (ppm)
	α-CH	3.77	t	56.9
Lysine	γ-CH_2	1.44	m	24.1
	γ-CH_2	1.50	m	24.1
	δ-CH_2	1.72	m	29.1
	β-CH_2	1.90	m	32.6
	ε-CH_2	3.02	t	42.1
	α-CH	3.75	t	57.5
Methionine	β-CH_2	2.13	m	16.6
	SCH_3	2.12	s	32.3
	γ-CH_2	2.63	t	31.4
	δ-CH	3.84	m	56.8
Pyruvate	α-CH_3	2.40	s	32.4
Taurine	S-CH_2	3.25	t	50.1
	N-CH_2	3.42	t	38.3
Threonine	γ-CH_3[a]	1.31	d	68.9
	α-CH	3.61	d	63.2
	β-CH_2	4.25	m	68.7
Trimethylamine	CH_3	2.87	s	47.3
Tyrosine	ε-CH_2	3.04	dd	38.2
	ε'-CH_2	3.18	dd	38.5
	ζ-CH	3.99	dd	54.0
	α-CH	6.90	m	118.6
	β-CH	7.20	m	132.9
Valine	γ-CH_3	0.98	d	19.4
	γ'-CH_3	1.03	d	20.5
	β-CH	2.26	m	31.8
	α-CH	3.58	d	63.3
α-glucose	C4H	3.39	m	72.5
	C2H	3.53	m	73.9
	C3H	3.73	m	63.4
	C6,6'H_2	3.84/3.77	dd	63.1
	C1H	5.25	d	95.0
β-glucose	C2H	3.27	dd	75.2

Metabolite	Position	^{1}H (ppm)	m (multiplicity)	^{13}C (ppm)
	C4H	3.45	m	72.7
	C5H[a]	3.48	m	76.4
	C3H	3.53	m	73.9
	C3H	3.72	m	73.8
	C6,6'H_2	3.90/3.72	dd	63.5
	C1H	4.66	d	98.7

Chapter Four Conclusion and Future Work

4.1. Conclusions

4.1.1. Optimal Preparation Protocol for NMR Analysis of Lamb Drip and Meat

Bead beating was found to be the easiest, most reliable technique for meat homogenisation. For comparison of both drip and meat samples solvent precipitation using a mix of acetonitrile, methanol and acetone was found to provide consistent results across technical replicates as well as sample types, and achieved a high level of protein removal with a low risk of preparation induced metabolism. The evaporation method which was easiest to complete and produced the best reproducibility of the technical replicates was vacuum centrifugation.

4.1.2. Confinement Odour Metabolites in Lamb Drip and Meat

Analysis of the drip data showed that there were statistically significant differences between the metabolite concentrations of the two odour classes. This analysis demonstrated that butyrate and a combination of leucine and lysine were more abundant in spoiled samples, while lactate was more abundant in samples exhibiting confinement odour. This is consistent with the theory that there is a predominance of butyric acid producing bacteria (such as certain species of *Clostridia*) in spoiled samples, compared with a higher proportion of LAB in the samples with confinement odour (bacterial analysis of the samples to confirm the presence of these species was outside the scope of this work). Overall it may suggest that confinement odour could be an early indicator of the onset of spoilage. No statistically significant differences in the metabolomic profiles of meat samples with confinement or spoilage odours were found.

4.1.3. Metabolites Differentiating Processing Plant of Lamb Drip and Meat

It was found that analysis of both drip and meat data showed that there were significant metabolite differences between samples originating from different processing plants. The differences found in both sample types were in good agreement. In samples from Plant 2 pyruvate was higher in concentration while glucose was lower in concentration. This is suggestive of the bacteria on these samples being at a later stage of growth than the bacteria on samples from Plant 1, they had moved on from metabolising glucose to metabolising amino acids.

4.1.4. Metabolites Differentiating Lamb Drip and Lamb Meat

Analysis of the drip and meat data suggests that drip may prove to be a useful tool in metabolomic analysis of meat considering the ease with which it can be sampled and prepared for analysis. Similar conclusions can be made from analysis of drip or meat samples for large sources of variation, as shown by the analysis of processing plant differences, but only drip was able to be differentiated based on odour status. This is potentially due to the increased homogeneity of drip when compared to meat.

4.2. Future Work

4.2.1. Sampling of Lamb Drip and Meat at Various Time Points for Time Series Analysis of Confinement Odour Metabolites

While it has been shown that meat with confinement odour cannot be differentiated by NMR analysis of its metabolome at the end of its storage life it is possible that the effect may be visible at other stages in the storage process. Therefore the investigation of the meat and drip metabolomes of packaged lamb at various time points in the storage process may yield information about the development of confinement odour. A multivariate time series statistical approach would be potentially beneficial to elucidating the changes in the metabolome of lamb during storage. This could then be related to the bacterial populations over the same period. The predominant bacterial species could change many times over the storage time (Davies et al., 1998). It would be particularly useful to sample the meat at the beginning of the storage period in order to compare the starting condition with the end state.

4.2.2. Mass Spectrometry Analysis of Meat and Drip for Confinement Odour Metabolites

Combining the output of NMR and MS analysis is becoming very popular for metabolomic studies as the techniques are highly complementary, with NMR detecting high concentration metabolites and MS covering low concentration constituents as well. Biological samples

contain metabolites covering a wide range of concentrations; a more complete picture of the metabolome can be attained through the use of both techniques.

Gas Chromatography MS of the headspace gases produced by stored lamb meat is another potential avenue for investigation. There is potential for this sensitive technique to better emulate the function of the nose in detecting confinement odour. The analysis of gas phase metabolites would be complementary to the analysis of those recovered in solution.

Metabolomics may include the analysis of lipids, an important class of compounds that form a subset of the metabolome. For a more comprehensive analysis of the differences between samples with and without CO the lipid profile (lipidome) should be considered alongside the water soluble low molecular weight compounds.

4.2.3. Treatment Changes to Increase Control Samples

The treatment conditions that were applied to the lamb meat in this work were designed to encourage bacterial growth and potentially the production of CO. This resulted in many of the samples becoming spoiled and very few samples with no odour to be used as a control for the statistical analysis. To determine whether meat with CO can be discriminated from meat which has no odour a different set of treatment conditions could be enacted to produce more 'control' samples. Shorter times at the highest temperature would allow less bacterial activity and so could lead to more 'control' samples. It has been shown that vacuum and MA packaged lamb meat do not develop unacceptable odours when held at -0.3°C for up to twelve weeks (Kiermeier et al., 2013). It would also be worthwhile to store the meat in identical conditions as this may make it easier to discover the effects of the other differences (such as between processing plants or drip and meat) when comparing their metabolite profiles.

Bibliography

Atherton, H. J., Bailey, N. J., Zhang, W., Taylor, J., Major, H., Shockcor, J., . . . Griffin, J. L. (2006). A combined 1H-NMR spectroscopy- and mass spectrometry-based metabolomic study of the PPAR-α null mutant mouse defines profound systemic changes in metabolism linked to the metabolic syndrome. *Physiological Genomics, 27*(2), 178-186. doi: 10.1152/physiolgenomics.00060.2006

Barnes, E. M. (1983). Meat microbiology (1982) Edited by M. H. Brown. Applied Science Publishers Ltd, London and New York. 529 pp. Price: pound46.00. *Meat Sci, 9*(3), 231-233. doi: 10.1016/0309-1740(83)90005-0

Beef and Lamb NZ. (2015). Lamb Exports by Region. Retrieved from http://www.beeflambnz.com/Documents/Information/Export%20lamb%20volume%20by%20region.pdf

Bill B.A., E. I. J., Gibbons, R.A., Grau F. H. and Vanderlinde P.B. *Effect of Carbon Dioxide on Storage of Lamb Carcasses*. CSIRO Australia.

Broadhurst, D., & Kell, D. (2006). Statistical strategies for avoiding false discoveries in metabolomics and related experiments. *Metabolomics, 2*(4), 171-196. doi: 10.1007/s11306-006-0037-z

Broda, D. M., De Lacy, K. M., Cook, R. L., & Bell, R. G. (1997). Prevalence of cold-tolerant clostridia associated with vacuum-packed beef and lamb stored at abusive and chill temperatures. *New Zealand Journal of Agricultural Research, 40*(1), 93-98. doi: 10.1080/00288233.1997.9513236

Broda, D. M., Delacy, K. M., Bell, R. G., Braggins, T. J., & Cook, R. L. (1996). Psychrotrophic Clostridium spp. associated with 'blown pack' spoilage of chilled vacuum-packed red meats and dog rolls in gas-impermeable plastic casings. *International Journal of Food Microbiology, 29*(2–3), 335-352. doi: http://dx.doi.org/10.1016/0168-1605(95)00070-4

Cajka, T., Danhelova, H., Zachariasova, M., Riddellova, K., & Hajslova, J. (2013). Application of direct analysis in real time ionization–mass spectrometry (DART–MS) in chicken meat metabolomics aiming at the retrospective control of feed fraud. *Metabolomics, 9*(3), 545-557. doi: 10.1007/s11306-013-0495-z

Chenomx. (2001). Preparing a Nanosep 3K Da Omega Microcenrtifuge Filter Tube for Filtration.

Davies, A. R., Board, R., & Board, R. (1998). *Microbiology of Meat and Poultry*: Springer Science & Business Media.

del Pulgar, J. S., Soukoulis, C., Biasioli, F., Cappellin, L., García, C., Gasperi, F., . . . Schuhfried, E. (2011). Rapid characterization of dry cured ham produced following different PDOs by proton transfer reaction time of flight mass spectrometry (PTR-ToF-MS). *Talanta, 85*(1), 386-393. doi: http://dx.doi.org/10.1016/j.talanta.2011.03.077

Devine, C. (2014). *Encyclopedia of Meat Sciences: 3-volume set*: Elsevier Science.

Dixon, R. A., Gang, D. R., Charlton, A. J., Fiehn, O., Kuiper, H. A., Reynolds, T. L., . . . Seiber, J. N. (2006). Applications of Metabolomics in Agriculture. *Journal of Agricultural and Food Chemistry, 54*(24), 8984-8994. doi: 10.1021/jf061218t

Efron, B., & Gong, G. (1983). A Leisurely Look at the Bootstrap, the Jackknife, and Cross-Validation. *The American Statistician, 37*(1), 36-48. doi: 10.1080/00031305.1983.10483087

Ercolini, D., Ferrocino, I., Nasi, A., Ndagijimana, M., Vernocchi, P., La Storia, A., . . . Villani, F. (2011). Monitoring of Microbial Metabolites and Bacterial Diversity in Beef Stored under Different Packaging Conditions. *Applied and Environmental Microbiology, 77*(20), 7372-7381. doi: 10.1128/aem.05521-11

Gill, C. (1989). Packaging meat for prolonged chilled storage: the Captech process. *British Food Journal, 91*(7), 11-15.

Gill, C., & Penney, N. (1986). Packaging conditions for extended storage of chilled dark, firm, dry beef. *Meat Science, 18*(1), 41-53.
Graham, S. F., Kennedy, T., Chevallier, O., Gordon, A., Farmer, L., Elliott, C., & Moss, B. (2010). The application of NMR to study changes in polar metabolite concentrations in beef longissimus dorsi stored for different periods post mortem. *Metabolomics, 6*(3), 395-404. doi: 10.1007/s11306-010-0206-y
Griffin, J. L., & Shockcor, J. P. (2004). Metabolic profiles of cancer cells. *Nat Rev Cancer, 4*(7), 551-561.
Jeremiah, L. E., & Gibson, L. L. (2001). The influence of storage temperature and storage time on color stability, retail properties and case-life of retail-ready beef. *Food Research International, 34*(9), 815-826. doi: http://dx.doi.org/10.1016/S0963-9969(01)00104-1
Johnson, B. Y. (1991). *Weep and other characteristics of stored and transported chilled meat*.
Kiermeier, A., Tamplin, M., May, D., Holds, G., Williams, M., & Dann, A. (2013). Microbial growth, communities and sensory characteristics of vacuum and modified atmosphere packaged lamb shoulders. *Food Microbiology, 36*(2), 305-315. doi: http://dx.doi.org/10.1016/j.fm.2013.06.016
Liland, K. H. (2011). Multivariate methods in metabolomics – from pre-processing to dimension reduction and statistical analysis. *TrAC Trends in Analytical Chemistry, 30*(6), 827-841. doi: http://dx.doi.org/10.1016/j.trac.2011.02.007
Lindon, J. C., & Nicholson, J. K. (2008). Spectroscopic and Statistical Techniques for Information Recovery in Metabonomics and Metabolomics. *Annual Review of Analytical Chemistry, 1*(1), 45-69. doi: 10.1146/annurev.anchem.1.031207.113026
Liu, C., Pan, D., Ye, Y., & Cao, J. (2013). 1H NMR and multivariate data analysis of the relationship between the age and quality of duck meat. *Food Chemistry, 141*(2), 1281-1286. doi: http://dx.doi.org/10.1016/j.foodchem.2013.03.102
Mannina, L., Sobolev, A. P., Capitani, D., Iaffaldano, N., Rosato, M. P., Ragni, P., . . . Coppola, R. (2008). NMR metabolic profiling of organic and aqueous sea bass extracts: Implications in the discrimination of wild and cultured sea bass. *Talanta, 77*(1), 433-444. doi: http://dx.doi.org/10.1016/j.talanta.2008.07.006
McMullen, L. M., & Stiles, M. E. (1994). Quality of fresh retail pork cuts stored in modified atmosphere under temperature conditions simulating export to distant markets. *Meat Science, 38*(2), 163-177. doi: http://dx.doi.org/10.1016/0309-1740(94)90107-4
Meat and Livestock Australia.). Retrieved from http://www.mla.com.au/Prices-markets/Overseas-markets/Competitors/Sheepmeat
Mills, J., Donnison, A., & Brightwell, G. (2014). Factors affecting microbial spoilage and shelf-life of chilled vacuum-packed lamb transported to distant markets: A review. *Meat Science, 98*(1), 71-80.
Næs, T., Brockhoff, P., & Tomic, O. (2011). *Statistics for Sensory and Consumer Science*: Wiley.
Nuclear Magnetic Resonance.). Retrieved 14/07 2015 from https://en.wikipedia.org/wiki/Nuclear_magnetic_resonance
NZ Trade and Enterprise.). Invest - Statistics. Retrieved from https://www.nzte.govt.nz/en/invest/statistics/
Osorio, M. T., Moloney, A. P., Brennan, L., & Monahan, F. J. (2012). Authentication of beef production systems using a metabolomic-based approach. *Animal, 6*(1), 167-172.
Psaraftis, H. N., & Kontovas, C. A. (2013). Speed models for energy-efficient maritime transportation: A taxonomy and survey. *Transportation Research Part C: Emerging Technologies, 26*(0), 331-351. doi: http://dx.doi.org/10.1016/j.trc.2012.09.012
random.org. List Randomizer.
Ray, B., & Bhunia, A. (2007). *Fundamental Food Microbiology, Fourth Edition*: CRC Press.
Richie, T. (2014). *Meat Industry Update.* Paper presented at the NZ Cold Storage Association 82nd Annual Conference, Wellington.

Ritota, M., Casciani, L., Failla, S., & Valentini, M. (2012). HRMAS-NMR spectroscopy and multivariate analysis meat characterisation. *Meat Science, 92*(4), 754-761. doi: http://dx.doi.org/10.1016/j.meatsci.2012.06.034

Rochfort, S. (2005). Metabolomics Reviewed: A New "Omics" Platform Technology for Systems Biology and Implications for Natural Products Research. *Journal of Natural Products, 68*(12), 1813-1820. doi: 10.1021/np050255w

Sacco, D., Brescia, M. A., Buccolieri, A., & Caputi Jambrenghi, A. (2005). Geographical origin and breed discrimination of Apulian lamb meat samples by means of analytical and spectroscopic determinations. *Meat Science, 71*(3), 542-548. doi: http://dx.doi.org/10.1016/j.meatsci.2005.04.038

Savorani, F., Picone, G., Badiani, A., Fagioli, P., Capozzi, F., & Engelsen, S. B. (2010). Metabolic profiling and aquaculture differentiation of gilthead sea bream by 1H NMR metabonomics. *Food Chemistry, 120*(3), 907-914. doi: http://dx.doi.org/10.1016/j.foodchem.2009.10.071

Savorani, F., Rasmussen, M. A., Mikkelsen, M. S., & Engelsen, S. B. (2013). A primer to nutritional metabolomics by NMR spectroscopy and chemometrics. *Food Research International, 54*(1), 1131-1145. doi: http://dx.doi.org/10.1016/j.foodres.2012.12.025

Seman, D. L., Drew, K. R., Clarken, P. A., & Littlejohn, R. P. (1988). Influence of packaging method and length of chilled storage on microflora, tenderness and colour stability of venison loins. *Meat Science, 22*(4), 267-282. doi: http://dx.doi.org/10.1016/0309-1740(88)90066-6

Small, A., Sikes, A., & Doral, D. (2008). Preliminary investigations into the use of deepchill temperatures for prolonged storage of beef. *Food Science Australia, www. icomst. helsinki. fi/icomst2008/Paper% 20CD/General% 20speakers+ posters-3p% 20papers/Session3 A, 3*.

Straadt, I. K., Aaslyng, M. D., & Bertram, H. C. (2011). Assessment of meat quality by NMR—an investigation of pork products originating from different breeds. *Magnetic Resonance in Chemistry, 49*, S71-S78. doi: 10.1002/mrc.2805

The Lamb Company.). Retrieved from http://www.thelambcompany.ca/index.php/en/about-us/faq

The Meat Industry Association. (2011). Submission to the New Zealand Productivity Commission On the International Freight Transport Services Issues Paper Retrieved 29 June 2015 from http://www.productivity.govt.nz/sites/default/files/Sub%20052%20-%20Meat%20Industry%20Assocation%20Submission.pdf

Trout, G. R. (1988). Techniques for measuring water-binding capacity in muscle foods—A review of methodology. *Meat Science, 23*(4), 235-252. doi: http://dx.doi.org/10.1016/0309-1740(88)90009-5

Trygg, J., Holmes, E., & Lundstedt, T. (2006). Chemometrics in Metabonomics. *Journal of Proteome Research, 6*(2), 469-479. doi: 10.1021/pr060594q

Ulrich, E. L., Akutsu, H., Doreleijers, J. F., Harano, Y., Ioannidis, Y. E., Lin, J., . . . Markley, J. L. (2008). BioMagResBank. *Nucleic Acids Research, 36*(suppl 1), D402-D408. doi: 10.1093/nar/gkm957

van der Greef, J., & Smilde, A. K. (2005). Symbiosis of chemometrics and metabolomics: past, present, and future. *Journal of Chemometrics, 19*(5-7), 376-386. doi: 10.1002/cem.941

Viant, M. R., Rosenblum, E. S., & Tjeerdema, R. S. (2003). NMR-Based Metabolomics: A Powerful Approach for Characterizing the Effects of Environmental Stressors on Organism Health. *Environmental Science & Technology, 37*(21), 4982-4989. doi: 10.1021/es034281x

Warner, R. D., Jacob, R. H., Rosenvold, K., Rochfort, S., Trenerry, C., Plozza, T., & McDonagh, M. B. (2015). Altered post-mortem metabolism identified in very fast chilled lamb M.

longissimus thoracis et lumborum using metabolomic analysis. *Meat Science, 108*, 155-164. doi: http://dx.doi.org/10.1016/j.meatsci.2015.06.006

Weljie, A., Bondareva, A., Zang, P., & Jirik, F. (2011). 1H NMR metabolomics identification of markers of hypoxia-induced metabolic shifts in a breast cancer model system. *Journal of Biomolecular NMR, 49*(3-4), 185-193. doi: 10.1007/s10858-011-9486-4

Westerhuis, J., Hoefsloot, H. J., Smit, S., Vis, D., Smilde, A., Velzen, E. J., . . . Dorsten, F. (2008). Assessment of PLSDA cross validation. *Metabolomics, 4*(1), 81-89. doi: 10.1007/s11306-007-0099-6

Wishart, D. S., Jewison, T., Guo, A. C., Wilson, M., Knox, C., Liu, Y., . . . Scalbert, A. (2013). HMDB 3.0--The Human Metabolome Database in 2013. *Nucleic Acids Res, 41*(Database issue), D801-807. doi: 10.1093/nar/gks1065

Worley, B. P., R. (2013). Multivariate Analysis in Metabolomics. *Current Metabolomics, 1*(1), 92-107.

Xia, J., Sinelnikov, I. V., Han, B., & Wishart, D. S. (2015). MetaboAnalyst 3.0—making metabolomics more meaningful. *Nucleic Acids Research*. doi: 10.1093/nar/gkv380

Young, O., Berdagué, J.-L., Viallon, C., Rousset-Akrim, S., & Theriez, M. (1997). Fat-borne volatiles and sheepmeat odour. *Meat Science, 45*(2), 183-200.

Zanardi, E., Caligiani, A., Palla, L., Mariani, M., Ghidini, S., Di Ciccio, P. A., . . . Ianieri, A. (2015). Metabolic profiling by 1H NMR of ground beef irradiated at different irradiation doses. *Meat Science, 103*, 83-89. doi: http://dx.doi.org/10.1016/j.meatsci.2015.01.005

Appendix A PLS-DA Analysis and Validation

Analysis of Drip for Confinement Odour Metabolites

PLS-DA models were built using the odour groups as the two classes to be differentiated; this resulted in a model (Figure 50) with only one component which had an R^2 value of 0.294 and a Q^2 of 0.084. There was no clear separation of the classes across the single component, and there was therefore no need to perform a test for validity.

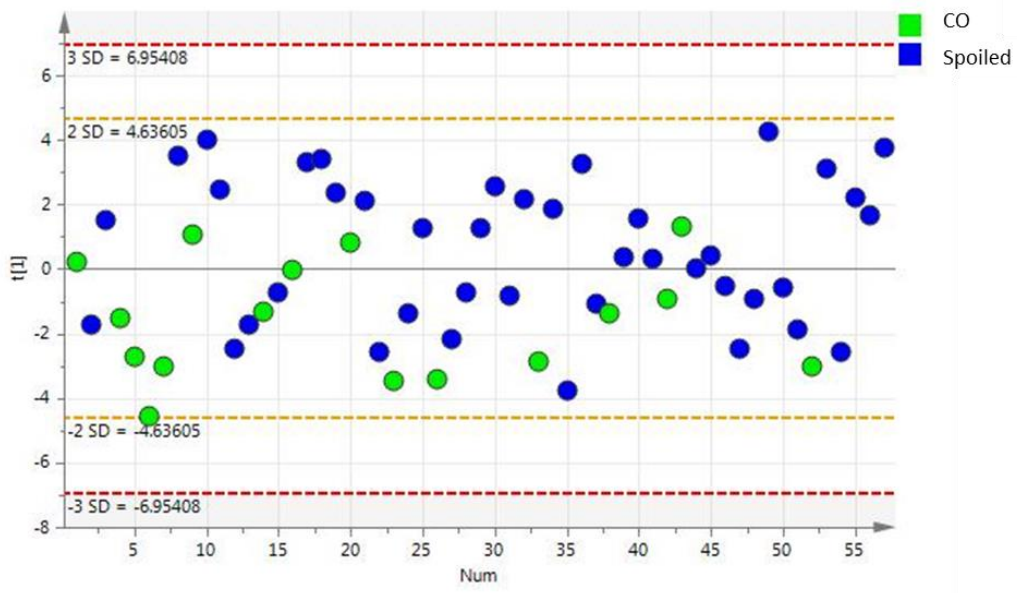

Figure 50 PLS-DA Scores Plot of Drip Classified by Odour Status

Analysis of Drip for Processing Plant Metabolites

A PLS-DA model was built using the processing plant from which the samples came as the discriminating classes. This produced a two component model (Figure 51) with an R^2Y of 0.73 and a Q^2 of 0.602. The validity of the model was assessed by permutation testing, and fit all criteria for validity.

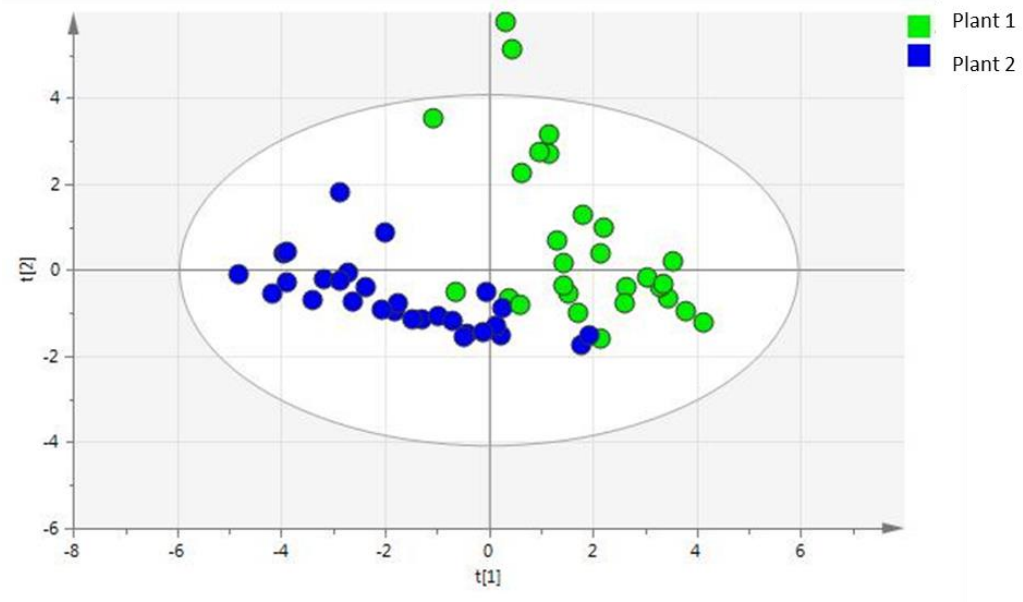

Figure 51 PLS-DA Scores Plot of Drip Samples Classified by Processing Plant

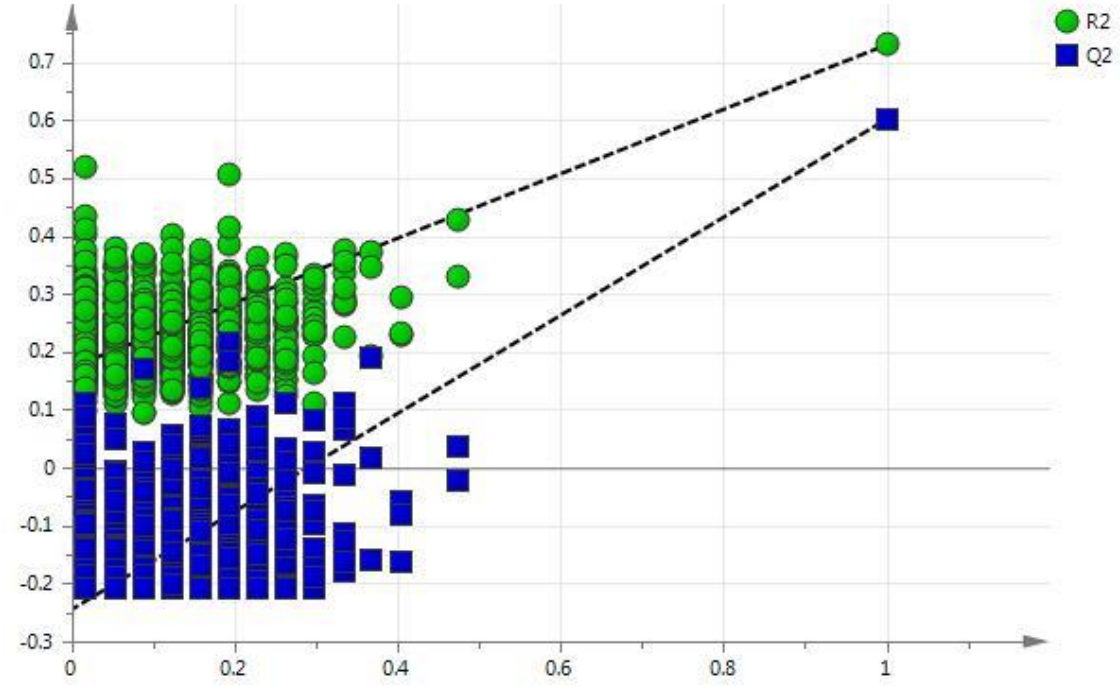

Figure 52 Permutation Test Plot Showing 999 Permutations for PLS-DA model of Drip Samples Classified by Processing Plant

The bins (circled in dashed red in Figure 53) corresponding to the peaks for isoleucine and pyruvate, as well as an unidentified peak and a bin with multiple peaks, had the greatest influence along the first component. Along the second component the bins with the greatest influence corresponded to the peaks for isoleucine, lactate, lipid, acetate, glutamine, choline and three unidentified metabolites.

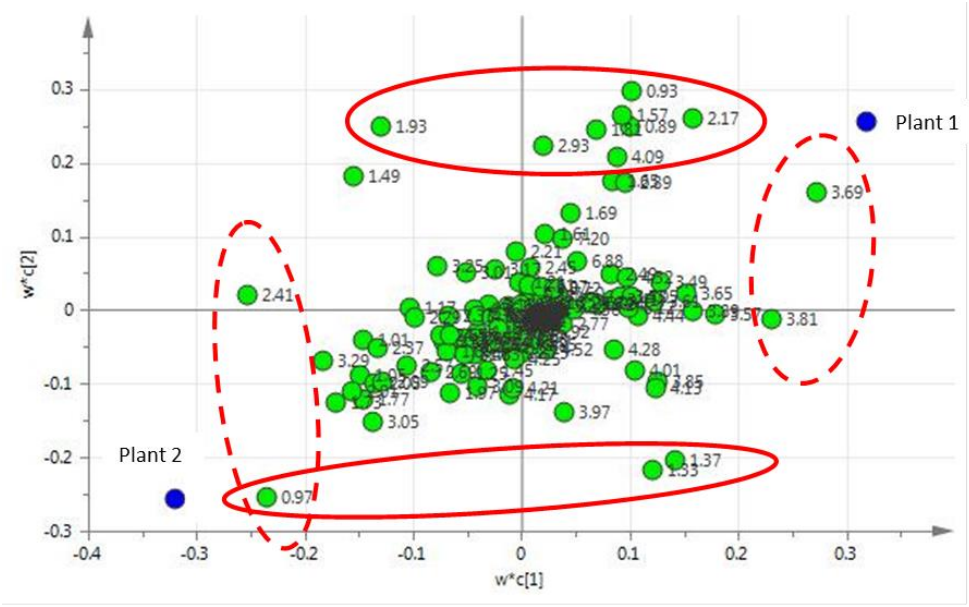

Figure 53 PLS-DA Loadings Plot of Drip Samples Classified by Processing Plant

Analysis of Meat for Confinement Odour Metabolites

No parameters for PLS-DA model building using the two odour types as classes produced a model with any components.

Analysis of Meat for Processing Plant Metabolites

A one component PLS-DA model was built with meat samples differentiated by their processing plant of origin. The R^2Y value was 0.33 while the Q^2 value was 0.253. There was a differentiation of the samples across the principal component roughly corresponding to the processing plant from which they came. There was some separation of the two classes of samples across the principal component, although it was not complete. The validity of the model was assessed using permutation testing and met all the criteria.

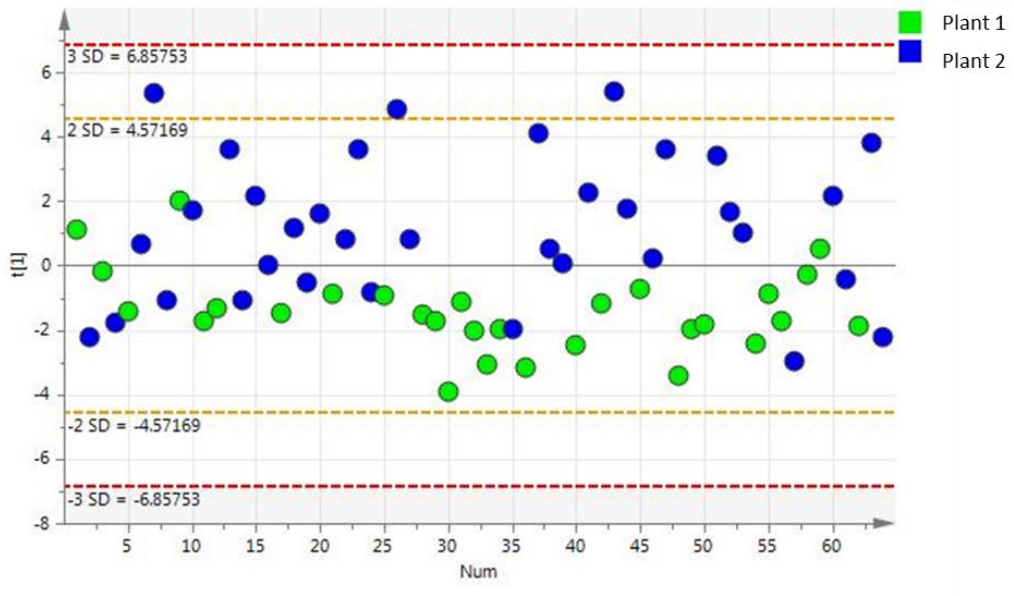

Figure 54 PLS-DA Loadings Plot of Meat Samples Classified by Processing Plant of Origin

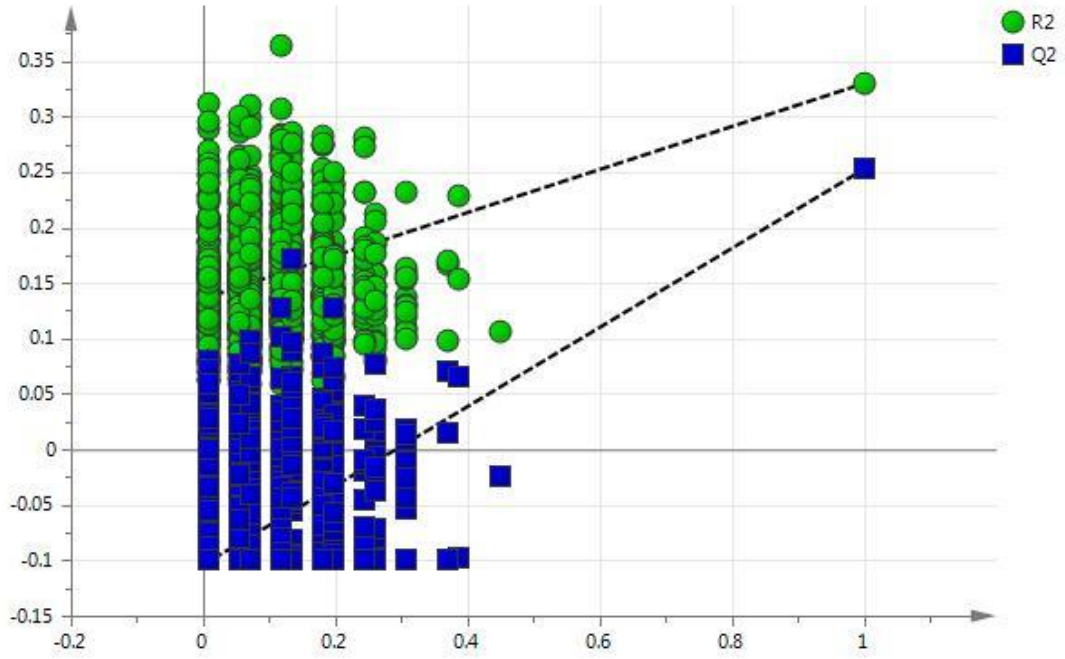

Figure 55 Permutation Test Plot Showing 999 Permutations for PLS-DA model of Meat Samples Classified by Processing Plant

The bins which contain the peaks for alanine, pyruvate and an unidentified metabolite at 3.20 ppm were the largest in the samples which came from plant S, while those containing the peaks for two unidentified metabolites at 3.67 and 3.76 ppm respectively were the largest in the samples which came from plant A.

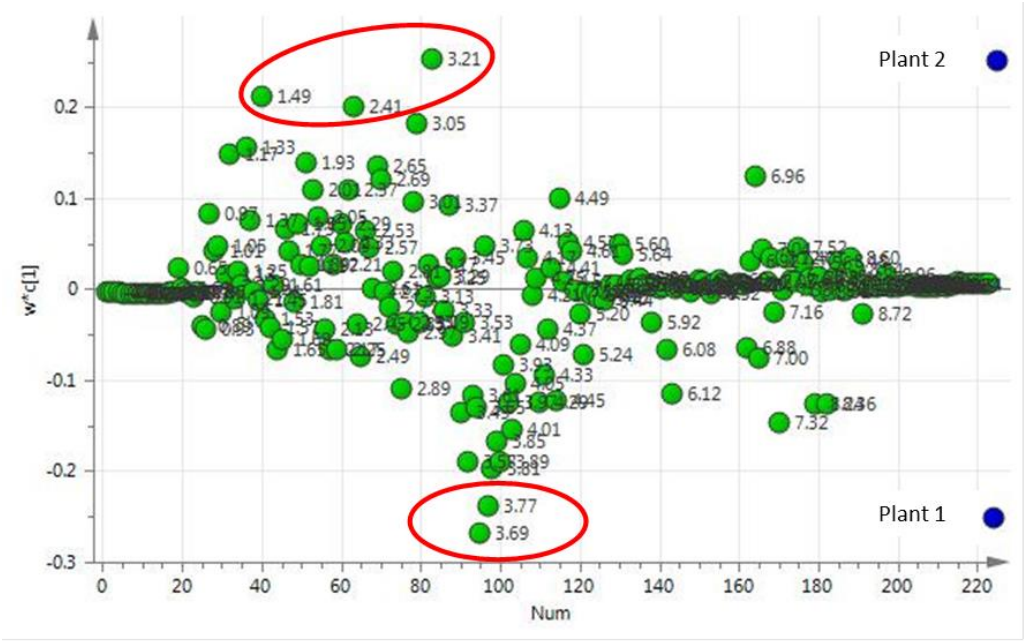

Figure 56 PLS-DA Loadings Plot of Meat Samples Classified by Processing Plant

Analysis of Drip and Meat for Confinement Odour Metabolites

PLS-DA models were built using the odour groups as the two classes to be differentiated; this resulted in a model with only one component which had an R^2 value of 0.08 and a Q^2 of 0.005. There was no clear separation of the classes across the single component, and there was therefore no need to perform a test for validity.

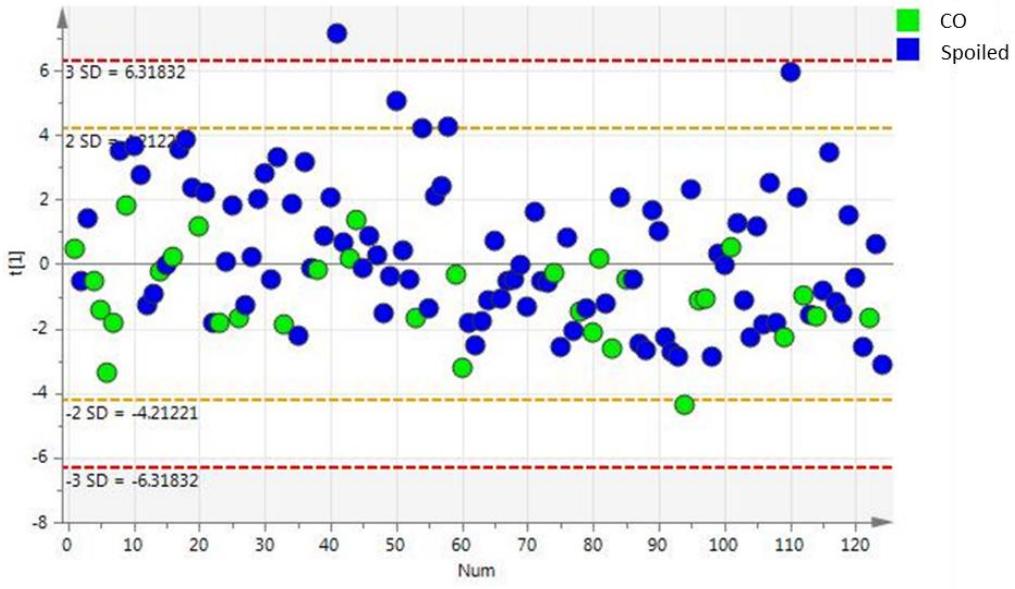

Figure 57 PLS-DA Scores Plot of Drip and Meat Classified by Odour Status

Analysis of Drip and Meat for Partitioning of Metabolites

PLS-DA models were built with the sample type (drip or meat) as the class, a four component model was built with an R^2 value of 0.968 and a Q^2 value of 0.933. A very clear separation was achieved and permutation testing validated this result.

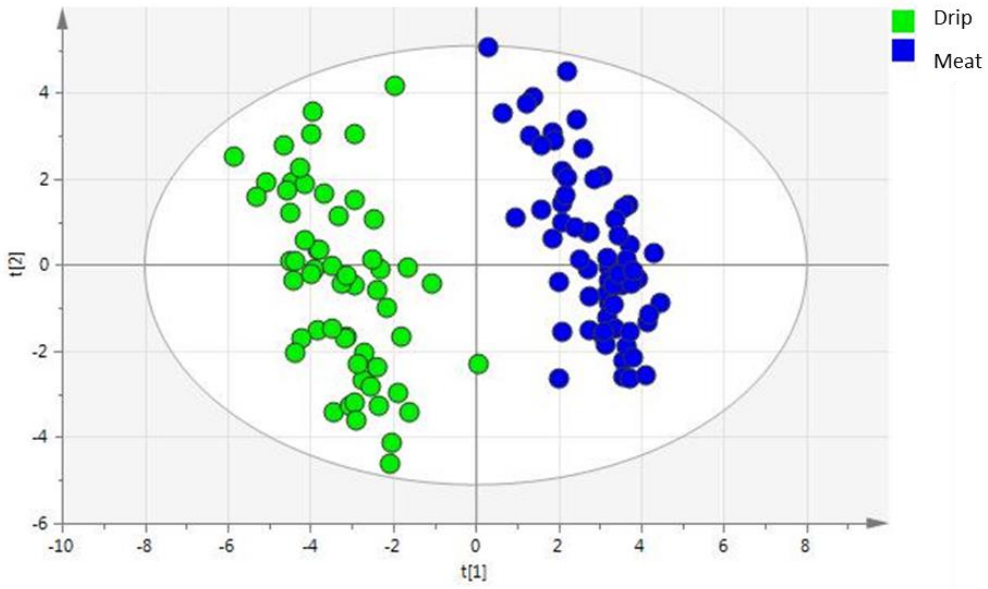

Figure 58 PLS-DA Scores Plot of Drip and Meat Classified by Sample Type

The loadings plot was inspected and the bins which had the greatest effect on the separation of the classes were noted. The bins corresponding to the peaks for creatine and inosine (circled in solid red on the left of Figure 60) had much higher values in the drip samples, while the bins corresponding to the peaks for lactate and choline (circled in dashed red on the right of Figure 60) had much higher values in the meat samples.

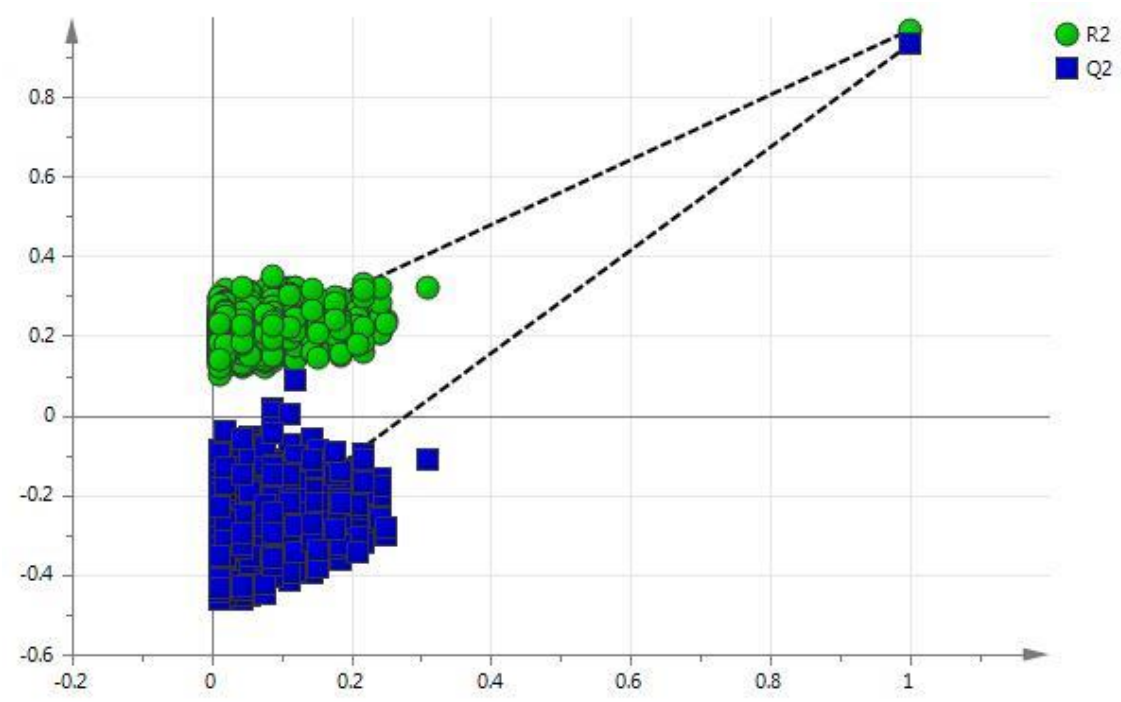

Figure 59 Permutation Test Plot Showing 999 Permutations for PLS-DA model of Drip and Meat Samples Classified by Sample Type

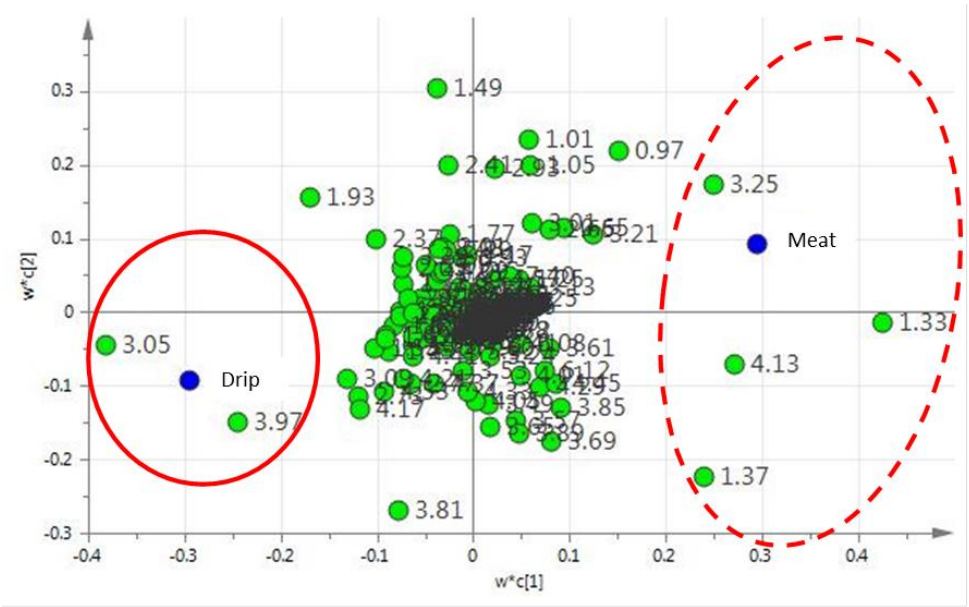

Figure 60 PLS-DA Loadings Plot of Drip and Meat Classified by Sample Type

Analysis of Drip and Meat for Processing Plant Metabolites

Using all drip and meat samples which produced an odour, a PLS-DA model was constructed using the processing plant of origin as the class. A three component model was produced with

an R^2 value of 0.58 and a Q^2 value of 0.472. There was a separation of the majority of the samples; however there were a few samples which crossed over into the other class. This result was validated by permutation testing and fulfilled all criteria.

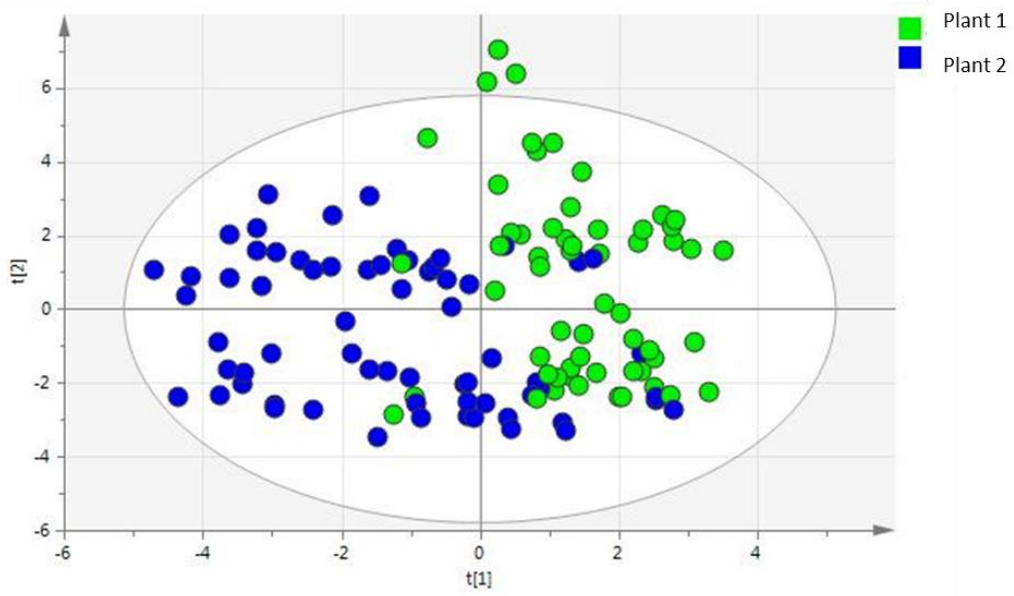

Figure 61 PLS-DA Scores Plot of Drip and Meat Classified by Processing Plant of Origin

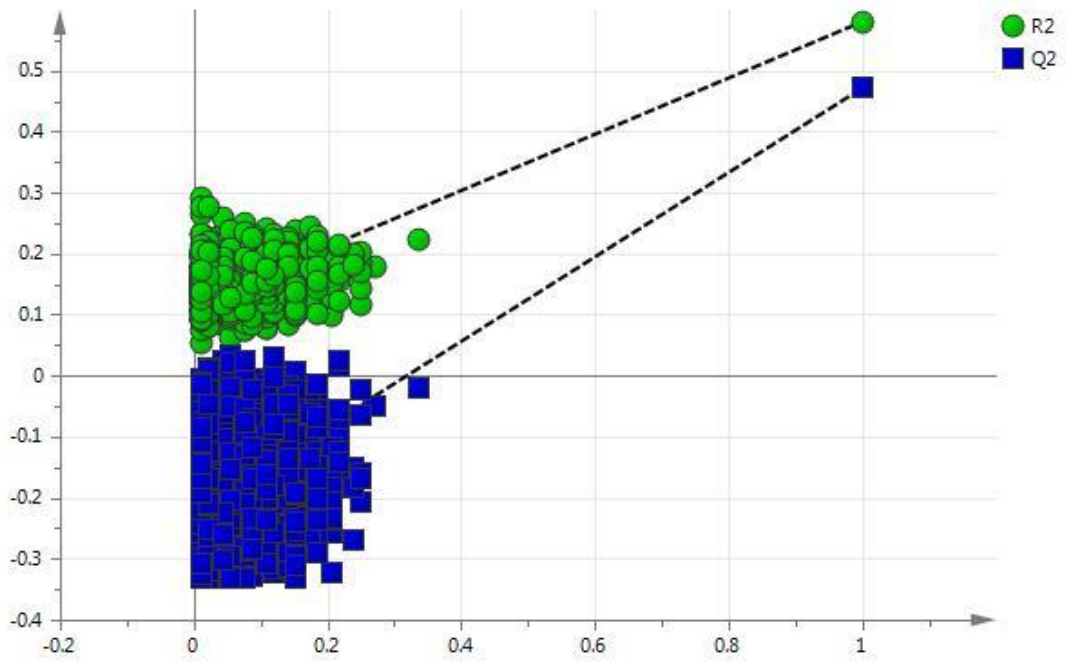

Figure 62 Permutation Test Plot Showing 999 Permutations for PLS-DA model of Drip and Meat Samples Classified by Processing Plant

The bins with the greatest effect on the classification of the two classes based on processing plant of origin are shown in Figure 63 circled in red. The bins corresponding to the peaks for

alanine and pyruvate (circled in solid red) were higher in the samples from plant S, while the bins which had higher values in samples from plant A (circled in dashed red) contained either peaks which could not be assigned to a known metabolite or peaks corresponding to a number of different metabolites.

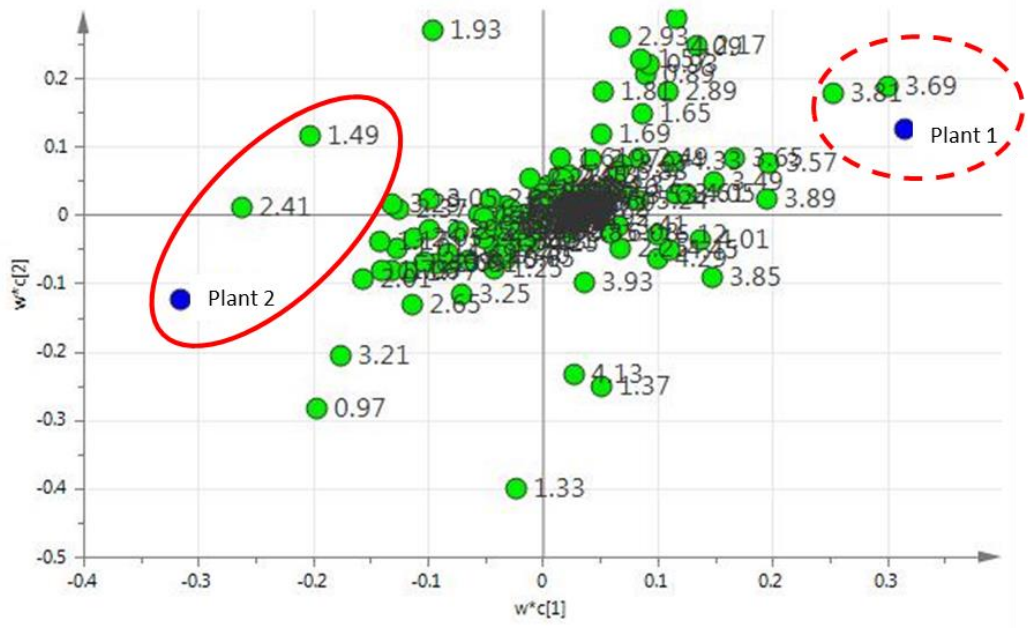

Figure 63 PLS-DA Loadings Plot of Drip and Meat Classified by Processing Plant of Origin